UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

CANOPY MICROCLIMATE MANIPULATION IN THE SUSTAINABLE MANAGEMENT OF 'PINOT NOIR' VINEYARD IN THE VIPAVA VALLEY

DISSERTATION

Melita Sternad Lemut

Mentors:
Prof. dr. Urška Vrhovšek
Dr. Klemen Lisjak

Nova Gorica, 2014

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

LR	Leaf removal
VE (LR)	Leaf removal, done at veraison
BS (LR)	Leaf removal, done at berry-set
PF (LR)	Pre-flowering leaf removal
UN	Untreated / no leaf removal (= control)
BBCH	Scale for description of phenological growth stages of grapes
BBCH 57	Inflorescences fully developed; flowers separating
BBCH 71	Fruit set: young fruits begin to swell, remains of flowers lost
BBCH 77	Berries beginning to touch
BBCH 83	Berries developing colour (veraison)
Mal-3-G	Malvidin-3-glucoside
Del-3-G	Delfinidin-3-glucoside
Peo-3-G	Peonidin-3-glucoside
Pet-3-G	Petunidin-3-glucoside
Cy-3-G	Cyanidin-3-glucoside
Qu	Quercitin
My	Myrcetin
Ka	Kaempferol
Is	Isorhametin
Sy	Syringetin
CM	Cold maceration
AF	Alcoholic fermentation
YWAP	Young wines after pressing
HCAs	Hydroxycinnamic acids
PAs	Pyranoanthocyanins
T_c	Temperature whiten canopy
T_{bs}	Temperature on berry surface
RH_c	Relative humidity whiten canopy
SWR	Transmitted global radiation
MeOH extract	Metanolic extract of grape berry skins according to Mattivi et al. (2006)
M:MeOH extract	Must:Methanol mixture (20:80)
MC	Microbial count
SNK test	Student-Newman-Keul test (statistics)

T-HSD test	Tukey's Honest Significance Difference test (statistics)
FDR	False-discovery rate correction (statistics)
PCA	Principal component analyses (statistics)
DAD	Diode array detector
(U)HPLC	(Ultra) high-performance liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
QqQ	Quadrupole mass spectrometer
Q-TOF	Quadrupole time-of-flight mass spectrometer
SMs	Secondary metabolites
a.a.t	In average of all three treatments (PF, UN, VE)
PBC	Potential biomarker candidate

1 INTRODUCTION

The public's growing concern for health and environment are leading towards more sustainable approaches in all sectors of agriculture, including viticulture. In all forms of sustainable viticulture (integrated, organic and biodynamic), the aim is to limit the type, quantity and timing of pesticide and fertilizer application. One of the first steps that could / should be taken towards higher quality and better sanitary conditions of the grapes is improving the control of the grapevine canopy microclimate, carefully adjusted to each individual site.

The term "microclimate" as a term used in viticulture was first adopted by Smart (1985), aiming to define the environmental conditions within the vine canopy. Depending on the canopy architecture, leaves and bunches, grapes can develop under conditions varying from heavily shaded through to fully sun-exposed (Haselgrove et al., 2000), thus differing berry-surface temperature, light intensity and relative humidity are achieved in the cluster area. It is known that the microclimate of the vine canopy can have a major effect on the physiology of the vine, bud differentiation, fruit growth and maturation.

Furthermore, dense canopies that minimize air circulation lead to a relatively humid environment, which increases the potential for mould growth. To protect their crop from *Botrytis cinerea* Pers. and similar microbial infections, farmers would usually apply fungicides, but controlling spore germination and growth in vineyard with fungicides may only be part of the solution.

In general, the vineyard can be considered an ecosystem influenced by multiple human interactions, including viticultural practices. Leaf removal is a viticulture technique used worldwide in order to improve the microclimate for clusters. It is traditionally performed to reduce grape rot and to obtain better ripening. In the Vipava Valley, it is usually applied at veraison (berry-coloration), whereas the quality potential of earlier leaf removal has not yet been explored.

Plants are very sessile systems that are unable to escape (natural and/or human induced) environmental pressures. As a result, they have evolved a dazzling array of flexibility in their responses to environmental conditions such as light/dark, drought, temperature, nutritional supply, microbial invasion, etc. (Weckwerth, 2003). When exposed to environmental stresses, most animals can move or run away, but plants have developed their own defence system. To

protect themselves against various stresses, plants (grapevines) normally react by producing a wide range of secondary metabolites (Sudha & Ravishankar, 2002).

Secondary metabolites are products of plant metabolism that are not directly included in the primary biochemical pathways of cell development, though they hold some very important functions in living plants such as protection, attraction or signalling (Wink, 2003). Beside enrolment in many important biological processes, they are crucial in the determination of grape and wine quality attributes (*e.g.* colour, taste and flavour) (Verpoorte et al., 2002).

Environmental metabolomics is the application of metabolomics (a novel analytical approach) that can characterize the interactions of plants with their environment. This approach has many advantages for studying organism–environment interactions and for assessing organism function and health at the molecular level (Bundy et al., 2009). Metabolomics in the environmental sciences ranges from understanding organism responses to abiotic pressures (e.g. climatic) to understanding biotic responses (e.g. diseases).

Although many recent research efforts are already focused on grapevine secondary metabolites, few have been performed in real purposely-induced microclimate scenarios and even fewer by implementing metabolomics in the vineyard environment.

In the presented research work, a field trial was thus designed to compare different leaf removal treatments (performed at berry set, at veraison and in the pre-flowering stage - later as less known technique in modern viticulture) with untreated grapevines, studying with classical and metabolomic analytical approaches how the induced microclimate modifications could affect a wide group of secondary metabolites in grape berries during maturation and at harvest and how a better understanding and control of sunlight, temperature and humidity exposure can contribute to future, high quality productive and more sustainable viticulture practices.

1.1 Research goals

The general objective of the presented work was to evaluate grape quality potentials (focusing on secondary metabolites and sanitary state) in four different real microclimate scenarios, induced by three different leaf removal performances: at (*i*) pre-flowering (BBCH 57), at (*ii*) berry-set (BBCH 71) and at (*iii*) veraison (BBCH 83), all compared with untreated vines. The aim was to define the preferential canopy microclimate - and therefore the most beneficial timing for leaf removal from the quality point of view but also leading to the more sustainable

management of observed vineyards in the future.

In addition, we aimed to gather some new valuable information regarding vine genotype-phenotype interactions in the case of abiotic (climate) and biotic stresses (pathogens - *B. cinerea* Pers.) by implementing novel analytical approaches into sample evaluations.

Within the general objective of the presented dissertation topic, there were four individual but closely connected objectives:

➲ Together with the basic grape quality parameters, we planned to evaluate the occurrence of several secondary metabolites in grapes during maturation and at harvest time in all samples from different leaf removal treatments under examination. Qualitative and quantitative profiles of anthocyanins, flavonols and hydroxycinnamic acids (probably the most important phenolics from a technological point of view) were aimed to be initially screened using classical analytical approaches but later upgraded with targeted metabolomic analyses to cluster a much wider range of information about specific groups within the observed grape metabolomes.

➲ Furthermore, we planned to supplement the classical and metabolomic targeted analyses with untargeted analyses of different polyphenol classes (untargeted metabolomics approach), aiming to define some new potential biomarkers involved in the plant bioprocess under the given conditions. Metabolomics would thus represent a new way to describe the biochemical response of the grapevine to canopy management and consequently a potential for the knowledge-based improvement of vineyard management.

➲ Based on the field trial results, we planned to investigate possible relationships between the detected grape metabolic behaviours with the induced microclimate conditions (temperature - berry surface temperature, light intensity and humidity). We aimed to explore some key combinations that could lead to a significant improvement in grape berry composition in an environmentally friendly manner, as well as to re-asses some existing information regarding plant-environment interactions by upgrading them with new analytical possibilities.

➲ To support the main hypothesis, we additionally planned to investigate the microbial ecology of the observed grapes in the different microclimate conditions and *B. cinerea* Pers. occurrence with or without a last Cyprodinil+Fludoksinil (switch®) application in parallel trial, in order to reveal the influence of environmental parameters and of viticulture practice (leaf removal) on the species composition and population dynamics of microbial community inhabiting 'Pinot Noir' variety grapes during ripening and at harvest. An important sub-

objective of the parallel trial was to evaluate the effectiveness of optimal leaf removal performances compared to fungicide application in the final stages of grape ripening, and therefore its possible contribution to the reduced use of pesticides.

2 THEORETICAL BACKGROUND

2.1 The Vineyard and the Environment

Like any other plant, the grapevine (and its performance) is highly dependent on the geo-climatic conditions in which the plant is grown (Gladstone, 1992). In viticulture, the term *terroir* is strongly adopted to describe the spatial and temporal entity characterized by the interaction between the grapevine and the environmental potentials, significant for the characteristics of grapes and related wines (Deloire et al., 2005; Vaudour, 2002). The concept of *terroir* can therefore be understood as a complex of natural environmental factors (topography, climate, geology and soil) that cannot easily be modified by the winegrower (van Leeuwen & Seguin, 2006; Laville, 1993). However, while the simplest terroir approaches mainly consider soil and climate and/or the interaction between them (Ubalde et al., 2010; Morlat, 2001), others also include non-environmental factors such as variety (van Leeuwen et al., 2004), viticultural and winemaking technology (Carbonneau, 2001), or even historical and sociological wine-growing factors (Vaudour, 2002).

2.1.1 Conventional vs. Sustainable viticulture approach

In nearly all parts of the world, pest and disease control in grapevines is generally achieved by the widespread application of pesticides, which results in high costs and a negative environmental impact (Ali et al., 2010). In conventional viticulture, the performance of the vineyard would normally be controlled by using a series of (toxic) insecticides, fungicides and herbicides, primarily (and with short-term consideration) to keep the vines healthy by destroying anything that may be harmful to them. In addition, many synthetic fertilizers would be used to promote vine production (Bell & Robson, 1999). However, when badly targeted and/or overdosed, such substances can promote many problems over time. Pesticides used in agro-ecosystems may be a hazard to non-targeted organisms (Kula & Kokta, 1992). Over-dosage of plant protection products can lead to economic losses and to reduced efficiency and/or an increased risk of resistance (Siegfried et al., 2007). The long-term use of organic and inorganic pesticides in vineyards has already resulted in increased concentrations of those

pollutants in soils and other environmental compartments (Komarek et al., 2010). Related problems such as an impaired natural balance, the presence of multiple toxic residuals (Renwick, 2002), the development of more and more pesticide resident strains of phytopathogens (Leroux et al., 2010), the lack of continued approval of some of the most effective protective substances (Spadaro & Gullino, 2004) and finally the public's growing awareness and concern for health and the environment (Pimente, 2005) are all leading towards more sustainable approaches in all sectors of agriculture, including viticulture.

In general, we can describe sustainable agro-ecosystems as those that maintain the resource base upon which they depend, rely on the minimum artificial inputs from outside the farm system, manage pests and diseases through internal regulating mechanisms, and are able to recover from the disturbances caused by cultivation and harvest (Gliessman, 1998; Altieri, 1995).

The so-called sustainable viticulture approach incorporates aspects of organic production to reduce the degradative factors of conventional viticulture (Tassie et al., 1990). Broadly defined, sustainable viticulture is an agricultural system that is environmentally sound, economically viable and socially responsible (Ingels, 1992).

Furthermore, sustainable vitiviniculture is defined by the OIV as a "Global strategy on the scale of the grape production and processing systems, at the same time incorporating the economic sustainability of structures and territories, producing quality products, considering the requirements of precision in sustainable viticulture, risks to the environment, product safety and consumer health and the valuing of heritage, history, culture, ecology and landscape."

In recent years, the term "sustainable" has been used for various methods of culturing (Howell, 2001). However, in all forms of sustainable viticulture (integrated, organic and biodynamic), it aims to limit the type, quantity and timing of pesticide and fertilizer application with more attention to the secondary effects on humans and the environment.

Integrated grape production can be defined as a nature-friendly form of vineyard management, using natural resources and mechanisms that reduce the negative impacts of farming on the environment and human health. An important aim and requirement of an integrated production in viticulture is the conservation of the vineyard environment, its habitats and wildlife, which must not be detrimentally altered or polluted (Corino & Calo, 2000). Biodynamic agriculture/viticulture is a method implemented by the Austrian scientist and philosopher

Rudolf Steiner in the early 1920s. Often compared to Organic agriculture/viticulture, biodynamic farming is different in a few distinct ways: it prohibits synthetic pesticides and fertilizers in the same way as certified organic farming - however, while organic farming methods focus on eliminating pesticides, growth hormones and other additives for the benefit of human health, biodynamic farming emphasizes creating a self-sufficient and healthy ecosystem (Magali & Grant, 2010; Reeve, 2005).

However, sustainable viticulture is not mainly about following the regulations, but more about lifestyle and the ability to adapt. The challenge for effective management is even greater in organic viticulture, where many effective chemical treatments cannot be used (Magarey et al., 2000). Unfortunately, many wine producers still associate the term sustainability solely with the environmental dimension and only few of them apply a more complete approach of sustainability, not only including the environment, but also the economic and social dimensions (Szolnoky, 2013). As the most sustainable approaches are those that are the least toxic, least environmentally harmful and least energy intensive, and yet which maintain productivity and profitability, there may be situations where the use of synthetic chemicals would/could be more "sustainable" than a strictly nonchemical approach (Ingels, 1992).

According to Ingels (1992), sustainable agriculture/viticulture should be viewed as goal-oriented rather than representing a specific set of practices. In his work, he says that the practices that can be used to achieve those goals may differ based on site-specific factors such as climate, site and soil characteristics, pests, irrigation method and the local availability of inputs. To sum up, the main practices that can enable growers to act with a higher level of sustainability would be:

➲ Soil management - monitoring, nutrient management, soil quality and pollution prevention.

➲ Water management - management strategies, off-site water movement and irrigation practices.

➲ Pest and pesticide management - insect, disease and weed monitoring and management practices along with the effectiveness and safety of their application practices.

➲ Viticultural practices - vine balance through canopy management and crop development practices (Landers, 2008; Reeve, 2005; Corino & Calo, 2001).

Vine balance therefore obviously has a close relationship with sustainability. The adjustment of the balance of grapevine yield and vine growth for the constant (sustainable) production of high-quality grapes must be considered central through the following rules (Failla, 2010; Howell, 2001):

➲ For each combination of genotype x environment, there is a technique to achieve the optimum crop production of grapes with a constant quality;

➲ The good growing practices should result from knowledge of the physiology of the growth and development of the grapevine;

➲ Sustainable levels of high quality grapes associated with high productivity can only be achieved through an adequate fruit yield and vine growth of a suitable bunch thermal and radiation microclimate.

The goal of sustainable winegrowing should be continual improvement, in other words moving along the continuum toward a higher level of sustainability (Ingels, 1992), but the successful conversion to sustainable practices not only requires time, but a substitution of beliefs and enhanced management as well as improved scientific knowledge to support it.

2.2. Metabolic constituents of the grapevine/grapes

The quality of the grapevine crop mainly depends on its metabolites (Ali et al., 2010). Plant metabolites are principally products of various series of enzyme-catalysed reactions (pathways) that occur naturally within each living cell; in any living cell, the carbon and energy source for the cell is first converted into a set of so-called precursor metabolites, which are subsequently converted to metabolites that can serve as building blocks for biomass synthesis, but can also be secreted from the cell (Villas Bôas et al., 2007).

Plants, including grapevines, can thus synthesize (through their metabolism) an extremely diverse range of organic compounds that can be essentially classified as primary and secondary metabolites, however, the precise boundary between those two groups is not always very clear (Crozier et al., 2006; Croteau et al., 2000). For example, some classes of secondary metabolites can also include several primary metabolites, so whether a compound is a primary or secondary metabolite is actually a distinction that cannot simply be based on its chemical structure but it is based more on its function (and sometimes its distribution within the plant kingdom) (Villas Bôas et al., 2007). However, primary and secondary plant metabolites are generally known and considered to perform completely

different tasks in plants (Ali et al., 2010; Villas Bôas et al., 2007).

2.2.1 Primary metabolites

Primary metabolism is generally related to energy and the production of core structures in the cell and is therefore mostly influenced by the nutritional environment (Villas Bôas et al., 2007). Primary metabolites can hence be found in all plant species where they are performing essential metabolic routines (Croteau et al., 2000) by being directly involved in normal plant growth, development and reproduction (Ali et al., 2010). Moreover, they are the products of fundamental metabolic cycles, such as the Krebs cycle, the Calvin cycle and glycolysis and also play important roles associated with plant photosynthesis and respiration (Villas Bôas et al., 2007; Crozier et al., 2006). However, grapevine primary metabolites can make up a higher percentage of the total mass in the leaves, buds, flowers and other non-reproductive parts compared to the fruit - the grape, while the attributes that make grapes desirable to us (and to certain pest species) are mainly accumulated in the last stages of grape development (Creasy GL & Creasy LL, 2009). In general, primary plant metabolites mainly (but not exclusively) include ubiquitous compounds, such as sugars, amino acids and organic acids (Villas Bôas et al., 2007), whereas in grape tissue particularly, beside carbohydrates (15-25%) and water (70-85%), there are about 0.5-1% of organic acids, about 0.01% of nitrogenous compounds and about 0.3–to-0.6% mineral compounds (Creasy GL & Creasy LL, 2009; Conde et al., 2007; Winkler, 1974).

2.2.2 Secondary metabolites

Plant compounds that are not directly involved in the primary biochemical pathways of cell development, and which (in contrast to primary plant metabolites) are not considered essential for plant functioning, are commonly classified as secondary metabolites. However, even though these are often regarded as the products of biochemical "side tracks" in the plant cells, many of them are found to hold very important functions in living plants such as protection, attraction or signalling (Wink, 2003). This includes compounds involved in plant/insect and plant/pathogen interactions, compounds preventing UV-B damage and compounds involved in hormone homeostasis (Kliebenstein, 2004). Secondary metabolites can be accumulated in relatively high concentrations, especially when a plant is subjected to different forms of stress (Gatto et al., 2008; Zhao et al., 2005; Cantos et al., 2001) or elicitors (Zamboni et al., 2006), hence they are strongly involved in the adaptation of plants to the environment (Kliebenstein et

al., 2005; Bourgaud et al., 2001). Beside enrolment into many important biological processes, they are important in the determination of food quality attributes (*e.g.* colour, taste and flavours) (Verporte et al., 2002).

In general, plant secondary metabolites are produced by pathways derived from primary metabolic routes (Villas Bôas et al., 2007) and are characterised by the enormous diversity of (often species specific) chemicals - many representing great value to the agrochemical and food industry (Ali et al., 2010) or as pharmaceuticals (Ramachandra Rao & Ravishankar, 2002). Despite their substantial diversity, secondary metabolites are essentially synthesized from a small number of key primary metabolites, which is also the basis of their general classification into three major groups: terpenoids (also called isoprenoids), alkaloids and phenolics, all synthesized within so-called isoprenoid, alkaloid and phenylpropanoid biosynthetic routes (Villas Bôas et al., 2007). Whereas terpenoids and related plant secondary metabolites (sesquiterpenoids and sterols) are derived from the five-carbon precursor isopentenyl diphosphate (IPP), alkaloids on the other hand are principally synthesized from amino acids. Finally, phenolic compounds are derived from the phenylpropanoid biosynthetic pathway - linked with the shikimic acid pathway which produces the phenyl propanoid derivatives (C6–C3) or the malonate/acetate (melavonate) pathway that produces the side-chain-elongated phenyl propanoids, including the large group of flavonoids (C6–C3–C6). The common precursor of phenol-containing amino acids and phenolic compounds is the aromatic amino acid phenylalanine (Villas Bôas et al., 2007; Dewick, 2002; Bennett & Wallsgrove, 1994).

Also grapevine (*V. vinifera* L.) products are a valuable source of secondary metabolites that are ultimately stored in various plant tissues (Iriti & Faoro, 2006). However, the technological value of wine grapes (precisely their berries) is largely determined by the presence of specific aroma compounds as well as the presence of a large group of molecules called phenolic compounds. Particularly grape/wine phenolics are of special interest to researchers not only due to their significant effects on grapevine crop quality (Ribereau-Gayon et al., 2006) but also due to the various associations of moderate wine drinking with health-promoting effects (Bertelli et al., 2009; Halliwell et al., 2005).

2.2.2.1 The phenolic composition of grapes and wines

(Poly)phenols are broadly distributed in the plant kingdom and are known as the most abundant plants secondary metabolites (Macheix et al., 1990). In grapes, they represent the third most abundant constituent (excluding water) after carbohydrates and fruit acids (Singleton, 1980). Technologically important phenolics are mainly present in the berry skins and seeds (Cheynier et al., 1996), however, there are some exceptions such as the presence of hydroxycinnamic acids in the pulp. The percentage of the total extractable phenolics in grape tissues is reported up to 10% in the pulp, 60–70% in the seeds and 28–35% in the skin; whereas the phenolic content of seeds may range from 5 to 8% of FW (Nawaz et al., 2006; Zoecklein et al., 1995). Mazza et al. (1999) reported 915-955 mg/kg ot total phenolics (expresed as galic acid) in 'Pinot Noir' grape skins at harvest. All phenolics found in grapes are product of phenylpropanoid biosynthetic pathway (Figure 1).

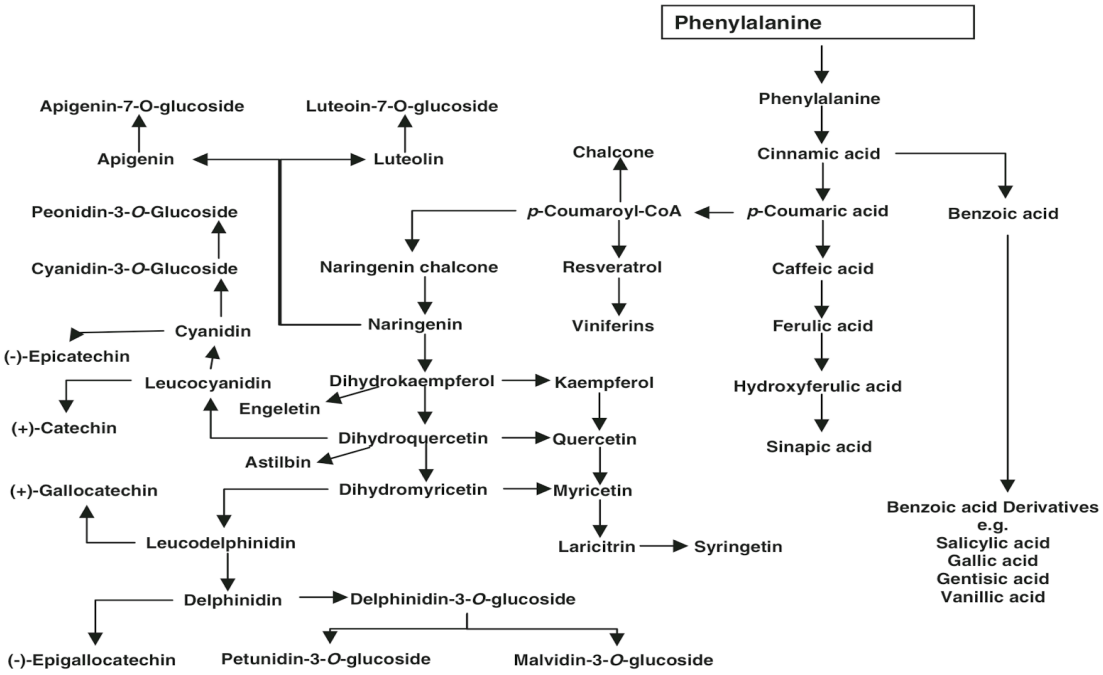


Figure 1: The biosynthetic pathway from phenylalanine to different classes of phenolics found in grapevine and wine (Ali et al., 2010: 4)

The name of the group "phenol" derives from a 6-carbon aromatic ring with at least one hydroxyl group (Creasy GL & Creasy LL, 2009). Phenolics can range from simple, low-molecular weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols (crozier et al., 2006). the most common classification of phenolic compounds is based on a division into flavonoid compounds with a C3-C6-C3 skeleton and

non-flavonoid compounds (C6-C1 the *p*-hydroxybenzoic group, C6-C3 the cinnamic group and C6-C2-C6 stilbenes or stilbenoids). Quantitatively, grapes contain most of flavonoid compounds in the skins (and/or seeds), whereas most of non-flavonoids are normally present in the pulp and are less abundant in other tissues (Adams, 2006; Kennedy et al., 2006; Monagas et al., 2005).

2.2.2.1.1 Non-flavonoids

The main group members of the non-flavonoid compounds present in grapes/wines are phenolic acids (including their derivates) and stilbenes. Various non-flavonoid phenolics can be detected in grapes and wines, but with the exception of hydroxycinnamic acids (and some hydroxybenzoic acids) they are normally present at relatively low concentrations (Kennedy et al., 2006). According to different authors, the levels of hydroxycinnamic acids in the pressed juice of red grapes were reported to vary from app. 20-120 mg/L (and from 100-230 mg/L in the juice of white grapes, whereas a typical level of total phenolic acids was reported between 100 and 200 mg/L in red wines (135 mg/L in Pinot Noir wine, expressed as caffeic acid) and around 20-60 mg/L in white wines (Rothwell et al., 2012; Ribéreau-Gayon et al., 2006; Mazza et al., 1999; Vrhovsek, 1998; Mattivi & Nicolini, 1997).

Phenolic acids are aromatic secondary metabolites that are widespread throughout the plant kingdom (Robbins, 2003). They are actually derivates of hydroxybenzoic and hydroxycinnamic acids, the most numerous of which are esterified to sugars, organic acids or alcohols (Monagas et al., 2005; Zoeklein, 1995).

Hydroxycinnamic acids are located in the vacuoles of the skin and pulp cells (Kennedy et al., 2006). Cinnamic acid can be converted to various ranges of hydroxycinnamates, which are collectively referred as phenylpropanoids (Crozier et al., 2006). Hydroxycinnamic acids derived from caffeic, ferulic, *p*-cumaric and sinapic acid are commonly found in grapes as tartaric acid esters, but may be found in their free forms in wine due to enzyme hydrolyses during vinification (Robbins, 2003). The principal hydroxycinnamic acids present in *vinifera* grapes are caftaric, coutaric and fertaric acids in the *trans* form, although low quantities of the *cis* isomers can also be detected (Singleton et al., 1978).

Hydroxybenzoic acids are synthesized in plants from the corresponding hydroxycinnamic acids. They are derived from benzoic acid and possess a C6-C1 skeleton with the principal

component being gallic acid (Crozier et al., 2006). Grapes are reported to contain gallic acid in the pulp (Ali et al., 2010; Lu and Foo, 1999), while a ester-linked form with flavan-3-ols was also reported in the seeds (Su & Singleton, 1969). However, other hydroxybenzoic acids (e.g. *p*-hydroxybenzoic, vanillic, syringic acid) and their derivatives can also be found in wines (Monagas et al., 2005), but their origin is normally more associated with oak wood.

Stilbenes are a group of compounds that contain 1,2-diphenylethylene as a functional group, with their skeleton based on a *trans*-resveratrol structure (Jeandet et al., 2002). These compounds are commonly present in the soft tissues (fruits, leaves, root tips and other herbaceous organs) (Baversco et al., 2007). In grapes, they are essentially located in the grape skins (Jeandet et al., 1991), but have also been reported in grape seeds (Sun et al., 2006; Pezet & Cuenat 1996) and stems (Bavaresco et al., 1997; Pezet & Cuenat 1996). The most abundant family representative is resveratrol (3,5,4′-trihydroxystilbene), a monomeric stilbene. Beside simple stilbenes (e.g. *trans*-pterostilbene, a dimethylated resveratrol derivative, 3,5-dimethoxy-4′-hydroxystilbene, *trans*- and *cis*-piceid, a 3-*O*-*β*-D-glucoside of resveratrol, *trans*- and *cis*-astringin, a 3-*O*-*β*-D-glucoside of 3′-hydroxy-resveratrol, and *trans*- and *cis*-resveratrol, a 4′-*O*-*β*-D-glucoside of resveratrol), Jeandet et al. (2002) reports that stilbenes can also be present in the grapevine in their oligomeric and polymeric forms - *e.g.* the so-called viniferins: *ε*-viniferin (dimeric resveratrol) or *α*-viniferin (trimeric resveratrol). They also identified stilbenoids like ampelopsin A (dimeric resveratrol) and hopeaphenol (dimeric ampelopsin A). Furthermore, stilbene group members piceatannol and astringinin, present in grapes (Bavaresco et al., 2002) and pallidol (dimmer), present in red wines (Landrault et al., 2002), were also reported previously.

The occurrence of stilbenes in plant tissues is largely associated with the resistance of plant to fungal diseases such as *Botrytis cinerea* Pers., although they can also occur due to abiotic stresses, such as UV irradiation (Gatto et al., 2008; Sun et al., 2006; Jeandet et al., 2002). Stilbenes are also of interest to researchers due to the fact that they are considered phytoalexins - a group of plant chemicals of low molecular mass that are inhibitory to microorganisms and their accumulation in plants is initiated by the interaction of the plant with microorganisms. Although phytoalexins are a large group present in plants, those from the *Vitaceae* seem to constitute a rather restricted group of molecules belonging to the stilbene family (Jeandet et al., 2002). However, despite the fact that grapes and red wines are two of the major dietary sources of stilbenes, their concentration in grapes is relatively low comparing to other phenolics present

(Ali et al., 2010; Sun et al., 2006).

Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development are listed by Landrault et al. (2002). In their study in Italy, Gatto et al. (2008) defined 'Pinot Noir' as a high producer of stilbenes/resveratrol, achieving the highest content among the 78 observed varieties with a stilbene (*trans* resveratrol and *trans* and *cis* piceid) level of around 20 mg/kg of FW. Also Bavaresco et al. (2007) found the levels of stilbenes in grapes to be variety specific, while Jeandet et al. (2002) additionally discuses resveratrol concentration in grape skins being negatively correlated with the developmental stage of the berries.

2.2.2.1.2 Flavonoids

Flavonoids are probably the most important group of grape phenolics. They are characterized by a basic skeleton of 15 C atoms (C6-C3-C6) of the 2-phenyl-benzopyrene type (= 2 phenolic groups: rings A and B) joined by an oxygen-containing ring. Their polyphenolic flavan skeleton can be variously substituted with hydroxyl, methyl, galloyl, glucosyl and acyl conjugations, and degree of polymerization (Aherne et al., 2002).

Flavonoids can be sub-divided into several sub-classes – in most classifications, into flavonols *(i),* flavanonols and flavones *(ii),* flavanols (flavan-3-ols) *(iii)* and anthocyanins *(iv)*.

Flavonols are known to be the products of the flavonoid biosynthetic pathway, which in red grapevine varieties also engenders anthocyanins and condensed tannins. They constitute a group of flavonoids that vary in colour from white to yellow (Makris et al., 2006). These pigments are essentially characterized by two benzene cycles bonded by an oxygenated heterocycle derived from the 2-phenyl chromone nucleus (Ribereau-Gayon et al., 2006). They are also known as important plant protectors against ultraviolet light irradiation and free radical scavengers (Downey et al., 2004a,b; Flint et al., 1986), so their concentration is highly influenced by the light exposure of observed plant tissues.

In grapes, they can be mainly found in the berry skins (Mattivi et al., 2006; Cheynier and Rigaud, 1996), but have also been detected in the pulp (Pereira et al., 2006). Two distinct periods of flavonol synthesis are reported in grapes, the first around flowering and the second during the ripening of the berries (Downey et al., 2003b).

Within their class, the flavonols are further differentiated by the substitution of the lateral nucleus, producing kaempferol (1OH), quercetin (2OH) and myrcetin (3OH) (Ribereau-Gayon et al., 2006), and are usually present in the glycosilated form of six main aglycones (quercetin, kampferol, isorhamnetin myrcetin, laricitrin and syringetin) (Mattivi et al., 2006). Glucose is the common sugar attached to the C-3 position of those main aglicons, but glucuronic acid has also been found as the glycosylation sugar of kaempferol, quercetin and myricetin, the nonmethoxylated flavonols (Cheynier and Rigaud, 1996). Furthermore, many other flavonols (glucosides) are also reported as present in grapes, such as kampferol galactoside, kampferol glucosylarabinoside, quercetin glucosylgalactoside, quercetin glucosylxyloside and quercetin 3-rhamnosylglucoside (i.e. rutin) etc. (Jefferey et al., 2008; Mattivi at al., 2006; Cheynier et al., 2000). Although predominant in their glycosilated form, the aglycones can be observed in wines, but probably originate from the hydrolysis of the glycosilated forms during the vinification processes (Cheynier et al., 2006). During red wine making, flavonols are extracted from the grape skins and the absolute concentrations of the free forms of flavonols following acid hydrolysis are reported to be three times higher in the red-skinned varieties than in the white-skinned varieties (Mattivi et al., 2006), varying from 3.81 to 80.37 mg/kg in the red varieties (50 mg/kg in 'Pinot Noir') and from 1.36 to 30.21 mg/kg in the white varieties. Even though they are not directly responsible for red wine colour, the flavonols are involved in the stabilization of the flavilium form of the anthocyanins in young red wines through the copigmentation phenomena (Boulton, 2001).

Flavanones and flavones: flavanones can be formed from the chalcone structure, whereas flavones are synthesized at a branch point of the anthocyanidin/proanthocyanidin pathway from flavanones as direct biosynthetical precursors (Martens & Mithöfer, 2005). Apart from other biological roles linked to them to date (e.g. flavone glycosides acting as co-pigments), they may also act as UV protectants (Iwashina, 2000).

The flavanonols astilbin (dihydroquercitin-3-rhamnoside) and engeletin (dihydrokampferol-3-rhamnoside) are the most common compounds of this group that are reported in grapes. They have been identified in grape skins and stems (Souquet et al., 2000; Trousdale & Singleton, 1983), in wine from white grapes and in grape pomace (Lu & Foo, 1999), as well as in red wines (Vitrac et al., 2001). Among the flavones, two flavone glycosides especially luteolin-7-glucoside and apigenin-7- glucoside have already been reported in grapes (Vrhovsek et al., 2012).

Also flavonols and flavan-3-ols share a similar biosynthetic pathway to flavonols and anthocyanins. They are usually colourless (Macheix et al., 1990) and can be found in grapes in monomeric, oligomeric or polymeric forms (or their esters with galactic acid or glucose). The most common monomeric flavanols are (+)- catechin and (+)- epicatechin, often in high concentrations in grapes skins, seeds and stems (Souquet et al., 2000; Escribano-Bailón et al., 1995; Su & Singleton, 1969). Less abundant are (+)-gallocatechin (Sun & Spranger, 2005) (-)-epigallocatechin (Escribano-Bailón et al., 1995), (+)-catechin-3-gallate and (+)-gallocatechin-3-gallate (Lee and Jaworski et al., 1990), which can also be detected in grape berries. Flavan-3-ols monomers represent only a small proportion of the total since the major part of flavan-3-ols are in oligomeric and polymeric forms (Monagas et al., 2005). Oligomeric flavonols typically consist of two to five monomeric units. The dimeric procyanidins are often referred to as the B-series and the trimeric procyanidins as the C-series. Five different dimers (procyanidin B1, B2, B3, B4 and B5) and two trimers (C1 and C2) have already been identified from grape skins and seeds (Nawaz et al., 2006). Flavan-3-ols oligomers and polymers (more than 5 monomeric units) are also known as proanthocyanidins or condensed tannins. Tannins are the most abundant class of phenolics in grapes and red wines (Kennedy et al., 2006) and they play important roles in the colour stability and the mouthfeel properties of wines (Kennedy et al., 2006; Singleton & Trousdale, 1992). They are typically classified as either condensed tannins or hydrolyzable tannins. Condensed tannins are oligomeric and polymeric compounds composed of flavanol units and they primarily originate from grape skins and seeds. On the other hand, hydrolyzable tannins mainly originate from oak and are actually gallic acid or ellagic acid esters of glucose (Edelmann and Lendl, 2002). The levels of tannins measured by protein precipitation are reported as varying from 30 to almost 1700 mg of catechin equivalents (Kennedy et al., 2006; Harbertson, 2003). Cortell et al. (2006) reported 1500 mmol/seed of monomeric flavan-3-ols and 5000 m/mol/seed of procyanindins in exposed 'Pinot Noir' samples.

Anthocyanins are synthesized through an anthocyanin biosynthetic pathway regulated by enzyme activities and gene expressions (Boss et al., 1996). In addition to the enzymes required for the synthesis of flavan-3-ols, two additional enzymes (LDOX and UFGT) are required for anthocyanin biosynthesis (Boss et al., 1996). Anthocyanins are the largest group of water-soluble pigments among plants (Mateus & Freitas, 2009). These pigments are primarily responsible for the colour of red grapes and their young wines and are almost exclusively located in the skins of the grapes. They can differ from each other in the number and position

of the hydroxyl and methoxyl groups located in the B-ring of the molecule (Monagas & Bartolomé, 2009). In red grapes, they exist exclusively as monomers (Gutierrez et al., 2005) and can be present as 3-monoglucosides of five anthocyanidins: cyanidin, delphinidin, peonidin, petunidin and malvidin. Except for 'Pinot Noir', red grape pigments are bound or acylated with acetic, caffeic or *p*-cumaric acids (Mazza, 1995). Although the anthocyanin profiles in grapes and consequently in wines can vary considerably between the different varietes and agro-environmental factors, (Revilla et al., 2013; 2009; Diago et al., 2012); Tarara et al, 2008; Spayd et al., 2002), malvidin-3-glucoside is typically the most abundant anthocyanin in red grape skins. The reported levels of anthocyanins in red grapes thus range from 300 to 7500 mg/kg (Mazza, 1995) and are reported 0.15-0.22 mg/berry in 'Pinot Noir' (Cortel, 2006) For monomeric anthocyanins, differences in the substitutions on the B ring result in differences in the wine colour properties. However, anthocyanins can also be incorporated into dimeric or larger units. Nevertheless, as very highly reactive pigments, they can easily react with other compounds present in wine such as tannins, acetaldehyde, keto acids or hydroxycinnamates (Harbertson & Spayd, 2006). Anthocyanin in co-pigmentation gives brighter, stronger and more stable colours than those expressed by anthocyanin alone (Eiro & Heinonen, 2002). Wines made from grapes low in co-factors will not be able to perform much co-pigmentation (Boulton, 2001) and will thus have low and unstable pigment contents, as seems to be the case with Pinot Noir wines.

2.3 Factors influencing phenolic synthesis and accumulation during grape berry ripening

The synthesis and accumulation of phenolic compounds in grape berries is a very complex process that can be affected by numerous factors such as season, variety, clone, soil structure and composition, maturity level, climatic and geographical conditions, as well as different viticulture practices (Revilla et al., 2013; 2009; Xu et al., 2011; Tian et al., 2009; Kennedy et al., 2002; 2000; Ramos et al., 1999; Dokoozlian & Kliewer, 1996; Jackson & Lombard, 1993). These multiple co-affecting factors are probably most transparent if organized into three major influential groups: genotype, environment and cultural practices. However, beside abiotic factors, there are also biotic factors (e.g. biotic stresses such as pathogen infections) that can have significant impact on the profiles of grape phenolic compounds (Gould & Lister, 2006; Jeandet et al., 2002).

2.3.1 Genotype

It is known that *vinifera* varieties themselves can determine the biosynthesis and accumulation of the phenolic compounds of grapes (Gatto et al, 2008; Mattivi et al., 2006). Grape varieties as well as their clones (due to intravarietal heterogeneity) determine both the quantitative and qualitative grape phenolic composition. As the rootstock type is related to vine performance, e.g. canopy growth (Koundouras et al., 2008), vine yield (Sathisha et al., 2010) and water exchange status (Ozden et al., 2010), the rootstock material can also be important to the final phenolic composition of the grape (Koundardas et al., 2009).

2.3.2 Abiotic environment related factors

Various environmental and related factors (e.g. topographical, climatic, agro-pedological), commonly described in viticulture using the French term terroir, have been acknowledged to have a significant influence on grape quality (Tarr et al., 2013; van Leeuwen & Seguin, 2005).

2.3.2.1 Climate

In viticulture, three levels of climate are usually distinguished (Keller, 2010). Smart (1985), defined three levels of climate that affect the quality and quantity of the grapevine crop: macro or regional climate, meso or site climate and micro or canopy climate. According to Keller (2010), those levels of climate can be further defined/described as:

Macroclimate: The climate of a region, which is ordinarily described by data collected at one or several weather stations. This is sometimes viewed as the mean of all the mesoclimates in a region. It is mainly determined by the geographic location (i.e. latitude, elevation and distance from large bodies of water), but it is independent of local topography, soil type and vegetation). The size of the region may extend for hundreds of kilometres.

Mesoclimate: The climate of a site or large vineyard. This is local variant of the macroclimate modified by topography (and hence also called the topoclimate). It may differ from the macroclimate due to elevation from the valley floor. The extent of a particular mesoclimate may range from hundreds of meters to several kilometres. This is the climate that is relevant to vineyard site selection.

Microclimate: The climate within and immediately surrounding the canopy or within the vineyard. This may differ from the mesoclimate due to aspect, slope and even soil type. Due largely to the presence of leaves, differences in microclimate may occur over as little as a few centimetres or up to hundreds of meters. This is the climate that can be manipulated by cultural practices (Keller, 2010).

Temperature and/or light are probably the most important climatic characteristics affecting grape phenolics. Although it is very difficult to separate the individual climatic influences of such single factors, there have been a lot of research projects undertaken in this direction, though fewer of them in real/field conditions.

2.3.2.1.1 Temperature

Temperature is an important factor that has been proven to significantly affect flavonoid biosynthesis in grapes. Changes in temperature have an effect on almost every aspect of the vine's functioning (Coombe, 1987). The inhibitory effect of (too) high temperatures on anthocyanin accumulation in the berry skins especially has been reported many times (Tarara et al., 2008; Kliewer & Torres, 1972; Kliewer, 1970). Many authors also reported (cool) night temperatures having considerable effects on grape coloration (e.g. Tomana et al., 1979; Kliewer & Torres, 1972). In addition, Cohen et al. (2008) and Mori et al. (2005) discuss the importance of the day/night temperature regime. In their studies they confirmed that the anthocyanin accumulation in skins grown under high night temperatures was lower than that in berries grown under low night temperatures (problem in case of Vipava Valley). Therefore, in the hotter regions, grape berries often tend to be poorly coloured and night temperatures have a considerable influence. The exact definition of "high temperature" varies between different authors but the critical temperatures are reported to be between 30°C and 35°C. Anthocyanin synthesis was greatly reduced in 'Cardinal' and 'Pinot Noir' grapes with 35°C daytime temperatures according to Kliewer (1970) and Kliewer & Torres (1972). The critical temperature for net anthocyanin accumulation in 'Merlot' berries was discussed to lie between 30 and 35°C and the temperatures (>35°C) found in some sun-exposed berries were detrimental to anthocyanin accumulation according to Spayd et al. (2002). However, the decrease in anthocyanins in grape skins under high temperatures could be caused by many factors, such as chemical (pH, temperature, light, oxygen) and/or enzymatic degradation and/or due to the inhibition of anthocyanin biosynthesis (Mori et al., 2007).

Recent studies on the biosynthesis of grape flavonoids depending on temperature have focused primarily on the accumulation and composition of anthocyanins, although Goto-Yamamoto et al. (2010) observed in their study that high temperatures also caused a moderate reduction in proanthocyanidin and quercetin concentrations in the skins. Cohen et al. (2008), in their study, reported flavonol content to be slightly higher in day-cooled berries at veraison, but with no significant differences to other treatments. At commercial harvest, there was a lower concentration of the total flavonols following daytime cooling and a slightly higher proportion of flavonols with di-hydroxylation following cooled and double-damped treatments. No significant difference between flavonol levels in the skin of the berries following low and high night temperature conditions were observed (Mori et al., 2005). There was only a slight difference reported in seed phenolics material yet a considerable variation in skins within the temperature regimes produced (Cohen et al., 2008). In contrast, the temperature in the last phase of ripening played an important role in the observed *cis*-piceid levels in 'Barbera', as described by Bavaresco et al. (2007). Few research projects have focused on other skin phenylpropanoids and their responses to changes in temperature, thus little is still known about the accumulation trends related to such climatic issues. Due to global warming, which is already affecting viticulture areas across the world, a better understanding of temperature impacts on vine functioning is becoming of great importance (Jones, 2005).

2.3.2.1.2 Light / sun exposure

Sunlight exposure is another very important factor with a certain impact on the phenolic composition of grapes (Spayd et al., 2002). Despite many investigations into anthocyanins, a considerable amount of contradictory data exists about light impact on red grape colour, most likely because it is very difficult to separate temperature/light effects. Some authors therefore report no changes in the total anthocyanins, while others reported some declines. However, apart from the concentration, ultraviolet light exposure is reported to be in relation to alterations in anthocyanin composition (Downey et al., 2006; Spayd et al., 2002; Haselgrove et al., 2000; Price et al., 1995). Even a short exposure of the berries to high temperature extremes during ripening appears to alter the partitioning of the anthocyanins between acylated and nonacylated forms and between the dihydroxylated and trihydroxylated branches of the anthocyanin biosynthetic pathway, as reported by Tarara et al. (2008).

On the other hand, exposure to UV light has a very evident effect on flavonols. Sun-exposed

bunches are reported to have higher flavonol contents than shaded ones (Ristic et al., 2007; Spayd et al., 2002; Haselgrove et al., 2000; Price et al., 1995). Almost no flavonols are formed if the bunches are completely shaded - with less than 10% of sun/light exposure (Downey et al., 2004), however if they are exposed later, there a rapid increase is detected. Thus, synthesis of flavonols is obviously very light dependent.

UV light is also reported to be able to induce the production of resveratrol / stilbenes (Adrian et al., 2000). In their study, UV-C induced berries, if exposed for long enough (48h), responded by the production of phytoalexins. In addition, in their study, UV elicitation was less efficient on biotically stressed plants compared to healthy ones. Regarding tannin accumulation in grape berries, these appear to be quite unaffected by sun exposure (Downey et al., 2004). In the case of 'Shiraz' (Dry, 2009), at harvest there were no significant changes observed in either the seeds or the skins. However, the study examined proanthocyanidin content and composition throughout berry development in both shaded and sun-exposed grapes, revealing significant differences throughout the intermediate stages of berry development, with shaded grapes reaching a much lower maximum in proanthocyanidin content than exposed ones. In the same study, the peak in proanthocyanidin accumulation in vinegrapes occurred around the time of veraison. Ristic et al. (2007) reported that shaded grapes showed an increase in seed tannins and a decrease in skin tannins but the decrease in flavonols in the shaded grapes was the biggest change observed, matching previous studies. However, as both of those trials were actually field trials there are also temperature (and probably other) effects present, revealing once again the difficulties of separating light and temperature effects, especially in the native environment.

While manipulating a single factor helps to understand its direct effects on phenolic biosynthesis, studies on the effects of the actual conditions on grape quality may be more useful in viticulture practice (Xu et al., 2011).

2.3.2.1.3 The synergistic effects of light and temperature

Emphasizing the effect of light and/or temperature in this perspective, their “symbiotic” functioning should be taken into account. Based on their field study, Haselgrove at al. (2000) indicate that light is not necessarily a limiting factor for anthocyanin synthesis if the bunches receive sufficient light of moderate intensity. In conditions where bunches are heavily shaded, it is likely that light is a limiting factor in the accumulation of anthocyanins during the early

stages of ripening and it seems that when berries are receiving adequate light, the temperature conditions may be a limiting factor. Later Tarara et al. (2008) reported that both the anthocyanin accumulation and their profiles in 'Merlot' were determined by a synergistic combination of solar radiation and berry temperature. Also Spayd et al. (2002*)* discussed that sun exposure differences can occur due to temperature or light effects. It is therefore reasonable that the findings could be interpreted as changes in the exposure to light/temperature around the fruit (Kennedy, 2008). Thus, when the influences of climatic conditions on grape quality are observed in real scenario conditions, multiple factors must be co-ordinately considered at the same time (Xu et al., 2011). While many of climatic factors/synergies have been investigated in vines (with the primary focus on grape colour), much of the published research has been conducted on model plant species (Downey et al., 2006).

2.3.2.1.4 Water regime

Rainfall distribution and efficiency are of great importance for the accumulation dynamics of phenolics in grape berries. Water stress is one of the factors that may greatly influence vine metabolism (Bahar et al., 2011). Moderate water stress has been reported to increase anthocyanin concentrations (Sivilotti et al., 2005; Ojeda et al., 2002) and the skin/pulp ratio by decreasing berry size (Kennedy et al., 2002). Water deficit has also been observed to increase the anthocyanin concentration even if the berry sizes were the same as the control (Roby et al., 2004). In parallel, water stress increases the expression of the anthocyanin biosynthetic pathway genes. There were limited effects reported on the biosyntheses of other phenolic compounds e.g. flavonols and proanthocyanidins (Castellarin et al., 2007). In contrast to moderate stress, extreme water stress can have a negative effect on berry quality, as reported by Bahar et al. (2011) and Sivilotti et al. (2005).

2.3.2.1.5 Wind

Although the wind may not have much of a direct impact on grape phenolics, it is obvious that it can significantly affect the vineyard's mezo- and microclimate. The wind chill effect can moderate too-high temperatures (Kobriger et al., 1984) during the summer and/or during the night. In contrast, it can has negative effects if present during excessive dry periods when it is connected to water stress. Very strong wind (e.g. the Bora in the Vipava Valley) can additionally cause considerable damage to the vine (e.g. leaves, buds, shoots, grapes), so it is

normal to expect the plant to react with the formation of secondary metabolites. Not much research work has been done on the influences of wind as a single factor on grape composition. Most probably because it is difficult to separate wind impact from topography and local airflows, as discussed by Bonnardot et al. (2002). However, some researchers evaluated wind effects in parallel with observations of other climatic parameters, e.g. temperature or leaf water potential (Coombe, 1997; Freeman et al., 1992). On the other hand, there have been more studies done in connections with humidity and correlated incidences of grape diseases (Tixier et al., 1998; Thomas et. al., 1987).

2.3.2.1.6 Humidity

Changes in the humidity of the canopy can particularly have two major effects. Firstly, by lowering the vapour pressure deficit, transpiration and photosynthesis are decreased, causing a further reduction in growth and subsequently in flavonoid accumulation (Downey et al., 2006). Secondly, high humidity increases the risk of pathogenesis through fungal or bacterial infection (Emmett et al., 1992; Thomas et al., 1987; Savage & Sall, 1984), which may cause a general wound response inducing flavonoid (Downey et al., 2006; Vogt et al., 1994) as well as stilbene occurrences (Jeandet et al., 2002). *Botrytis cinerea* Pers. (a frequent problem for 'Pinot Noir') and other ubiquitous mould genera (e.g. *Cladosporium* spp., *Aspergillus* and *Penicillium* spp.) (Serra and Peterson, 2007) are spread throughout the plant tissues and germinate when the humidity (and temperature) are appropriate (Barata et al., 2012). The relative humidity in the last phase of ripening also plays an important role in *cis*-piceid levels, according to Bavaresco et al. (2007). According to Keller (2000), humidity can also affect leaf growth; consequently leaves growing in low humidity remain smaller than leaves growing in high humidity. Transpiration from the leaves and, to a lesser extent, from the fruit/grape can cause humidity build-up in the centres of canopies. The extent that this occurs depends on canopy ventilation (e.g. wind speed) Smart (1985).

2.3.2.2 Other abiotic environmental factors

2.3.2.2.1 Soil

Many soil characteristics, such as soil depth; structure, texture, water storage capacity and related fertility can affect grapevine phenolics (Downey et al., 2006). Soil may also affect

moisture and nutrient availability to the plant, affect the microclimate due to its heat-retaining and light-reflecting capacity, and affect root growth due to its penetrability (Jackson & Lombard, 1993), hence it can have both a direct and an indirect impact on all the classes of phenolic compounds. In addition, some practices, such as periodic soil tillage can cause an increase in vine vigour and yield (Vršič et al., 2011) and can thus consequently affect the phenolics in the grapes as well.

2.3.2.2.2 Vineyard site selection and vineyard design

As the climate itself is an important factor in choosing a site for vineyard development, it is obvious that position (e.g. topography and latitude) significantly affects grape composition. An increase in anthocyanin concentration has already been observed with increased elevation. The altitude was reported also to affect flavan-3-ols (Mateus et al., 2001) and stilbenes (Bavaresco et al., 2007). Vineyard design characteristics such as planting density (Smart, 1985), training system (Zoecklein et al., 2008) and row orientation (Berquist et al., 2001) are also known to be influential.

2.3.2.2.3 Vintage / Season

Many studies have confirmed that the concentration of phenolics in the grape berry can vary significantly with vintage (its conditions) (Kennedy, 2008; Cortell et al., 2007; Pastor del Rio, 2006; Downey et al., 2003). The variability of the weather inside the existing geo-climatic frame plays a crucial role in determining productivity, as well as the quality of the viticultural crops. Grapevine is commonly grown in temperate areas where the culture practice is one-crop-a-year that allows 3-5 months of vine dormancy from late fall to early spring, however in recent years, grape production has been expanded to warmer subtropical areas as well, where the climate conditions allow the production of two crops per year (Xu et al., 2011).

2.3.2.2.4 Harvest time / Grape maturity level

Although it is known that anthocyanins accumulate in the berry from the beginning of veraison and increase during fruit ripening, there is also evidence that suggests a decline in anthocyanins later on in berry development (Kennedy et al., 2008; Kennedy, 2002). According to Perez-Magariaeo & Gonzalez (2006), the harvesting dates of grapes (directly correlated with the degree of maturity of the grapes),

influenced the wine phenolics compositions especially in case of 'Cabernet Sauvignon'. Wines made from grapes harvested one week later than the usual date of harvest, generally had higher contents of several phenolics, which can act as co-factors maintaining the colour intensity and violet tonalities in aged wines. Besides that, those wines had lower levels of caftaric and coutaric acids, which are two of the main substrates for oxidation and browning processes. Nevertheless, the results of Tian et al. (2009) showed that the concentrations of majority of the phenolic acids in musts increased with harvest time delay. However, on the other side climate change can cause earlier winegrape ripening (Vršič et al., 2014).

2.3.2.2.5 Plant hormones

Beside exogenous environmental factors (e.g. water and temperature stress, light, fertilizer, etc.), endogenous (e.g. plant hormones) can also decrease flavonoid biosynthesis when their quantity is either limiting or excessive (Braidot et al., 2008). In particular, plant hormones could affect flavonoid biosynthesis in a complex way. It has been basically reported that abscisic acid, auxin and ethylene are responsible for an increase in flavonoids, while on the other hand gibberellic acid and inhibitors of the ethylene receptor decrease their synthesis (Dan & Lee, 2004; Joeng et al., 2004; Deikman & Hammer, 1995).

Finally, some other factors that may influence the phenolic composition of grape berries are the age of vines (Reynolds et al., 2007) and pathogenesis (Amati et al., 1996) = biotic stresses.

2.3.3 Biotic environmental factors

Biotic stress occurs as a result of damage done to the plants by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and harmful insects, weeds or cultivated/native plants (Keller, 2010). Grapevines share their living space - both above and below ground - with a myriad of other organisms, mainly arthropods (e.g. spiders, insects) and microorganisms (e.g. bacteria, yeasts). Most of them do not try to harm the vines or grapes or are unable to overcome the plant protection mechanisms, although they are always present in the vineyard ecosystem (Keller, 2010). However, in certain conditions, several of them can act as pathogens. In the case of biotic stresses, the plant usually reacts by producing specific secondary metabolites, so it is important to study the microbial ecology of certain *terroir*, the occurrences of microorganisms and their behaviour in specific microclimatic conditions, as

well as the risk they represent.

Some pathogens are known to effect grape colour (e.g. *Botrytis cinerea* Pers.) and the wines from infected grapes were also reported to contain more hydroxycinnamic tartaric esters (Amati et al., 1996), but in general biotic stress is most observed in connection with stilbene increase in the plants (Bavaresco et al., 2008; Versari, 2001).

2.3.3.1 Grape microbial ecology

Microbial species present on the surface of grape berries, especially in the last stages of berry ripening, play an important role in the grape's sanitary situation that is later on reflected in the resulting wines (Fleet et al., 2003; Fugelsang 1997). Each vineyard with its specific characteristics can give rise to different microflora with quantitative and qualitative species heterogeneity (Renouf et al., 2005; Pretorius et al., 1999). Different yeast, bacteria and fungi cover the grape berry surface, forming a complex, large and diverse microbial community, fundamentally changing depending on the stage of grape berry development (Renouf et al., 2005; Coombe, 1992; Rosini et al., 1982). Quantitatively, mature sound grapes harbour microbial populations at levels of 10^3–10^5 CFU/g, mostly consisting of yeasts and various species of lactic and acetic bacteria (Fleet, 1999) with some filamentous moulds.

2.3.3.1.1 Factors affecting grape microbial ecology

Beside changes connected to the grape development stage, there are several factors reported to have a significant impact on grape microbial communities: various environmental factors such as vineyard geography (Fleet et al., 2002) and climatic stresses (changes in temperature, rainfall regime and humidity) (Longo et al., 1991); various wind events and microclimate as affected by canopy management (Pretorius, 1999); UV radiation and nutritive limitations (Sabate et al., 2002; Renouf et al., 2005) and the application of cultural practices like fertilization, irrigation and spraying with fungicides (Renouf et al., 2005; Lindow & Brandl, 2003; Sabate et al., 2002; Pretorius et al., 1999). Finally, the grape variety itself as well physical damage to the grapes (Pretorius et al., 1999) and the presence of fallen dried berries, dead canes, vine bark, dried bunch stems and dead cover crop trash (Zahavi et al., 2000) can leverage the presence of microorganisms. In a positive way, yeasts can perform the alcoholic fermentation of grape musts into wines, while lactic acid bacteria can contribute to wine flavour and stability through malolactic fermentation. Furthermore, noble rot, caused by the

fungi *Botrytis cinerea* Pers. (at the final stages of maturation), is appreciable in some styles of wine. However, microorganisms can also have various negative impacts on grape and/or wine quality. They can significantly depreciate must chemical and sensorial characteristics, contribute to sluggish and/or stuck or even failed fermentations and thus spoil the wine during fermentation and in the later stages of vinification (Fleet, 2001).

In general, grapevine pathogens can be sub-divided into main pathogens of high economic importance that are predominant, like downy mildew (*Plasmopora viticola* Berk. e Curtis, Berl. & de Toni), powdery mildew (*Erysiphe necator* Schwein.) and bunch rot (*Botrytis cinerea* Pers.), and those that occur only locally or temporarily (Ribéreau-Gayon et al., 2005). There are several types of rot attacking *vinifera* grapes, although the most frequent (and/or the most observed is that caused by the mould *Botrytis cinerea* Pers.

2.3.3.2 *Botrytis cinerea* Pers.

Botrytis cinerea Pers. is a very common fungus in nature that causes diseases on a variety of agricultural crops, including grapes. When *Botrytis cinerea* Pers. infects vines/grapes, the disease can be named with different synonyms such as Gray mould (also gray mould) or *Botrytis* Bunch Rot (bunch rot) (Rosslenbroich & Stuebler, 2000). *Botrytis cinerea* Pers. is by far the most important of the 22 *Botrytis* species *sensu stricto* (Beever & Weeds, 2007; Hennebert, 1973) and it possesses some unique characteristics: it can live pathogenically but also saprophytically and it can be devastating in some crops but beneficial under certain conditions – e.g. the so-called noble rot (Rosslenbroich & Stuebler, 2000). A *Botrytis cinerea* Pers. infection can attack leaves; it can cause blossom blight or shoot blight, although the most common phase of this disease is the infection and rot of ripening berries (Ellis et al., 2004). The disease occurs throughout the whole viticultural world but is most common in regions with cool to moderate temperatures (Percival et al., 1994). It can significantly reduce both yield and quality (Mullins et al., 1992), so it is responsible for significant economic damage in vineyards worldwide (Elmar & Reglinski, 2006).

In general, all grape varieties are susceptible to bunch rot, although the losses can be greater on tight-clustered varieties (e.g. 'Pinot Noir') and French hybrids (Mullins et al., 1992). The severity of the disease can also increase with late maturing virieties (e.g. Riesling), especially in the case of late season rain and very dense canopies (Broome et al., 1995; Thomas et al., 1988).

2.3.3.2.1 Symptoms of infection with *Botrytis* Bunch Rot

Symptomless infections, established early in the spring, can give rise to the expression of symptoms under wet conditions later in the season, by and large from the inner bunch (Carey et al., 2004; du Preez, 2002). The most destructive phase of *Botrytis* bunch rot occurs in the last stages of grape berry ripening. The first symptoms of the disease are usually evident by the time of berry-coloration, veraison (Hed et al., 2009; Percival et al., 1994). Areas of skins that slip freely from the grape characterize the early stage of *Botrytis* rot, thus the first signs of the disease in coloured grapes are 2-3 mm wide, circular, faintly cleared spots, usually beginning to show about 72 hours after inoculation (Winkler et al., 1974). After attacking individual berries, the infection can easily progress to whole clusters. The affected berries first appear soft and watery, then split open and develop a greyish mould on the surface while often shrivelling at the same time. While berries of white varieties usually become brownish when infected, those of red varieties develop a reddish colour. As the disease progresses, the fungus produces abundant aerial mycelia that spreads further from infected berries to healthy ones (Gabler et al., 2006).

2.3.3.2.2 Conditions and factors predisposing the disease

Warm and humid conditions in the bunch area greatly promote disease development. *B. cinerea* can germinate at temperatures between 2 and 37°C, although most of the germination (98 to 100%) occurs between 10 and 25°C (Guetsky et al., 2001), with warmer conditions being favourable (optimum 20.8°C according to Nair & Hill, 1992). Germination can be stimulated by the presence of water and nutrients from organic materials e.g. pollen, leaf/wood; or debris (Sivilotti et al., 2013). The probability of infection increases in general with the timing and severity of wet conditions, even though it can also occur in the absence of water if the relative humidity is very high (> 94%) (Kassemeyer & Berkelmann-Lohnerz, 2009). In brief, the fungus thrives best in high humidity or damp conditions and still air. In such optimal conditions, germination can occur in less than 24 hours.

Any berry surface wound can provide an infection site for the fungus even in the absence of favourable conditions, so any factor causing damage to grape tissues will exacerbate the incidence of the disease. Grape berries can be wounded by various climatic events like frost, hail, strong winds and direct sun exposure or due to various biotic factors e.g. insects, birds and other pathogen attacks, as well as fruit swelling (due to excessive rain) or fruit split and/or

berry compression due to grape compactness (Hed et al., 2009; Elmer & Michailides, 2004; Coertze et al., 2002; Nair & Hill 1992).

2.3.3.2.3 Controlling the disease

The disease control strategies are several and in many cases co-joined in order to achieve better results. During the last 60 years, controlling *Botrytis cinerea* Pers. has relied heavily upon the use of protective synthetic chemicals (Rosslenbroich & Stuebler, 2000). Fungicide spraying was shown to be the most important way to manage *Botrytis* bunch rot (grey mould), also reducing the problems of another disease - sour rot - at the same time. The active substances in use for the defence against those diseases are boskalid, ciprodinil + fludioksonil, fenheksamid and pirimetanil. This approach however cannot be regarded sustainable. Regulations governing botryticides residue have severely restricted chemical control options in conventionally managed vineyards, particularly during the preharvest period (Elmer & Reglinski, 2006). *B. cinerea* also frequently becomes resistant to chemical fungicides. For those and for environmental reasons, research is targeted at limiting fungicides inputs (Elmer & Reglinski, 2006). It is known that prevention is a first step toward possible fungicide reduction (e.g. promoting good air circulation and light/sun penetration by proper pruning, controlling weeds and suckers, positioning or removing shoots for uniform leaf development as well as avoiding wounds by controlling insects, birds, microbes etc.). However, there are some other known techniques that can be implemented and coupled with chemicals to reduce the occurrence of rots - e.g. leaf removal (discussed later in this chapter), calcium applications (Easterwood, 2002), see weeds treatments (Aziz et al., 2003) and hormone application (Bigot et al., 2008). Biological control as an alternative option to reduce *Botrytis* infection has been reported to be effective for many different crops (Elad et al., 1996), with plant defence stimulants being shown to increase host resistance and microorganisms to suppress disease epidemics, for example (Elmer & Reglinski, 2006). Furthermore, cultural practices such as modification of cluster compactness and the removal of floral debris, which is believed to be a principal source of primary bunch rot infection (Nair et al., 1988; Wolf et al., 1997), may also be an effective tools in the integrated control of bunch rot. On the other hand, in organically managed vineyards there are very few options for bunch rot suppression (Elmer & Reglinski, 2006), so disease control could be dependent upon varieties with an inherent resistance, carefully applied canopy management (Zoecklein et al., 1992) or the application of "soft control options" such as compost teas (Ryan et al., 2005).

2.3.4 Cultural practices

It is known that viticultural practices modulate basic grape quality and the expression of the phenolic composition of grapes throughout the grape ripening process. Cultural practices such as the training system, pruning, bunch thinning, vigour management, etephon applications, bud and leaf removal, water management and fertilization (Poni et al., 2009; Guidoni et al., 2008; Zoecklein et al., 2008; Cortel et al., 2007; Delgado et al., 2004; González-Neves et al., 2002; Esteban et al., 2001; Jackson & Lombard, 1993) are reported to have an influence on phenolic biosynthesis and accumulation. Beside this, agronomic approaches (e.g. organic or biodynamic) are also discussed as having an effect on grape phenolic composition (Vian et al., 2006; Reeve et al., 2005). According to Smart (1985), the cultural practices together with soil and climate, can have direct effects, while alterations to canopy microclimate can have additional indirect effects by altering vine physiology.

2.3.4.1 Canopy management

The canopy, in the viticulture sense, can be defined as the aboveground parts of the vine (i.e. shoots, leaves, fruit, trunk and cordon) (Keller, 2010). According to Smart et al. (1990), the canopy is described by the dimensions of the boundaries in a space (i.e. width, height, length etc.) as well as by the amount of the shoot system within this volume (typically leaf area). Canopies can be continuous (without large gaps) or discontinuous (if canopies are separated from vine to vine) and/or divided (with discrete foliage walls); they can be crowded or dense, where there is much area within the volume bounded by canopy surfaces (Smart et al., 1990).

Grapevine canopies can impact several canopy microclimate factors: the amount of photosynthetic photon flux density (PPFD), light exposure, wind speed, evaporation, air temperature and air humidity (Dry, 2000). Open canopies can also contribute to a better distribution of applied pesticides (Poni et al., 2006) and consequently impact the microbial population on the grapes.

Canopy management is generally viewed as positioning and maintaining bearing shoots and their fruit in a microclimate that is optimal for grape quality, inflorescence initiation and cane maturation (Jackson, 1994). The term canopy management thus includes a range of techniques that can be applied to a vineyard to alter the position or amount of leaves, shoots and fruit in a space, and to achieve some desired arrangement (i.e. microclimate) (Smart et al., 1990). Efforts to reduce existing canopy shade and increase grape exposure include viticultural practices such

as leaf removal and cluster thinning (Vasconcelos & Castagnoli, 2000; Zoecklein et al., 1992; English et al., 1990).

The term bunch exposure has become well established when discussing different levels of canopy shading. Dense, shaded grapevine canopies are adverse environments for quality grape production (Zoecklein et al., 1992). The quality of grapes maturing within dense canopies can be critically reduced by rots promoted by poor ventilation and/or related reduced pesticide penetration (Zoecklein et al., 1992; English et al., 1989; Gubler et al., 1987). Regarding chemical composition, berries that develope in open canopy conditions (high level of bunch exposure) generally have a higher sugar concentration in the grape juice (measured as total soluble solids), an improved acidity balance (lower pH and higher titratable acidity), less incidence of unripe herbaceous fruit characters and often increased concentration of phenolics in the grape berries (Main & Morris, 2004; Berqvist et al., 2001; Haselgroove et al., 2000; Dokoozlian & Kliever, 1996), as opposed to those that develop in shaded (less exposed) canopy conditions.

The aim of studding the effects of canopy management/canopy microclimate manipulation on overall plant functioning within different *terroirs* is not only to improve grape composition from a technological point of view but also (or even mainly) to improve the knowledge and the understanding of plant biosynthetic behaviour behind it. This is even more important in the frame of predicted climate change and/or global warming scenarios, as any global atmospheric warming trend might seriously affect grape berry ripening in the future (Vršič et al., 2014; Jones et al., 2005).

2.3.4.1.1 Leaf removal

One of the most frequently applied summer canopy management operations in viticulture is so-called fruit-zone leaf removal (Poni et al., 2006). Leaf removal (also called defoliation) is a well-known viticulture technique, initially performed in colder production areas in order to improve grape ripening and reduce the risk of grape infections (Smart, 1990; 1985). In this technique, some or all of the leaves surrounding the shoot base are eliminated in order to improve the illumination and the air circulation around the clusters, leading to some improvements in the canopy microclimate (Dry, 2000). In the case of phenolics, selective leaf removal became especially beneficial in crowded canopies (Vasconcelos & Castagnoli, 2000), and/or when the clusters are completely or nearly completely shadowed by the leaves. This is

particularly important due to the fact that flavonols and, to a certain degree, also anthocyanins are known to be dependent on cluster light exposure (Chorti et al. 2010; Haselgroove et al., 2000; Price et al., 1995). The absorption of solar radiation by the leaves leads to low levels of it within the canopy itself (Smart, 1985), thus the number of leaf layers within grapevine canopies basically determines the degree of shading/sun exposure (Smart, 1985), which consequently may alter the grape composition significantly.

However, if clusters are already exposed efficiently to the sunlight (> 40%), too much leaf removal might even be harmful (so-called over-exposure), especially in warmer and/or hot climates, where phenolic declines in such cases are observed more often than in cooler regions (Berqvist et al., 2001, Haselgrove et al., 2000). Too high summer temperatures (> 30°C or even > 35°C) are reported to have a negative impact on anthocyanin synthesis (Mori et al., 2007), whereas canopy microclimate manipulation can also alter wine aroma compounds (Marais et al., 1999). Excessive absolute fruit temperatures, rather than the difference between the grape surface temperatures and the ambient temperatures, have significantly reduced anthocyanin concentrations in sun-exposed 'Merlot' grape berries as reported by Spayd et al. (2002). Furthermore, the exposure of berries to high temperature extremes for relatively short periods during ripening was observed to alter the partitioning of anthocyanins between acylated and nonacylated forms and between dihydroxylated and trihydroxylated branches of the anthocyanin biosynthetic pathway (Tarara et al., 2008), leading to important changes in anthocyanin qualitative and quantitative profiles. Therefore, improving the control of the grapevine canopy microclimate, carefully adjusted to each individual site, is of great importance for better plant functioning as well as for better grape quality.

As well as the climatic conditions (cooler or warmer) of the wine region, row orientation is also an important factor reported to have significant influences on the temperature/light situation on different sides of the vine (Shrestha & Fidelibus, 2005; Berquist et al., 2001). This should be taken into account before any leaf removal decisions. Spayd et al. (2002), for example, reported that the highest absolute fruit temperatures occurred in west-exposed 'Merlot' grapes. Leaf removal is thus often performed from just one side of the vine, (e.g. the northern in warmer climates) in order to avoid the risks, but promote the potential benefits and thus optimize grape quality (Bergqvist et al., 2001). In warm to hot viticultural regions, strong exposure of grapes to full sunlight on west- and south-facing canopies is therefore best avoided unless some other method is used for reducing fruit temperature, however complete fruit shading is also not recommended (Spayd et al., 2002). Potential sunburn of grape berries (Corti

et al., 2010), especially of sensitive varieties with thinner skins, is another issue that needs to be taken into account before leaf removal decisions. It is therefore obvious that a carefully applied, site adjusted and knowledge-based leaf removal approach is of great importance for the final composition and quality of the grape. However, due to the very complex reactions of the plant to different stresses, there are no simple answers available regarding the best combination of light and temperature exposure. In addition, sunlight exposure of individual clusters on the same vine may vary dramatically, based on cluster location within the canopy (Berqvist et al., 2001).

Finally, the amount of leaves (severity of leaf removal) and the decision on which leaves to remove (location of leaf removal) depends on the individual canopy structure. Excessive leaf removal or the removal of the wrong leaves may cause a reduction in berry weight and soluble solids, probably because too much leaf area has been removed (Bledsoe et al., 1988). On the whole, leaf removal (its severity, location and timing) should be very carefully evaluated in any geo-climatic scenario and for each variety in terms of: quality benefits (and risks) (e.g. changes of anthocyanins and aroma compounds); sanitary benefits (e.g. reduction of *Botrytis cinerea* Pers. *infection*); and sunburn risk (notably in sensitive varieties).

2.3.4.1.2 Leaf removal timing

Beside the location and severity of leaf removal, its timing is probably the most important factor for its final effectiveness (Main & Morris, 2004). Leaf removal performance is particularly related to the phenological stages of grape berry development during which the practice is adopted. It is commonly performed in the post-flowering period (between the phenological growth stages BBCH 69 and BBCH 83), but earlier implementation (e.g. around flowering) and the effects on the secondary metabolism due to such early alternations in the microclimate conditions have not yet been extensively explored in the Vipava Valley.

The most widely adopted form of leaf removal - at veraison - has already been reported to affect primary and secondary metabolite syntheses of many observed *vinifera* varieties, and its effects are mainly related to leaf layer number, photosynthesis rate and canopy surface area (Bavaresco et al., 2008). Veraison defoliation of very dense canopies especially was reported to increase sugars, flavours, total and phenol-free glycosides and flavonoids, while on the other hand it can decrease the acidity and gray mould incidence, compared with untreated vines (Poni et al., 2006; Percival et al., 1994; Reynolds et al., 1994; Zoecklein et al., 1992; 1998).

However, the viticultural practice of late (veraison) leaf removal is now losing many of its advantages due to warmer vineyard conditions. Many winegrowers are already facing (global warming related) problems as a result of higher temperatures and severe UV exposure after canopy opening, which can lead to harmful sunburn on the grape berry skin tissue (Chorti et al., 2010), as well as inhibition of the biosynthesis of some important phenolic compounds (e.g. anthocyanins) (Tarara et al., 2008) and aroma compunds ((Marais et al., 1999).

Even though earlier leaf removals have been much less studied in the past, there are some recent reports existing with promising results regarding grape phenolic improvement for 'Pinot Noir' in the case of leaf removal at earlier (berry-set) stages (Sternad Lemut et al., 2011 – results shown in the chapter Results & Discussion), and recently also promising results for related wine phenolics (Diago et al., 2012).

One aspect of the canopy structure that should not be underestimated in any leaf removal decisions is the age distribution of leaf population as the grapevine leaves are net importers of sugars until they reach 50% to 80% of their final size (Kurooka & Fukunaga, 1990; Kriedemann, 1970). Working on 'Pinot Noir', Koblet et al. (1994) reported a significant reduction in the main leaf area between control and defoliated in case of increased defoliation severity. The most efficient leaves in the canopy are those that are recently expanded, indicating that the age of the canopy can be manipulated with selective leaf removal and shoot tipping at appropriate growth stages (Vasconcelos & Castagnoli, 2000).

2.3.4.1.2.1 Pre-flowering leaf removal

Leaf removal during flowering or shortly after flowering has been implemented before in some vineyards, whereas so-called pre-flowering leaf removal (sometimes also called pre-bloom leaf removal) is a more viticultural practice. Unlike traditional leaf removal, it is carried out even before vine flowering (Tardaguila et al., 2010; Poni et al., 2009; 2006). Since the basal leaves are the main source of assimilates at flowering, their removal initially reduces berry set, leading to smaller and looser clusters with fewer berries but of better and more homogeneous quality (higher soluble solids and/or anthocyanin concentrations) as reported for some varieties such as 'Sangiovese' and 'Trebianno' (Gatti et al., 2012; Poni et al., 2009) or 'Graciano' and 'Carignan' (Tardaguila et al. 2010). Due to reduced grape compactness, fruit health can be additionally improved by early leaf removal through the reduction of *Botrytis* occurrence as reported by Diago et al. (2010a, 2010b), studying 'Graciano' and 'Carignan' but also doing trials on 'Tempranillo' and 'Granache'. Beside grape composition alternations and grape health benefits,

significant crop reduction was also observed on very early-defoliated vines through reduced fruit set (Gatti et al., 2012; Poni et al., 2009, 2006; Intrieri et al., 2008). Furthermore, recent work on 'Lambrusco' and 'Barbera' berries showed that pre-bloom defoliation induced a consistent increase in their relative skin mass (Poni et al., 2009). Sensory differences in wine aroma attributes between control and defoliation treatments were also observed, although inconsistent trends between the two seasons were noted as a result of the vintage effect (Diago et al., 2010a). The existing very early leaf removal research was mainly conducted using manual defoliation (Poni et al., 2009, 2006), but the effects of mechanical early leaf plucking on the composition of 'Sangiovese' grapes have been also investigated (Intrieri et al., 2008).

Although the first scientific observations of pre-flowering leaf removal treatments indicate many promising results, a lot of existing knowledge relies only on the observation of a very few grapevine varieties and in even fewer geo-climatic conditions. In other words, these very early leaf removal alternatives need to be well explored on a 'from grapes to wine' basis before their adaptation to any grapevine variety can be justified.

Yield management is one of the key techniques in modern viticulture and is widely recognized as an important factor in the composition of grapes and the resultant wines as it is regulated by law in many European countries (Diago et al., 2010). Planned crop reduction is usually achieved by winter pruning and cluster thinning. Hoverwer, cluster thinning is very labour intensive and expensive (Poni et al., 2009) and probably less effective for grape quality improvements compared to early defoliation. Whereas cluster thinning was already reported to improve grape composition at harvest (Reynolds et al., 2007; Guidoni et al., 2002), several studies failed to clearly demonstrate such outcomes, even showing compensatory growth of the retained clusters, which end up thicker and with larger berries (Keller et al., 2005; Chapman et al., 2004). Pre-flowering leaf removal with its side effects on plant berry set and consequently on the yield (in the case of the several varieties studied to date) could hense become a (cheaper and potentially more effective) yield management alternative in the future - an idea that became one of our objectives to explore on 'Pinot Noir'.

2.4 Metabolomics

Metabolomics is a novel, recently developed analytical technology that is applied to research in order to acquire complex biological information in a more comprehensive manner. This rapidly emerging field in analytical biochemistry can be regarded as the newest of the "omics"

research areas, following genomics, transcriptomics and proteomics (Wishart, 2008, Dettmer & Hammock, 2004); however, it has been suggested several times that metabolomics may in fact provide the most "functional" information of the all "omics" technologies (Ryan & Robards, 2006). In general, metabolomics focuses on high-throughput characterisation of plentiful, naturally occurring, low molecular weight organic metabolites within biological matrices such as various cells, tissues or bio-fluids (Bundy et al., 2009; Wishart, 2008). The quantitative complement of such molecules, present in cells in a particular psychological or developmental state, is named metabolome (Oliver et al., 1998; Goodarce et al., 2004).

The metabolome is formally defined as the collection of all small molecule metabolites or chemicals that can be found in cells, organs or organisms (Wishart, 2008). It comprises the complete set of (intracellular and extracellular) metabolites, the non-genetically encoded substrates, intermediates and products of various metabolic pathways (Nielsen & Jewett, 2007). These small molecules can include a range of endogenous and exogenous chemical entities or any other chemical that can be used or synthesised by a given organism or its cells (Wishart, 2008).

The plant metabolome is quite complex, with current estimates on the order of 15000 metabolites within a given species and over 200,000 different metabolites within the plant kingdom (Pichersky & Gang, 2000; Dixon, 2001; Hall et al., 2002).

Metabolomic approaches: Until recently, most metabolic analyses were restricted to only profiling several selected classes of compounds or to fingerprinting metabolic changes without sufficient analytical resolution enabling determination of metabolite levels and identities separately (Fiehn, 2002). Today, metabolomics, the study of ''as-many-small-metabolites-as-possible'' in a system, has thus become an important tool in gaining much wider information in numerous research areas. Metabolomics can be primarily classified as targeted or untargeted. Targeted metabolomics analysis focuses on a specific group of intended metabolites (targets) and in most cases requires identification and quantification of as many metabolites within the group as possible (Cevallos-Cevallos et al., 2009; Mapelli et al., 2008; Ramautar et al., 2006; Ryan & Robards, 2006). It is important for assessing the behaviour of a specific group of compounds in the sample under determined conditions, while in contrast, untargeted (comprehensive) metabolomics focuses on the detection of as many groups of metabolites as possible in order to obtain patterns or fingerprints, without necessarily identifying or quantifying a specific compound(s) (Cevallos-Cevallos et al., 2009). While

historically, target analysis has been reserved for interrogating relatively small numbers of metabolites, new developments enable quantitative analyses of much more expanded metabolome coverage (Nielsen & Jewett, 2007). Within targeted metabolomics, the term metabolite profiling is used to describe the quantitative and qualitative analysis of only the identified metabolites (Bino et al., 2004; Oikawa et al., 2008). The intention of so-called metabolic fingerprinting on the other hand is not to identify or quantify each individual observed compound but to rapidly classify numerous samples using multivariate statistics (e.g. compare fingerprint patterns of metabolites that change in response to different environmental stresses) (Dettmer & Hammock, 2004; Ryan & Robards, 2006).

Based on the specific objective of the analysis and data manipulation, most metabolomics studies can be classified as discriminative, informative and/or predictive (Cevallos-Cevallos et al., 2009). Discriminative metabolomics analyses are aimed at finding differences between sample populations without necessarily creating statistical models or evaluating possible pathways that may elucidate such differences. Discrimination is commonly achieved through the use of multivariate data analysis techniques, maximising the classification with principal components analysis being the most used tool. Informative metabolomics analyses are focused on the identification and quantification of targeted or untargeted metabolites to obtain sample intrinsic information (e.g. possible pathways, discovery of novel bioactive compounds, discovery of biomarkers, creation of specialised metabolite databases and metabolites functionality studies). Finally, some metabolomics reports are so-called predictive reports. In such cases, statistical models based on metabolite profile and its abundance is created to predict a variable that is difficult to quantify by other means (Cevallos-Cevallos et al., 2009).

Metabolomics, as an analytical approach, furthermore combines plentiful strategies to identify and quantify cellular metabolites using sophisticated analytical technologies with the application of statistical and multivariate methods for information extraction and data interpretation (Roessner & Bowne, 2009).

2.4.1 Applications of plant metabolomics

Secondary metabolites are mainly produced for the chemical defence of plants against climatic alterations or natural predators, however they can also be of great value to us as various nutrients or medicines. Besides providing an understanding of the metabolic state in plants under various circumstances, metabolomics techniques are also applicable for the clarification

of their functions (Oikawa, 2008). Furthermore, metabolomics is predicted to play a significant, if not indispensable role in bridging the phenotype-genotype gap and thus assisting in the desire for full genome sequence annotation as part of the quest to link gene to function (Fiehn, 2002; Sumner et al., 2003). It also represents a way to achieve possible (desired) modifying of plant metabolism and/or its physiology. Such capabilities are essential for breeding higher quality plant varieties that are disease resistant or that produce fruit of superior nutritional and sensory quality. In addition, through the identification of various biomarkers, metabolomics enables better definition of plant disease status as well as the role of environmental conditions and stresses on plant productivity and crop quality.

As such, metabolomics is gaining much interest in a variety of biological fields such as plant, animal, cellular, microbial, pharmaceutical, medical and genetic science as well as in food science, agriculture and environmental sciences. Last but not least, plant metabolomics is becoming invaluable in any multidisciplinary oriented plant research.

Within Food science, metabolomics represents an important potential in areas such as food quality, food safety, food microbiology, food processing as well as compliance with multiple food regulations (Cevallos-Cevallos et al., 2009). The food metabolome is characterised not only by considerable chemical diversity (>100 major chemical classes) but also by considerable variations in chemical abundance (Wishart, 2008). Many of the existing food metabolomics research reports are focusing on fruits and vegetables (e.g. Rudell et al., 2008; McDougall et al., 2008; Ursem et al., 2008; Moco et al., 2008; Luthria et al., 2008), with few implementing detailed metabolomic-based analysis to wine research (e.g. Son et al., 2008; Son et al, 2009; Rochfort et al., 2010; Arapitsas, 2012) or grape research (e.g. Ali et al., 2011; Toffali et al., 2011), however the interest of research groups in such upgrading and possible re-evaluating of existing knowledge is rapidly growing.

Environmental metabolomics is an application of metabolomics that can characterise the interactions of organisms with their environment. This approach has many advantages for studying organism–environment interactions and for assessing organism function and health at the molecular level (Bundy et al., 2009). Metabolomics in environmental sciences ranges from understanding organism responses to abiotic pressures (e.g. thermo tolerance, water deficit tolerance, light/circadian rhythm) to the understanding of biotic responses (e.g. diseases). Guy et al. (2008) published a review on the existing metabolomics efforts of temperature in plants, while Goodacre et al. (2003) investigated the subtle metabolic changes that occur when plants

are exposed to different photoperiods. Untargeted analyses have been used in the identification of possible fingerprints of biological phenomena such as plant diseases (Cevallos-Cevallos et al., 2009), although few of such researches have been focused on the vineyard environment. Recent metabolomics applications in Agricultural research (sometimes very difficult to be separated completely from environmental research) involve mainly assessments of genetic and environmental impacts on the metabolite composition of crop plant, which is principally a direct function of the desired crop quality (Memelink, 2005). Again, to date there have been few publications dealing with metabolomics based "grapevine" quality studies as related to different environmental factors, whereas the influence of *terroir* as a whole on grape/wine characteristics was recently investigated using metabolomics approaches (López-Rituerto et al. 2012, Tarr et al., 2013).

However, this new analytical tool, together with its enormous capacity, has to date still been very poorly explored within Food Sciences as well as within Environmental and Agricultural sciences.

3 EXPERIMENTAL - GENERAL

3.1 Materials

3.1.1 Plant material

'Pinot Noir' (*Vitis vinifera* L.), known as an early ripening red grapevine variety with genetically low phenolic (mainly anthocyanin) potential and high disease sensitivity due to its compact clusters, was chosen as the variety under observation in all our experiments. Although not very abundant, Pinot Noir wine from Vipava Valley is gaining very good results in local winemaking and is also successfully entering foreign wine markets. As a variety of normally cooler viticultural regions (Jones et al., 2006), 'Pinot Noir' is most likely reaching its upper limits regarding the optimal temperature regime in the Vipava Valley geo-climatic conditions. Consequently it is very sensitive to any climatic alternations, especially to variations in temperature in warm to hot climates (Webb et al., 2008) and is thus an effective variety for climate change related research. During the last year of observations (2011), the same experiment was performed not only in the Vipava Valley (Slovenia), but also on 'Pinot Noir' from Trentino (Italy) (Table 1) with the aim of comparing the experimental outcomes of both (different) *terroirs*.

Figure 2: 'Pinot Noir' (*Vitis vinifera* L.) (Photo: M. Sternad Lemut)

Berry skins and/or whole berry tissues, as a plant material, were subjected to various analytical procedures to reveal the effects of purposely induced microclimate shifts as a result of canopy microclimate manipulation. In 2009 and 2010, the ripe grapes from all the treatments were furthermore subjected to classical Pinot Noir vinification processes with the aim of studying the results of alleged grape quality improvements during alcoholic fermentation (must, young wine).

3.1.1.1 Experimental vineyards

Three different 'Pinot Noir' vineyards - two from the Vipava Valley (Slovenia) and one from Trentino (Italy) - were involved in the experiments during three years of observations. However, the vineyard in Potoce (Vipava Valley) was used as our main experimental site, while the Podraga vineyard (Vipava Valley) was involved in the experiment in 2009, whereas the Molini vineyard (Trentino, Italy) was involved in the experiment in 2011. The main characteristics of all the experimental vineyards can be seen in Table 1.

Table 1: Basic data on the experimental vineyards involved in the experiments.

Vineyard → / Parameters ↓	POTOCE	PODRAGA	MOLINI
Location	(Middle) Vipava Valley, Primorje Region, Slovenia	(Upper) Vipava Valley, Primorje Region, Slovenia	San Michele all'Adige Trentino Region, Italy
Elevation	95 m a.s.l.	220 m a.s.l	245 m a.s.l.
Variety	'Pinot Noir'	'Pinot Noir'	'Pinot Noir'
Clone	666	777	115
Rootstock	SO4	SO4	3309
Planting year	2004	2005	1989
Training system	Guyot (single)	Guyot (single)	Guyot (single)
Plant density	5682 plants/ha (0.8 m x 2.2 m)	6940 plants/ha (0.8 m x 1.8 m)	6178 plants/ha (0.9 m x 1.8 m)
Row orientation	E-W	N-S	70 ° NE (E-NE: W-SW)
Vintages in experiment	2009, 2010, 2011	2009	2011
Photos: Melita Sternad Lemut, Roberto Zorer			

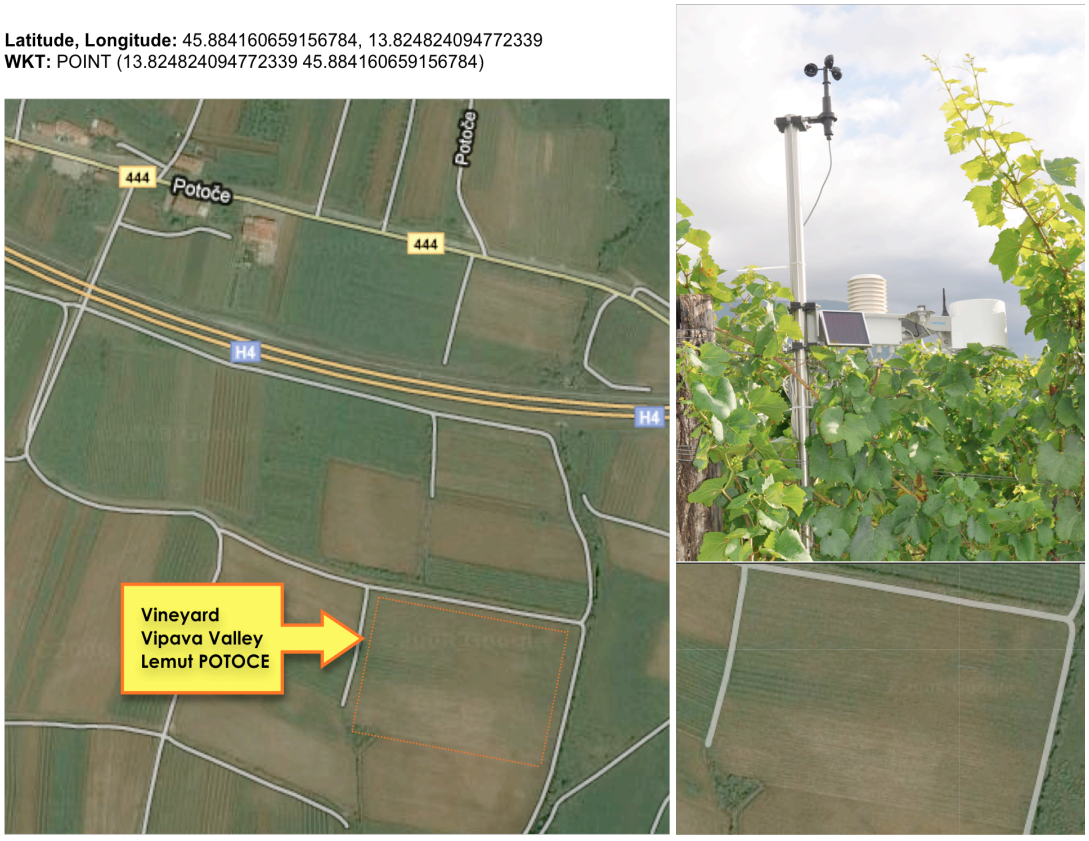


Figure group 3: The main experimental vineyard of Potoce in the Vipava Valley (Slovenia) with its exact location data (Photo: M. Sternad Lemut; Map: http://www.getlatlon.com/index.html, december, 2010)

3.1.1.2 Experimental design and sampling

In Slovenia, a completely randomized experimental design was set up in experimental vineyards (Figures 5 and 6) using plots of 5 vines. Leaf removal (LR) treatments were applied as follow: PF (pre-flowering), leaf removal (LR) performed before flowering, at phenological stage BBCH 57 (Lorenz et al., 1995) (in 2010 and 2011); BS (berry-set), LR applied at BBCH 71 (in 2009 and 2010); VE (veraison), LR performed at BBCH 83 (in all three vintages); UN (control treatment), LR not applied (untreated vines / leaves retained) (in all three vintages). Leaf removal was performed manually by removing the basal 4-to-6 leaves of all shoots as normally carried out for the pre-flowering defoliation (Poni et al., 2009). Particular microclimate scenarios within the canopy were achieved (and monitored) in the different leaf removal treatments (Figure 4).

In 2010 (all the treatments) and 2011 (only for PF), a parallel spraying trial was added to the Potoce vineyard experimental plan: 3 x 10 vines per treatment were randomly selected and observed for *B. cinerea* occurrence as well as for the total microbial count of other moulds, yeasts and bacteria (with and without fungicide application) (Figure 5).

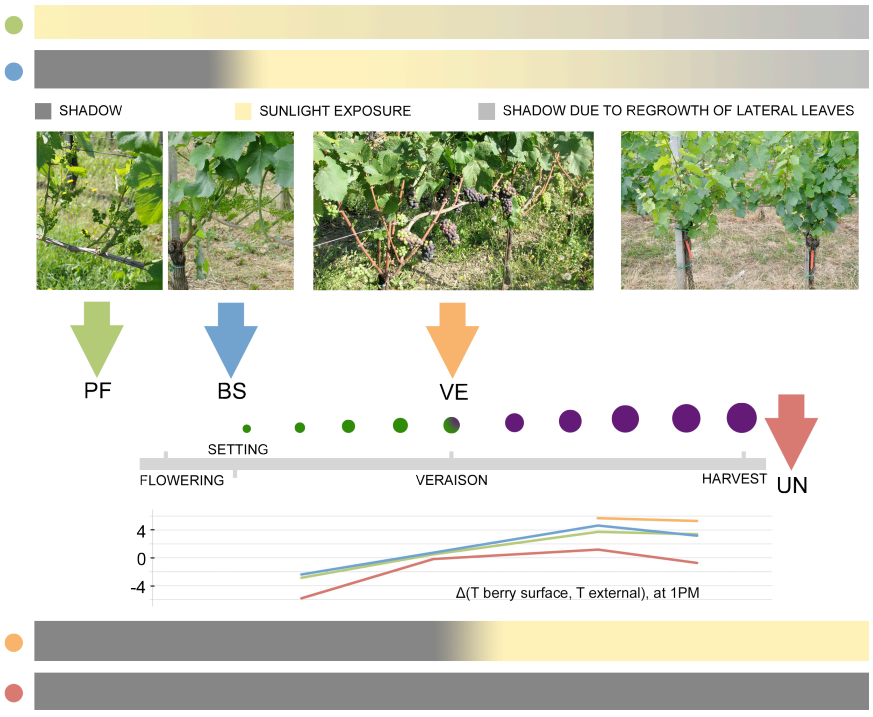


Figure 4: Scheme of four different microclimate scenarios as achieved through leaf removal at different phenological stages of grape berry development: PF, at pre-flowering; BS, at berry set; VE, at veraison; UN, untreated control (Graphic: N. Lemut).

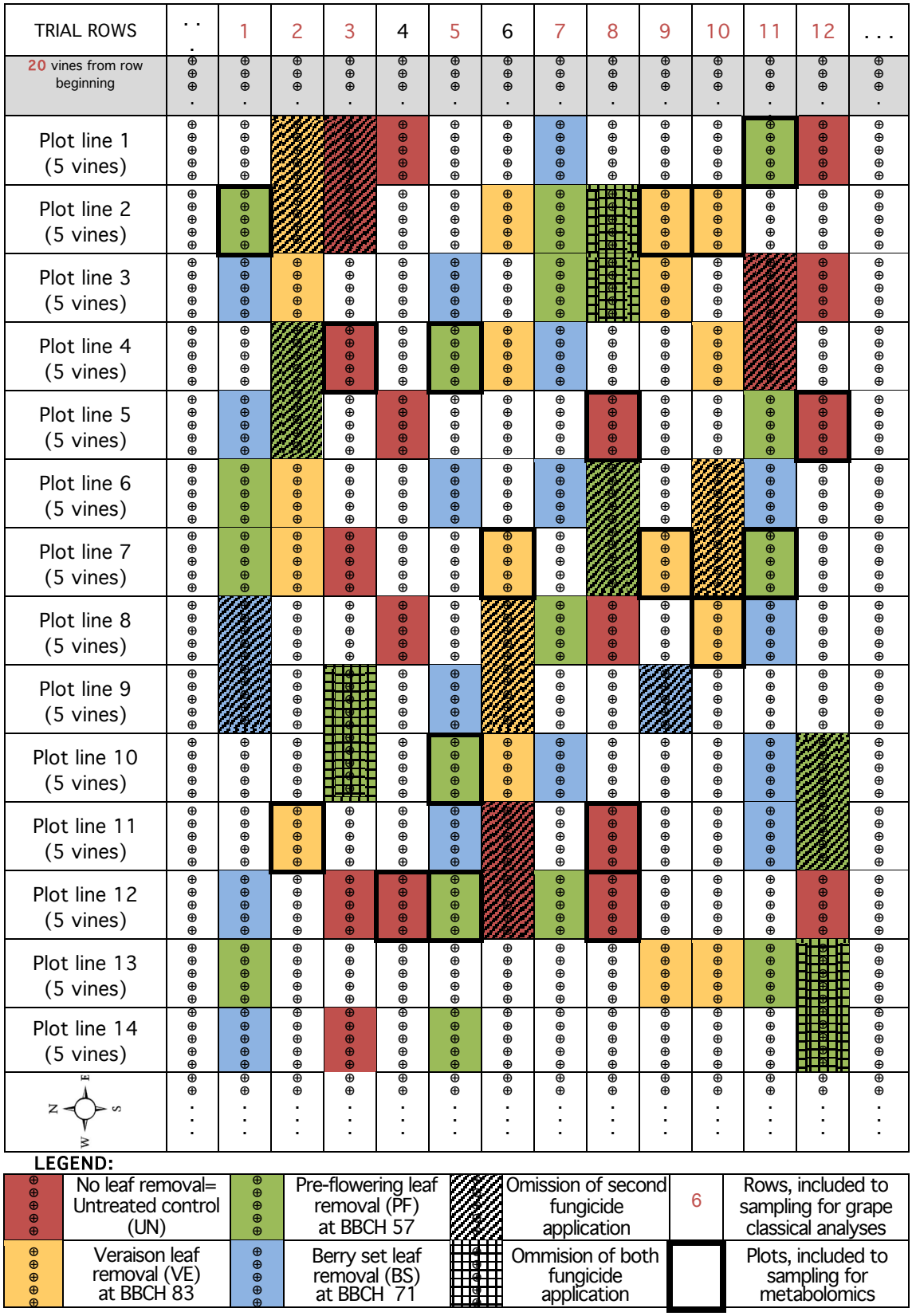


Figure 5: The main experimental design (plots: clear colour) and experimental design of subtrials (plots: diagonal, grid lines) in the Potoce vineyard (Slovenia); ⊕ = 1 vine.

The sampling regime and sampling sizes of plant material from each trial within the selected vineyards / designs (Figures 5, 6 and 7) are summarised in Table 2.

Table 2: Sampling regime and sample sizes within the main experiment and its subtrials in experimental vineyards (Treat, treatment; Biol. Rep, biological replicates; app, application).

	Subtrial Material	No of Treat.	No of Biol.Rep.	Sampling
Potoce 2009	Wines	4	3	From all the main plots (clear colour) → 16 plots/treatment - Sample size: all the grapes
Potoce 2009	Grapes	4	3	From the main plots (clear colour) of randomly collected trial rows (red numbers) → 12 plots/treatment - Sample size: 220 berries
Podraga 2009	Wines	4	3	From all the main plots (clear colour) → 16 plots/treatment - Sample size: all the grapes
Podraga 2009	Grapes	4	3	From the main plots (clear colour) of randomly collected trial rows (red numbers) → 12 plots/treatment - Sample size: 220 berries
Potoce 2010	Wines	4	3	From all the main plots (clear colour) → 16 plots/treatment - Sample size: all the grapes
Potoce 2010	Grapes	4	3	From the main plots (clear colour) of randomly collected trial rows (red numbers) → 12 plots/treatment - Sample size: 220 berries
Potoce 2010	Grapes/ Microbial Ecology & Spraying Trial	4	3	From all the subtrial plots (diagonal) for 1x fungicide app. and from major plots for 2x fungicide app. → 3 (double) plots/treatment (60 vines/tr.) - Sample size: all the grapes for visual inspection / 250 berries juice for plating
Potoce 2011	Gropes/ Microbial Ecology & Spraying Trial	3	3	From PF (green) subtrial plots (diagonal, grid) and from major PF plots for 2x fungicide app. → 3 (double) plots / treatment (60 vines/treat.) - Sample size: all the grapes for visual inspection / 250 berries juice for plating
Potoce 2011	Grapes Classical Analyses	3	3	From the main plots (clear colour) of randomly collected trial rows (red numbers) → 12 plots/treatment - Sample size: 220 berries
Potoce 2011	Grapes Metabolomics	3	6	From all the main plots (clear colour) framed with black border → 6 plots/treatment – Sample size: 500g of berries
Molini 2011	Grapes Metabolomics	3	6	From all the main plots (Figure 7; clear colour) → 6 plots/treatment (Figure 7)

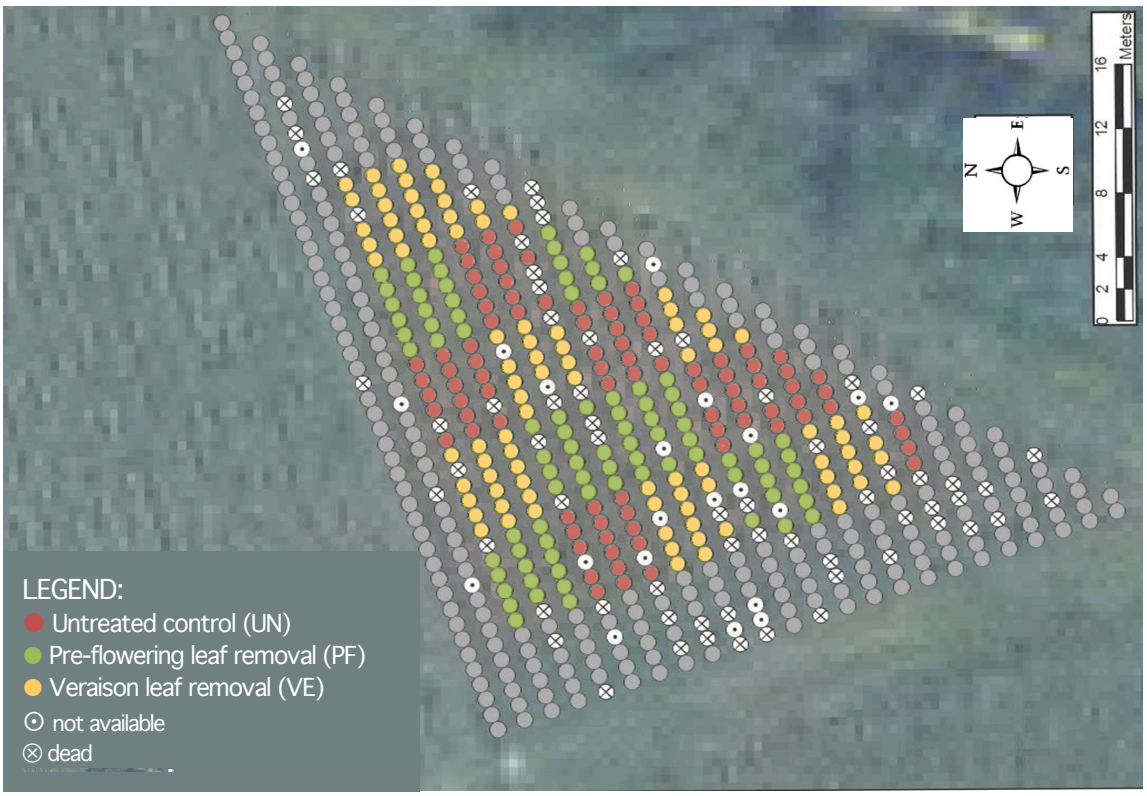
	Plot line 1 (5 vines)	Plot line 2 (5 vines)	Plot line 3 (5 vines)	Plot line 4 (5 vines)	Plot line 5 (5 vines)	Plot line 6 (5 vines)	Plot line 7 (5 vines)	Plot line 8 (5 vines)	Plot line 9 (5 vines)	Plot line 10 (5 vines)	Plot line 11 (5 vines)	Plot line 12 (5 vines)
1	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕
2	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕
3	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕
4	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕	⊕⊕⊕⊕⊕

LEGEND:

⊕⊕⊕⊕⊕	No leaf removal = Untreated control (UN)	⊕⊕⊕⊕⊕	Berry set leaf removal (BS) at BBCH 71	⊕⊕⊕⊕⊕	Veraison leaf removal (VE) at BBCH 83

Figure 6: Experimental design in the Podraga vineyard (Slovenia), vintage 2009.

Figure 7: Experimental design in the Molini vineyard (Italy), vintage 2011.



3.2 Methods – general

3.2.1. Grapes – evaluation of chemical composition

3.2.1.1 Sample preparation

3.2.1.1.1 Extraction of grape phenolic compounds from grape berry skins

The extraction of phenolic compounds from grape berry skins was performed as previously reported by Mattivi et al. (2006). In brief, the skins from 20 berries (3 lots for each treatment) were first extracted for 24 h in 100 mL of methanol (Sigma, Germany) without stirring. The extract was then separated and an additional 50 mL of methanol was added to the skins for 2 h. The two extracts were finally combined in dark glass containers (MeOH extract hereinafter) and stored at -20 °C until the analyses were performed (Mattivi et al., 2006).

3.2.1.1.2 Sample preparation for HPLC analyses of phenolic compounds

Before (HPLC) analysis of the anthocyanins, the MeOH extracts (3.2.1.1.1) were diluted with 1% trifluoracetic acid (TFA, Sigma, Germany) in water using a 1:1 (v:v) ratio. For (HPLC) analysis of flavonols and hydroxycinnamic acids, the MeOH extracts were diluted with 0.5% formic acid (Sigma, Germany) in water using the ratio 1:1 (v:v). All the dilutions with acidified water were done in order to maintain the symmetry of the chromatographic peaks. Prior to injection, the samples were filtered through a 0.45 μm Millipore HPLC filter.

3.2.1.1.3 Sample preparation for the metabolic profiling of grape berry skins

Before UHPLC/QqQ-MS/MS analysis of phenolic compounds, an aliquot of 10 mL of MeOH extract (3.2.1.1.1) was first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd., UK) under reduced pressure at 45°C. The sample was then reconstructed in a quantitative flask up to 1 mL of the final volume with methanol (FLUKA Sigma-Aldrich, Germany) and filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, USA). Additional dilution with MeOH was carried out if needed for the compounds present at higher concentrations.

3.2.1.1.4 Sample preparation for metabolomics analyses of grape berries

The preparation of grape berry samples (6 biological lots of 500 g berries), previously stored at -80 °C, was adopted according to Theodoridis et al. (2012). First the samples were ground in liquid nitrogen using an IKA analytical mill (Staufen, Germany) in order to obtain a homogenous frozen powder. Then, in brief, 2 g of frozen powder from each sample were extracted in sealed glass vials using 5 mL of water/methanol/chloroform (20:40:40) mixture. After vortexing for 1 min, the samples were put in an orbital shaker for 15 min (at room temperature). Samples were then centrifuged at 1000g and 4 °C for 10 min, and the upper phases constituted of aqueous methanol extract were collected. Extraction was repeated by adding another 3 mL of water:methanol (1:2) to the pellet and chloroform fractions, and by shaking for another 15 min. After centrifugation, the upper (organic) phases from the two extractions were combined, brought to 10 mL in quantitative flasks, and filtered through a 0.2 μm PTFE filter prior to analysis (Theodoridis et al., 2012; Vrhovsek et al., 2012).

3.2.1.2 Analytical methods

3.2.2.2.1 Analyses of basic grape quality parameters

The parameters of the grape ripening curve were followed on a weekly basis from veraison until harvest using standard procedures (Commission Regulation..., 1990) for evaluating the total soluble solids (°Brix), pH value and total titratable acidity (eq. of Tartaric Acid in g/L) in the grape juice. All analyses were performed using fresh samples and in three biological replicates.

3.2.2.2.2 HPLC analysis of anthocyanins and flavonols

The analysis was carried out according to previously published protocols (Sternad Lemut et al., 2011). The separation and quantification of individual anthocyanins and flavonols was performed using a Waters chromatographic system (Waters, Milford, MA, USA) comprising two Waters 510 pumps, a Waters 717+ autosampler and a Waters 2487 UV–visible (VIS) dual-wavelength detector. Individual anthocyanins were separated on the Atlantis column C18, 3.9 mm x 150 mm, 3 μm (Waters, USA).

Flavonols were separated using a Phenomenex Luna, C18, 4.6 mm x 150 mm, 5 μm column (Phenomenex, USA). All analyses were performed in three biological replicates. The method is described in detail in the published article by Sternad Lemut et al. (2011), presented in the Results and Discussion section (4.1.3.3 and 4.1.3.4).

3.2.2.2.3 HPLC determination of hydroxycinnamic acids

The analysis was carried out according to the previously published protocol (Sternad Lemut et al., 2011). Selected hydroxycinnamic acids (HCAs) were identified using the standard addition of the observed HCAs. Since coutaric acid is not commercially available, it was identified as described in Mozetic et al. (2006). Quantification was carried out using external calibration curves plotted by means of gradient high performance liquid chromatography with UV detection (HPLC-VIS). The instrumentation used and the separation parameters were the same as for flavonol determination, the chromatogram was recorded at 320 nm. All analyses were performed in three biological replicates. The method is described in detail in the published article by Sternad Lemut et al. (2011), presented in the Results and Discussion section (4.1.3.5).

3.2.2.2.4 The metabolic profiling (targeted metabolomics) of polyphenols

A comprehensive targeted metabolomic analytical approach according to Vrhovsek et al. (2012) was applied. The method was developed with the potential to perform the qualification and quantification of 135 phenolics belonging to different chemical groups that are typically present in fruits, such as benzoates, phenylpropanoids, coumarins, stilbenes and flavonoids (flavones, isoflavones, flavanones, flavan-3-ols flavonols and dihydrochalcones). Ultra high-performance liquid chromatography (UHPLC-MS/MS) was performed using a Waters Acquity UHPLC system (Milford, MA, USA) consisting of a binary pump, online vacuum degasser, autosampler and column compartment. The separation of the observed phenolic compounds was achieved using a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Samples were kept at 6 °C during the analysis. Mass spectrometry detection was performed using a Waters Xevo TQMS (Milford,

MA, USA) instrument equipped with an electrospray (ESI) source (Vrhovsek et al., 2012). All analyses were performed in three biological replicates.

3.2.2.2.5 Untargeted metabolomics analyses

Analyses were performed according to the published method by Theodoridis et al. (2012) using a Waters Acquity ultra high-performance liquid chromatography (UHPLC), coupled to a Synapt High Definition Hybrid Quadrupole/Time-of-Flight Mass Spectrometer (HDMS QTOF-MS) (Waters, Manchester, UK) via an electrospray interface (ESI), operating in W-mode and controlled by MassLynx 4.1. For reversed phase chromatography (RP), an ACQUITY UPLC 1.8 μm 2.1 x 100 mm HSS T3 column (Waters, Milford, MA, USA) at 30 °C was used. Spectra were collected in positive ESI mode over a mass range of 50-3000 amu with a scan duration of 0.3 s in centroid mode. The transfer collision energy and trap collision energy were set to 6 and 4 V. External calibration with sodium formate and Lock Mass calibration with leucine enkephaline solution was applied (Theodoridis et. al., 2012). All the analyses were performed in six biological replicates.

3.2.2 Wines - vinification procedure and the evaluation of chemical composition

3.2.2.1 Must fermentation

Grapes from all the treatments were collected separately (in three biological replicates) at optimum maturity (based on the maturity level registered on control (UN) grapes: 22 $^{\circ}$Brix and 5.5 g L^{-1} titratable acidity on average. All grape samples were initially de-stemmed, crushed, protected with 0.1 g kg^{-1} K_2S2O_5 and mixed, before 20 L of the pomace was separated and placed into 25 L stainless steel experimental tanks. Experimental cold maceration and alcoholic fermentation were performed in the temperature-controlled chambers of the Wine Research Cellar (University of Nova Gorica, Slovenia). Each 20 L batch was initially cooled to 5 °C and then subjected to a typical Pinot Noir winemaking procedure, starting with 48 h of cold maceration (in a chamber set to 5 °C), followed by induced alcoholic fermentation once the temperature of the pomace had been brought back to room

temperature (in a chamber set to 20 °C). The yeast strain *Saccharomyces cerevisiae* 'Fermol Premier Cru' type (AEB Group, Brescia, Italy) was used to start fermentation (0.2 g L^{-1}) and the yeast nutrient Fermoplus Starter (AEB Group) was added (0.3 g L^{-1}) to achieve better continuation of the fermentation processes. Skin caps were pushed down and mixed every 12 h, while the fermentation curve data (temperature, sugar level/density) was checked every 24 h. Samples were collected after cold maceration, every 48 h during alcoholic fermentation (after mixing the fermenting juice and skin caps) and finally after pressing in a pressure-controlled experimental (30 L) mechanical press (Skrlj d.o.o., Batuje, Slovenia), following the protocol of four (4 x 5 min) cycles (0.4/0.6/0.8/1.0 bar respectively). Then 20 mL of each must/wine under observation was added to 80 mL of methanol (hereinafter mMeOH extracts) in a dark glass container and stored at −20 °C until the analysis was performed.

3.2.2.2. Sample preparation

3.2.2.2.1 Sample preparation for flavonol determination

Methanol was first removed from the samples (mMeOH extracts - 3.2.2.1.) using a rotary evaporator (Buechi R-210, Flawil, Switzerland). Individual flavonols in the musts/wines were then subjected to the acid hydrolysis of flavonol glycosides as previously described by Mattivi et al. (2006).

3.2.2.2.2 Sample preparation for anthocyanin determination

Before the analysis, 20:80 methanol extracts (mMeOH extracts - 3.2.2.1) were filtered (0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, USA)) and diluted with 1% trifluoracetic acid (TFA, Sigma, Germany) in water using a 1:9 (MeOH extract:TFA) (v:v) ratio.

3.2.2.2.3 Sample preparation for determining hydroxycinnamic acids

A 10 mL aliquot of mMeOH extract (3.2.2.1) was first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd, Ipswich, UK) under reduced pressure at

45°C. The sample was then reconstructed in a quantitative flask to a final volume of 1mL with 5mL L^{-1} formic acid and filtered through 0.45 μm, 13 mm polytetraflouroethylene syringe-tip filters (Millipore, Bedford, MA, USA) into vials.

3.2.2.2.4 Sample preparation for determining pyranoanthocyanins

Sample preparation for the analyses of pyranoanthocyanins was performed in accordance with the report of Rosetto et al. (2004), although instead of wine, its 20:80 mMeOH extract (3.2.1.1.1) was used with a 20 mL aliquot of mMeOH extract (3.2.2.1), first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd, Ipswich, UK) under reduced pressure at 35°C. The sample was then reconstructed in a quantitative flask to a final volume of 1mL with diluted methanol (27% in water and 0.3% $HClO_4$). Finally samples were filtered through 0.22 μm, 13 mm PTFE syringe tip filters (Millipore, Bedford, USA) into a 2 mL autosampler amber LCMS certified vial (Waters) and injected (Arapitsas et al., 2012; Roseto et al., 2004).

3.2.2.3 Analytical methods

3.2.2.3.1 Analyses of flavonols

Individual flavonols in musts/wines were analysed after subjection to the acid hydrolysis of flavonol glycosides, as previously described by Mattivi et al. (2006). The further separation and quantifications of flavonol aglicons were performed using a Waters Alliance 2695 high-performance liquid chromatograph (HPLC) equipped with a Waters 2996 diode array detector (DAD) (Milford, MA, USA) and using a reversed-phase column Purospher RP18 250 mm × 4 mm (5 μm) with precolumn. Previously reported chromatographic procedure (Mattivi et al., 2006) was used. Each detected flavonol myricetin, kaempferol, quercetin, syringetin and isorahmnetin was expressed as mg/L in must/wine using the external standard method, specific for each compound. Standards were purchased from Sigma (Steinheim, Germany) or from Roth (Karlsruhe, Germany) in the case of isorahmnetin. All analyses were performed in triplicates.

3.2.2.3.2 Analysis of anthocyanins

The analytical method as previously presented by Sternad Lemut et al. (2011) was used. The separation and quantification of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside was performed using gradient high performance liquid chromatography with UV-VIS detection at 520 nm (Waters, MA, USA). Individual anthocyanins were separated on the Atlantis column C18, 3.9 × 150 mm, 3 μm (Waters, MA, USA) and quantified as malvidin-3-glucoside (mg/L) equivalent. All analyses were performed in triplicates.

3.2.2.3.3 Analyses of hydroxycinammic acids

Esters of hydroxycinnamic acids were determined based on a previously reported method (Nardini et al., 2009). The separation and quantification of hydroxycinnamates was performed using a Waters Alliance 2695 HPLC equipped with a Waters 2996 DAD (Milford, MA, USA). The chromatographic separations were performed on a Luna C-18 column (5.0 μm particle size, 250 × 3.0 mm i.d., Phenomenex, Torrance, CA) including a guard column. All analyses were done in triplicates.

3.2.2.3.4. Analyses of pyranoanthocyanins

The occurrence of pyranoanthocyanins during cold maceration and alcoholic fermentation in must/wine samples was monitored using a Waters ACQUITY ultra-high performance liquid chromatograph (UHPLC) (Miliford, MA, USA) coupled to a Waters Xevo tandem quadrupole mass spectrometer (TQ-MS) (Waters, UK). The published analytical procedure by Arapitsas et al. (2012) was applied. In brief, all the samples were analysed on a reverse phase (RP) Acquity UHPLC BEH C18, 1.7 μm, 2.1 × 150 mm column (Miliford, MA, USA), protected with an Acquity UHPLC BEH C18, 1.7 μm, 2.1 × 5 mm precolumn (Miliford, MA, USA), at 40 °C. The column eluent was directed to the mass spectrometer, and analyte detection was performed by multiple reactions monitoring (MRM) using the MS/MS transitions. Electrospray positive ionization mode (ESI) was applied for all compounds with the parameters in the source set. The cone voltage and collision energy were optimized

for each analyte. The MRM conditions were optimized for the standards by direct infusion into the ES ionization source. For compounds that standards were not available for, the MRM transitions found in the literature were used and optimized through multiple injections of the same sample under various ion source parameters. The chromatographic data reported in the literature was also used for identification. The quantification of individual compounds was performed using UHPLC-MS/MS. If the authentic standard was not available, the analytes were quantified relative to malvidin-3-glucoside, by the malvidin-3-glucoside calibration curve (Arapitsas et al., 2012). All analyses were performed in three biological replicates.

3.2.2.3.5 UV-VIS spectrophotometric analyses of total polyphenols and colour properties

Analyses of the total polyphenols were carried out according to a previously published method employing the Folin-Ciocalteu reagent (Di Stefano and Guidoni, 1989). Absorbance at 765 nm was measured with a Lambda 35 UV–VIS spectrophotometer (PerkinElmer, Waltham, MA, USA). The final results were expressed in mg of gallic acid equivalents (GAE) per L of must/wine. Colour measurements were performed on the same instrumentation, using the procedure described previously (Gómez-Míguez et al., 2007). The CIELab parameters *L*, a** and *b** were determined following the recommendations of the Commission Internationale de l'Eclairage (CIE, 1986).

3.2.3 Evaluation of the basic viticulture parameters and cluster compactness

One sample of 50 leaves of different sizes was collected randomly in order to assess leaf area. For each leaf, the main vein length was measured and leaf area estimated by scanning the leaves and processing them throughout binarization using the Image package (softonic®, Barcellona, Spain). A curvilinear regression model was fitted to determine leaf area (LA) based on main vein length (L) and leaf area ($LA = 0.5435*L^2 + 0.7494*L$). At the harvest point, three plants per plot were measured for leaf area. During data collection, main leaves and lateral leaves were kept separate, with the aim of evaluating the relative percentage of both groups. Within the same plant, the shoots were also kept separate, with the aim of ascertaining a second

relationship between the number of leaves x shoot and leaf area. In one plant x plot, the length of all the leaves was measured, while for the other two plants/plots, the leaves were only counted by separating the main and lateral leaves for each shoot. With the data obtained from the measured plants, a curvilinear model was then obtained by plotting number of leaves/shoot against leaf area/shoot, both for main ($LA= -1.6141*L^2 + 119.92*L$) and lateral leaves ($LA= -0.9537*L^2 + 87.269*L$), and using them further for final leaf area calculations. At the same time (at harvest), a yield/plant and cluster number was determined, and furthermore the average cluster weight and leaf area-to-yield ratio was computed. A 100-berry weight of each plot was also evaluated using standard procedures (Commission Regulation..., 1990). In addition, 50 randomly selected clusters from each treatment were weighted and sized (max length) at harvest in order to calculate the grape compactness ratio by rating cluster weight and length.

3.2.4 Monitoring climatic characteristics

The mesoclimatic characteristics of the main experimental site were followed using an IMT 300 meteorological station (Metos Instruments, New Delhi, India), collecting data for global radiation, rainfall, wind speed, leaf wetness, air temperature, relative humidity and dew point. In addition, further meteorological data was collected from a nearby weather station of the National Meteorological Service of Slovenia (EARS, Slovene public information, EARS-meteo.si), aiming to confirm and support the data downloaded from our meteo station at the vineyard site. The canopy microclimate conditions in the cluster area (temperature and relative humidity) were monitored during the critical - hottest period before harvest via i-Button sensors / mini-data loggers (SPR Hygrochron Temperature / Humidity Logger i-Button with 8KB Data-Log Memory, Maxim, CA, USA), collecting and storing the data on an hourly basis (Figure 8). I-button sensors were placed directly in the cluster area of all the treatments, half of them facing north and half of them facing south. The temperature on the berry surfaces from the vines receiving the different treatments was measured every 2 h during three typical hot (August) days. Twenty-five measurements per treatment were performed at each observation point/time using a Voltcraft IR-360 IR Thermom infrared thermometer (Conrad Electronics, Hirschau, Germany).

The evaluation of the incoming solar radiation in the cluster area was computed by taking hemispherical photos (using fish-eye lens Sigma, EX DG fisheye 8mm 1:3.5) from below the grapevines and facing exactly to the north. The images were then processed with the Gap Light Analyser 2.0 software (Frazer et al., 1999) with the goal of determining the daily trend of solar radiation. The basic idea of the technique is to identify solar radiation relevant to open sky areas, and then merging them with a radiation and sun-path model in order to compute the total annual solar radiation for a grapevine (Schwalbe et al., 2009).

Figure 8: Mini-Data-Loggers, located in the cluster area (Photo: M. Sternad Lemut).

3.2.5 Monitoring the microbial ecology

3.2.5.1 Visual examination of grey mould and sour rot occurrences

A week before harvest (2010) and at harvest time (2010 and 2011), all the clusters were visually examined for bunch rots, but mainly for *Botrytis cinerea* Pers. (gray mould). Grey mould incidence and severity averages were then calculated in both years for all the plots under observation. Since it is difficult to precisely observe cluster infections just by visual inspection, we also performed a monitoring of the microbial population as affected by different treatments by direct plating on selective media (3.2.5.2), as well as monitoring microbial activity by the quantification of selected microbial metabolites (bellow) in order to obtain a clear picture of the situation from multiple aspects (3.2.5.3).

3.2.5.2 Microbial enumeration

To reveal the main grape berry related microbial groups as affected by the different timing of leaf removal (and using different spraying treatments - subtrial), a week before harvest (2010) and at harvest time (2010 and 2011), a quick monitoring was performed using a set of selective media for the detection of filamentous fungi, yeasts, lactic and acetic acid bacteria. The microbial enumeration was performed by plating 100 μL of a dilution series of juice, obtained from 250 randomly collected berries from all the treatments, aseptically picked, squashed in sterile bags and plated immediately. The berries (in three biological replicates) were collected from several locations in the vineyard so that spatial fluctuations dictated by the uneven microbial distribution were minimized (Barata et al., 2012). Plating was performed in duplicate, on selective culture media as follows: WL (Walerstein Laboratory) nutrient agar (Fluka) supplemented with 0.03% chloramphenicol for yeast enumeration (Pallmann et al., 2001) and WL-Differential medium (WLD; WL with added cycloheximide) was also included in 2011 for the easier detection of non-Saccharomyces yeasts (Fugelsang and Edwards, 2007); Potato dextrose agar (PDA) (Biolife) for mould enumeration; Glucose–yeast extract–carbonate medium (GYCM) (50 g L^{-1} glucose, 10 g L^{-1} yeast extract, 30 g L-1 CaCO3, 25 g L^{-1} agar) for acetic acid bacteria and MRS medium (Biolife) supplemented with 2% tomato juice and 0.1 mg mL^{-1} cycloheximide for lactic acid bacteria enumeration (Fugelsang and Edwards, 2007). The plates were incubated at 25°C for up to 14 days. Colonies were counted and expressed as colony-forming units (CFU) per mL. In general, bacterial and fungal identification was performed to the genus/species level on the basis of micro- and macromorphology. For the faster detection of yeasts on the WL medium, the guidelines described by Pallman et al. (2001) were also followed.

3.2.5.3 Determining the microbial indicator metabolites

In order to evaluate the relationship between the presence and actual activity of microbes, we performed a quick monitoring of selected microbial indicator metabolites (like gluconic acid, glycerol and volatile acids in the grape juice), which can serve as an indicator of grape deterioration. The analyses of gluconic acid and glycerol were performed as previously described by Larcher et al. (2009). Separation

and quantification were performed with the help of a high performance liquid cromatography (HPLC) Alliance 2695 (Waters, Milford, MA, USA) equipped with an autosampler of same producer mod. 540. An RCX/10 anion exchange column (250 x 4.6 mm; Hamilton; Bonaduz, Switzerland) was used in the system. The PAD detector was a Coulochem II 5200A used in pulsed mode and equipped with a 5040 gold electrode cell (ESA Inc.; Chelmsford, MA, USA). Volatile acids were analysed with the help of a Quattro Continuous Flow Analyzer (Bran+Luebbe, Norderstedt, Germany). The automated method uses automatic online distillation of the volatile acid medium and the subsequent reaction of the distillate with potassium iodide. After acetic acid liberates iodine, its absorbance is measured at 410 nm. The sample preparation and analytical procedure itself was performed according to the producer's instructions and volatile acids were finally expressed as acetic acid. All analyses were performed in three biological replicates.

3.2.6 Costs and energy calculations

Calculations on energy consumption were made in order to compare energy investments for all treatment variants. Costs were calculated based on the accounting records of the wine estate owner and manager of the vineyard under experiment (cost of human work, tractor hour, diesel and spraying agent), data obtained in this study and on data from literature. They included: Energy of human work: 1.96 MJ/hour (Ozkan et al., 2007); number of working-hours: data from this study, see Table 17; Diesel consumption for tractor work and spraying: diesel density 0.855 kg/L (Bosch, 2007), diesel heating value: 46.9 MJ/kg (Audsley et al., 1997); diesel consumption: 7.2 L/spraying (data from this study); Tractor and sprayer depreciation: tractor lifetime: 10,000 hours, sprayer lifetime: 3000 hours (Glithero et al., 2012); energy for machinery production: 142.7 MJ/kg (Litskas et al., 2013), tractor weight 2360 kg, sprayer weight: 200 kg and number of invested working hours: data from this study, see Table 17); the energy of the spraying agent (Switch®): active substance content: data from producers; energy for production of active substance/kg: 288.88 MJ/kg (West et al., 2002); Gibbs free energy of water: 4.94 kJ/kg; spraying agent consumption: 0.75 kg/ha/spraying and water consumption: 200 L/ha/spraying (data from this study).

3.2.7 Data processing and statistical analyses

All the data was initially processed with the help of Microsoft Excel 2008 for Mac, Version 12.2.3 (Microsoft Corporation, WA, USA), however, the chromatographic raw data sets were first processed using the Mass Lynx Target Lynx Application Manager (Waters MA, USA) or Empower software (Waters). The data was then processed running one-way ANOVA, with the exception of the spraying subtrial data, where a two-ways ANOVA was used. In the cases where the number of tested parameters was very high, a multiple-testing correction was performed for ANOVA computing (false-discovery rate correction, FDR) (Benjamini et al., 1995). Once the differences were significant ($P < 0.05$), the Student–Newman–Keuls (SNK) or Tukey Honest Significant Difference (HSD) test was selected to search for the differences between treatments. In case of the observation of *Botrytis cinerea* Pers. incidence and severity, the angular arcsine transformation of calculated indexes was adopted since the percentage data distribution is binomial and the transformation is needed in order to make the distribution normal. The overall differences among treatments were evaluated by means of multivariate Principal Component Analysis (PCA) on the autoscaled data; however, for the microbial ecology trial the data was preliminarily subjected to logarithmic transformation prior to PCA analysis. In the case of the targeted metabolomics analyses of harvest samples in 2011, together with ANOVA of single parameters, a Canonical analysis was performed in order to ascertain the multidimensional differences among the treatments keeping the classes of different phenolic compounds separated (ellipses representing a 95% confidence interval). All these statistical tests were performed with the help of the Statistica software package (StatSoft,Tulsa, OK, USA) and/or »R« (R Core Team, 2013).

4 RESULTS AND DISCUSSION

4.1. Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley (Sternad Lemut et al., 2011)

4.1.1 Abstract

Wine colour depends directly on the quantitative and qualitative profiles of anthocyanins, flavonols and hydroxycinnamic acids, as well as on their involvement in polymerization and co-pigmentation reactions. 'Pinot Noir' is a grape variety with a low natural colour potential, often combined with problems of low colour stability for its wines during aging. The influence of leaf removal timing on grape colour related polyphenols was observed during maturation and at harvest time for the 2009 vintage "real scenario" conditions in two different vineyards in the Vipava Valley (Slovenia), known for its warm but windy climate. With different timing of leaf removal, a different microclimate can be achieved, with different light exposure for the specific plant tissue and different temperatures on berry surfaces. Phenolic compounds were characterised and quantified using HPLC/Vis. Three classes of polyphenols were affected by leaf removal timing, but with differing intensities and with some differences between locations. Hydroxycinnamic acids were affected only slightly, mostly at the beginning of the maturation period. Anthocyanins were significantly affected, while flavonols were the most affected by treatments in both vineyards and their concentration was clearly related to modification of the light within the canopy.

Keywords: *Vitis vinifera* L., 'Pinot Noir', leaf removal, canopy microclimate, berry size, polyphenols, anthocyanins, flavonols, hydroxycinnamic acids, food composition, food analysis.

4.1.2 Introduction

There are different classes of polyphenols in *Vitis vinifera* grape berries and related wines, however three classes of flavonoids - anthocyanins, flavonols and flavan-3-ols (monomers and polymeric proanthocyanidins) - usually account for 80 to 90% of the phenolic content in conventionally red wines (Jeong et al., 2008). Their presence and/or absence can contribute greatly to wine quality (Downey et al., 2006). Anthocyanins are primarily responsible for the colour of red grapes and wines. While many grape varieties have very complex anthocyanin profiles with up to 20 different anthocyanins (Mattivi et al., 2006), the 'Pinot Noir' profile has only five 3-monoglucoside anthocyanins: cyanidin, delphinidin, petunidin, peonidin and malvidin (Cortell and Kennedy, 2006).

Flavonols are generally considered to act as UV protectors and free radical scavengers (Downey et al., 2004). Although colourless, flavonols are known to contribute to wine colour as co-pigments (Asen et al., 1972; Boulton, 2001). Mattivi et al. (2006) reported certain correlations between their metabolic pathways, implying that any attempt to optimize the pattern of one, might also be expected to affect the patterns of other metabolites. They also reported that some biosynthetic analogies depend on the same parallel enzymatic activities for both classes of flavonoids: anthocyanins and flavonols. Guidoni et al. (2008) indicate that the anthocyanin profile is not strictly dependent on genotype but can be modulated by environmental conditions. Among other factors, regional climate and the methods and timing of some viticulture practices can have an important effect on canopy microclimate (Haselgrove et al., 2000; Shaulis et al., 1966). Temperature has been reported to have a major role in berry growth and ripening (Tarara et al., 2008). Haselgrove et al. (2000) discussed the fact that light is not necessarily a limiting factor for anthocyanin synthesis, if bunches receive sufficient light of moderate intensity. In conditions where bunches are heavily shaded, it is likely that light is a limiting factor in the accumulation of anthocyanins during the early stages of ripening and it seems that while berries are receiving adequate light, the temperature conditions may be a limiting factor. In the case of flavonols it has been pointed out that UV-light barriers contribute significantly towards decreasing flavonol glycoside content in berry tissues (Spayd et al., 2002), while in the group of non-flavonoid phenolic acids, the *trans* isomers (of tartaric esters of hydroxycinnamic acids) are assumed to be a natural phenomena, while *cis*-forms are thought to be the product of UV-induced isomerization (Singleton et al., 1978).

Leaf removal is a viticulture practice used worldwide in order to improve the microclimate for clusters. It is traditionally performed in order to improve bunch microclimate, reduce grape rot (*Botrytis cinerea* and sour rot) and to obtain a better ripening. Occurring as a direct effect of the climate, grapevine growth can be determined by phenological events. Understanding the phenology of a given plant system is important in determining the ability of a region to produce a crop within the confines of its climatic regime. In terms of cultivation, knowledge of plant growth stages is advantageous, as cultural and chemical practices can be applied at optimum times in the plant's annual growth cycle (Jones and Davis, 2000).

This study was thus designed to compare two leaf removal treatments (performed at berry set and at veraison) with untreated grapevines, studying how induced microclimatic modification could affect polyphenol occurrence in grape berries during grape maturation.

4.1.3 Material and methods

4.1.3.1 Plant material and experimental design

The experiment was carried out in 2009, observing two 'Pinot Noir' vineyards located in the Vipava Valley (Slovenia):

Location in Podraga: vineyard planted in 2005, Guyot trained, plant density 6940 plants/ha (0.8 m x 1.8 m), altitude 220 m a.s.l., row orientation N–S;

Location in Potoce: vineyard planted in 2004, Guyot trained, plant density 5682 plants/ha (0.8 m x 2.2 m), altitude 95 m a.s.l., row orientation E–W.

A completely randomized experimental design was set up in both vineyards with 16 plots of 5 vines. Leaf removal was performed at berry set (BS) and at veraison (VE), removing the basal 5-to-6 leaves of all shoots manually, thus allowing the grapes to be better exposed to the sunlight. Untreated vines were used as a control (UN). Leaf area was assessed in order to evaluate the total amount per plant, but also the contribution of main and lateral shoots. Leaf area (measured only at harvest) was estimated by measuring the maximum length (L) of all leaves, and then by applying curvilinear regression to determine the area (0.5435 x L2 + 0.7494 x L). At harvest time the yield/plant and cluster number were determined and the average cluster weight calculated. 100 berry-samples were randomly collected from each treatment at different stages of maturity during the period of berry ripening and at harvest time.

The berries were removed from bunches together with pedicels and frozen immediately (in order to avoid oxidation). Three 20-berry lots were randomly taken from each 100-berry treatment sample, each lot was weighed and frozen prior to extraction and phenolic analysis. A similar sampling method was previously presented by Haselgrove et al. (2000).

4.1.3.2 Extraction of anthocyanins and flavonols

Skins from 20 berries (3 lots for each treatment) were extracted for 24 h in 100 mL of methanol (Sigma, Germany) without stirring. The extract was then separated and an additional 50mL of methanol was added to the skins for 2 h. Both extractions were performed in the darkness; the two extracts were combined in dark glass containers and stored at 20°C until analysis (Mattivi et al., 2006).

Before analysis of the anthocyanins, the extracts were diluted with 1% trifluoracetic acid (TFA, Sigma, Germany) in water using a 1:1 (v:v) ratio. For analysis of flavonols and hydroxycinnamic acids the methanolic extracts were diluted with 5% formic acid (Sigma, Germany) in water using the ratio 1:1 (v:v). The samples were diluted with acidified water in order to maintain the symmetry of chromatographic peaks. Prior to injection the samples were filtered through a 0.45 mm Millipore HPLC filter. All analysis was carried out in duplicate. All chemical reagents used were HPLC grade.

4.1.3.3 Analysis of anthocyanins

The analytical method applied was previously presented by Trost et al. (2008), although slightly modified for the purpose of this study. Separation and quantification of 'Pinot Noir' anthocyanins: delphinidin-3-glucoside (Del-3-Glu), cyanidin-3-glucoside (Cy-3-Glu), petunidin-3-glucoside (Pet-3-Glu), peonidin-3-glucoside (Peo-3-Glu) and malvidin-3-glucoside (Mal-3-Glu) were performed using gradient high performance liquid chromatography with UV–Vis detection at 520 nm. Analysis was carried out with a Waters chromatographic system (Waters corporation, USA) made up of two 510 pumps, auto-sampler 717+, UV–Vis dual wavelength 2487 detector and Empower Millennium software. Individual anthocyanins were separated on the Atlantis column C18, 3.9 mm x 150 mm, 3 μm (Waters, USA). Individual anthocyanins were quantified as malvidin-3-glucoside (purity 95%) purchased from Extrasynthese, France (k = 130,528, R2 = 99.67%, DL = 0.03 mg/L, QL = 0.1 mg/L) and identified using HPLC–MS, as previously reported by Košir et al. (2004). The

samples were stable for at least 48 h. In order to determine method repeatability the same sample was injected six times. The relative standard deviations of the observed peak area for individual anthocyanins were: Del-3-Glu, 2.1%; Cy-3-Glu, 2.2%; Pet-3-Glu, 1.0%; Peo-3-Glu, 0.7% in Mal-3-Glu, 0.7%. Analysis was performed at room temperature. The injection volume was 20 mL. For anthocyanin, the separation gradient of mobile phases was used. Mobile phase A was made up of acetonitrile (Merck, Germany) and water 10:90 (v:v). The mobile phase B was made up of acetonitrile and water 50:50 (v:v). Both mobile phases were acidified with 0.2 vol.% TFA (Sigma, Germany). The gradient of mobile phase B changed from 10% to 25% in 20 min. In the next 20 min the percentage of mobile phase B increased from 25% to 55%, then to 90% B in 1 min. Equilibration to the initial gradient conditions lasted for 12 min. A flow rate of 0.5 mL/min through the gradient was used.

4.1.3.4 Analysis of flavonols

The analytical method applied was as previously presented by Robins and Bean (2004), slightly modified for the purpose of this study. For quantitative determination of individual flavonols, high performance liquid chromatography was used with UV detection at 360 nm. An external calibration curve was plotted using quercetin-3-glucoside (Qu 3-Glu) (k = 45,318, R2 = 99,47%, DL = 0.2 mg/L, QL = 0.6 mg/L) and kaempherol-3-glucoside (Ka-3-Glu) (k=40,231, R2 =99,76%, DL=0.3mg/L, QL=1mg/L). Both Qu-3-Glu (purity 90%) and Ka-3-Glu (purity 97%) standards were purchased from Sigma (Germany). Myricetin-3-glucoside (My-3-Glu) was quantified as quercetin-3-glucoside equivalent. Samples were stable at room temperature for at least 48 h. Repeatability was determined by six injections of the same sample. The relative standard deviations of the observed peak areas for flavonols were: My-3-Glu, 3.75%; Qu-3-Glu, 2.02% and Ka-3-Glu, 6.40%. Analysis was performed using the Waters chromatographic system (Waters Corporation, USA) made up of two 510 pumps, auto-sampler 717 +, dual UV–Vis 2487 detector and Empower Millennium software. Flavonols were separated using a Phenomenex Luna, C18, 4.6 mm x 150 mm, 5 μm column (Phenomenex, USA). Analysis was carried out at room temperature; the injection volumes were 20 mL. Mobile phase A was water, and mobile phase B was methanol (Merck, Germany). Both phases were acidified with 1vol.% of formic acid. The following gradient was used: the percentage of mobile phase B increased in 33 min from 5% to 38% and in the next 10 min from 38% to 50%. In the last 3 min mobile phase B reached 90%. Finally the system was equilibrated to initial conditions for 14 min. A flow rate of 1 mL/min was used.

4.1.3.5 Hydroxycinnamic acid determination

Selected hydroxycinnamic acids were identified using standard addition of observed acids. Since coutaric acid is not commercially available, it was identified as described in Mozetic et al. (2006). The concentrations of *cis* and *trans*-coutaric acid were expressed as caffeic acid equivalent. The following hydroxycinnamic acids were identified: *trans*-caftaric acid and *cis*- and *trans*-coutaric acid. Quantification was carried out using external calibration curves plotted using gradient high performance liquid chromatography with UV detection. The chromatogram was recorded at 320 nm. Calibration curves were plotted using *trans*-caftaric acid (purity 97%), purchased from Sigma, Germany (k = 96,945, R2 = 99.99%, DL = 0.05 mg/L, QL = 0.15 mg/L) and caffeic acid (purity 98%), purchased from Sigma, Germany (k = 122,265, R2 = 99.83%, DL = 0.03 mg/L, QL = 0.1 mg/L). The stability of prepared samples was shown to be at least 48 h. The relative standard deviations of observed peak areas made by six injections of the same sample were: *trans*-caftaric acid, 1.73%, *cis*-coutaric acid, 5.12% and *trans*-coutaric acid, 3.64%. The instrumentation used and the separation parameters were the same as for flavonol determination.

4.1.3.6 Statistical analysis

Phenolic composition data were statistically analysed using one-way ANOVA to test the significance of treatments at different stages of berry ripening ($P < 0.05$). Student–Newmann–Keuls test of multiple comparisons of mean values was performed to separate the averages. In order to perform these tests the Statistica software package was used to carry out calculations.

4.1.4 Results and discussion

The effects of leaf removal were related to yield and canopy density, thus the vineyards at the two locations showed different characteristics most probably linked to the soil, row orientation, exposition and plant winter/green management as reported by de Andrés-de Prado et al. (2007), Intrieri et al. (1996) and Jackson and Lombard (1993).

The values of leaf area and yield revealed a good equilibrium between the parameters in both vineyards and for all treatments, although differences between the two locations were found (data not reported). In the Potoce vineyard leaf area was about 48% higher than in Podraga, with abundant growth of lateral shoots in Potoce. Leaf removal did not significantly affect

grape production. The values of leaf area-to-yield ratio were in the optimal range as reported by Smart (1985).

4.1.4.1 Berry size and ripening degree

One very important parameter that needs to be taken into account when comparing leaf removal treatments and their final influence on wine colour characteristics is berry weight, since there are stresses and sink/source effects that can significantly modify the increase in volume (Coombe, 1989). Metabolite concentration can change in relation to berry size as well as skin to flesh ratio (and therefore skin to must ratio).

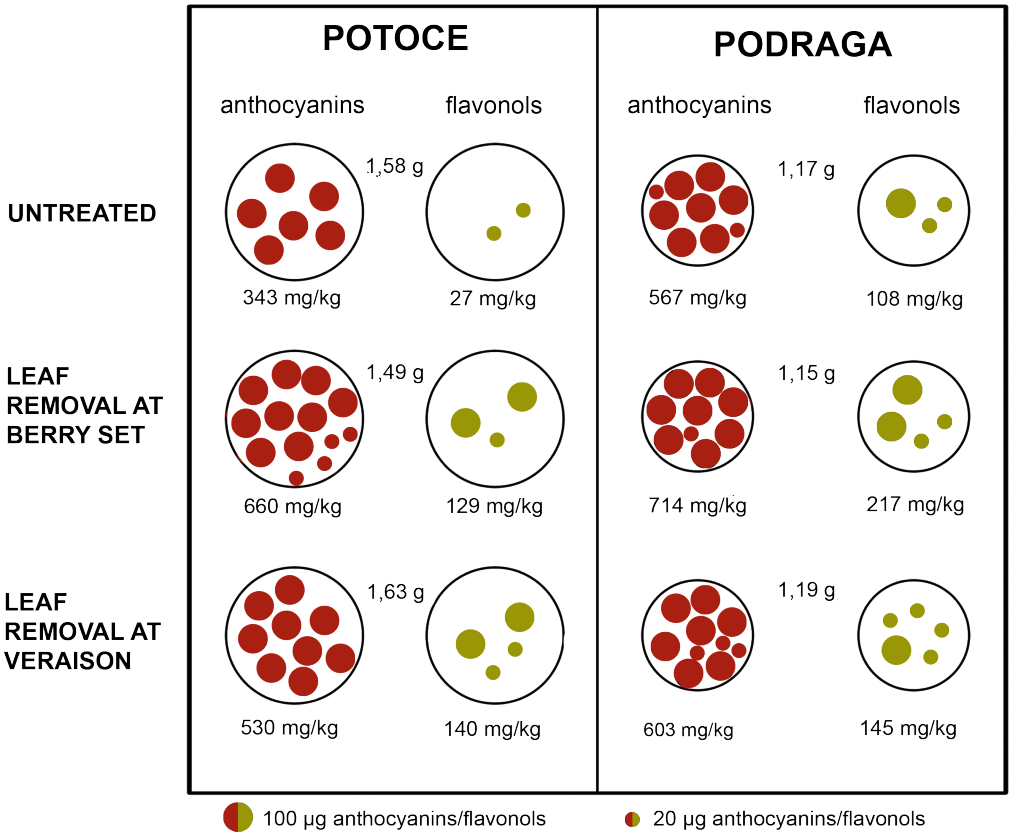


Figure 9: Effects of leaf removal treatments on anthocyanin and flavonol occurrence in grapes. Large white circles represent berries (size-proportional). Black circles represent x-berry amounts of anthocyanins and flavonols. The concentration is given outside the circles together with berry size.

When leaf removal was performed at véraison in both of the observed vineyards an increase in berry weight was observed (harvest data are reported in Figure 9), while an opposite result was obtained for BS treatment. There are contrasting results in the literature as regards the effects of

leaf removal on berry size. Poni et al. (2006) and Ollat and Gaudillere (1998) reported a reduction in berry weight when leaf removal was carried out at berry set, but other authors did not find any differences (Tardaguila et al., 2008; Cortell and Kennedy, 2006; Chorti et al., 2010). Belvini et al. (2010) highlighted that leaf removal could be stressful for grapevines when coupled with shoot trimming (too strong reduction in leaf area).

Coombe (1989) also reported that a strong reduction in leaf area could positively affect berry weight, since solutes are redirected to berries, thus allowing water to enter them in order to equilibrate osmotic potential. The sugar loading values support this suggestion, being higher with VE treatment in both locations (data not reported).

With the aim of supporting this suggestion, a small trial was carried out in 2010, comparing leaf removal at veraison with or without coupling to shoot trimming, thus demonstrating that an increase of berry weight could happen (unpublished data).

The trend of sugar accumulation and acidity degradation was comparable between both locations, although significant differences in values were highlighted. Ripening degree observed in Potoce was 22.4 Brix and 5.31 g/L of titratable acidity, while in Podraga was 23.6 Brix and 5.98 g/L of titratable acidity. Most probably the higher vineyard altitude, the lower yield and smaller berry size can help to explain the better ripening degree showed for Podraga grapes.

4.1.4.2 Anthocyanins, flavonols and hydroxycinnamic acids

Leaf removal significantly affected the concentration of anthocyanins in berries, both when performed at berry set and at véraison, but was more significant in the former case.

Looking at the profile of anthocyanins, in both locations the effect of leaf removal timing was similar, although in Podraga (Table 3) the highest values of Del-3-Glu and Pet-3-Glu were shown in the berry skins with BS treatment, followed by VE and UN. The concentration of Peo-3-Glu was higher with UN treatment (although not significant at harvest), while BS showed the highest values of Mal-3-Glu as compared to both VE and UN treatments.

Comparing these trends with Potoce (Table 4), the exception was Cy-3-Glu, since its concentration was higher with BS treatment as compared to both UN and VE treatment. Peo-

3-Glu was higher during maturation with UN and BS treatments, but at harvest time higher values were maintained only for BS.

The trends for single anthocyanins as shown in the two locations may explain the differences in total anthocyanins (Figure 10), probably related to the canopy structure (Cortell et al., 2005).

In Podraga, the leaf area was smaller and light therefore entered the canopy more easily, reaching the clusters. Leaf removal probably increased the amount of light to clusters (data not available), but was not so effective as in Potoce, where leaf area nearly double.

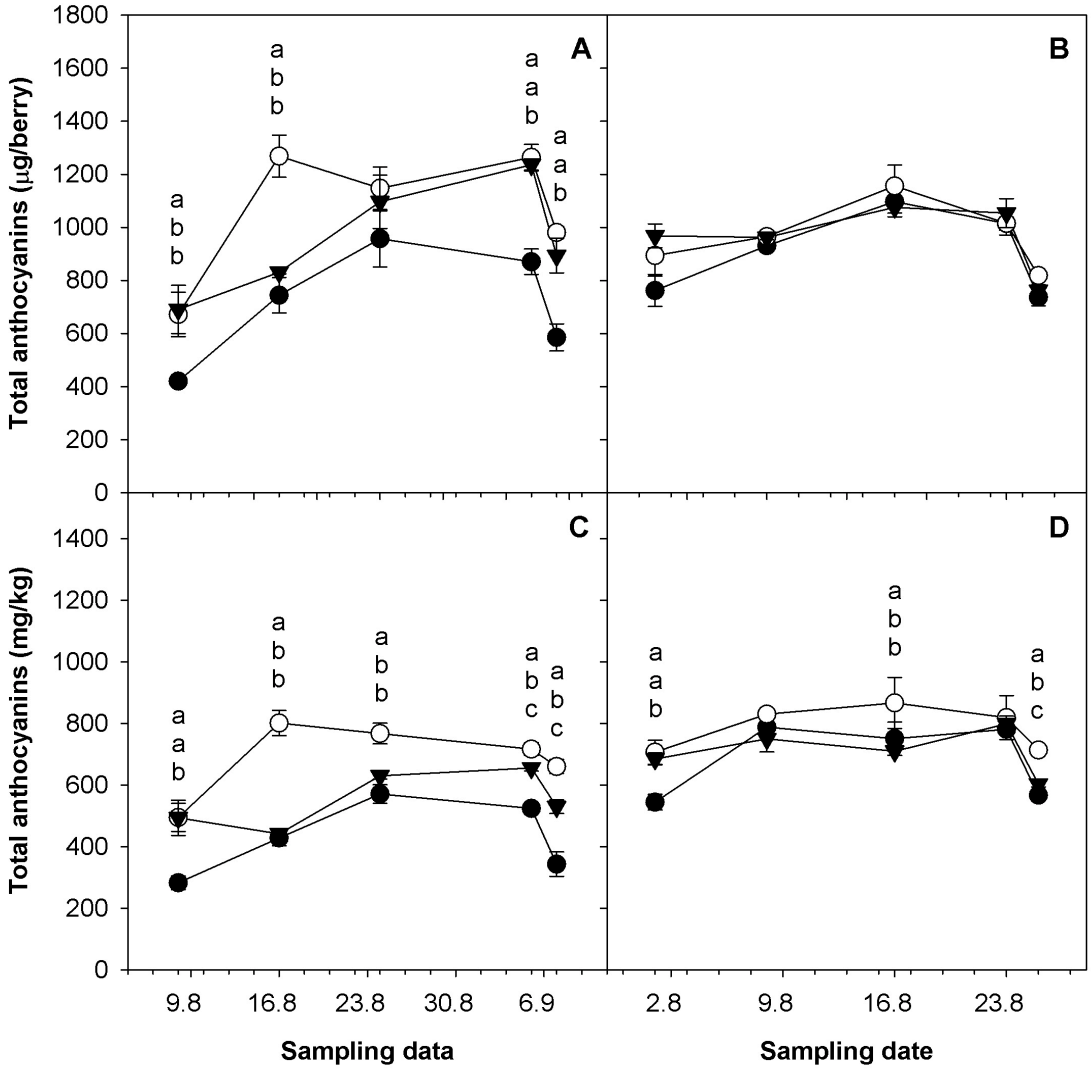


Figure 10: Effect of leaf removal treatments on anthocyanins amounts x-berry (A and B) and concentration (C and D) at the two vineyards. ANOVA to compare data (P indicated); n = 3. Means separated using Student–Newmann–Keuls test.

Table 3: Anthocyanin profiles (mg/kg) as affected by leaf removal at the Podraga vineyard. ANOVA to compare data (P indicated); n=3. Means were separated using Student-Newmann-Keuls test.

	Sampling dates				
Delphinidin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	33.1 b	48.6	32.3 b	35.4 b	24.1 c
Berryset leaf removal (BS)	59.0 a	49.8	50.5 a	41.8 a	34.7 a
Veraison leaf removal (VE)	58.5 a	44.9	39.3 b	39.6 a	29.3 b
F	70.5	1.42	12.5	7.49	15.1
sign F	0.000 ***	0.341 n.s.	0.007 **	0.023 *	0.005 **
Cyanidin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	5.15 c	21.4 a	20.1	25.5 a	11.3
Berryset leaf removal (BS)	9.92 b	10.7 c	18.8	19.2 c	11.5
Veraison leaf removal (VE)	12.3 a	15.5 b	20.1	23.3 b	11.8
F	320	30.7	0.69	34.4	0.25
sign F	0.000 ***	0.004 **	0.538 n.s.	0.000 ***	0.786 n.s.
Petunidin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	41.3 b	62.8	44.5 b	48.0	32.4 c
Berryset leaf removal (BS)	69.2 a	67.2	67.0 a	55.0	46.5 a
Veraison leaf removal (VE)	68.3 a	59.7	51.9 b	51.8	38.3 b
F	66.6	1.10	10.5	4.41	27.3
sign F	0.000 ***	0.416 n.s.	0.011 *	0.066 n.s.	0.001 ***
Peonidin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	80.4 c	179 a	215 a	258 a	169
Berryset leaf removal (BS)	91.5 b	101 b	166 b	182 b	164
Veraison leaf removal (VE)	103 a	113 b	167 b	204 b	164
F	20.2	52.9	18.4	21.1	0.19
sign F	0.002 **	0.001 **	0.003 **	0.002 **	0.835 n.s.
Malvidin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	385 b	477 b	439 b	414 b	331 b
Berryset leaf removal (BS)	477 a	602 a	565 a	521 a	457 a
Veraison leaf removal (VE)	443 a	518 b	431 b	480 b	359 b
F	16.4	14.0	9.44	10.4	48.8
sign F	0.004 **	0.016 *	0.014 *	0.011 *	0.000 ***

Asterisks indicate significant differences among the treatments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Means followed by the same letter or no letters are not significantly different.

Table 4: Anthocyanin profiles (mg/kg) as affected by leaf removal at the Potoce vineyard. ANOVA to compare data (P indicated); n=3. Means were separated using Student-Newmann-Keuls test.

	Sampling dates				
Delphinidin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	13.4 b	19.0 b	21.3 c	13.9 b	8.31 b
Berryset leaf removal (BS)	29.9 a	52.1 a	44.2 a	28.5 a	30.0 a
Veraison leaf removal (VE)	24.7 a	23.8 b	36.4 b	29.6 a	26.6 a
F	*23.8*	*83.1*	*36.1*	*96.5*	*47.0*
sign F	*0.001* **	*0.000* ***	*0.000* ***	*0.000* ***	*0.000* ***
Cyanidin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	2.24 b	5.10 b	10.7 b	15.9	8.49 b
Berryset leaf removal (BS)	4.33 a	13.1 a	13.5 a	16.0	11.8 a
Veraison leaf removal (VE)	3.56 a	5.13 b	10.8 b	17.6	9.61 b
F	*20.9*	*69.0*	*6.23*	*2.44*	*8.03*
sign F	*0.002* **	*0.000* ***	*0.034* *	*0.168 n.s.*	*0.020* *
Petunidin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	17.6 b	24.7 b	26.4 c	18.6 b	12.6 b
Berryset leaf removal (BS)	38.1 a	64.6 a	56.3 a	37.8 a	38.5 a
Veraison leaf removal (VE)	31.5 a	29.1 b	46.3 b	38.9 a	34.0 a
F	*16.0*	*168.3*	*55.0*	*175*	*57.2*
sign F	*0.004* **	*0.000* ***	*0.000* ***	*0.000* ***	*0.000* ***
Peonidin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	45.4 b	86.4 b	172 a	201 a	125 b
Berryset leaf removal (BS)	78.7 a	134 a	168 a	213 a	180 a
Veraison leaf removal (VE)	73.0 a	83.6 b	121 b	179 b	113 b
F	*16.2*	*35.7*	*71.6*	*13.4*	*32.6*
sign F	*0.004* **	*0.027* *	*0.000* ***	*0.006* **	*0.000* ***
Malvidin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	204 b	293 b	341 c	274 c	189 c
Berryset leaf removal (BS)	344 a	537 a	485 a	421 a	399 a
Veraison leaf removal (VE)	361 a	300 b	416 b	390 b	346 b
F	*26.1*	*251*	*34.7*	*210*	*69.6*
sign F	*0.001* **	*0.000* ***	*0.000* ***	*0.000* ***	*0.000* ***

Asterisks indicate significant differences among the treatments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Means followed by the same letter or no letters are not significantly different.

In order to describe the light environment around clusters in both vineyards, another class of polyphenols more sensitive to light changes - flavonols (Haselgrove et al., 2000; Makris et al., 2006; Price et al., 1995) was examined. As expected, the differences in flavonol contents among treatments were amplified as compared to anthocyanins. As flavonols are synthesised earlier than anthocyanins (Boss et al., 1996), they can profit more by better cluster exposure (Downey et al., 2006).

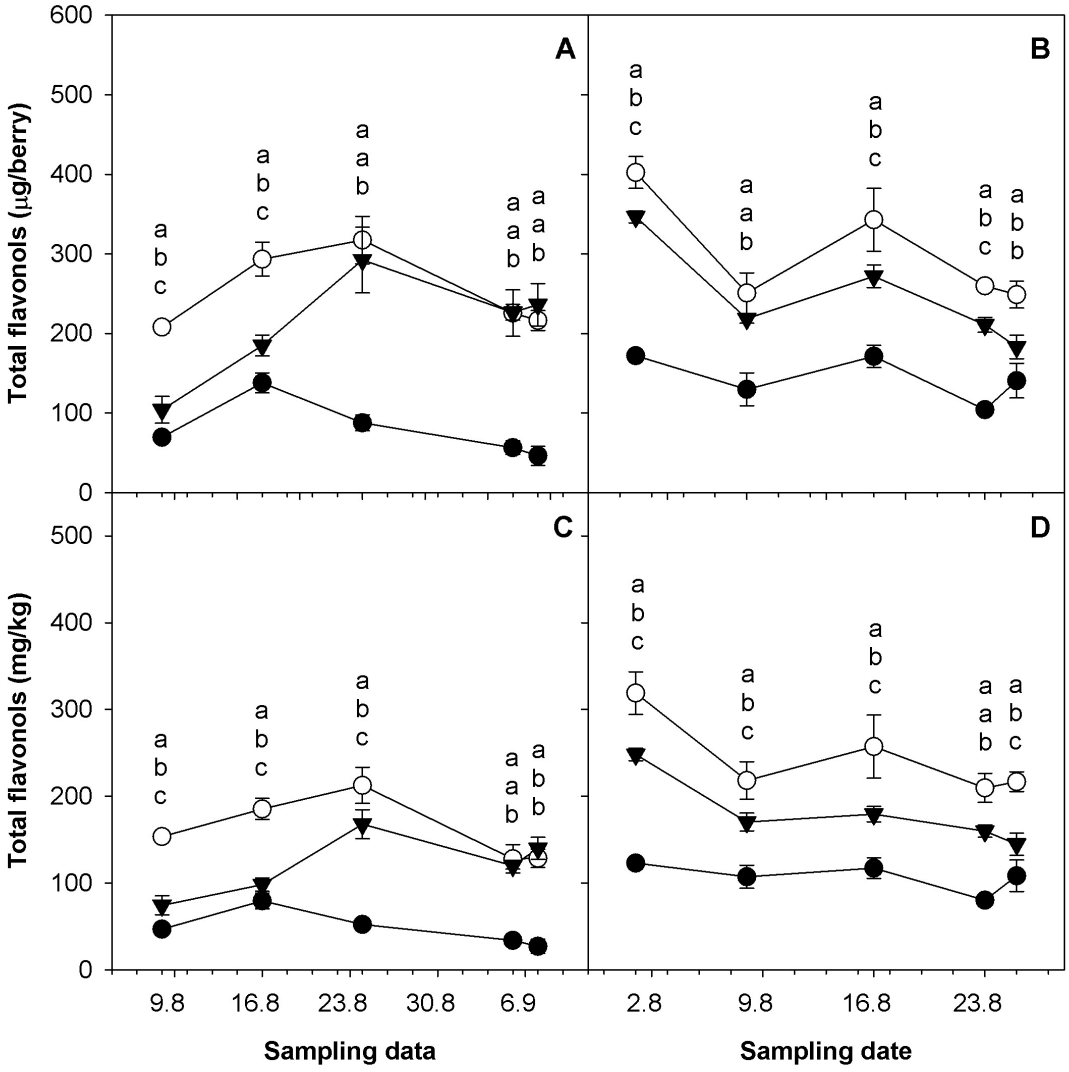


Figure 11: Effect of leaf removal treatments on flavonols amounts x-berry (A and B) and concentration (C and D) at the two vineyards. ANOVA to compare data (P indicated); n = 3. Means separated using Student–Newmann–Keuls test.

In the Podraga vineyard, Qu-3-Glu was shown to be more enhanced by BS leaf removal, however VE had less effect (Table 5). My-3-Glu and Ka-3-Glu both profited in the case of BS and VE leaf removal treatments with higher values as compared to UN. Observing the total amount of flavonols for this location (Fig 11 B and D), the concentration reduced slowly

during maturation, being the highest for BS, followed by VE and UN treatments. In Potoce Qu-3-Glu and Ka-3-Glu were shown to be the highest in BS at the beginning of maturation, but thereafter the concentration increased significantly for VE treatment, reaching comparable values at harvest time (Table 5). At the last stages of maturation, My-3-Glu occurred with higher values for VE and lower for BS treatment.

The total concentration of observed flavonols highlighted the differences as shown for Qu 3-Glu, thus the amount was the highest for BS treatment at the beginning of ripening, while at the end VE and BS were found to be similar, because the concentration increased significantly with VE treatment (Figs. 11A and C).

Hydroxycinnamic acids were the third group of compounds analysed. Both in Podraga and Potoce, BS treatment was again demonstrated to be very effective in enhancing the concentration of trans-caftaric but also cis- and trans-coutaric acids throughout maturation (Tables 4 and 5 – supplementary data). For this class of polyphenols, leaf removal was less effective than for anthocyanins and flavonols.

The differences highlighted between treatments and locations may affect both berry size (Rodriguez Montealegre et al., 2006) and changes in flavonoid biosynthesis (Castellarin et al., 2007). In order to verify synthesis changes, anthocyanins and flavonols were calculated both in terms of concentration (mg/kg) and on a x-berry basis (mg/berry). Looking at anthocyanins, only in the Potoce vineyard (Figs. 10 A and C) it was possible to attribute the concentration change to higher synthesis, while in Podraga (Figs. 10 B and D) the differences were mainly related to changes in berry size. Increased berry size occurred with VE treatment could account for the specific trends between amount x berry (Figs. 10A and B) and concentration (Figs. 10C and D).

For flavonols (Fig. 11), both more synthesis and berry size effects must be taken into account. Again in this case, bigger berries with VE treatment can explain some differences in both vineyards, but increased synthesis occurred for both leaf removal treatments, being higher for BS. An attempt to obtain overall characterisation of flavonol and anthocyanin occurrence in grapes at harvest can be seen in Fig. 9. As previously reported, the extent of changes to anthocyanins and flavonols due to leaf removal were shown to be related to the characteristics of the two vineyards, thus

Table 5: Flavonol profiles (mg/kg) as affected by leaf removal at the Podraga and Potoce vineyards. ANOVA to compare data (P indicated); n = 3. Means separated using Student–Newmann–Keuls test.

PODRAGA VINEYARD	Sampling dates				
Myricetin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	13,1 c	11,1 b	11,1 b	8,25 b	10,1 b
Berryset leaf removal (BS)	25,6 a	21,0 a	25,5 a	21,2 a	19,8 a
Veraison leaf removal (VE)	37,6 b	22,8 a	21,9 a	22,0 a	17,2 a
F	271	31,5	15,9	138	27,1
sign F	0,000 ***	0,000 ***	0,004 **	0,000 ***	0,000 ***
Quercetin-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	90,5 c	73,6 c	82,2 c	52,3 c	77,2 c
Berryset leaf removal (BS)	262 a	169 a	198 a	157 a	166 a
Veraison leaf removal (VE)	175 b	120 b	130 b	107 b	101 b
F	204	43,6	33,0	107	43,2
sign F	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
Kaempferol-3-glucoside	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	4,57 b	7,01 b	8,03 b	6,09 b	9,94 b
Berryset leaf removal (BS)	11,8 ab	7,12 b	13,3 a	12,4 a	14,0 a
Veraison leaf removal (VE)	16,8 a	9,55 a	14,4 a	12,7 a	13,2 a
F	7,23	6,81	31,1	69,2	18,3
sign F	0,025 *	0,029 *	0,000 ***	0,000 ***	0,003 **

POTOCE VINEYARD	Sampling dates				
Myricetin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	3,11 b	5,20 b	4,95 c	4,09 c	2,52 c
Berryset leaf removal (BS)	8,48 a	26,71 a	14,2 b	11,4 b	11,2 b
Veraison leaf removal (VE)	8,45 a	5,17 b	20,4 a	16,8 a	20,5 a
F	68,6	216	36,6	168	41,1
sign F	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
Quercetin-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	35,2 c	61,2 c	34,5 c	19,8 c	15,4 b
Berryset leaf removal (BS)	124 a	128 a	164 a	92,2 a	87,8 a
Veraison leaf removal (VE)	47,2 b	79,4 b	117 b	74,4 b	90,9 a
F	288	49,2	82,6	67,6	86,9
sign F	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
Kaempferol-3-glucoside	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	0,84 c	3,48 b	4,06 b	3,14 c	2,58 b
Berryset leaf removal (BS)	7,32 a	13,4 a	16,8 a	12,2 b	13,8 a
Veraison leaf removal (VE)	4,64 b	3,77 b	17,4 a	15,4 a	14,0 a
F	24,6	101	60,2	97,6	57,3
sign F	0,001 **	0,000 ***	0,000 ***	0,000 ***	0,000 ***

Asterisks indicate significant differences among the treatments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Means followed by the same letter or no letters are not significantly different.

highlighting once again the importance of light on phenolic events (Bergqvist et al., 2001; Chorti et al., 2010; Cortell et al., 2005; Downey et al., 2006). Comparing UN with the other two treatments (amount x berry) in Potoce, a significant increase in both anthocyanin and flavonol synthesis could be seen. At this location anthocyanin content increased by 63% and 49% with BS and VE treatments, respectively.

For flavonols (Fig. 11), both more synthesis and berry size effects must be taken into account. Again in this case, bigger berries with VE treatment can explain some differences in both vineyards, but increased synthesis occurred for both leaf removal treatments, being higher for BS. An attempt to obtain overall characterisation of flavonol and anthocyanin occurrence in grapes at harvest can be seen in Fig. 9. As previously reported, the extent of changes to anthocyanins and flavonols due to leaf removal were shown to be related to the characteristics of the two vineyards, thus highlighting once again the importance of light on phenolic events (Bergqvist et al., 2001; Chorti et al., 2010; Cortell et al., 2005; Downey et al., 2006). Comparing UN with the other two treatments (amount x berry) in Potoce, a significant increase in both anthocyanin and flavonol synthesis could be seen. At this location anthocyanin content increased by 63% and 49% with BS and VE treatments, respectively.

In Podraga, a lower increase of anthocyanins was obtained with BS (+10%) or VE (+0.01%) treatments as compared with UN. For both vineyards, leaf removal treatments enhanced flavonol synthesis to a greater extent as compared to anthocyanins. The increase of synthesis in Potoce was very significant for BS (+450%) and VE (+500%) treatments as compared to UN, while in Podraga the magnitude of differences with UN were reduced both for BS (+71%) and VE (+29%) treatments. A ratio describing the relative increase in flavonols and anthocyanins (FLA/ANT) during maturity was calculated. At harvest the index was higher in leaf removal treatments, thus highlighting the greater influence of light on flavonols. The index values were much lower in Potoce than Podraga (except for VE treatment = 0.26 and 0.24 in Potoce and Podraga, respectively), supporting the suggestion that the canopy could significantly affect the light environment thus reducing flavonol accumulation (Cortell et al., 2005). In Potoce, for BS and UN treatments the index was lower than in VE (BS = 0.19; UN = 0.08), probably due to new growth of lateral shoots causing clusters to be shadowed again. In Podraga the index values were higher than in Potoce (not for VE). With BS the value was the highest (0.30) as compared to VE (0.24) or UN (0.19). During maturation the ratio was similar for VE

and BS treatments but much higher than for UN. These results agree with the trends explained for anthocyanins and flavonols on a x-berry basis (Figs 10 A and B; 11A and B).

4.1.5 Conclusions

Viticultural practices affecting cluster microclimate may result in different phenolic accumulation trends related to site, row orientation, exposition and canopy architecture. In Potoce and Podraga, the relative importance of leaf area created particular characteristics in terms of canopy light environment, thus promoting site effects in terms of phenolic occurrence. In Potoce, the grapes responded significantly to leaf removal, allowing anthocyanins and flavonols to accumulate; in Podraga only flavonols were affected significantly, probably due to higher sensitivity to light enhancement. Leaf removal at berry set was shown to affect both anthocyanin and flavonol concentration very positively. Cluster exposure was shown to affect the three classes of polyphenols differently. Flavonols were found to respond significantly to leaf removal, while anthocyanins were affected to an intermediate extent and hydroxycinnamic acids changed only slightly. These results demonstrate that this practice can promote vineyard-specific outcomes in different locations within the same viticultural area, once again revealing the importance of individual canopy management in order to improve grape quality characteristics.

4.1.6 Acknowledgments

We thank Tilia and Burja Wine Estates for the use of their vineyards.

4.1.7 Supporting information

Apendix A / Figure 1_S1: Hydroxycinnamic acid profiles (mg/kg) as affected by leaf removal at the Podraga vineyard. Means were separated using Student-Newmann-Keuls test (P<0,05).

Appendix B / Figure 1_S2: Hydroxycinnamic acid profiles (mg/kg) as affected by leaf removal at the Potoce vineyard. Means were separated using Student-Newmann-Keuls test (P<0,05).

4.1.8 References

de Andrés-de Prado, R., Yuste-Rojas, M., Sort, X., Andrés-Lacueva, C., Torres, M., Lamuela-

Raventós, R.M., 2007. Effect of soil type on wines produced from *Vitis vinifera* L. cv. Grenache in commercial vineyards. *Journal of Agricultural and Food Chemistry* 55 (3), 779–786.

Asen, S., Stewart, R.N., Norris, K.H., 1972. Co-pigmentation of anthocyanins in plant tissue and its effect on color. *Phytochemistry* 11, 1139–1144.

Belvini, P., Pascarella, G., Dalla Costa, L., Sivilotti, P., 2010. Con interventi al verde su vite aumenta l'efficienza delle foglie. *Informatore Agrario* 66 (17), 46–50.

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. *American Journal of Enology and Viticulture* 52 (1), 1–6.

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 for the pathway regulation. *Plant Physiology* 111, 1059–1066.

Boulton, R., 2001. The copigmentation of anthocyanins and its role in color of red wine: a critical review. *American Journal of Enology and Viticulture* 52 (2), 67–87.

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

Chorti, E., Guidoni, S., Ferrandino, A., Novello, V., 2010. Effect of Different cluster sunlight exposure levels on ripening and anthocyanin accumulation in Nebbiolo grapes. *American Journal of Enology and Viticulture* 61 (1), 23–30.

Coombe, B.G., 1989. The grape berry as a sink. *Acta Horticulturae* 239, 149–158.

Cortell, J.M., Halbleib, M., Gallagher, A.V., Righetti, T.L., Kennedy, J.A., 2005. Influence of vine vigor on grape (*Vitis vinifera* L. Cv. Pinot Noir) and wine proanthocyanidins. *Journal of Agricultural and Food Chemistry* 53 (14), 5798–5808.

Cortell, J.M., Kennedy, J.A., 2006. Effect of shading on accumulation of flavonoid compounds in (*Vitis vinifera* L.) Pinot Noir fruit and extraction in a model system. *Journal of Agricultural and Food Chemistry* 54, 8510–8520.

Downey, M.O., Harvey, J.S., Robinson, S.P., 2004. The effect of bunch shading on berry development and flavonol accumulation in Shiraz grapes. *Australian Journal of Grape and Wine Research* 10 (1), 55–73.

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. *American Journal of Enology and Viticult*ure 57 (3), 257–268.

Guidoni, S., Ferrandino, A., Novello, V., 2008. Effects of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. *American Journal of Enology and Viticulture* 59 (1), 22–29.

Haselgrove, D., Botting, B., Heeswijck, R., Høj, P.B., Dry, P.R., Ford, C., Land, P.G.I., 2000. Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of *Vitis vinifera* L. cv Shiraz grape berries. *Australian Journal of Grape and Wine Research* 6 (2), 141–149.

Intrieri, C., Silvestroni, O., Rebucci, B., Poni, S., Filippetti, I., 1996. The effects of row orientation on growth yield, quality and dry matter partitioning in Chardonnay vines trained to simple curtain and spur-pruned cordon. In: Proceedings of the 4th International Cool Climate

Viticulture & Enology Symposium, Rochester, NY, USA, pp. 10–15.

Jackson, D.I., Lombard, P.B., 1993. Environmental and management practices affecting grape composition and wine quality. *American Journal of Enology and Viticulture* 44 (4), 409–430.

Jones, G.V., Davis, R.E., 2000. Climate influences on grapevine phenology, grape composition, and wine production and quality for Bordeaux, France. *American Journal of Enology and Viticulture* 51 (3), 249–261.

Jeong, S.T., Goto-Yamamoto, N., Hashizume, K., Esaka, M., 2008. Expression of multicopy flavonoid pathway genes coincides with anthocyanin, flavonol and flavan-3-ol accumulation in grapevine. *Vitis* 47 (3), 135–140.

Košir, I.J., Lapornik, B., Andrenšek, S., Golc-Wondra, A., Vrhovsek, U., Kidrič, J., 2004. Identification of anthocyanins in wines by liquid chromatography, liquid chromatography–mass spectrometry and nuclear magnetic resonance. *Analytica Chimica Acta* 513 (1), 277–282.

Makris, D.P., Kallithraka, S., Kefalas, P., 2006. Flavonols in grapes, grape products and wines: burden, profile and influential parameters. *Journal of Food Composition and Analysis* 19, 396–404.

Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., Velasco, R., 2006. Metabolite profiling of grape: flavonols and anthocyanins. *Journal of Agricultural and Food Chemistry* 54, 7692–7702.

Mozetič, B., Trebše, P., Hribar, J., 2006. Determination and quantitation of anthocyanins and hydroxycinnamic acids in different cultivars of sweet cherries (*Prunus av*ium L.) from Nova Gorica region (Slovenia). *Food Technology and Biotechnology* 40 (3), 207–212.

Ollat, N., Gaudillere, J.P., 1998. The effect of limiting leaf area during stage I of berry growth on development and composition of berries of *Vitis vinifera* L. cv Cabernet Sauvignon. *American Journal of Enology and Viticulture* 49 (3), 251–258.

Poni, S., Casalini, L., Bernizzoni, F., Civardi, S., Intrieri, C., 2006. Effects of early defoliation on shoot photosynthesis yield components, and grape composition. *American Journal of Enology and Viticulture* 57 (4), 397–407.

Price, S.F., Breen, P.J., Valladao, M., Watson, B.T., 1995. Cluster sun exposure and quercetin in Pinot Noir grapes and wine. *American Journal of Enology and Viticulture* 46 (2), 187–194.

Robins, R.J., Bean, S.R., 2004. Development of a quantitative high-performance liquid chromatography–photodiode array detection measurement system for phenolic acids. *Journal of Chromatography A* 1038, 97–105.

Rodriguez Montealegre, R., Romero Peces, R., Chaco`n Vozmediano, J.L., Martinez

Guascuenã, J., Garcia Romero, E., 2006. Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in a warm climate. *Journal of Food Composition and Analysis* 19, 687–693.

Shaulis, N.J., Amberg, H., Crowe, D., 1966. Response of concord grapes to light exposure and Geneva double curtain training. In: Proceedings of the American Society for Horticultural Science, vol. 89. pp. 268–280.

Singleton, V.L., Timberlake, Z.F., Lea, A.G.H., 1978. The phenolic cinnamates of white grapes and wines. *Journal of the Sciences of Food of Agriculture* 29, 403–410.

Smart, R.E., 1985. Principles of grapevine canopy microclimate manipulation with implications for yield and quality: a review. *American Journal of Enology and Viticulture* 36 (3), 230–239.

Spayd, S.E., Tarara, J.M., Mee, D.L., Fergusson, J.C., 2002. Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. *American Journal of Enology and Viticulture* 53 (3), 171–182.

Tarara, J.M., Lee, J., Jungmin, L., Spayd, S.E., Scagel, C.F., 2008. Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in Merlot grapes. *American Journal of Enology and Viticulture* 59 (3), 235–247.

Tardaguila, J., Diago, M.P., Martinez de Toda, F., Poni, S., Vilanova, M., 2008. Effects of timing of leaf removal on yield, berry maturity, wine composition and sensory properties of cv. Grenache grown under non irrigated conditions. *Journal Internation de la Science de la Vigne et du Vin* 42 (4), 221–229.

Trost, K., Golc-Wondra, A., Prošek, M., Milivojević, L., 2008. Anthocyanin degradation of blueberry-aronia nectar in glass compared with carton during storage. *Journal of Food Science* 73 (8), 405–411.

4.2 Early versus late leaf removal strategies for 'Pinot Noir' (*Vitis vinifera* L.): effect on colour-related phenolics in young wines following alcoholic fermentation (Sternad Lemut et al., 2013a)

4.2.1 Abstract

BACKGROUND: The widely adopted viticultural practice of late (véraison) leaf removal is now losing many of its advantages as a result of today's warmer vineyard conditions. With the aim of seeking a good alternative, the influence of earlier leaf removals (at pre-flowering and berry-set) on colour-related phenolics in young Pinot Noir wines was investigated in the years 2009 and 2010.

RESULTS: Total flavonols in 2009 wines were 71 and 52% higher in case of véraison and berry-set treatments respectively as compared with untreated controls, while in 2010 the average content of flavonols was highest with pre-flowering leaf removal (75% higher than controls). The anthocyanin content in 2009 wines was 18 and 11% higher in case of véraison and berry-set treatments respectively and was favoured by early leaf removals in 2010 (50 and 43% higher in case of berry-set and pre-flowering treatments respectively) as compared with controls. Changes in hydroxycinnamic acid profiles were shown to be greatest in 2010 wines resulting from early leaf removal treatments. Promoted formation of vitisin A-like pigments in 2010 leaf removal treatments was observed during fermentation.

CONCLUSIONS: The phenolic profiles of grapes/wines were affected by leaf removal timing, although differently in two (extremely different) seasons. Earlier leaf removal strategies showed some promising results, with good proportions mainly of flavonols and anthocyanins, retained also in young wines. Vitisins A in wines were positively affected by all leaf remova

Keywords: 'Pinot Noir', global warming, pre-flowering leaf removal, berry-set leaf removal, phenolic profiles, pyranoanthocyanins.

4.2.2 Introduction

The profiles of the main technologically important phenolic compounds in grapes are not only genotype-dependent but can also be significantly affected by the manipulation of grapevine environmental conditions.[1–3] Although winegrowers obviously cannot influence the vintage/regionmacroclimate or site/vineyard mesoclimate conditions, it is intheir power to significantly improve microclimate conditions within cluster areas by carefully selecting viticulture techniques and moreover choosing the timing of their implementation at different grape development stages.

Leaf removal (defoliation) is known to be one of the viticultural practices enabling successful canopy microclimate manipulation, leading to some important grape quality improvements,[1,4,5] although earlier application (at pre-flowering and berry-set) has been less studied and there is still little information on its influence on wines from different grapevine varieties.[6] Within the context of global warming, many winegrowers are already facing the problem of a loss of effectiveness of widely adopted late (véraison) leaf removal, due to higher temperatures and severe UV exposure after canopy opening, leading to harmful sunburns on the grape berry skin tissue[7] as well as inhibition of the biosynthesis of some important phenolic compounds (e.g. anthocyanins).8 On the other hand, earlier leaf removal alternatives need to be well explored on a 'from grapes to wine' basis before their adaptation to any grapevine variety can be justified.

Any significant vineyard management-related improvements in grape composition would normally be aimed to be reflected also in the wines,[9] but it is known that they can significantly or even totally lose their importance during the winemaking processes.The initial quantitative and qualitative input of flavonols, anthocyanins and hydroxycinnamic acids is probably most relevant for the development of red wine colour characteristics, which are particularly important for the 'Pinot Noir' (*Vitis vinifera* L.) grapevine variety, known for its poor (genetically derived) anthocyanin potential. Thus any improvement in anthocyanin quantitative and/or qualitative profiles may be of considerable importance for Pinot Noir, even in terms of the simple anthocyanin glucosides normally found in relatively large quantities in other young red wines.[9] Better anthocyanin yield combined with improvements in other colour-related phenolics in young wines can offer greater potential for further formation of complex and more stable pigments such as pyranoanthocynanins and other related pigments,[10–13] thus improving wine colour stability over time. According to the literature,[14] there are two crucial factors for

the formation of pyranoanthocyanins: anthocyanins (with a minimum concentration corresponding to 18.5 mg L−1 as malvidin-3-glucoside[15]) and their reaction partners (such as hydroxycinnamic acids, vinylphenols, acetone and some further enolisable molecules).

Apart from several oenological variables affecting the final yield of phenolics in wine during maceration/fermentation, their location in grape berry tissue and their chemical interactions (with each other and with other grape berry compounds) are known to limit the results in wine. Furthermore, some phenolic compounds are reported to be absorbed by the yeasts during fermentation, although with different intensities for individual compounds.[16] However, very little is known about the direct and/or indirect influence of early defoliation on reaction dynamics/chemical formation during coldmaceration and alcoholic fermentation and finally the yield of colour-related phenolics in young 'Pinot Noir' wines. This experiment was thus designed to reveal the reflection of vineyard microclimate modification efforts on the fate of related phenolics during typical Pinot Noir vinification processes.

4.2.3 Material and methods

4.2.3.1 Experimental vineyard and plant material

The experiment was carried out in two consecutive years (2009 and 2010), observing 320 (240 in 2009) 'Pinot Noir' (*V. vinifera* L.) grapevines in total, located in the Vipava Valley (Slovenia) at the Potoce vineyard. The vineyard was planted in 2004 and is Guyot trained, with a plant density of 5682 plants ha^{-1} (0.8 m x 2.2 m), an altitude of 95 m a.s.l. and E–W row orientation. A completely randomised experimental design was set up with 16 plots of five vines per treatment. Leaf removal was performed manually, removing the basal four to six leaves from all shoots. In 2009, defoliation was performed at berry-set (BS) (phonological stage BBCH 71) and at véraison (VE) (BBCH 83), while one-third of the experimental vines was left untreated (UN) and used as a control. In 2010 a new and innovative viticulture technique of preflowering (PF) leaf removal (BBCH57) was added to the observation and compared with the berry-set, véraison and control treatments.

4.2.3.2 Must fermentation and sampling

Grapes from all treatments were collected (in biological triplicates) separately at optimum maturity (based on the maturity level registered on UN grapes: 22 °Brix and 5.5 g L^{-1} titratable

acidity on average), which was reached on 6th and 20th September in 2009 and 2010 respectively. All grape samples were first de-stemmed, crushed, protected with 0.1 g kg^{-1} K_2S2O_5 and mixed, before 20 L of the pomace was separated and placed in 25 L stainless steel experimental tanks (in total, 3 tanks x 20 L of pomace for each treatment, corresponding to biological triplicates collected from the vineyard). Experimental cold maceration and alcoholic fermentation were performed in the temperature-controlled chambers of the Wine Research Cellar, University of Nova Gorica, Slovenia. Each 20 L batch was initially cooled to 5 °C and then subjected to typical Pinot Noir winemaking treatments, starting with 48 h of cold maceration (in a chamber set to 5 °C) and followed by induced alcoholic fermentation once the temperature of the pomace had been brought back to room temperature (in a chamber set to 20 °C). The yeast strain Saccharomyces cerevisiae 'Fermol Premier Cru' type (AEB Group, Brescia, Italy) was used to start fermentation (0.2 g L^{-1}) and the yeast nutrient Fermoplus Starter (AEB Group) was added (0.3 g L^{-1}) to achieve better continuation of the fermentation processes. Skin caps were pushed down and mixed every 12 h, while the fermentation curve data (temperature, sugar level/density) were checked every 24 h. Samples were collected after cold maceration, every 48 h during alcoholic fermentation (after mixing the fermenting juice and skin caps) and finally after pressing in a pressure-controlled experimental (30 L) mechanical press (Skrlj d.o.o., Batuje, Slovenia), following the protocol of four (4 x 5 min) cycles (0.4/0.6/0.8/1.0 bar respectively). Then 20 mL of each must/wine under observation was added to 80 mL of methanol (hereinafter MeOH extracts) in a dark glass container and stored at −20 °C until the analysis was performed.

4.2.3.3 Climate/microclimate (berry surface temperature) monitoring

The temperature on berry surfaces from vines receiving the different treatments was measured every 2 h during three typical hot (August) days for both vintages under observation. Twenty-five measurements per treatment were done at each observation point/time using a Voltcraft IR-360 IR Thermom infrared thermometer (Conrad Electronics, Hirschau, Germany). Data on site climate conditions were collected with the help of an IMT300 iMetos meteorological station (Pessl Instruments, Weiz, Austria) located in the experimental vineyard. Furthermore, data from the National Meteorological Service of Slovenia ("Slovene public information" EARS-meteo.si) were used to evaluate climatic conditions.

4.2.3.4 Analysis of grape samples

In the case of grape samples, MeOH extracts of berry skins were first prepared[17] and the analysis was then carried out according to previously published protocols.[3] The separation and quantification of individual anthocyanins and flavonols was done using a Waters chromatographic system (Waters, Milford, MA, USA) comprising two Waters 510 pumps, a Waters 717+ autosampler and a Waters 2487 UV–visible (VIS) dual-wavelength detector. Total amounts were calculated as the sum of the individual compounds detected. All analyses were performed in technical duplicates.

4.2.3.5 Analysis of must/wine samples

4.2.3.5.1 HPLC-DAD determination of flavonols

Methanol was first removed from theMeOH extracts using a rotary evaporator (Büchi, Flawil, Switzerland). Individual flavonols in musts/wines were then analysed after flavonol glycosides had been subjected to acid hydrolysis, as described previously.[17] Further separation and quantification of flavonol aglycons was performed using a Waters Alliance 2695 high-performance liquid chromatograph (HPLC) equipped with a Waters 2996 diode array detector (DAD). Total flavonols were calculated as the sum of the individual flavonols detected.

4.2.3.5.2 HPLC-VIS analysis of anthocyanins

The analytical method described previously[3] was used to detect anthocyanins in the MeOH extracts of the musts and wines under observation. The separation and quantification of individual anthocyanins was performed using a Waters chromatographic system comprising two Waters 510 pumps, a Waters 717+ autosampler and a Waters 2487 UV–VIS dual-wavelength detector. Total anthocyanins were calculated as the sum of the individual anthocyanins detected. All analyses were performed in technical duplicates.

4.2.3.5.3 UHPLC/TQ-MS determination of pyranoanthocyanins

The occurrence of pyranoanthocyanins was monitored using a Waters ACQUITY ultra-high performance liquid chromatograph (UHPLC) coupled to a Waters Xevo tandem quadrupolemass spectrometer (TQ-MS), following the published analytical procedure.[13]

Sample preparation for the analysis was previously carried out in accordance with the reported protocol,[18] although a 20:80 (v/v) MeOH extract was used instead of wine, and dilution with water was avoided.

4.2.3.5.4 HPLC-DAD determination of hydroxycinnamic acids

Esters of hydroxycinnamic acids were determined based on a previously reported method,[19] with a minor adjustment to sample preparation for the present sample material. A 10 mL aliquot of MeOHextract was first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd, Ipswich, UK) under reduced pressure at 45 °C. The sample was then reconstructed in a quantitative flask to a final volume of 1 mL with 5 mL L^{-1} formic acid and filtered through 0.45 μm, 13 mm polytetraflouroethylene syringe-tip filters (Millipore, Bedford, MA, USA) into vials. The separation and quantification of hydroxycinnamates was performed using a Waters Alliance 2695 HPLC equipped with a Waters 2996 DAD.

4.2.3.5.5 UV–VIS spectrophotometric determination of colour properties

Colour measurements were performed with a Lambda 35 UV–VIS spectrophotometer (PerkinElmer, Waltham, MA, USA) using the procedure described previously.[20] The CIELab parameters L^*, a^* and b^* were determined following the recommendations of the Commission Internationale de l'Eclairage (CIE).[21]

4.2.3.6 Statistical analysis

Grape, must and wine composition datawere statistically analysed using one-way analysis of variance (ANOVA) to test the significance of treatments at harvest (grapes) and at different stages of cold maceration/alcoholic fermentation (musts/wines) ($P < 0.05$). The Student–Newman–Keuls (SNK) test of multiple comparisons of mean values was performed to separate the averages. In order to perform these tests, the STATISTICA software package (StatSoft, Tulsa, OK, USA) was used for all calculations.

4.2.4 Results and discussion

4.2.4.1 Vintage conditions and berry surface temperatures

Climate change is dramatically affecting the course of the seasons, with many fluctuations between years in terms of temperature and rainfall.[22] In our experiment, carried out in a 'Pinot Noir' vineyard in the Vipava Valley, the two successive (2009 and 2010) seasons were also markedly different from each other, with the former being very warm and dry throughout the summer (mainly in May, August and September) and the latter much colder (during the same months), primarily owing to extensive rainfall (Table 6).

Table 6: Basic seasonal macroclimate characteristics[46] of the two observed vintages (2009 and 2010).

	CLOUDINESS (%)		SUN HOURS		RAINFALL (mm)		AVERAGE T (°C)	
↓ Month / Year →	2009	2010	2009	2010	2009	2010	2009	2010
May	41	64	248	166	26	258	18.7	16.0
June	59	48	214	272	80	108	20.5	20.7
July	36	33	301	319	123	200	22.7	23.5
August	32	43	326	283	82	75	23.8	21.1
September	38	51	221	190	65	367	19.5	16.7

Climate variability significantly determines vintage-to vintage quality differences.[23] During our experiment the berry surface temperature was therefore monitored after véraison, aiming to ascertain the differences in the actual microclimate obtained by leaf removal performed at different phonological stages. In both years the grapes from VE leaf removal showed the highest berry surface temperatures when compared with the untreated (UN) control and with earlier leaf removal treatments (at berry-set (BS) in 2009 and 2010 and at pre-flowering (PF) in 2010) (Figures 12 A and 12 B). During the day, berry surface temperatures were lowest on UN grapes, where no leaves had been removed from the cluster area. After VE leaf removal the grapevines obviously did not have enough time for re-growth of the lateral leaves to the same extent as in the case of earlier leaf removals. Thus VE clusters were more exposed to sunlight, leading to higher berry temperatures being confirmed for this treatment. Early leaf removal, performed either at PF or at BS, triggered both earlier and higher production of lateral leaves, which partially re-shaded the bunches[6,24] during the hottest (August) days. New lateral leaves of early leaf removals were therefore protecting the berries from already reported destructive

sunburns[7] and from possibly harmful too high temperatures.[25,26] The BS and PF berry surface temperatures were shown to be higher than on UN grapes (since lateral leaves are smaller than main leaves) but lower than on VE grapes.

However, in our experiment we observed that colour-related phenolic profiles (quantitative and/or qualitative) were affected by leaf removal timing in both years (although to a different extent), even though the two years observed were extremely different in terms of temperature, sun hours and rainfall (Table 6).

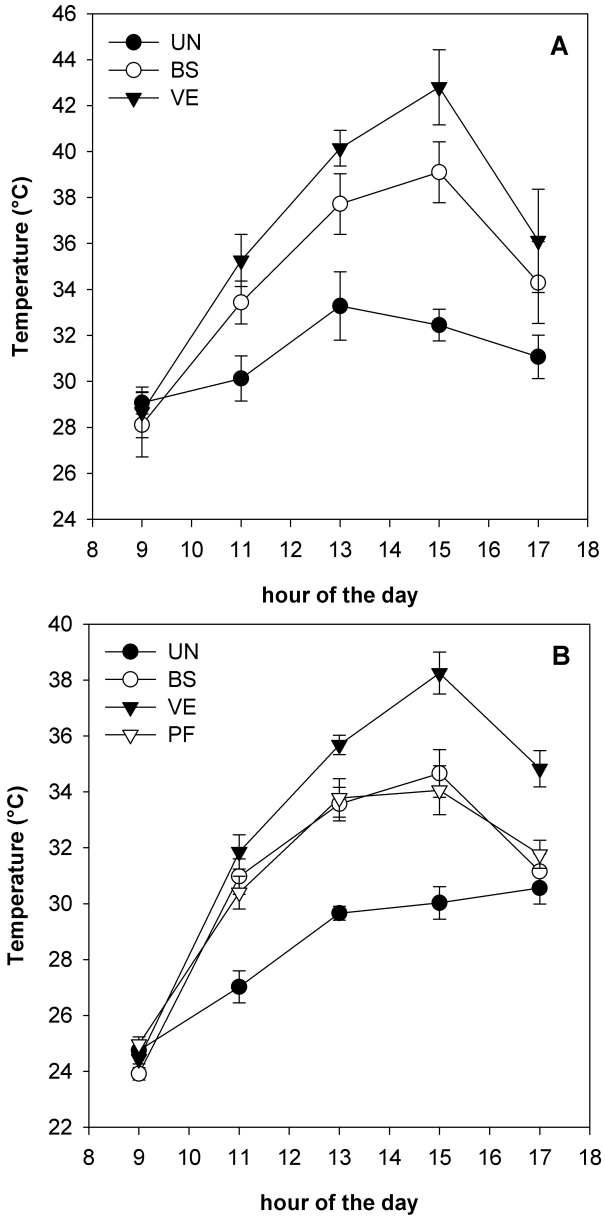


Figure 12: Average berry surface temperatures in mid (10–22) August 2009 (A) and 2010 (B) as related to different leaf removal treatments: UN, untreated (control); PF, pre-flowering leaf removal; BS, berry-set leaf removal; VE, v´eraison leaf removal. Means were separated using the SNK test ($P < 0.05$).

4.2.4.2 Flavonols in grapes and related musts/wines

Sun-exposed bunches have already been reported to have significantly higher flavonol levels than shaded bunches.[1,4,6,26,27] Almost no flavonols are formed if the bunches are completely shaded (with less than 10% sunlight exposure);[28] however, if exposed later, a rapid increase is detected. Flavonol occurrence can therefore be considered as a biomarker for a sun exposure regime achieved in a bunch area within the canopies following microclimate manipulation management. Furthermore, the vintage characteristics in terms of actual bunch light exposure within the canopy can be better evaluated by monitoring the behaviour of flavonol biosynthesis during grapevine maturation.

In our experiment, in accordance with the seasonal climatic characteristics (fewer sun hours in 2010), the total flavonol levels in grapes were lower in 2010 than in 2009. In 2009 the flavonol content in UN grapes at harvest was 21 mg kg^{-1}, while it was significantly higher with leaf removal treatments, reaching 124 mg kg^{-1} in VE (+490%) and 113 mg kg^{-1} in BS (+438%) grapes.[3] The following 2010 season showed 22 mg kg^{-1} total flavonols in UN, 77 mg kg^{-1} in VE (+250%), 105 mg kg^{-1} in BS (+377%) and 101 mg kg^{-1} in PF (+359%) grapes (with no corresponding PF value in 2009). Subsequently, a lower total amount was also found in 2010 wines compared with 2009 wines (Figs 13 C and 13 D). Although not significant, in 2009 the concentration of flavonols was higher in VE than in BS grapes, while in 2010 the opposite was noted between BS/PF and VE grapes. By comparing temperatures and sun hours during the two summers (Table 6), it is possible to observe that August 2009 was hot and sunny, so VE leaf removal led to an important (vintage-related) increase in flavonol synthesis. In contrast, the 2010 season was sunny in June and July, resulting in a higher occurrence of flavonols for early-opening BS and PF grapes, while the later summer was cloudier, leading to a lower performance of late VE leaf removal treatment in 2010. Two distinct periods of flavonol synthesis are already reported in grapes, the first around flowering and the second during ripening of the developing berries.29 Regardless of climatic differences between the seasons observed, the shaded grapes of untreated vines (UN) consistently provided significantly lower amounts of total flavonols as compared with those subjected to leaf removal treatments, proving once again the importance of berry light exposure for flavonol biosynthesis.[1,4,26] After the experimental grapes had been subjected to vinification processes, it was observed that only small amounts of total flavonols were already extracted during the cold maceration period, and only in the 2010 vintage were there some differences between treatments already evident at that point (Figs 13 C and 13 D). After yeast inoculation the concentration of total flavonols

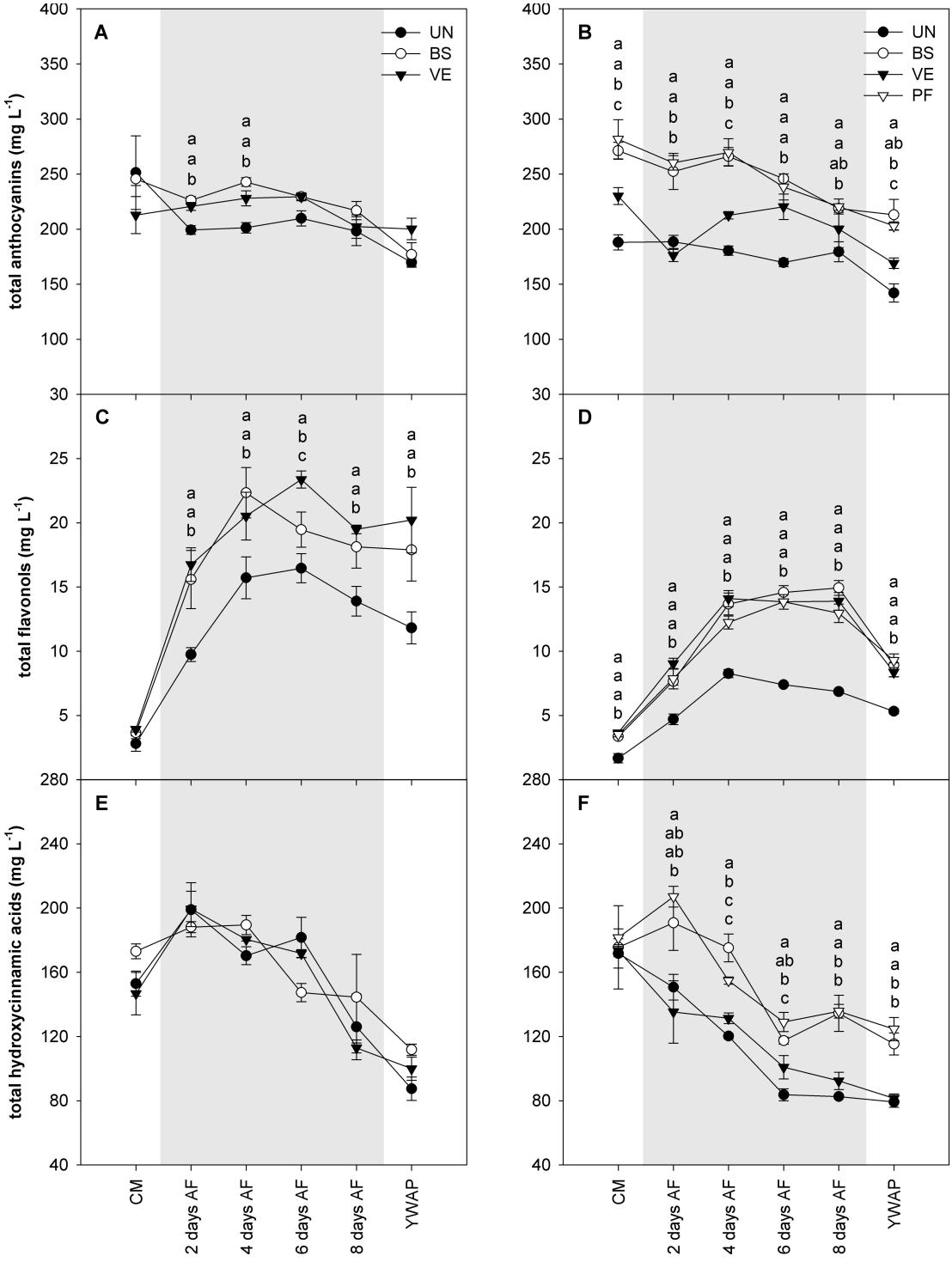


Figure 13: Total anthocyanins (A, 2009; B, 2010), total flavonols (C, 2009; D, 2010) and total hydroxycinnamic acids (E, 2009; F, 2010) in musts and wines as related to different leaf removal treatments: UN, untreated (control); PF, pre-flowering leaf removal; BS, berry-set leaf removal; VE, veraison leaf removal; CM, cold maceration; AF, alcoholic fermentation; YWAP, young wine after pressing. Means were separated using the SNK test ($P < 0.05$).

increased during fermentation and the differences between the control and treated grapes were more clearly shown. The 2009 musts showed minor differences in terms of the total flavonol

concentration detected during fermentation, with the control treatment presenting the lowest amount. In contrast, the 2010 musts coming from vines subjected to all leaf removal treatments (PF, BS and VE) showed a more significant enhancement of flavonols as compared with the control (UN), but there were still no significant differences highlighted among nthem. In young wines after pressing (YWAP) and in both seasons the amount of total flavonols was also similar in the case of all leaf removal treatments, but significantly higher than in the control (Figures 13 C and 13 D). The findings are very much in agreement with the published observations of Diago et al.,[6] although their experiment was done on the Tempranillo grapevine variety. In their trial, early leaf removal also resulted in a significant increase in total flavonols in grapes, while the timing of defoliation did not seem to significantly affect the flavonol concentration in wines. Furthermore, they observed different flavonol yields between two different vintages, implying that seasonal discrepancies are partially responsible for this outcome. Although they did not show meteorological data, their discussion fits in with our findings, supported by vintage condition data, which in our case can also explain seasonally related changes interms of preferable results for early (in 2010) or late (in 2009) defoliation. In addition to differentn regional geoclimatic characteristics (La Rioja versus Vipava Valley), the lower total amount of flavonols obtained in our experiment can be partly explained by the different variety under observation and partly by the fact that much more severe leaf removal was carried out in the case of Tempranillo, with eight basal leaves being removed for early defoliation,[6] while a maximum of four to six basal leaves were removed in the case of 'Pinot Noir' leaf removal in 2009[3] and 2010.

Looking at the occurrence of individual flavonols in the must/wines under observation (Table 7), quercetin (Qe) concentration levels during fermentation of both vintages were significantly higher following leaf removal treatments in comparison with the control (UN), this being maintained in YWAP. Any successful vineyard-related improvements in Qe yield are of great importance for the 'Pinot Noir' grapevine variety (often hampered by low wine colour intensity and stability), as Qe has been shown to be involved in co-pigmentation reactions, and its quantitative level and ratio with other phenolics may be crucial for better development of wine colour characteristics.[15] Few differences were observed in the case of myricetin (My) concentration during fermentation of 2009 musts, but in related young wines after pressing (YWAP) the amount was significantly higher in BS as compared with the control (UN). On the other hand, in the 2010 vintage, all leaf removal treatments showed significantly higher concentrations as compared with the control (UN) during fermentation, although this

Table 7: Individual flavonols (mg L^{-1}) in musts and wines as related to different leaf removal treatments: UN = untreated (control); PF = pre-flowering leaf removal; BS = berry-set leaf removal; VE = veraison leaf removal; CM = cold maceration; AF = alcoholic fermentation; YWAP = young wine after pressing.

	VINTAGE 2009						VINTAGE 2010					
	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP
My												
UN	0.9 ± 0.17	2.8 ± 0.13	4.5 ± 0.46 b	3.5 ± 0.42	3.1 ± 0.24	2.7 ± 0.33 b	0.6 ± 0.20 b	1.2 ± 0.28 b	2.0 ± 0.09 b	1.5 ± 0.05 b	0.9 ± 0.41 b	0.9 ± 0.07
PF							1.1 ± 0.05 a	2.3 ± 0.59 a	2.8 ± 0.36 a	2.9 ± 0.44 a	2.7 ± 0.22 a	1.0 ± 0.06
BS	1.2 ± 0.10	3.1 ± 0.91	6.2 ± 0.31 a	4.0 ± 0.33	3.9 ± 0.13	4.6 ± 0.80 a	0.9 ± 0.01 a	2.1 ± 0.05 a	3.4 ± 0.55 a	2.9 ± 0.32 a	3.0 ± 0.10 a	1.1 ± 0.40
VE	1.1 ± 0.04	3.8 ± 0.32	4.9 ± 0.76 b	4.0 ± 0.12	3.6 ± 0.47	3.8 ± 0.39 ab	0.9 ± 0.06 a	1.9 ± 0.22 a	3.3 ± 0.22 a	3.1 ± 0.30 a	2.7 ± 0.75 a	1.0 ± 0.15
sign. F	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*	**	*	**	**	**	*n.s.*
Qe												
UN	0.9 ± 0.23 b	3.7 ± 0.23 b	6.0 ± 0.56 b	6.6 ± 0.38 c	5.4 ± 0.62 b	4.1 ± 0.38 b	0.5 ± 0.26 b	1.8 ± 0.25 b	3.3 ± 0.26 b	2.8 ± 0.03 c	2.8 ± 0.14 b	1.9 ± 0.15 b
PF							1.4 ± 0.07 a	3.3 ± 0.61 a	5.3 ± 0.35 a	5.9 ± 0.26 a	5.3 ± 1.43 a	4.2 ± 0.17 a
BS	1.3 ± 0.15 a	8.1 ± 0.94 a	9.5 ± 1.56 a	8.1 ± 0.52 b	8.2 ± 1.14 a	6.8 ± 1.29 a	1.4 ± 0.08 a	3.1 ± 0.26 a	5.9 ± 0.64 a	6.3 ± 0.46 a	6.4 ± 0.38 a	3.4 ± 1.64 a
VE	1.1 ± 0.13 a	7.6 ± 0.60 a	8.6 ± 0.50 a	10.2 ± 0.29 a	9.2 ± 0.04 a	8.2 ± 1.10 a	1.4 ± 0.10 a	3.7 ± 0.33 a	5.6 ± 0.43 a	5.2 ± 0.17 b	5.5 ± 0.56 a	3.3 ± 0.25 a
sign. F	*	***	*	***	**	**	***	**	***	***	**	*
La												
UN	0.3 ± 0.03 b	0.6 ± 0.01	0.9 ± 0.07 b	1.1 ± 0.06 b	0.9 ± 0.08 b	0.9 ± 0.11 b	0.2 ± 0.03 b	0.3 ± 0.03 c	0.5 ± 0.01 c	0.5 ± 0.01 b	0.5 ± 0.01 b	0.4 ± 0.01 b
PF							0.3 ± 0.01 a	0.4 ± 0.05 b	0.6 ± 0.03 b	0.7 ± 0.03 a	0.7 ± 0.01 a	0.6 ± 0.01 a
BS	0.3 ± 0.03 b	0.8 ± 0.27	1.1 ± 0.04 a	1.2 ± 0.12 b	1.0 ± 0.06 b	1.1 ± 0.11 b	0.3 ± 0.03 a	0.4 ± 0.03 b	0.7 ± 0.05 ab	0.8 ± 0.05 a	0.8 ± 0.06 a	0.7 ± 0.05 a
VE	0.4 ± 0.01 a	0.9 ± 0.07	1.1 ± 0.10 a	1.6 ± 0.06 a	1.2 ± 0.07 a	1.4 ± 0.13 a	0.3 ± 0.03 a	0.5 ± 0.03 a	0.7 ± 0.05 a	0.7 ± 0.05 a	0.8 ± 0.03 a	0.6 ± 0.03 a
sign. F	*	*n.s.*	*	**	*	**	**	**	***	***	***	***
Ka												
UN	0.1 ± 0.02 c	0.3 ± 0.01 b	0.5 ± 0.15 b	0.4 ± 0.03 c	0.4 ± 0.04 c	0.4 ± 0.05 b	0.1 ± 0.01 c	0.2 ± 0.01 c	0.2 ± 0.01 c	0.3 ± 0.01 c	0.3 ± 0.01 c	0.2 ± 0.01 b
PF							0.2 ± 0.01 b	0.3 ± 0.03 b	0.4 ± 0.01 b	0.5 ± 0.01 b	0.5 ± 0.01 b	0.4 ± 0.01 a
BS	0.1 ± 0.01 b	0.4 ± 0.15 b	0.6 ± 0.11 ab	0.5 ± 0.04 b	0.6 ± 0.11 b	0.5 ± 0.07 b	0.2 ± 0.01 b	0.3 ± 0.03 b	0.5 ± 0.03 b	0.5 ± 0.03 b	0.5 ± 0.03 b	0.4 ± 0.03 a
VE	0.2 ± 0.01 a	0.8 ± 0.07 a	0.8 ± 0.04 a	1.0 ± 0.03 a	0.9 ± 0.02 a	0.9 ± 0.12 a	0.2 ± 0.01 a	0.5 ± 0.01 a	0.6 ± 0.05 a	0.6 ± 0.03 a	0.6 ± 0.06 a	0.4 ± 0.03 a
sign. F	***	**	*	***	***	**	***	***	***	***	***	***
Is												
UN	0.4 ± 0.11	1.7 ± 0.13	2.9 ± 0.32 b	3.6 ± 0.21 c	3.1 ± 0.32	2.7 ± 0.26 c	0.2 ± 0.06 b	0.9 ± 0.08 c	1.6 ± 0.16 c	1.7 ± 0.03 b	1.7 ± 0.01 b	1.2 ± 0.03 b
PF							0.5 ± 0.18 a	0.9 ± 0.20 c	2.4 ± 0.15 b	2.9 ± 0.22 a	2.5 ± 0.76 ab	2.2 ± 0.15 a
BS	0.5 ± 0.15	2.2 ± 0.71	4.0 ± 0.11 a	4.3 ± 0.30 b	3.3 ± 0.22	3.6 ± 0.23 b	0.5 ± 0.13 a	1.4 ± 0.16 b	2.5 ± 0.23 b	3.0 ± 0.20 a	3.2 ± 0.30 a	2.3 ± 0.42 a
VE	0.6 ± 0.03	2.8 ± 0.18	4.0 ± 0.51 a	5.0 ± 0.14 a	3.5 ± 0.14	4.5 ± 0.63 a	0.7 ± 0.05 a	1.9 ± 0.16 a	3.0 ± 0.23 a	3.2 ± 0.22 a	3.2 ± 0.36 a	2.1 ± 0.15 a
sign. F	*n.s.*	*n.s.*	*	***	*n.s.*	**	**	***	***	***	*	**
Sy												
UN	0.2 ± 0.03	0.5 ± 0.02 b	0.9 ± 0.09	1.2 ± 0.08 b	1.0 ± 0.01	1.0 ± 0.09 b	0.2 ± 0.05	0.3 ± 0.05	0.6 ± 0.01 b	0.7 ± 0.01 b	0.7 ± 0.01	0.7 ± 0.05 b
PF							0.1 ± 0.07	0.6 ± 0.25	0.7 ± 0.03 b	1.0 ± 0.05 a	1.3 ± 0.56	0.9 ± 0.05 a
BS	0.2 ± 0.05	0.9 ± 0.13 a	1.0 ± 0.14	1.4 ± 0.08 a	1.1 ± 0.03	1.3 ± 0.02 ab	0.2 ± 0.06	0.4 ± 0.03	0.8 ± 0.07 ab	1.1 ± 0.06 a	1.1 ± 0.15	1.0 ± 0.08 a
VE	0.3 ± 0.01	0.9 ± 0.06 a	1.0 ± 0.07	1.5 ± 0.06 a	1.1 ± 0.20	1.5 ± 0.23 a	0.2 ± 0.03	0.5 ± 0.05	0.9 ± 0.08 a	1.0 ± 0.06 a	1.1 ± 0.06	1.0 ± 0.10 a
sign. F	*n.s.*	**	*n.s.*	**	*n.s.*	*	*n.s.*	*n.s.*	**	***	*n.s.*	**

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly different.

significance disappeared in YWAP. Furthermore, the concentration trends of laricitrin (La) and kaempferol (Ka) were mostly preferable in VE musts during fermentation in both vintages. Intermediate, similar values were observed for BS and PF leaf removal treatments, while, as expected, the lowest values were again detected for UN samples. At the end of fermentation, higher concentrations of both La and Ka (in VE) were still shown in 2009, while only the difference between treatments and the control was found in the following 2010 season. Similar behaviour was observed for isorhamnetin (Is) and syringetin (Sy). In the first season (2009), no differences among leaf removal treatments were detected during the first part of fermentation, but thereafter the concentration of both flavonols was found to be significantly increased in VE YWAP. In 2010, on the other hand, significantly higher Is values were already shown at the beginning of fermentation with BS and VE leaf removal treatments as compared with the control. Finally, in YWAP, all treatments were shown to lead to a better performance in terms of Is and Sy occurrence as comparedwith the control (UN).

4.2.4.3 Anthocyanins in grapes and related musts/wines

In 2009 the anthocyanin content in control grapes at harvest was 343 mg kg^{-1}, while it was significantly higher in those subjected to leaf removal treatments, reaching 530 mg kg^{-1} in VE (+54%) and 660 mg kg^{-1} in BS (+92%) grapes.[3] Although the following 2010 season was much colder, consequently with a longer total maturation time, the concentration of total anthocyanins in grapes was comparable, being 364 mg kg^{-1} in UN grapes, but once again enhanced following defoliation treatments, with 520 mg kg^{-1} in VE (+43%), 509 mg kg^{-1} in BS (40%) and 590 mg kg^{-1} in PF (+62%) grapes. The consistently lower amount of total anthocyanins in UN grapes (in both vintages) corroborates previous studies,[1,30] discussing that the synergistic effect of both light and temperature is crucial for the synthesis of these pigments. Furthermore, this is also in accord with a study done on Merlot grapes,[31] which resulted in vintage-dependent differences in anthocyanin concentration when comparing different leaf removal severities (moderate and severe), but consistently the lowest concentration (in both observed vintages) for nil treatment (without any leaf removal).

When processing the grapes, we observed that the amounts of total free anthocyanins in the wines were higher with VE leaf removal treatment in 2009 than in 2010, with BS performing better in 2010 and PF (having no corresponding values for 2009) showing similar dynamics to BS in 2010. In the case of UN wines, we observed lower final concentrations of total free

anthocyanins in 2010 wines than in 2009 wines (Figures 13 A and 13 B) (despite similar results in grapes). The skin tissue maturation of UN was probably less complete in the colder 2010, thus not allowing the anthocyanins to be dissolved equally during fermentation. In the case of a longer ripening period (as took place in 2010), the grapes were already reported to have higher levels of berry skin break force and energy.[32] Not only has a trend for a reduced anthocyanin concentration been reported for bunches less exposed to sunlight,[33] but pigment extractability timing from such grapes/skins in winemaking has already been discussed, showing a significant delay in pigment release.[34] The relatively low UN anthocyanin content in 2010 YWAP consequently led to the treated grapes showing a slightly higher proportional change when compared with the control, although the total amounts were basically comparable in both vintages. Furthermore, any PF results should probably also be considered in relation to the significantly lower total yield (kg per vine) as already reported for some varieties.[35–37]

Individual anthocyanin profiles in musts/wines (Table 8) showed basically similar trends in both vintages, with the exception of petunidin-3-glucoside (Pet-3G). While in 2009 the concentration of Pet-3G decreased continuously during fermentation (finally reaching comparable values in YWAP for all treatments), in 2010 the Pet-3G concentration in musts initially increased (reaching a peak after 4 days of fermentation) and then decreased during the final stages of fermentation. Thus, at the end of fermentation, PF gave the highest value, followed by BS and VE, while the control (UN) showed the lowest Pet-3G concentration in YWAP. Regardless of the timing of leaf removal, the concentration of malvidin-3-glucoside (Mal-3G) during fermentation (as well as in YWAP for both vintages) was higher (but not constantly significant) than in the control (UN), in agreement with a previous study[38] showing a higher yield of Mal-3G from light-exposed bunches. Furthermore, we detected interesting extraction dynamics in the case of cyanidin-3-glucoside (Cy-3G) for both vintages: although extracted during cold maceration, the Cy-3G level dropped down below the detection limit after 2 days of alcoholic fermentation in 2009 and after 4 days in 2010. The Cy-3G was probably either absorbed by the yeasts16 or subjected to oxidation processes before the appropriate reductive environment (CO2) was achieved within the fermentation processes[9,39] and/or was rapidly involved in co-pigmentation reactions.[11] Anthocyanins are known to be highly unstable, but if an analogy between the catechol groups and the most common anthocyanidins is made, it would be expected that those with an *o*-dihydroxyl substitution (including Cy) are the most susceptible to oxidation.[40]

Table 8: Individual anthocyanins (mg L^{-1}) in musts and wines as related to different leaf removal (LR) treatments: UN, control; PF, pre-flowering LR; BS, berry-set LR; VE, veraison LR; CM, cold maceration; AF, alcoholic fermentation; YWAP, young wine after pressing.

	VINTAGE 2009						VINTAGE 2010					
	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP
Del-3G												
UN	10.1 ± 2.65	3.7 ± 0.16	2.4 ± 0.43	2.1 ± 0.17	1.2 ± 0.29	0.7 ± 0.24	5.2 ± 0.37 c	4.7 ± 0.40 b	3.5 ± 0.18 b	2.8 ± 0.21 b	2.6 ± 0.31 b	1.6 ± 0.05 d
PF							9.1 ± 1.47 ab	8.2 ± 0.43 a	6.7 ± 0.60 a	4.3 ± 0.43 a	3.9 ± 0.48 a	2.8 ± 0.07 a
BS	5.6 ± 0.34	4.5 ± 0.35	2.8 ± 0.31	2.6 ± 0.17	1.4 ± 0.51	0.7 ± 0.07	8.9 ± 0.49 a	7.9 ± 1.11 a	6.1 ± 0.49 ab	4.3 ± 0.39 a	3.3 ± 0.14 ab	2.4 ± 0.21 b
VE	8.4 ± 3.56	4.6 ± 0.54	3.2 ± 0.56	2.5 ± 0.17	1.9 ± 1.12	0.5 ± 0.19	7.3 ± 0.44 b	4.5 ± 0.26 b	3.9 ± 0.17 b	3.4 ± 0.28 b	2.8 ± 0.38 b	1.3 ± 0.08 c
sign. F	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	**	***	***	**	**	***
Cy-3G												
UN	12.4 ± 2.44 a	–	–	–	–	–	5.4 ± 0.41 b	0.8 ± 0.03	–	–	–	–
PF		–	–	–	–	–	7.7 ± 1.08 a	1.0 ± 0.18	–	–	–	–
BS	6.0 ± 0.35 b	–	–	–	–	–	7.1 ± 0.41 a	0.9 ± 0.15	–	–	–	–
VE	7.9 ± 3.23 ab	–	–	–	–	–	6.6 ± 0.38 a	0.8 ± 0.04	–	–	–	–
sign. F	*						*	*n.s.*				
Pet-3G												
UN	14.1 ± 3.34	7.3 ± 0.32 b	6.3 ± 0.52	5.5 ± 0.86	5.1 ± 0.77	3.5 ± 0.27	6.5 ± 0.46 c	7.9 ± 0.45 b	8.4 ± 0.67 c	6.7 ± 0.36 b	7.2 ± 1.00 c	3.6 ± 0.30 c
PF							12.0 ± 1.76 a	12.8 ± 0.56 a	14.1 ± 1.17 a	11.2 ± 0.51 a	10.3 ± 0.70 a	8.4 ± 0.37 a
BS	7.6 ± 0.41	8.6 ± 0.39 a	6.4 ± 0.84	6.1 ± 0.71	4.6 ± 0.39	3.7 ± 0.86	11.4 ± 0.97 a	11.4 ± 1.30 a	13.4 ± 0.79 a	11.1 ± 0.09 a	9.5 ± 0.14 ab	6.9 ± 0.95 ab
VE	11.7 ± 5.18	8.6 ± 0.46 a	6.0 ± 0.59	5.9 ± 0.24	3.8 ± 0.44	3.9 ± 0.45	8.8 ± 0.98 b	7.5 ± 0.77 b	9.8 ± 0.38 b	8.9 ± 0.76 a	7.9 ± 1.17 b	4.8 ± 0.82 b
sign. F	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	**	***	***	***	**	***
Peo-3G												
UN	90.4 ± 20.2	24.1 ± 2.96 a	18.4 ± 0.98 a	18.3 ± 2.13 a	13.9 ± 2.94	12.5 ± 0.89 a	75.3 ± 4.71 b	45.3 ± 3.82 ab	26.2 ± 1.16 b	21.8 ± 1.07 ab	22.7 ± 2.05 a	15.3 ± 1.12 b
PF							105.0 ± 10.6 a	57.3 ± 3.98 a	30.4 ± 3.04 a	24.4 ± 2.38 a	21.6 ± 1.55 ab	19.2 ± 0.46 a
BS	90.1 ± 7.41	21.5 ± 0.51 a	18.2 ± 0.83 a	16.6 ± 0.65 ab	12.6 ± 0.96	8.9 ± 1.99 b	97.9 ± 4.41 a	50.9 ± 9.62 a	26.6 ± 2.17 ab	22.0 ± 0.42 ab	18.8 ± 0.74 ab	15.5 ± 1.38 b
VE	66.1 ± 5.83	17.2 ± 1.11 b	14.3 ± 1.51 b	13.8 ± 1.13 b	9.8 ± 1.71	11.1 ± 0.94 ab	84.9 ± 4.69 b	34.7 ± 2.23 b	21.1 ± 0.32 c	19.7 ± 1.61 b	17.6 ± 2.33 b	13.5 ± 0.49 b
sign. F	*n.s.*	*	**	*	*n.s.*	*	**	**	**	*	*	***
Mal-3G												
UN	124.2 ± 29.2	164.0 ± 4.23 b	174.0 ± 6.55 b	183.8 ± 8.78 b	177.9 ± 18.90	152.8 ± 6.15	95.5 ± 6.16 c	128.5 ± 5.53 b	141.0 ± 5.12 c	136.8 ± 4.98 b	145.3 ± 12.1 c	118.6 ± 8.26 c
PF							147.9 ± 15.9 a	179.4 ± 6.65 a	214.9 ± 16.7 a	196.3 ± 17.0 a	183.2 ± 9.14 ab	170.1 ± 3.66 a
BS	136.1 ± 4.24	191.4 ± 5.07 a	215.4 ± 4.95 a	204.1 ± 4.71 a	198.1 ± 13.2	175.3 ± 15.6	145.7 ± 7.17 a	179.8 ± 16.51 a	216.8 ± 10.7 a	206.2 ± 3.03 a	185.8 ± 5.02 a	182.8 ± 14.1 ab
VE	118.6 ± 11.5	190.2 ± 4.52 a	204.5 ± 9.31 a	207.2 ± 3.97 a	186.8 ± 17.2	184.4 ± 15.7	122.3 ± 6.88 b	127.5 ± 6.39 b	175.9 ± 4.95 b	186.4 ± 17.21 a	169.9 ± 25.3 b	145.5 ± 4.68 b
sign. F	*n.s.*	***	**	*	*n.s.*	*n.s.*	***	***	***	***	*	***

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly diffe

Also the levels of peonidin-3-glucoside (Peo-3G) in both vintages decreased significantly (by nearly 80%) as soon as fermentation started. It was previously reported (although based on Nebbiolo grapes) that cultural practices such as modifying the fruit zone microclimate might also modify the (colour-important) Peo/Mal ratio,[2] with Peo-3G being more reactive and less stable than Mal-3G. After a rapid decrease during the initial stages of fermentation, the concentration of Peo- 3G then decreased only slightly and with low differences between treatments in 2009, while in 2010 the PF wines (YWAP) showed significantly higher Peo-3G values as compared with all other treatments. Similar delphinidin-3-glucoside (Del-3G) extraction dynamics was detected during the fermentation of both vintages; however, only in 2010 were the differences between treatments significant. In 2010 the concentration of Del-3G was initially significantly higher in PF and BS treatments, with no differences between VE and UN; however, at the end of fermentation, young wines showed significant differences between all treatments, with PF treatment giving the highest result.

Specifically, Del-3G, Cy-3G and Peo-3G were mostly already extracted during coldmaceration, while their concentrations later decreased. On the contrary, Pet-3G (2010) and Mal-3G levels continued to increase during the first days of fermentation.

4.2.4.4 Pyranoanthocyanins in musts/wines

To learn more about the fate of free monomeric anthocyanins (and some other phenolics) during cold maceration and fermentation, we also investigated the formation of pyranoanthocyanins (PAs) in 2010 musts/wines. The trends observed and the presence of detected PAs within the vinification processes suggest that something interesting happens during must fermentation (Table 9). Vitisins A, the adducts normally resulting from the cycloaddition of a molecule of pyruvic acid to one of the anthocyanins,[13,41,42] were the most widely detected PAs in our samples. All leaf removal treatments showed significantly higher synthesis/content of vitisin A-like pigments as compared with the control during fermentation (except VE after 8 days of fermentation) and in YWAP. Although the initial trend was obviously towards early leaf removals (PF and BS) during fermentation, the differences between the three leaf removal strategies were not statistically confirmed in YWAP owing to high variations among the biological triplicates. However, the trends observed were in agreement with the detected phenolics in grapes coming from different leaf removal treatments, suggesting that carefully selected leaf removal (and its timing) may also improve

the formation patterns of more stable pigments in Pinot Noir wine, making an important contribution to its problematic wine colour stability. Beside vitisins A, pyrano malvidin-3-glucoside was also detected in all samples, but only in traces after cold maceration, and later reached similar values for all treatments in YWAP (Table 9).

Anthocyanin pattern recognition, genetically controlled by the plant variety, has already been shown to be inherited by the pigments formed during wine ageing,[13] but our results suggest for the first time that the phenolic patterns resulting from early leaf removal may alsomake an important contribution to PA formation patterns.

Table 9: Pyranoanthocyanins (mg L^{-1}) detected in musts and wines as related to different leaf removal treatments: UN = untreated (control); PF = pre-flowering leaf removal; BS = berry-set leaf removal; VE = veraison leaf removal; CM = cold maceration; AF = alcoholic fermentation; YWAP = young wine after pressing.

	CM	4 days AF	8 days AF	YWAP
vitisin A				
UN	4.0 ± 0.53	19.9 ± 1.36 c	20.2 ± 1.00 b	18.9 ± 0.64 b
PF	4.0 ± 0.19	26.5 ± 0.39 a	26.1 ± 2.44 a	26.4 ± 1.10 a
BS	3.3 ± 0.80	25.5 ± 0.22 ab	26.1 ± 2.11 a	24.7 ± 1.48 a
VE	3.4 ± 0.12	24.3 ± 0.33 b	22.1 ± 1.18 b	24.0 ± 4.29 a
sign. F	*n.s.*	***	**	*
pyrano malvidin-3-glucoside				
UN	0.1 ± 0.05	0.9 ± 0.07 c	0.8 ± 0.15	0.9 ± 0.06
PF	0.1 ± 0.01	1.3 ± 0.04 a	1.1 ± 0.19	1.1 ± 0.14
BS	0.1 ± 0.05	1.1 ± 0.12 bc	1.1 ± 0.34	1.1 ± 0.10
VE	0.1 ± 0.03	1.1 ± 0.04 b	1.1 ± 0.03	1.2 ± 0.12
sign. F	*n.s.*	**	*n.s.*	*n.s.*

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly different.

4.2.4.5 Hydroxycinnamic acids in musts/wines

In a similar way to anthocyanins, we observed less significant reflections of vineyard-related improvements in 2009 wines, but the significance of total hydroxycinammic acids (HCAs) composition in must/wine, in relation to early leaf removal treatments, was much better retained in 2010 wines (Figs 13 E and 13 F). The overall levels of total HCAs detected in wines from both seasons were in agreement with the existing literature,[43] with similar achievements for UN and VE in both years. However, the colder conditions in 2010 seemed to favour HCAs yield (in terms of qualitative and quantitative profiles) in wines made following earlier leaf removal. This could be explained either by the negative contribution of too high temperature (in the case of 2009) or by the positive effect of longer/more intense light

Table 10: Individual hydroxycinnamic acids (mg L^{-1}) in musts and wines as related to different leaf removal treatments: UN = untreated (control); PF = pre-flowering leaf removal; BS = berry-set leaf removal; VE = veraison leaf removal; CM = cold maceration; AF = alcoholic fermentation; YWAP = young wine after pressing.

	VINTAGE 2009						VINTAGE 2010					
	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP	CM	2 days AF	4 days AF	6 days AF	8 days AF	YWAP
GRP												
UN	3.5 ± 0.09 ab	5.0 ± 0.61	5.9 ± 0.10 a	7.5 ± 0.90 b	4.2 ± 1.22	4.1 ± 0.36	15.1 ± 2.77	9.5 ± 0.34	7.7 ± 0.63 c	8.4 ± 0.43 b	9.6 ± 0.80	6.9 ± 0.61
PF							11.1 ± 1.53	10.7 ± 0.61	9.3 ± 0.44 ab	9.2 ± 1.07 b	10.8 ± 1.33	8.7 ± 0.81
BS	5.0 ± 1.07 a	5.2 ± 0.57	5.9 ± 0.44 ab	6.7 ± 0.24 b	4.9 ± 1.12	4.9 ± 0.77	11.1 ± 3.81	9.8 ± 1.59	10.0 ± 0.98 a	8.5 ± 0.28 b	11.2 ± 2.11	8.4 ± 1.00
VE	3.7 ± 0.29 b	6.1 ± 0.34	5.2 ± 0.23 b	8.9 ± 0.54 a	4.8 ± 0.12	4.6 ± 0.80	11.4 ± 0.76	8.0 ± 2.06	8.2 ± 0.53 bc	10.7 ± 0.40 a	9.9 ± 1.06	7.4 ± 0.27
sign. F	*	*n.s.*	*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*	**	*n.s.*	*n.s*
t-caftaric acid												
UN	124.2 ± 10.8	157.7 ± 24.0	131.8 ± 7.52	139.7 ± 17.0 a	97.3 ± 27.4	66.1 ± 9.40	126.5 ± 10.4	111.1 ± 10.7 bc	87.4 ± 1.88 c	57.1 ± 5.37 b	53.9 ± 2.48 b	53.3 ± 4.18 b
PF							138.5 ± 8.41	154.7 ± 8.42 a	113.3 ± 3.24 b	91.5 ± 6.93 a	94.1 ± 3.93 a	88.1 ± 8.96 a
BS	137.0 ± 7.22	147.6 ± 3.22	145.9 ± 8.90	110.4 ± 7.67 b	109.6 ± 37.1	81.6 ± 5.08	133.5 ± 33.5	142.3 ± 22.2 ab	128.8 ± 10.9 a	82.8 ± 2.60 a	89.9 ± 15.0 a	80.4 ± 8.28 a
VE	120.4 ± 20.6	155.6 ± 14.9	140.7 ± 0.89	126.1 ± 2.82 ab	86.0 ± 7.16	73.6 ± 8.90	132.6 ± 6.21	100.2 ± 24.9 c	95.8 ± 4.32 c	68.2 ± 9.70 b	61.8 ± 6.03 b	54.3 ± 3.25 b
sign. F	*n.s.*	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*	***	**	***	***
c-coutaric												
UN	5.0 ± 0.45 b	7.8 ± 1.16	8.0 ± 0.22 b	8.0 ± 0.67 b	6.5 ± 1.49	5.1 ± 0.87 b	5.8 ± 0.64	6.9 ± 0.67	6.5 ± 0.10 c	5.4 ± 0.20 b	5.8 ± 0.40 b	6.4 ± 0.41 b
PF							6.1 ± 0.03	9.3 ± 0.58	8.1 ± 0.30 ab	8.1 ± 0.81 a	8.8 ± 0.98 a	8.7 ± 0.87 a
BS	7.4 ± 1.38 a	7.9 ± 0.76	9.2 ± 0.57 a	7.7 ± 0.35 b	8.2 ± 1.54	8.2 ± 0.22 a	5.7 ± 1.61	8.4 ± 1.36	8.8 ± 0.86 a	7.5 ± 0.37 a	10.0 ± 0.42 a	8.4 ± 0.83 a
VE	4.9 ± 0.49 b	8.7 ± 0.54	8.8 ± 0.36 ab	9.3 ± 0.37 a	7.1 ± 0.06	6.9 ± 0.86 a	5.6 ± 0.06	6.5 ± 1.63	7.3 ± 0.22 bc	6.8 ± 0.79 a	6.3 ± 0.91 b	6.9 ± 0.42 b
sign. F	*	*n.s.*	*	*	*n.s.*	**	*n.s.*	*n.s.*	**	**	***	**
t-coutaric acid												
UN	20.1 ± 2.20 ab	28.4 ± 3.47	24.5 ± 2.00	26.4 ± 3.30	18.0 ± 5.33	13.1 ± 2.00	24.1 ± 2.42	23.1 ± 2.19 b	18.5 ± 0.31 c	12.7 ± 0.90 b	13.2 ± 0.26 b	12.6 ± 0.79 b
PF							25.7 ± 1.47	32.4 ± 1.90 a	24.1 ± 0.87 b	20.1 ± 1.62 a	21.9 ± 2.13 a	19.0 ± 1.89 a
BS	23.6 ± 1.22 a	27.2 ± 1.99	28.4 ± 1.75	22.6 ± 1.49	21.7 ± 6.46	17.1 ± 1.06	25.1 ± 6.43	30.3 ± 4.72 a	27.3 ± 2.41 a	18.5 ± 0.74 a	23.1 ± 4.79 a	18.0 ± 1.76 a
VE	17.6 ± 2.01 b	29.0 ± 3.21	25.7 ± 0.57	27.3 ± 0.42	17.2 ± 1.75	14.8 ± 1.97	23.7 ± 0.51	20.6 ± 5.05 b	19.9 ± 0.67 c	15.0 ± 1.81 b	14.2 ± 1.55 b	12.7 ± 0.71 b
sign. F	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*	***	***	**	***

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly different.

exposure on HCAs biosynthetic behaviour (in the case of leaf removal treatments) or (most likely) by the combined effect of light and temperature on the HCAs profiles in grapes and consequently in wines. This theory cannot, however, be supported by other findings, since there is a serious lack of relevant research in the field. However, our existing 2010 data show that the concentration of total HCAs after cold maceration was similar for all treatments (Figs 13 E and 13 F). Thereafter (during fermentation) the concentration of total HCAs in the case of PF and BS continued to increase up to the second day of fermentation, while in the case of VE and UN the concentration started to decrease immediately. This was probably due to a lower initial content in the grapes and/or a different qualitative profile, e.g. the ratio between *trans* caftaric and *trans*- and *cis*-coutaric acids, as both are reported to have different losses due to incomplete extraction from the berry and may be oxidised to a different extent.[44] Individually detected *trans*-caftaric as well as *cis*- and *trans*-coutaric HCAs in 2010 all showed similar (lower) extraction trends in VE and UN, and higher (but similar) extraction trends in wines from earlier leaf removal treatments (BS and PF) (Table 10). It seems that earlier leaf removal (PF and BS) may provide a better HCAs yield in wines. In contrast with these findings, 2-S-glutathionyl caftaric acid (GRP) occurrence did not show any significant differences between YWAP samples for either the 2009 or the 2010 vintage. HCAs are believed to be involved in many co-pigmentation reactions, with a possible favourable influence on wine colour stability,[45] making our findings very important for the variety under observation. Very little is known about the effects of climate/microclimate on HCAs formation in grapes (especially when compared with existing anthocyanin and flavonol data in the field).

However, it is good to know that even if the differences achieved in the vineyard are relatively small, they can be retained in young Pinot Noir wines.

4.2.4.6 Wine colour properties

Colour density (AU) values in YWAP (measured in 2010) were shown to be favourable in earlier (PF and BS) leaf removal treatments (33 and 28% higher respectively) as compared with late (VE) (8% higher) and in all cases as compared with the control (UN) (14.60 ± 0.43 AU). On the contrary, the hue values in YWAP did not show any significant differences, although a slightly higher trend was observed in the case of VE (0.79 ± 0.09). These results correspond with those of similar experiments carried out on Tempranillo,[6] although slightly lower colour density (and higher hue) values were observed, as could be expected for Pinot

Noir. Furthermore, the results are also in agreement with existing reports stating that alterations insomespecific phenolic combinations (e.g. Qe/Mal-3G ratio) may cause important wavelength shifts.[15] The dimension *a** coordinate (of CIELab colour space) indicated significantly higher (preferable) "magenta colour-opponent" dimensions in the case of YWAP from earlier leaf removals PF and BS, with 41 and 39% higher values respectively as compared with the control (with no significance highlighted between them), followed by VE (14% higher than the control). The dimension *b** coordinate was found to be significantly lower only in the case of the untreated control, while *L** observations of the lightness/darkness of the colour did not show any significant differences between treatments in YWAP (data not shown). In particular, the wine colour properties obtained were consistent with the better yield of some colour-related phenolics observed in the case of earlier leaf removals as comparedwith late leaf removal performance and control.

4.2.5 Conclusions

Despite differences between the two seasons observed, several important shifts in the phenolic profiles of the grapes and consequently in their wines were detected, affected by the leaf removal technique itself or promoted even more by the specific timing of its implementation. Both early leaf removals showed good potential to become an alternative to véraison application in the case of Pinot Noir, especially because of some positive indications regarding colour improvements. The flavonol profiles were closely linked to the climatic conditions during the year (mainly in terms of sun hours and their distribution over summer), and the synthesis of quercetin, probably the most important flavonol involved in co-pigmentation, was positively affected by leaf removals in both seasons. The qualitative and/or quantitative profiles of anthocyanins and hydroxycinnamates, both involved in the formation of more complex/stable pigments (during fermentation but mainly during wine aging), were both affected by microclimate manipulation. However, some favourable results in response to early leaf removal were statistically confirmed only in the 2010 season. Promoted formation of vitisin A-like pyranonathocyanins was detected already during fermentation of 2010 must if leaf removal was performed at any time.

4.2.6 Acknowledgements and financial support

We would like to thank the owners of the Tilia Estate for allowing us to carry out the experimental work in their vineyards, and Domenico Masuero, Daniele Perenzoni, Cesare

Lotti and Dejan Glavan for their help with sampling and/or analytical work.

4.2.7 References

(1) Haselgrove D, Botting B, Heeswijck R, Høj PB, Dry PR, Ford C and Land PGI, Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of *Vitis vinifera* L. cv. Shiraz grape berries. *Aust J Grape Wine Res* 6: 141-149 (2000).

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

(3) Sternad Lemut M, Trost K, Sivilotti P and Vrhovsek U, Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. *J Food Comp Anal* 24: 777-784 (2011).

(4) Price SF, Breen PJ, Valladao M and Watson BT, Cluster sun exposure and quercetin in Pinot Noir grapes and wine. *Am J Enol Vitic* 46: 187-194 (1995)

(5) Poni S, Casalini L, Bernizzoni F, Civardi S and Intrieri C, Effects of early defoliation on shoot photosynthesis, yield components and grape composition. *Am J Enol Vitic* 57: 397-407 (2006).

(6) Diago MP, Ayestara B, Guadalupe Z, Garrido A and Tardaguila J, Phenolic composition of Tempranillo wines following early defoliation of the vines. *J Sci Food Agric* 92: 925-934 (2012).

(7) Chorti E, Guidoni S, Ferrandino A and Novello V, Effect of different cluster sunlight exposure levels on ripening and anthocyanin accumulation in Nebbiolo grapes. *Am J Enol Vitic* 61: 23-30 (2010).

(8) Tarara JM, Lee J, Jungmin L, Spayd SE, Scagel CF, Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in Merlot grapes. *Am J Enol Vitic* 59: 235-247 (2008).

(9) du Toit WJ, Marais J, Pretorius IS and du Toit M, Oxygen in must and wine: A review. *S Afr J Enol Vitic* 27: 76-94 (2006).

(10) Fulcrand H, Dueñas M, Salas E and Cheynier V, Phenolic reactions during winemaking and aging. *Am J Enol Vitic* 57: 289-297 (2006).

(11) Castañeda-Ovando A, de Lourdes Pacheco-Hernández M, Páez-Hernández ME, Rodríguez JA and Galán-Vidal CA, Chemical studies of anthocyanins: A review. *Food Chem* 113: 859-871 (2009).

(12) de Freitas V and Mateus N, Formation of pyranoanthocyanins in red wines: a new and diverse class of anthocyanin derivatives: A review. *Anal Bioanal Chem* 401: 1467-1477 (2011).

(13) Arapitsas P, Perenzoni D, Nicolini G and Mattivi F, Study of Sangiovese wines pigment profile by UHPLC-MS/MS. *J Agric Food Chem* DOI: 10.1021/jf302617e (2012)

(14) Rentzsch M, Schwartz M, Winterhalter P, Pyranoanthocyanins - an overview on structures, occurrence, and pathways of formation. *Trends Food Sci Technol* 18: 526-534 (2007).

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

(16) Medina K, Boido E, Dellacassa E and Carrau F, Yeast interactions with anthocyanins during red wine fermentation. *Am J Enol Vitic* 56: 104-109 (2005).

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

(18) Rossetto M, Vanzani P, Zennaro L, Mattivi F, Vrhovsek U, Scarpa M and Rigo A, Stable free radicals and peroxyl radical trapping capacity in red wines. *J Agric Food Chem* 52: 6151-6155 (2004).

(19) Nardini M, Forte M, Vrhovsek U, Mattivi F, Viola R and Scaccini C, White wine phenolics are absorbed and extensively metabolized in humans. *J Agric Food Chem* 57: 2711-2718 (2009).

(20) Gómez-Míguez M, González-Miret ML and Heredia FJ, Evolution of colour and anthocyanin composition of Syrah wines elaborated with pre-fermentative cold maceration. *J Food Eng* 79: 271-278 (2007).

(21) CIE, Colorimetry, Publication No. 15.2 (2nd ed.). Central Bureau of the CIE, Vienna, Austria (1986).

(22) Schultz HR, Climate change and viticulture: A European perspective on climatology, carbon dioxide and UV-B effects. *Aust J Grape Wine Res* 6: 2-12 (2000).

(23) Jones GV and Hellman E, Site Assessment: in "Oregon Viticulture" Hellman, E. (ed.), 5th 1st Edition, Oregon State University Press, Corvallis, Oregon, pp. 44–50 (2003).

(24) Percival DC, Fisher KH and Sullivan JA, Use of fruit zone leaf removal with *Vitis vinifera* L. cv. Riesling grapevines. II. Effect on fruit composition, yield, and occurrence of bunch rot (*Botrytis cinerea* Pers.: Fr.). *Am J Enol Vitic* 45: 133-140 (1994).

(25) Kliewer WM and Torres RE, Effect of controlled day and night temperatures on grape coloration. *Am J Enol Vitic* 23:71-77 (1972).

(26) Spayd SE, Tarara JM, Mee DL and Fergusson JC, Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. *Am J Enol Vitic* 53: 171-182 (2002).

(27) Ristic R, Downey MO, Iland PG, Bindon K, Francis IL, Herderich M and Robinson SP, Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. *Aust J Grape Wine Res* 13: 53-65 (2007).

(28) Downey MO, Harvey JS and Robinson SP, The effect of bunch shading on berry development and flavonol accumulation in Shiraz grapes. *Aust J Grape Wine Res* 10: 55-73 (2004).

(29) Downey MO, Harvey JS and Robinson SP, Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Aust J Grape Wine Res* 9:110-121 (2003).

(30) Cortell JM, Halbleib M, Gallagher AV, Righetti TL and Kennedy JA, Influence of vine vigor on grape (*Vitis vinifera* L. Cv. Pinot Noir) anthocyanins. 1. Anthocyanin concentration and composition in fruit. *J Agric Food Chem* 55: 6575-6584 (2007).

(31) King PD, McClellan DJ and Smart RE, Effect of severity of leaf and crop removal on grape and wine composition of Merlot vines in Hawke's Bay vineyards. *Am J Enol Vitic.* DOI: 10.5344/ajev.2012.12020 (2012).

(32) Rolle L, Gerbi V, Schneider A, Spanna F and Segade SR, Varietal relationship between instrumental skin hardness and climate for grapevines (*Vitis vinifera* L.). *J Agric Food Chem* 59: 10624-10634 (2011).

(33) Cortell JM and Kennedy JA, Effect of shading on accumulation of flavonoid compounds in (*Vitis vinifera* L.) Pinot Noir fruit and extraction in a model system. *J Agric Food Chem* 54: 8510-8520 (2006).

(34) Rustioni L, Rossoni M, Calatroni M and Failla O, Influence of bunch exposure on anthocyanins extractability from grapes skins (*Vitis vinifera* L.). *Vitis* 50: 137-143 (2011).

(35) Poni S, Bernizzoni F, Civardi S and Libelli N, Effects of pre-bloom leaf removal on growth of berry tissues and must composition in two red *Vitis vinifera* L. cultivars. *Aust J Grape Wine Res* 15: 185-193 (2009).

(36) Intrieri C, Filippetti I, Allegro G, Cetinati M and Poni S, Early defoliation (hand vs mechanical) for improved crop control and grape composition in Sangiovese (*Vitis vinifera* L.). *Aust J Grape Wine Res* 14: 25-32 (2008).

(37) Tardaguila J, Diago MP, Martinez de Toda F, Poni S and Vilanova M, Effects of timing of leaf removal on yield, berry maturity, wine composition and sensory properties of cv. Grenache grown under non irrigated conditions. *Journal Internation de la Science de la Vigne et du Vin* 42: 221-229 (2008).

(38) Matus JT, Loyola R, Vega A, Peña-Neira A, Bordeu E, Arce-Johnson P and Alcalde JA, Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. *J. Exp. Bot*. 60: 853-867 (2009).

(39) Waterhouse AL and Laurie VF, Oxidation of wine phenolics: A critical evaluation and hypotheses. *Am. J. Enol. Vitic* 57: 306-313 (2006).

(40) Kennedy JA, Saucier C and Glories Y, Grape and wine phenolics: history and perspective. *Am J Enol Vitic* 57: 239-248 (2006).

(41) Fulcrand H, Benabdeljalil C, Rigaud J, Cheynier V and Moutounet M, A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* 47: 1401–1407 (1998).

(42) Marquez A, Dueñas M, Serratosa MP and Merida J, Identification by HPLC-MS of anthocyanin derivatives in raisins. *J Chem*. DOI: 10.1155/2013/274893 (2013).

(43) Waterhouse AL, Wine Phenolics. Annals of the New York Academy of Sciences 957: 21–36 (2002).

(44) Vrhovsek U, Extraction of hydroxycinnamoyltartaric acids from berries of different grape varieties. *J Food Comp Anal* 46: 4203-4208 (1998).

(45) Oliveira J, de Freitas V, Artur M, Silva AMS and Mateus N, Reaction between hydroxycinnamic acids and anthocyanin-pyruvic acid adducts yielding new portisins. *J Agric Food Chem* 55: 6349-6356 (2007).

(46) EARS. Slovene Public Information, EARS, meteo.si; http://meteo.arso.gov.si; October, 2013.

4.3 The use of metabolic profiling to study grape skin polyphenol behaviour as a result of canopy microclimate manipulation in a 'Pinot Noir' vineyard (Sternad Lemut et al., 2013b)

4.3.1 Abstract

Canopy microclimate manipulation can have a significant effect on grapevine gene expression behaviour in the phenylpropanoid biosynthetic pathway and can thus affect the yield of many important grape berry compounds. Focusing on only a few targeted phenolics in the past, new advanced analytical multimethod approaches are opening up a much wider range of possibilities to fill in the gaps of missing knowledge about plant secondary metabolism within purposely-induced microclimate shifts. Different leaf removal timings, leading to different microclimate scenarios, were therefore introduced in a 'Pinot Noir' vineyard to reveal related alterations of multiple classes of skin phenolics, including some rarely studied to date. Different accumulation trends during cluster development were detected not only between groups but interestingly also between individual compounds within groups. Although many significant changes were observed early in the season, these were often less significant by the end of maturation. However, at harvest, 31 of 72 detected compounds still showed significant differences in comparison to the control for at least one of the three leaf removal approaches.

Key words: metabolic profiling; canopy microclimate manipulation; leaf removal; pre-flowering leaf removal; secondary metabolism; phenolics; 'Pinot noir' (*Vitis Vinifera* L.)

4.3.2 Introduction

Grapevine constituents that are not directly involved in the primary biochemical pathways of cell development are commonly classified as secondary metabolites. Even though they are not considered as essential for normal plant functioning, many of them have important roles such as signaling and attraction, as well as protecting the plant from different biotic and/or abiotic stresses.[1] This concerns compounds involved in plant-pathogen (or insect) interactions, compounds preventing UV damage to different plant tissues and compounds implicated in hormone homeostasis.[2] Consequently, secondary metabolites are normally present in higher

concentrations when a plant is subjected to different stresses or elicitors.[3] However, not only are they important for plant adaptation and survival, but their occurrence and chemical diversity can also account for significant diversity in the quality of different agricultural crops.

In general, secondary metabolites consist of a wide array of species-specific chemicals, belonging to different phytochemical groups, such as alkaloids, terpenes, antibiotics, volatile oils, resins, cardiac glycosides, sterols, saponins, and phenolic compounds.[4] Phenolics in particular are currently under special research interest, not only due to crop plant quality issues, but also due to various associations with human health-promoting effects.

Apart from variety specifics, the occurrence of vine secondary metabolites, including phenolics, is largely determined by the geo-climatic conditions (terroir) in which the plant is grown.[5] Although regional macroclimate or site mesoclimate cannot be influenced, grapevine canopy microclimate conditions can, on the other hand, be manipulated by implementing some viticultural practices in the vineyard environment. Leaf removal is one of the techniques that can be employed to maneuver the microclimate in the cluster area; however, its performance is particularly related to the phenological stages of grape berry development at which the practice is adopted.[6] It is commonly performed in the post-flowering period (between phenological growth stages BBCH 69 and BBCH 83), whereas earlier implementation before flowering (at BBCH 57) and the effects on the secondary metabolism due to such early alterations in microclimate conditions have not yet been extensively explored.

When actually manipulating canopy microclimate in the field, temperature is one of the factors to be considered carefully. Apart from many effects on vine performance, a trend toward a reduction in total acidity in particular is confirmed also in Slovenia, as a result of higher temperatures during the growing season nowadays.[7] Temperature has also been shown to significantly affect flavonoid biosynthesis. Several experiments have revealed the inhibitory effect of (overly) high temperature on anthocyanin accumulation in berry skins,[8-10] whereas the critical temperature leading to the inhibition of anthocyanin synthesis is reported to be between 30 and 35 °C, varying according to different authors. Moreover, cool night temperatures and day/night temperature regimes can also lead to modifications in red grape color characteristics.[8,11,12] Recent studies on the biosynthesis of grape flavonoids as affected by temperature have indeed mainly focused on anthocyanins, however, ref 13 also highlighted high temperatures causing a moderate reduction in proanthocyanidin and quercetin concentrations in berry skins. Furthermore, the temperature in the last phase of ripening played

an important role in the observed *cis*-piceid levels in 'Barbera', as described in ref 14. Few research projects have focused on other skin phenylpropanoids and their responses to changes in temperature; thus, little is still known about accumulation trends related to such climatic issues.

Sunlight exposure is another important factor with a known impact on the phenolic composition of grapes.[10,15] There is much contradictory data about the impact of light on red grape colour, however, apart from concentration, ultraviolet light exposure has been reported to alter the anthocyanin composition of different grape varieties.[10,16] On the other hand, exposure to UV light has a very evident effect on flavonols.[10,15,16] UV light has also been reported to stimulate the production of the stilbene resveratrol,[17] whereas the accumulation of tannins in grape berries was shown to be relatively unaffected, with some changes being detected only early in the season.[18,19] Once again, little research has yet been done on many other grape skin phenolics in relation to light exposure or further single/multiple microclimate parameters.

Environmental metabolomics and related advanced multimethod approaches initially focused mainly on the whole fruit/crop metabolome as affected by environmental conditions. However, for its successful implementation into standard practice, an understanding of environmental effects on a range of compounds in a single plant tissue is also very important. Many grape phenolics are known to be synthesized exclusively in (*V. vinifera*) grape berry skins (e.g. anthocyanins, flavonols, stilbenes). Some others, such as flavan-3-ols, are also present in the seeds, whereas those from skins seem to be more affected by environmental factors and have a higher tendency for polymerization[19,20] as well are essentially more likely to be extracted in must/wine. Grape berry skins thus undoubtedly represent a very important tissue, to be considered separately using new analytical possibilities in order to improve knowledge and enable more successful grapevine canopy management. Whereas scientific studies manipulating a single controlled factor may indeed help to improve the understanding of the direct effects on plant biosynthesis, studies in realistic conditions may be more helpful for viticultural practice.[21] However, in the case of actual field observations, multiple factors should be considered together due to their known synergic effects. A field trial was thus designed to employ new advanced analytical possibilities to improve knowledge of grapevine phenylpropanoid biosynthetic behavior in the case of different "real case" (and up to certain level defined) microclimate scenarios, created by carrying out leaf removal at different phenological stages within 'Pinot noir' cluster development.

4.3.3 Material and methods

4.3.3.1 Experimental vineyard and plant material

The experiment was carried out in 2010, observing in total 320 'Pinot Noir' (*V. vinifera* L.) grapevines in a vineyard located in Potoce (Vipava Valley, Slovenia). The vineyard was planted in 2004 and the rows are oriented east-west, whereas its plant density is 5682 plants/ha (0.8 m vine spacing x 2.2 m raw spacing). It is situated at an altitude of 95 m above see level and the training system adopted is the single Guyot. A completely randomised experimental design was set up in the middle of the vineyard, with four treatments and three replicates (12 plots of 20 plants). The treatments were applied as follow: PF (pre-flowering), leaf removal (LR) performed before flowering, at phenological stage BBCH 57;[22] BS (berry-set), LR applied at BBCH 71; VE (veraison), LR performed at BBCH 83; UN (control treatment), LR not applied (untreated vines / leaves retained). Leaf removal was performed manually, removing the basal four-to-six leaves of all the shoots as normally carried out for pre-bloom treatments,[23] thus for each treatment the same leaf removal severity was applied (at pre-flowering (PF), berry set (BS) and veraison (VE) time, respectively). After initial performance of experimental defoliations, the leaf removed zones were not maintained leaf free.

4.3.3.2 Monitoring of microclimate conditions

The temperature and relative humidity in the cluster area of all the treatments were monitored during the hottest period (from August 14 to September 11, 2010) via DS1923 i-Button sensors (Maxim Integrated Products, CA, USA), collecting and storing data on an hourly basis.

4.3.3.3 Sampling and sample preparation

Grape berry samples from all plots were collected separately during maturation (from June to September 2010). Sampling was essentially carried out at 10-day intervals (with some adjustments due to rain events) and at harvest time (based on the maturity level recorded in the control grapes: 22 °Brix and 5.6 g L^{-1} titratable acidity on average). The first sampling was done when the berries were formed enough for successful separation of the skins; thus, it was done after the performance of the first two leaf removal strategies (at pre-flowering and berry set). Berry samples were carefully collected together with their pedicels to avoid any damage

and/or oxidation risks. Samples were then frozen immediately and methanol extracts (MeOH extracts hereinafter) of berry skins were prepared as previously described.[24] Briefly, the skins of previously weighed berries were carefully separated from flesh and seeds (while the berries were still frozen) and put directly into a dark glass container with 100 mL of MeOH. After 24 hours of initial extraction, the extract was separated and the second extraction for 2 hours in 50 mL of MeOH was performed. Both methanolic extracts were then combined, and the MeOH extracts were kept at -20 °C until the analyses were carried out.

Before UHPLC-QqQ-MS/MS analysis of phenolic compounds, an aliquot of 10 mL of MeOH extract was first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd., UK) under reduced pressure at 45 °C. The sample was then reconstructed in a quantitative flask up to 1 mL of the final volume with methanol (Fluka, Sigma-Aldrich) and filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA, USA). Additional dilution with MeOH was carried out if needed for the compounds present in higher concentrations.

Before HPLC analysis of grape anthocyanins, the MeOH extracts were first filtered (0.45 μm Millipore HPLC filter) and thereafter diluted with 1% trifluorocetic acid (TFA, Sigma, Germany) in water using a 1:1 (v/v) ratio to maintain the symmetry of chromatographic peaks.

4.3.3.4 UHPLC-QqQ-MS/MS (targeted metabolomics) analysis

A comprehensive targeted metabolomic analytical approach according to ref 25 was applied. The method was developed with the potential to perform qualification and quantification of 135 phenolics, belonging to different chemical groups present in fruit, such as benzoates, phenylpropanoids, coumarins, stilbenes, and flavonoids (flavones, isoflavones, flavanones, flavan-3-ols flavonols and dihydrochalcones). Ultra high performance liquid chromatography (UHPLC-MS/MS) was performed using a Waters Acquity UHPLC system (Milford, MA, USA). Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm , kept at 40 °C.[25] All analyses were performed in biological triplicates.

4.3.3.5 HPLC determination of anthocyanins

The analytical method as previously presented by ref 6 was applied for the detection of anthocyanins. Separation and quantification of delphinidin-3-glucoside (Del-3-Glu), cyanidin-3-glucoside (Cy-3-Glu), petunidin-3-glucoside (Pet-3-Glu), peonidin-3-glucoside (Peo-3-Glu) and malvidin-3-glucoside (Mal-3-Glu) were performed using gradient high performance liquid chromatography with UV-vis detection at 520 nm (Waters chromatographic system). Individual anthocyanins were separated on the Atlantis column C18, 3.9 × 150 mm, 3 μm (Waters, USA), and quantified as malvidin-3-glucoside (mg/L) equivalent. All analyses were performed in biological triplicates.

4.3.3.6 Data processing and statistical analysis

Processing of phenolic raw data sets was performed with the help of Mass Lynx Target Lynx Application Manager (Waters), except for anthocyanins, which were processed with the help of Empower software (Waters). Multivariate Principal Component Analysis (PCA) on the autoscaled data was performed to visualize the effects of the different leaf removal strategies. To get further insight on the metabolic effects of the different leaf removal strategies, separate ANOVA models for the different metabolites were performed. Because many tests were done, some form of multiple-testing correction was necessary. Here, we have used the false discovery rate (FDR) correction.[26] In those cases when the corrected p values were below 0.05, Tukey's Honest Significant Difference (HSD) was used to find which factor levels actually differ. All of these statistical tests were performed with R.[27]

4.3.4 Results and discussion

The vintage 2010 in Vipava Valley (macroclimate) was quite warm and sunny earlier in the season (June, July), but colder during late season (September), mainly due to frequent rainfall events (Supp. Inf. Table 3_S1). However, different leaf removal treatments led to four different canopy microclimate scenarios (sun exposure, temperature, humidity) in the cluster area. Earlier leaf removal (LR) treatments (pre-flowering (PF) and berry set (BS)) caused clusters to be more exposed to the sun during June and July (open canopies), whereas clusters of late - veraison (VE) leaf removal (LR) treatment were initially covered by leaves (closed canopy) and then opened in mid August. On the other hand, untreated control (UN) remained closed until harvest. Differences in temperature and humidity within cluster areas were thus recorded

in critical, hot August days, with basically lower relative humidity and higher temperature in the case of LR treatments when compared to control (Supp. Inf. Figures 3_S2 and S3). As the present field experiment is supported by measurements of microclimate conditions and thus four different purposely induced and, to certain level, defined microclimate scenarios were compared, this work is of great value on a more global level even if the statistical significance and reproduction of the results over more harvest seasons have not been completed. Within viticultural parameters (Supp. Inf. Table 3_S2) a reduced cluster weight and yield were observed for PF, as previously observed in case of 'Barbera' and 'Lambrusco salamino' (*Vitis vinifera* L.).[23]

4.3.4.1 Phenolic compounds

To adapt to ongoing changes in the environment, plants can use "their enormous metabolic capacity to produce a large variety of secondary metabolites,"[28] including phenolics. Of 140 phenolic compounds under observation, in total 72 different phenolics were detected in skin samples in the case of at least one sampling point during the maturation period (June-September 2010). The detected compounds were the group representatives of flavonols (22), stilbenes (18), flavan-3-ols (10), benzoates (7), anthocyanins (5), hydroxycinnamates (5), flavones (2), flavanones (2), and dihydrochalcones (1). Different accumulation trends (peaking behavior) during cluster berry development were detected, not only between groups but also between individual compounds within the same chemical group, signifying that the timing of leaf removal may play an important role in targeted promoting of specific compounds with either early or late biosynthetic behavior.

PCA was performed to visualize the effects of different leaf removal strategies and to highlight the evolution of the metabolic profiles over time. Figure 14 shows the projection of the data set on the PC1 x PC2 plane accounting for 65% of the total variance. The effects of the different treatments over time segments are graphically highlighted by connecting the median of the replicates.

Figure 14 highlights that at the earlier stage, the control was different from PF and BS, indicating that both types of early leaf removal (performed before first sampling, see Sampling) caused substantial changes in plant biosynthetic behavior soon after they were implemented. The global view obtained by PCA, however, does not allow clear separatation of the treatments at the latest time-point. To get further insight on the metabolic effects of the

different leaf removal strategies, separate ANOVA models for the different metabolites were performed. In the following they are discussed in details.

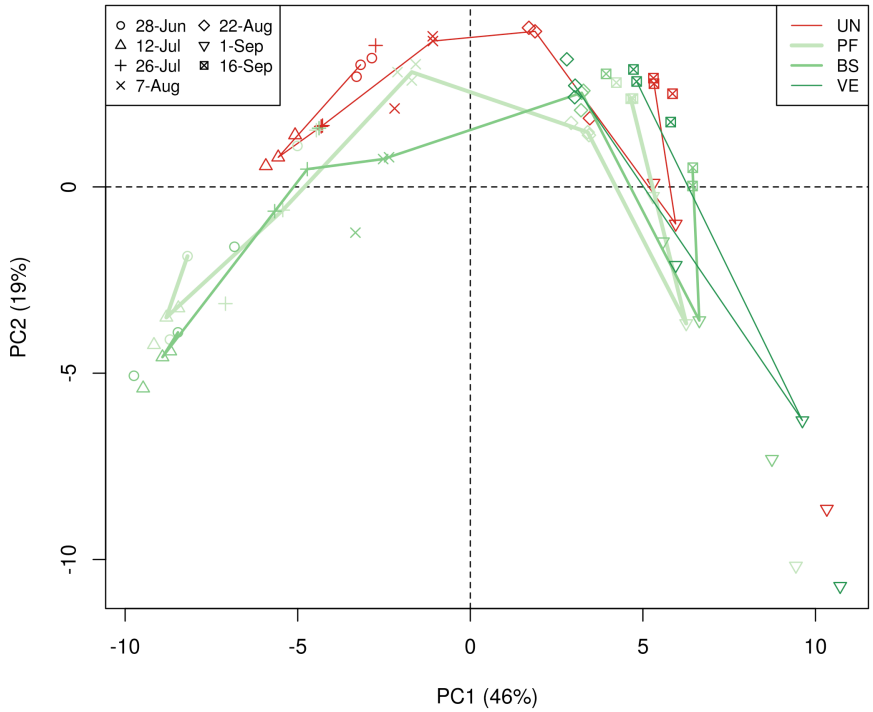


Figure 14: Multivariate Principal Component Analysis (PCA) on the autoscaled data showing the projection of the data set in the PC x PC2 plane as affected by different leaf removal strategies highlighting the evolution of the metabolic profiles over time (PF=pre-flowering; BS=at berry set; VE=at veraison; UN=control with no leaf removal). The segments connect the median of the biological replicates.

4.3.4.1.1 Flavonols

Flavonols are known to be the products of the flavonoid biosynthetic pathway, which in red grapevine cultivars also engenders anthocyanins and condensed tannins. In *V. vinifera* grapes they are mostly present as glycosides and are synthesized only in grape skins.[15,20] Different physiological functions of flavonols are reported in plants; however, their most widespread role still appears to be protection from excessive UV damages. The biosynthesis of total flavonols in our experiment was extensively triggered by leaf removal treatments, because the light environment within the grapevine canopy was considerably enhanced. A clear increase in flavonols following an increase in cluster sun exposure has already been reported.[10,15,16] As they can be almost entirely absent in the case of shaded bunches[19] and with a prompt increase after later exposure, flavonols can be considered as a biomarker for the sun exposure regime achieved in a bunch area following canopy microclimate manipulation.[29]

Two distinct periods in flavonol synthesis have been already reported in grapes, the first around flowering and the second during ripening of developing berries.[18] This "two peak" biosynthetic behavior in relation to total flavonols was also detected in our experiment (Figure 15A), although the increase in total flavonols (particularly in the case of earlier BS and PF leaf removal) was much higher early in the season and was followed by only a minor increase (in the case of VE/UN and PF) later in the season.

However, observing only total flavonol content seriously masks the interesting behavior of individual compounds. By focusing on the accumulation trends of individual flavonols, it was indeed revealed that their concentration peaks differ considerably. Although most of them showed the greatest synthesis later in the season, therefore signifying late peaking behavior, others on the contrary showed the greatest synthesis early in the season (early peaking behavior), whereas some of flavonols showed "no specific behavior" at all (Table 11). Finally, only two of flavonols showed "two peak behaviou" during berry development (Table 11). If the "two-hump" curve of total flavonols is occurring during grape berry development, this is most likely due to a combined reality of differently behaved individuals.

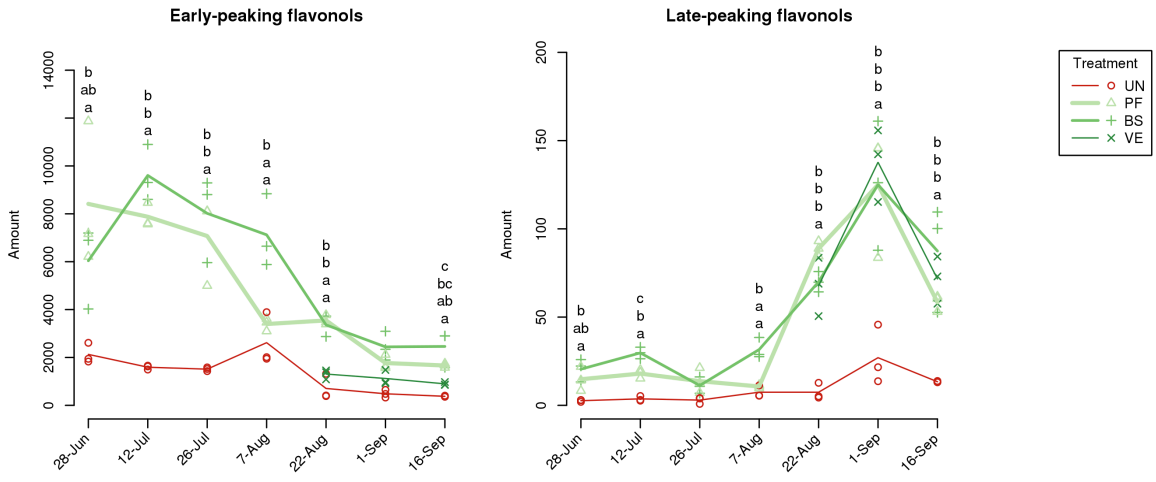


Figure 15: Total flavonols (μg/g skins) with early peak behavior and late peak behavior as affected by canopy microclimate manipulation through leaf removal at different phenological stages (PF=pre-flowering; BS=at berry set; VE=at veraison; UN=control with no leaf removal).

Despite the fact that most flavonols peaked relatively late, those same flavonols were present in relatively small concentrations (all ranging on average from 125 to 138 μg/g skins at the highest point in the case of LR treatments) (Figure 16). On the other hand, there were fewer peaking earlier, but they occurred in much higher concentrations (on average from 7878 to

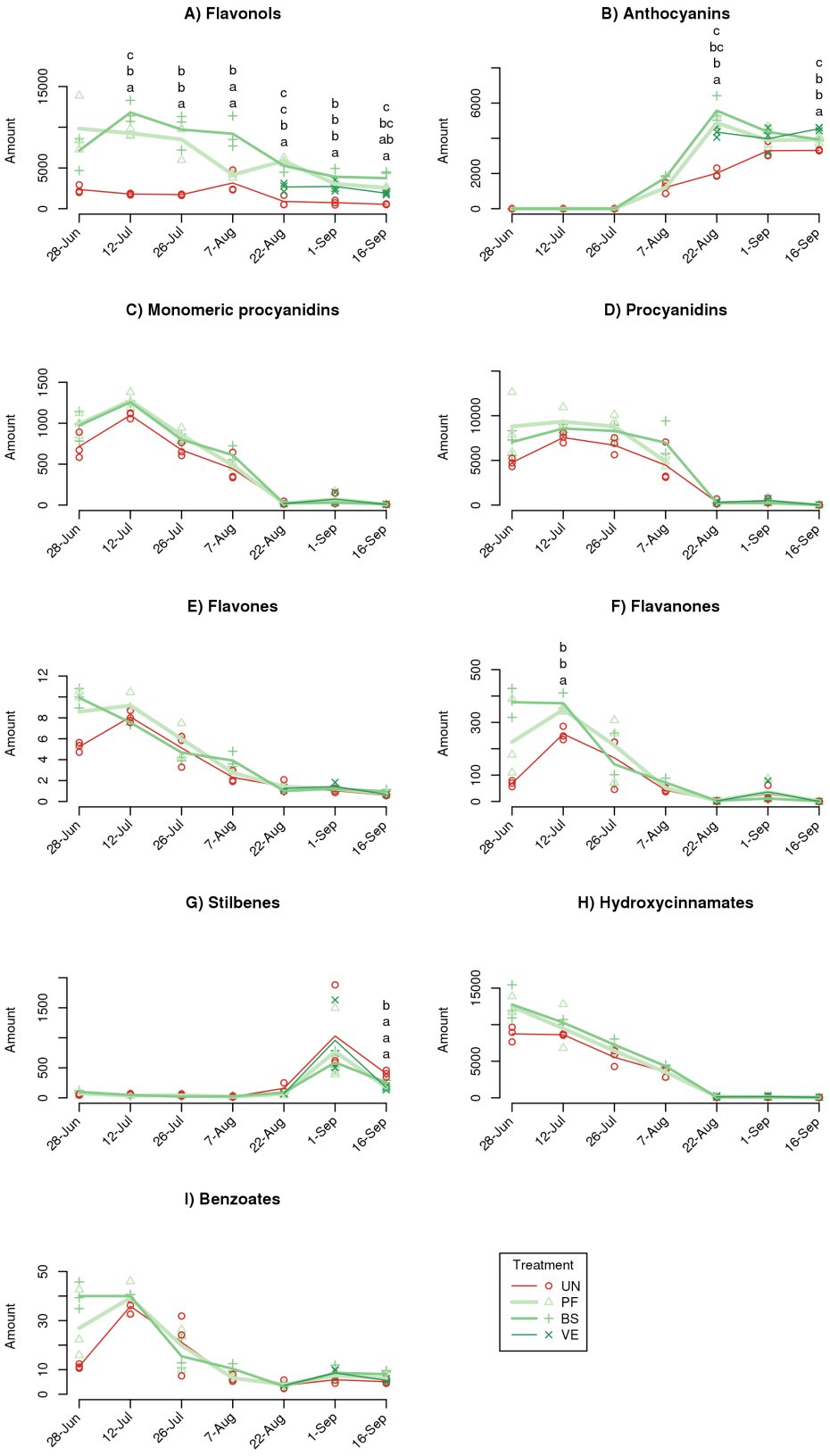


Figure 16: Total phenolics within different phenolic groups (μg/g skins) as affected by canopy microclimate manipulation through leaf removal at different phenological stages (PF=pre-flowering; BS=at berry set; VE=at veraison; UN=control with no leaf removal).

9604 µg/g skins at the highest point in the case of LR treatments). Moreover, early peaking flavonols in total were still present at harvest with a 12-28x higher concentration than in total late peaking flavonols and represented 65% of total flavonols in the case PF leaf removal, the same (65%) in case of BS leaf removal treatment but only 47% in the case of late leaf removal (VE).

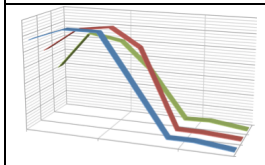
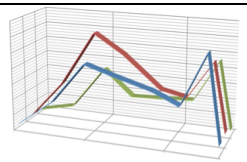
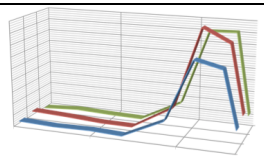
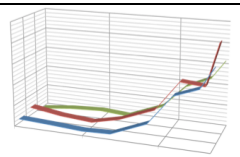
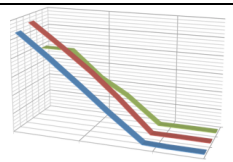
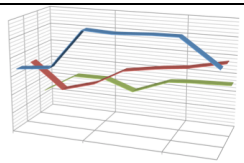
Considering different leaf removal treatments, we could also observe that earlier leaf removal treatments (PF and BS) fundamentally affected the occurrence of flavonols with early peaking behavior and that the related improvement in total flavonol content was also retained at harvest (3755±1174 and 2563±105 µg/g skins for BS and PF, respectively; as compared to 1902±212 and 540±51 µg/g skins for VE and UN, respectively). Late (veraison) leaf removal, on the other hand, caused only a minor increase in total flavonol concentration as compared to untreated control and with no significant results at harvest (Fig 15 A). This clearly indicates that early peaking flavonols can only be promoted by early leaf removal intervention. On the contrary, the accumulation of late peaking flavonols as compared to the control was similar at harvest (with no significant differences) for all the leaf removal treatments, regardless of the timing at which it twas carried out (Figure 16).

Mattivi et al. [24] have already reported certain correlations between the metabolic pathways of anthocyanins and flavonols, implying that any attempt to optimize the pattern of one might also affect the patterns of others. However, both groups are important for grape and wine quality. Anthocyanins mostly in terms of color intensity, whereas anthocyanins and flavonols are both involved into co-pigmentation reactions,[30] crucial for the development of wine color stability (a known problem for Pinot noir wines).[31] Therefore, if the biosynthesis of flavonols would be strongly promoted early in the season, even before anthocyanins start to accumulate, there would probably be a chance of improving the yield of both at harvest. This could/should indeed be one of the tasks of successful canopy microclimate manipulation.

4.3.4.1.2 Anthocyanins

Anthocyanins are synthesized from phenylalanine through an anthocyanin biosynthetic pathway regulated by enzyme activities and gene expressions.32 In addition to the enzymes required for the synthesis of flavan-3-ols, two additional enzymes (LDOX and UFGT) are required for anthocyanin biosynthesis.

Table 11: Grouping of detected skin phenolic compounds according to their biosynthetic behavior (their concentration peak within berry development period): early peak behavior (peak before veraison); late peak behavior (peak after veraison).

PEAK BEHAVIOUR	Early peak behaviour	Two peaks behaviour	Late peak behaviour	Mainly increasing behaviour	Mainly decreasing behaviour	No specific peak behaviour
						
SKIN PHENOLIC COMPOUNDS	• 4-hydroxybenzoic acid • syringaldehyde • caffeic acid • caffeic acid+catechin con. • phlorizin • luteolin-7-glucoside • naringenin-7-glucoside • catechin • gallocatechin • procyanidin B1 • procyanidin B2+B4 • rutin • quercetin-3-glucunoride • kaempferol-3-rutinoside • quercetin-3-Glc-Ara • taxifolin	• quercetin-3-rhamnoside • kaempferol-3-glucunoride	• methyl gallate • syringic acid • *trans*-resveratrol • *cis*-resveratrol • piceatannol • pterostilbene • *trans*-piceid • *cis*-piceid • astringin • isorhapontin • *cis*-ε-viniferin • *cis*-ω-viniferin • *trans*-ω-viniferin • ampelopsin D+ quadrangularin A • pallidol • isorhamnetin • myricetin • laricitrin • syringetin • kaempferol-3-glucoside • myricetin-3-rhamnoside • isorhamnetin-3-glucoside • isorhamnetin-3-rutinoside • quercetin-3,4'-diglucoside • delphinidin-3-glucoside • cyanidin-3-glucoside • petunidin-3-glucoside • malvidin-3-glucoside	• vanillic acid • *trans*-ε-viniferin • α-viniferin • Z-miyabenol C • isohopeaphenol • ampelopsin H + vaticanol C-like isomer • syringetin-3-glucoside+ syringetin-3-galactoside • peonidin-3-glucoside	• caftaric acid • fertaric acid • *trans*-coutaric acid • epicatechin gallate • epigallocatechin gallate • procyanidin B3	• vanillin • gallic acid • ferulic acid • apigenin-7-glucoside • naringenin • epicatechin • epigallocatechin • quercetin-4'-glucoside • kaempferol • quercetin • quercetin-3-glucoside • quercetin-3-galactoside

They are expressed mainly in the skins and typically from the onset of ripening (veraison), as confirmed again in our experiment. The role of anthocyanins in plants has been investigated in many studies, but compelling evidence is still lacking. However, they are mainly associated with protection from solar ultraviolet rays and attack by herbivores or pathogens.[33]

In the samples, anthocyanins were first detected on August 7 (Figure 15 B). From that sampling date, total anthocyanins increased rapidly until they became stable (or even declined) in late August (between samplings on August 22 and September 1). However, when looking at total anthocyanins, we observed that the accumulation in the case of the untreated control (in contrast to all of the leaf removal treatments) did not experience the "late August decline". This raises the question of what could briefly inhibit synthesis in the case of open canopy (leaf removal treatment) but not in the case of shaded grapes (untreated control UN), even the total amount in the case of UN was still lower than in LF treatments at the same time. This last fact is however consistent with the literature, stating that light exposure is required in addition to optimal temperature to promote anthocyanin synthesis.[16] On the other hand, with regard to temperature, it has previously been reported that the average daily temperature on the berry surface is higher in the case of leaf removal treatments than in the untreated control[29] and that too high temperature can inhibit anthocyanin synthesis[8] - which may be one of the reasons for a brief slowing biosynthetic behaviour. It seems that higher temperature within the cluster area of PF, BS and VE (Supp. Inf. Figure 3_S2) did briefly inhibit anthocyanin synthesis, but, on the other hand, light exposure still favored open canopies. This finally led to a situation at harvest that was clearly in favor to leaf removal treatments, with VE showing the best results (4545±99 μg/g skins), followed by BS and PF (3927±81 and 3921±197 μg/g skins respectively), whereas the lowest concentration was detected in UN (3313±33 μg/g skins). As 2010 was rather cold and rainy in the Vipava Valley, the results differ slightly from our previous observations.[6] In the warmer and drier 2009, the BS treatment (PF not performed) led to higher total anthocyanins than VE. This was probably due to higher berry surface temperatures after veraison leaf removal, which exposed the grapes directly to the sun in the hottest period, as August was very hot in 2009.[29] BS (earlier treated) grapes on the other hand were partially protected by the re-growth of lateral leaves by that time. However, the decrease in anthocyanins in grape skins with higher temperatures could be caused by many factors, such as chemical factors (pH, temperature, light, oxygen) and/or enzymatic degradation and not only due to inhibition of anthocyanin biosynthesis.[34]

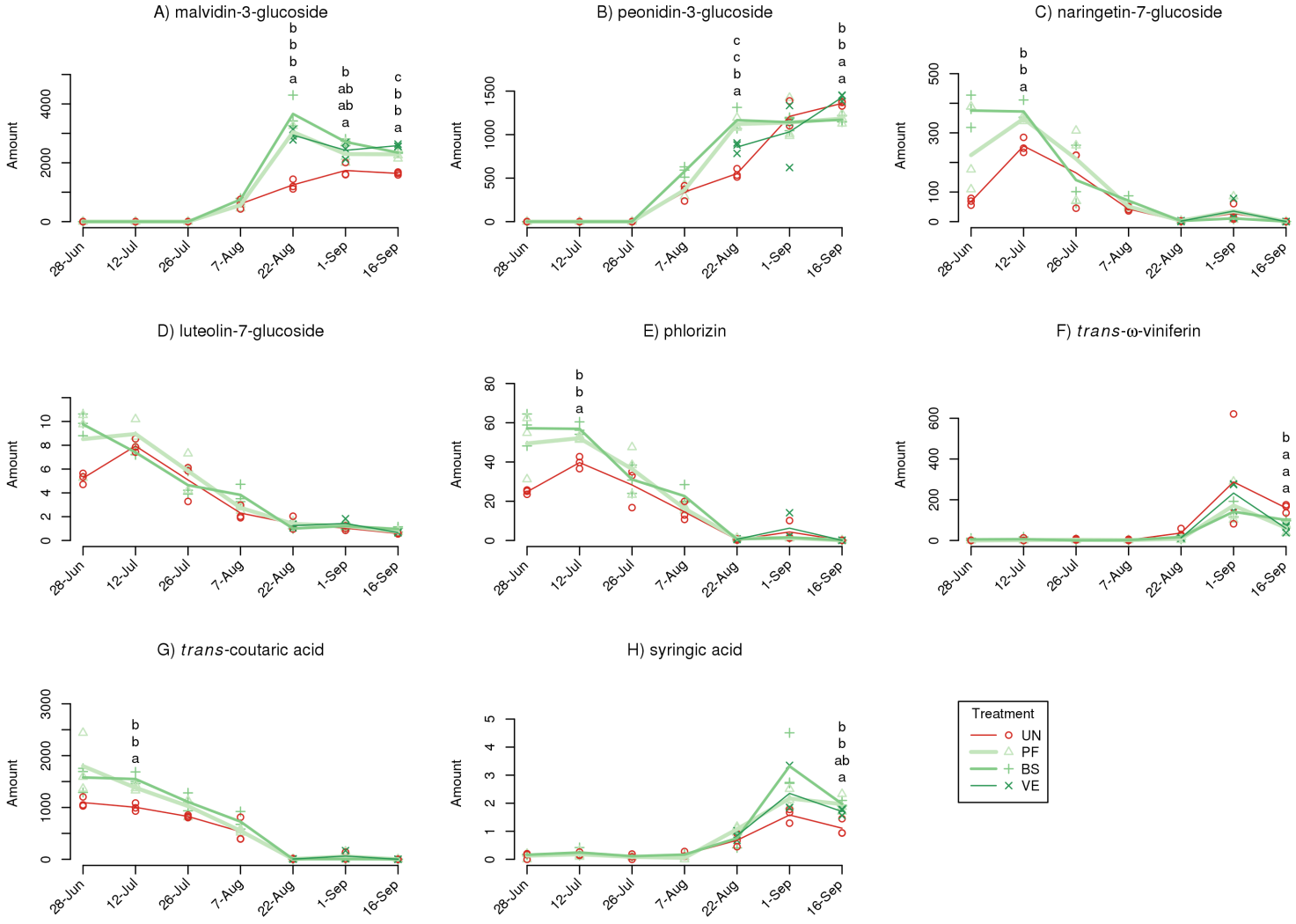


Figure 17: Accumulation dynamics (μg/g skins) of selected typical individual representatives from different phenolic groups as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal). Accumulation dynamics of the rest of the detected individual representatives can be seen within the Supporting Information.

Finally, by looking more closely into the accumulation trends for individual anthocyanins, in the case of leaf removal (LR) treatments as compared to untreated grapes (UN) we observed an increase in delphinidin, cyanidin, petunidin and malvidin glucosides (95, 34, 71, and 58% respectively at harvest in case of VE, reaching the highest values).

In case of early LR samples (BS and PF) a slight decrease in peonidin-3-glucoside (14 and 13%) as compared to UN was shown, but again an increase (although slight and not significant) as compared to VE (5 %). Malvidin and petunidin glucosides can be seen in Figures 17 A and B; others with similar behaviour to malvidin-3-glucoside can be found in the Supp. Inf. Figure 3_S1. These trends are similar to the observations of ref 20 in their light exclusion trial, so such behavior is probably more a consequence of light than of temperature.

4.3.4.1.3 Flavan-3-ol monomers and proanthocyanidins

Flavan-3-ols consist of both monomers and polymers with a different degrees of polymerization (proanthocyanidins), and share a biosynthetic pathway similar to that of flavonols and anthocyanins. In contrast to anthocyanins and flavonols, flavan-3-ols can be found not only in the skins but also in the seeds of the grape berry.[35] The generally accepted biological role of flavan-3-ols in plants is linked to protection against microbes, fungi, insects, and herbivorous animals,[36] whereas it is also believed that polymers, tannins, play a certain structural role in plants.

Flavan-3-ols were present with the highest concentrations around berry set, but started to decrease before veraison, finally remaining more or less stable in the last stages of maturation (Figures 15 C and D). This is in agreement with the observations of ref 18, highlighting that the synthesis of procyanidins in skins occurs early in berry development and reaches a maximum around veraison - in our case just before the veraison. Procyanidin B1, the most abundant flavan-3-ol in our samples (>6000 μg/g skins at the highest point for LR treatments) actually dictated the trend in terms of totals, whereas B3 showed a slightly more decreasing trend, in the case of PF already starting from the first sampling point. It is hard to arrive at conclusions regarding the exact trends shown for procyanidins B2 and B4, because it was not possible to separate them analytically due to their co-elution. However, their sum (B2 + B4) showed early peaking behavior, similar to B1, but with a much lower total concentration (68-80 μg/g skins on average at the highest point for LR treatments) (Supp. Inf. Figure 3_S1).

Many other authors have also observed skin and seed flavan-3-ols accumulation during berry development,[20,35,37] although their findings regarding total amounts and accumulation trends are not always consistent. Furthermore, the specific effects of grape cultivar[35] and vintage[37] seem to be significant. On the other hand, it has been reported that seed tannins are normally made up of monomeric flavan-3-ols (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate, whereas skin tannins can also accumulate (-)-epigallocatechin, (+)-gallocatechin and (-)-epigallocatechin gallate,[38] which is in accordance with our results for monomeric flavan-3-ols. If we compare total monomeric flavan-3-ols and total procyanidins from our trial (Figures 15 C and D) separately, we can observe that their (early) peaking behavior is similar, although procyanidins are present in much higher concentrations. In general, it has been reported that skin tannins tend to have a much higher degree of polymerization than tannins present in the seeds.[38] In the case of individual flavan-3-ol monomers, peaking behavior similar to total flavan-3-ols was observed for (+)-catechin and (+)-gallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate. (-)-Epicatechin and (-)-epigallocatechin, on the other hand, showed completely random behavior (Supp. Inf. Figure 3_S1). Furthermore, our study of canopy microclimate manipulation (through leaf removal treatments) revealed that the different trends between untreated (shaded) control and (sun exposed) leaf removal treatments could be observed only up to the point in which total flavan-3-ols concentrations become lowest but stable (last 3 weeks of maturation) (Figures 15 C and D). After that point (and at harvest) no significant differences were observed between the treatments or with the control. Light exposure in relation to skin flavan-3-ols has been previously studied in 'Shiraz' and 'Pinot noir'[19,20]. In their study, the authors observed a change in the abundance and/or polymerization level of flavan-3-ols in the skins of the shaded fruit at veraison in both 'Shiraz' and 'Pinot noir', whereas in the study of ref 20 (on 'Pinot noir') the differences also remained evident at harvest. However, both of the studies were done through artificial manipulation of light exposure, using boxes over the grapes to achieve very low exposure environment, probably much lower than in an actual situation in the case of vine canopies with no leaf removal. In addition to the research carried out on the effect of light, ref 12 also reported (in their experiment with controlled sun exposure) some changes in skin proanthocyanidin concentration at harvest as a result of berry temperature.

Focusing further on individual compounds affected by leaf removal, all procyanidins and some flavan-3-ol monomers: (+)-catechin, (+)-gallocatechin and particulary (-)-epicatechin gallate showed lower accumulation trends in the case of untreated vines as compared to leaf removal

treatments. In any case, the difference could again only be seen early in the season and had actually disappeared by harvest time. In the case of (-)-epicatechin and (-)-epigallocatechin no specific trends were generally observed in favor of any treatment (Supp. Inf. Figure 3_S1).

4.3.4.1.4 Flavones and flavanones

Flavanones can be formed from the chalcone structure, whereas flavones are synthesized at a branch point of the anthocyanidin/proanthocyanidin pathway from flavanones as direct biosynthetical precursors.[39] Flavone formation in various tissues of a wide range of plant species is catalyzed by the flavone synthase (mainly FSNII).[39] Apart from other biological roles linked to them to date (e.g. flavone glycosides acting as co-pigments) they may also act as UV protectants.[40]

Two representatives of flavones (luteolin-7-glucoside and apigenin-7-glucoside) and two members of flavanones (naringetin and naringetin-7-glucoside) were detected in our skin samples. They have previously been reported in grapes,[25] however, to our knowledge, flavones and flavanones have never been studied with the scope of showing changes during berry development and studying biosynthetic behavior, resulting from canopy microclimate manipulation. The most abundantly present naringetin-7-glucoside (on average 375 μg/g skins at highest point for BS) showed typical early peaking behavior (Figure 17 C), whereas luteolin-7-glucoside (Figure 4 D) showed similar behavior, but was present in lower concentrations (up to 10 μg/g skins at highest point). Together they are mainly responsible for the trends observed in total amounts (Figures 15 E and F). On the other hand, naringetin and apigenin-7-glucoside accumulated in very low concentrations (below 1.0 μg/g skins and 0.25 μg/g skins on average respectively) and consequently did not show particularly clear (trustable) trends during the observation period (Supp. Inf. Figure 3_S1). For all four compounds we detected the lowest concentration trends at the beginning of observation (28 June) for the control grapes, but the differences later disappeared, reaching similar values for all treatments at harvest (on average between 0.3 and 0.4 μg/g skins for naringetin; between 0.6 and 0.9 μg/g skins for luteolin-7-glucoside; between 0.2 and 0.6 μg/g skins for naringetin-7-glucoside and below the detection limit for apigenin-7-glucoside). As flavones share common precursors with anthocyanins, their levels are generally negatively correlated, which basically means that a reduction in flavones will probably cause "an increase in anthocyanins due to the precursor flowing in only one direction".[39] Although flavones and flavanones are both present in relatively small

concentrations, this is probably also something to be considered in future detailed canopy microclimate manipulation research.

4.3.4.1.5 Dihydrochalcones

Chalcones are of great significance biosynthetically as they are the immediate precursors of all other classes of flavonoids. However, very little is known about the biosynthesis of dihydrochalcones from chalcones.[41]

Phlorizin, as the only detected representative of dihydrochalcones, is a natural product and dietary constituent found in several fruits,[42] mainly in apples, but also in grapes.[25] The phlorizin concentration was highest (between ≈20 and 60 μg/g skins on average) at the first two sampling points during the observations (Figure 17 E). It decreased steadily thereafter, being hardly present at harvest time (<0.2 μg/g skins). Indeed, untreated grapes early in the season showed a lower accumulation of phlorizin than those subjected to leaf removal treatments, but later the difference was no longer significant.

4.3.4.2 Non-flavonoids

4.3.4.2.1 Stilbenes

Stilbene and flavonoid syntheses have a common origin, as both derive from the general phenylpropanoid metabolism, although stilbenes are synthesized by stilbene synthase from coumaroyl-coenzyme A (CoA) and three molecules of malonyl-CoA via cleavage of four carbon dioxide molecules.[43] In grapes, the synthesis of stilbenes takes place in berry skins,[44] and it is known that they play an important role in plant and environment interactions. Resveratrol, as the most studied stilbene-type compound and some of its derivatives, such as viniferins, pterostilbene and piceid, have already been reported to be involved in plant defense mechanisms against abiotic stress - such as UV light - and biotic stress.[43,45] The level of stilbenes produced has also been found to be cultivar specific,[14] and in their study ref 46 defined 'Pinot noir' as a high producer of resveratrol, reaching the highest content among 78 observed varieties. The total amount of stilbenes in our samples increased significantly and reached a peak during the berry-coloration (veraison) period (589, 769, 958, and 1030 μg/g skins on average in BS, PF, VE, and UN, respectively), but later decreased on approaching

harvest, reaching 257, 170, 171, and 402 μg/g skins on average in BS, PF, VE, and UN, respectively at harvest (Figure 15 G). The increase and peak within the last stages of development (from veraison until harvest) are consistent with the findings of ref 47, although their work was done on 'Corvina'. Moreover, even if they did not focus their observations on the stages before veraison, it is evident that the detected amounts of observed stilbenes were very low at veraison, as we found in our study. On the other hand, ref 44 also reported the presence of resveratrol earlier in the season (before veraison), but in their research they artificially applied UV-radiation to detached 'Pinot noir' grapes. Their findings can, however, explain some low concentration trends of several individual stilbenes (hardly seen, if only the totals are observed) around the time of early leaf removal (PF, BS), as the treatments opened up the developing berries more directly to the sun.

Then again, the biggest increase in total stilbenes in our case was undoubtedly observed in late August and early September, with the highest values being shown for untreated grapes (even though these grapes were the least exposed to UV light) and VE (where the clusters were highly exposed to light, because leaf removal in this case had just recently been performed). Some authors[14] already discussed a possible role of stilbene accumulation in responding to the changes in microclimate. In 2010 the Vipava Valley was basically warm and sunny in early summer (June and July), but cold later with extensive rainfall in August and September (Supp. Inf. Table 3_S1). In the present experiment the highest relative humidity within cluster area was detected in case of UN (Supp. Inf. Figure 3_S3) leading to the increased probability of *B. cinerea* infection.[48] Luczka[49] already pointed out that UV-light exposure induces similar amounts of resveratrol as mold *Botrytis cinerea*, thus in our experiment the occurrence of mold (due to wet conditions) could account for the higher content of stilbenes in UN grapes - even more than UV-exposure did.

Apart from resveratrol (*trans* isomer between 120 and 143 μg/g skins on average at the highest point), many other stilbene representatives were also detected in relatively large amounts in our 'Pinot noir' samples, such as *trans* and *cis*-piceid with ranges from 157-244 and 28-154 μg/g skins on average respectively at the highest points, as well as *trans*-ω-viniferin with ranges from 142 to 287 μg/g skins on average at the highest point (Figure 17 F). Furthermore, resveratrol can be transformed by *Botrytis cinerea* into resveratrol (E)-dehydrodimer, pallidol, leachinol F and restrytisols A-C,[50] which in our case can also explain the relatively high pallidol occurrence, especially in the case of untreated grapes (117 μg/g skins on average).

Finally, regardless of the abundance of other individually detected representatives (Supp. Inf. Figure 3_S1), all of them showed typical late peaking behavior (Table 11).

4.3.4.2.2 Hydroxycinnamates

Hydroxycinnamic acids (HCAs) are precursors for the synthesis of many other molecules, such as flavonoids and lignin[51] and are known to be located in the vacuoles of the skin and pulp cells. HCAs have important functions in maturation processes and in plant defense, and can also improve fruit flavor quality.[52] The principal hydroxycinnamic acids occurring in *V. vinifera* grapes are caftaric, coutaric and fertaric acids in *trans* form, although small quantities of the *cis* isomers can also be detected.[53] Furthermore, they are reported to be involved in the browning reactions of must and wine and carry out antimicrobial and antioxidant activities.[54] As can be seen for total HCAs (Figure 15 H) as well as for individual HCAs (Figure 17 G & Supp. Inf. Figure 3_S1), neither total nor any of the individual HCAs showed significantly higher values for any of the treatments at harvest time, although the trends were higher for early leaf removal at the beginning of the observations, which is similar to that reported previously.[29] In general, a decreasing trend was shown for HCAs. It appears that hydroxycinnamic acids in the skin cannot be easily manipulated through different timing of leaf removal treatments.

4.3.4.2.3 Benzoates

Hydroxybenzoic acids are synthesized in numerous plants from the corresponding hydroxycinnamic acids. The four most common plant hydroxycinnamic acids are *p*- coumaric, caffeic, ferulic, and sinapic acids, whereas the corresponding hydroxybenzoic acids are *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids.[55] In our experiment, seven compounds, classified as benzoates, were detected. By observing the total amount (Figure 15 I) we can see that they are more abundant earlier in the season, with a peak in the middle of July, but later decreasing until they became stable with a very low concentration during the last 3 weeks of maturation. Leaf removal did not cause any stable or clear accumulation trends; therefore from the totals alone we could not reach any conclusions in favor of any treatment. Individual representatives, vanillic, *p*-hydroxybenzoic, syringic and gallic acid have previously been reported in grapes with comparable values at harvest.[25,55] However, they were only analyzed in ripe grapes, whereas our results also show their behavior during grape berry

development. Whereas vanillic acid generally increased (reaching 1.6 (UN) to 2.2 (BS) μg/g skins on average at harvest), *p*-hydroxybenzoic acid on the other hand showed early peaking behavior, but has decreased later, being hardly detectable at harvest time. Neither compound showed any important differences between leaf removal treatments. On the contrary, syringic acid (Figure 17 H) (peaking late in the season with 1.6 to 3.3 μg/g skins on average at the highest point) was the only benzoate showing significant differences at harvest as compared with UN, essentially in favor of early leaf removal (BS and PF) (both around 2 μg/g skins on average). Vanillin was already detected in traces[56] in 'Pinot noir' grapes during berry development. Also in our grape skins vanillin (< 0.5 μg/g skins on average and with no specific behavior) as well as syringaldehyde were found - generally early in the season - with later up to 2 μg/g skins on average, decreasing later until harvest to < 0.5 μg/g skins on average. Finally, an ester of gallic acid - methylgallate was also detected in low amounts in the second half of our observation period.

When summarizing all of the results, we can see that changes in microclimate conditions in the cluster area affected many of the compounds observed. However, some were affected only early in the season, while many others retained significant alterations until harvest (Table 12). Canopy microclimate manipulation had a big effect in the case of flavonols, particularly early peaking flavonols. Higher synthesis of anthocyanins in the case of veraison leaf removal was detected, whereas early leaf removals showed a reduction in peonidin glucoside but an increase in all other individual compounds. Despite the fact that flavonols and anthocyanins are on the same biosynthetic pathway, it seems possible to positively affect both classes by taking into account their (different) peaking behavior. Furthermore, stilbenes showed late peaking behavior and were generally highest in the case of no leaf removal (closed canopy of control vines), most probably due to better conditions for *B. cinerea* development. Within benzoates, syringic acid was the only representative still showing significant changes in favor of early leaf removal at harvest. Many representatives of other classes of polyphenols, such as flavones, flavan-3-ols, flavanones, and hydroxycinnamic acids, essentially reduced their concentration from the time of veraison, with different trends between treatments often shown only in the first stages of maturation. One question was raised: Is the higher concentration obtained with early leaf removal at the beginning of maturation nevertheless still important within the complex pattern of physiological changes of the berry and finally for overall grape quality at harvest? In the future further research should be carried out, aiming to understand how early peaking could affect late peaking compounds in different canopy microclimate scenarios and

climatic conditions.

Table 12: Detected phenolic compounds (μg/g skins) showing significant alterations at harvest point in comparison to the control, as a result of canopy microclimate manipulation through leaf removal at different phenological stages (PF=pre-flowering; BS=at berry set; VE=at veraison; UN=control with no leaf removal).

At harvest	UN	PF	BS	VE	*p value*	*F*[a]
syringic acid	a[b]	b	b	b	*0.0276*	*
cis-resveratrol	a	b	b	b	*0.0400*	*
cis-ε-viniferin	a	b	b	b	*0.0345*	*
trans-ω-viniferin	a	b	b	b	*0.0041*	**
pallidol	a	b	b	b	*0.0024*	**
ampelopsin D + quadrangularin A	a	b	b	b	*0.0055*	**
isohopeaphenol	a	b	b	b	*0.0343*	*
ampelopsin H + vaticanol C-like isomer	a	b	b	b	*0.0095*	**
naringenin	a	b	b	b	*0.0262*	*
kaempferol-3-rutinoside	a	b	b	c	*0.0072*	**
quercetin-Glc-Ara	a	a	a	b	*0.0459*	*
rutin	a	b	c	d	*0.0024*	**
quercetin-3-glucuronide	a	b	b	c	*0.0071*	**
kaempferol	a	b	b	b	*0.0194*	*
myricetin	a	ab	b	b	*0.0306*	*
kaempferol-3-glucoside	a	b	b	b	*0.0170*	*
myricetin-3-rhamnoside	a	ab	bc	c	*0.0083*	**
isorhamnetin-3-glucoside	a	ab	b	b	*0.0136*	*
syringetin-3-glucoside + syr.-3-galactoside	a	ab	ab	b	*0.0262*	*
isorhamnetin-3-rutinoside	a	b	b	c	*0.0170*	*
taxifolin	a	ab	ab	b	*0.0412*	*
quercetin-3-rhamnoside	a	ab	b	b	*0.0337*	*
kaempferol-3-glucuronide	a	ab	ab	b	*0.0369*	*
quercetin	a	ab	b	b	*0.0412*	*
quercetin-3-glucoside	a	b	b	b	*0.0092*	**
quercetin-3-galatoside	a	b	b	b	*0.0120*	*
delphinidin-3-glucoside	a	b	c	d	*0.0006*	***
cyanidin-3-glucoside	a	ab	b	c	*0.0197*	*
petunidin-3-glucoside	a	b	c	d	*0.0001*	***
peonidin-3-glucoside	a	b	c	d	*0.0019*	**
malvidin-3-glucoside	a	b	c	d	*0.0004*	***

[a]Data were processed through ANOVA and means separated using Tukey's Honest Significant Difference (HSD) test (P<0.05) (* = p<0.05; ** = p<0.01; *** = p<0.001). [b]Means followed by the same letter are not significantly different.

4.3.5 Acknowledgements

We thank the owners of the Tilia Estate for allowing us to carry out the experimental work in their vineyards and to Domenico Masuero, Mattia Gasperotti and Dejan Glavan for their help with sampling and/or analytical work.

4.3.6 Supporting information

Appendix C / Figure 3_S1: Accumulation dynamics (μg/g skins) of 64 (data not shown) individual representatives from different phenolic groups as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal).

Appendix D / Figure 3_S2: Hourly temperatures (°C) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Top panel shows the comparison between control (UN) and pre-flowering leaf removal (PF, green line). Middle panel shows the comparison between control (UN) and berry set leaf removal (BS, green line). Bottom panel shows the comparison between control (UN) and veraison leaf removal (VE, green line).

Appendix E / Figure 3_S3: Hourly relative humidity (%) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Top panel shows the comparison between control (UN) and pre-flowering leaf removal (PF, green line). Middle panel shows the comparison between control (UN) and berry set leaf removal (BS, green line). Bottom panel shows the comparison between control (UN) and veraison leaf removal (VE, green line).

Appendix F / Table 3_S1: Basic seasonal (monthly) characteristics of the observed vintage (2010)[57].

Appendix F / Table 3_S2: Basic viticultural parameters as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal) (relative values)

4.3.7 References

(1) Wink, M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 2003, 64, 3–19.

(2) Kliebenstein, D.J.; Rowe, H.C.; Denby, K.J. Secondary metabolites influence Arabidopsis /Botrytis interactions: variation in host production and pathogen sensitivity. Plant J. 2005, 44, 25–36.

(3) Zamboni, A.; Vrhovsek, U.; Kassemeyer, H-H.; Mattivi, F.; Velasco, R. Elicitor-induced reservatrol production in cell cultures of different grape genotypes (Vitis spp.). Vitis 2006, 45, 63-68.

(4) Ali, K.; Maltese, F.; Choi, Y.H.; Verpoorte, R. Metabolic constituents of grapevine and grape-derived products. Phytochemistry Reviews 2010, 9, 357–378.

(5) Tarr, P.T.; Dreyer, M.L.; Athanas, M.; Shahgholi, M.; Saarloos, K.; Second, T.P. A metabolomics based approach for understanding the influence of terroir in Vitis Vinifera L. Metabolomics 2013, 9, 170–177.

(6) Sternad Lemut, M.; Trost, K.; Sivilotti, P.; Vrhovsek, U. Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. J. Food Compos. Anal. 2011, 24, 777–784.

(7) Vršič, S.; Vodovnik, T. Reactions of grape varieties to climate changes in North East Slovenia. Plant Soil Environ. 2012, 58, 34–41.

(8) Kliewer, W.M.; Torres, R.E. Effect of controlled day and night temperatures on grape coloration. Am. J. Enol. Viticult. 1972, 23, 71–76.

(9) Tarara, J. M.; Lee, J.; Spayd, S. E.; Scagel C. F. Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in Merlot grapes. Am. J. Enol. Viticult. 2008, 59, 235–247.

(10) Spayd, S.E.; Tarara, J.M.; Mee, D.L.; Fergusson J.C. Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries. Am. J. Enol. Viticult. 2002, 53, 171–182.

(11) Mori, K.; Sugaya, S.; Gemma, H. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. Sci. Hortic. Amst. 2005, 105, 319–330.

(12)Cohen, S.; Tarara, J.M.; Kennedy, J.A. Assessing the impact of temperature on grape phenolic metabolism. Anal. Chim. Acta. 2008, 621, 57–67.

(13)Goto-Yamamoto, N.; Mori, K.; Numata, M.; Kazuya Koyama, K.; Kitayama, M. Effects of temperature and water regime on flavonoid content and composition in the skin of red-wine grapes. J. Int. Sci. Vigne Vin. 2010, special issue Macrowine, 75–80 E.

(14) Bavaresco, L.; Pezzuto, S.; Gatti, M.; Mattivi, F. Role of the variety and some environmental factors on grape stilbenes. Vitis 2007, 46, 57–61.

(15) Price, S.F.; Breen, P.J.; Valladao, M.; Watson, B.T. Cluster sun exposure and quercetin in Pinot noir grapes and wine. Am. J. Enol. Viticult. 1995, 46, 187–194.

(16) Haselgrove, L.; Botting, D.; Van Heeswijck, R.; Hoj, P.B.; Dry, C.; Ford, C.; Iland P.G. Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of Vitis vinifera L. Cv. Shiraz grape berries. Aust. J. Grape Wine R. 2000, 6, 141–149.

(17) Adrian, M.; Jeandet, P.; Breuil, A. C.; Levite, D.; Debord, S.; Bessis, R. Assay of resveratrol and derivative stilbenes in wines by direct injection high performance liquid chromatography. Am. J. Enol. Viticult. 2000, 51, 37–41.

(18) Downey, M.O.; Harvey, J.S.; Robinson, S.P. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Aust. J. Grape Wine R. 2003, 9, 15–27.

(19) Downey, M.O.; Harvey, J.S.; Robinson, S.P. The effect of bunch shading on berry development and flavonol accumulation in Shiraz grapes. Aust. Journal Grape Wine R. 2004, 10, 55–73.

(20) Cortell, J.M.; Kennedy, J.A. Effect of shading on accumulation of flavonoid compounds in (Vitis vinifera L.) Pinot Noir fruit and extraction in a model system. J. Agric. Food Chem. 2006, 54, 8510–8520.

(21) Xu, C.; Zhang, Y.; Zhu, L.; Huang, Y.; Lu, J. Influence of growing season on phenolic compounds and antioxidant properties of grape berries from vines grown in subtropical climate. J. Agric. Food Chem. 2011, 59, 1078–1086.

(22) Lorenz, D.H.; Eichhorn, K.W.; Bleiholder, H.; Klose, R.; Meier, U.; Weber, E. Growth stages of the grapevine: phenological growth stages of the grapevine (Vitis vinifera L. ssp. vinifera) - codes and descriptions according to the extended BBCH scale. Austr. J. Grape Wine R. 1995, 1, 100–103.

(23) Poni, S.; Bernizzoni, F.; Civardi, S; Libelli, N. Effects of pre-bloom leaf removal on growth of berry tissues and must composition in two red Vitis vinifera L. cultivars. Aust. J. Grape Wine R. 2009, 15, 185–193.

(24) Mattivi, F.; Guzzon, R.; Vrhovsek, U.; Stefanini, M.; Velesco, R. Metabolite profiling of grape: flavonols and anthocyanins. J. Agric. Food Chem. 2006, 54, 7692–7702.

(25) Vrhovsek, U.; Masuero, D.; Gasperotti, M.; Franceschi, P.; Caputi, L.; Viola, R.; Mattivi, F. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. J. Agric. Food Chem. 2012, 60, 8831–8840.

(26) Benjamini, Y; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B. Met. 1995, 57, 289–300.

(27) R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing. Vienna, Austria, 2013, http://www.R-project.org

(28) Schijlen, E.G.W.M.; de Vos, C.H.R.; van Tunen, A.J.; Bovy, A.G. Modification of flavonoid biosynthesis in crop plants. Phyochemistry 2004, 65, 2631–2648.

(29) Sternad Lemut, M.; Trost, K.; Sivilotti, P.; Arapitsas, P.; Vrhovsek, U. Early vs. late leaf removal strategies for 'Pinot noir' (Vitis vinifera L.): effect on colour-related phenolics in young wines following alcoholic fermentation. J. Sci. Food Agric. 2013, doi: 10.1002/jsfa.6193.

(30) Boulton R; The copigmentation of anthocyanins and its role in the color of red wine: a critical review. Am. J. Enol. Viticult. 2001, 52, 67–87.

(31) Parley, A.; Vanhanen, L.; Heatherbell, D. Effects of pre-fermentation enzyme maceration on extraction and colour stability in Pinot Noir wine. Aust. J. Grape Wine R. 2001, 7, 146–152.

(32) Boss, P.K.; Davies, C.; Robinson, S.P. Analysis of the expression of anthocyanin pathway genes in developing Vitis vinifera L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol. 1996, 111, 1059–1066.

(33) Gould, K.S.; Lister, C. Flavonoid functions in plants. In: Flavonoids: chemistry, biochemistry and applications. Andresen, M.; Markham, K.R. Eds.; CRC Taylor & Francis: New York, 2006; 8, 397-443.

(34) Mori, K.; Goto-Yamamoto, N.; Kitayama, M.; Katsumi, H. Loss of anthocyanins in red-wine grape under high temperature. J. Exp. Bot., 2007, 58, 1935–1945.

(35) Harbertson, J.F.; Kennedy, J.A.; Adams, D.O. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. Am. J. Enol. Viticult. 2002, 53, 54–59.

(36) Aron, P. M.; Kennedy, J. A. Flavan-3-ols: nature, occurrence and biological activity. Mol. Nutr. Food Res. 2008, 52, 79–104.

(37) del Rio, J.L.P.; Kennedy, J.A. Development of proanthocyanidins in Vitis vinifera L. cv. Pinot noir grapes and extraction into wine. Am. J. Enol. Viticult. 2006, 57, 125-132.

(38) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J.M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. J. Agric. Food. Chem. 1999, 47, 2719–2723.

(39) Martens, S.; Mithöfer, A. Flavones and flavone synthases. Phytochemistry 2005, 66, 2399–2407.

(40) Iwashina, T. The structure and distribution of the flavonoids in plants. J. Plant Res. 2000, 113, 287–299.

(41) Veitch, N.C.; Grayer, R.J. Chalcones, dihydrochalcones, and aurones. In: Flavonoids: chemistry, biochemistry and applications. Andresen, M.; Markham, K.R. Eds.; CRC Taylor & Francis: New York, 2006; 16, 1003–1101.

(42) Ehrenkranz, J. R. L.; Lewis, N. G.; Kahn, C.R; Roth, J. Phlorizin: a review. Diabetes Metab. Res. Rev. 2005, 21, 31–38.

(43) Fornara, V.; Onelli, V.; Sparvoli, F.; Rossoni, M.; Aina, R.; Marino, G.; Citterio, S. Localization of stilbene synthase in Vitis vinifera L. during berry development. Protoplasma. 2008, 233, 83–93.

(44) Jeandet, P.; Bessis, R.; Gautheron, B.; The production of resveratrol (3,5,4'-trihydroxystilbene) by grape berries in different developmental stages. Am. J. Enol. Vitic. 1991, 42, 41–46.

(45) Jeandet, P.; Douillet-Breuil, A.; Bessis, R.; Debord, S.; Sbaghi, M.; Adrian, M. Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J. Agric. Food Chem. 2002, 50, 2731–2741.

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

(47) Versari, A.; Parpinello, G.P.; Tornielli, G.B.; Ferrarini, R.; Giulivo, C. Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. J. Agric. Food Chem. 2001, 49, 5531–5536.

(48) Kassemeyer, H-H.; Berkelmann-Lohnertz B. Fungi of Grapes. In: Biology of Microorganisms on Grapes, in Must and in Wine, Konig et al. (eds.), Springer-Verlag Berlin Heidelberg, 2009, pp 61-89.

(49) Luczka, C. J. Stilbene phytoalexins and susceptibility to Botrytis cinerea in Vitis. 1982, Thesis, Cornell University.

(50) Cichewicz, R.H.; Kouzi, S.A.; Hamann, M.T. Dimerization of resveratrol by the grapevine pathogen Botrytis cinerea. J. Nat. Prod. 2000, 63, 29–33.

(51) Bahri, M.; Hance, P.; Grec, S.; Quillet, M.; Trotin, F.; Hilbert, J.; Hendriks, T. A "Novel" protocol for the analysis of hydroxycinnamic acids in leaf tissue of chicory (Cichorium intybus L., Asteraceae). Scient. World J. 2012, doi: 10.1100/ 2012/142983.

(52) Fallico, B.; Lanza, M.C.; Maccarone, E.; Asmundo, C.N.; Rapisarda, P. Role of hydroxycinnamic acids and vinylphenols in the flavor alteration of blood orange juices. J. Agric. Food Chem. 1996, 44, 2654–2657.

(53) Singleton, V. L.; Timberlake, C. F; Lea, A. G. H. The phenolic cinnamates of white grapes and wine. J. Sci. Food Agric. 1978, 29, 403–410.

(54)Vrhovsek, U. Extraction of hydroxycinnamoyltartaric acids from berries of different grape varieties. J. Food Comp. Anal. 1998, 46, 4203–4208.

(55) Mattila, P.; Hellström, J.; Törrönen, R. Phenolic acids in berries, fruits, and beverages. Agric. Food Chem. 2006, 54, 7193−7199.

(56) Fang, F.; Li, J.; Zhang, P.; Tang, K.; Wang, W. Effects of grape variety, harvest date, fermentation vessel and wine ageing on flavonoid concentration in red wines. Food Res. Int. 2008, 41, 53–60.

(57) EARS. Slovene Public Information, http://meteo.arso.gov.si, October 2012.

Financial support:

This work was partially performed within the Creative Core programme (AHA-MOMENT) contract no. 3330-13-500031, co-supported by RS-MIZS and European Regional Development Fund Research; and partially by the European Union through the European Social Fund (Operational Programme for Human Resources Development for the period 2007-2013, 1st development priorities: Promoting entrepreneurship and adaptability, policy priorities 1.3: Scholarship Scheme). PF, RW and UV were supported by ADP 2012 project, funded by the Autonomous Province of Trento.

4.4 Pre-flowering leaf removal in 'Pinot Noir' (*Vitis vinifera* L.): potential for successful microbial control in the context of more sustainable, energy efficient and cost-effective vineyard management (Sternad Lemut et al., submitted, 2013c)

4.4.1 Abstract

Grapevine canopy management can have a major effect on canopy microclimate conditions. Particularly temperature and relative humidity conditions around the clusters may be of crucial importance in making these sites liable to a lower or higher incidence of microbial infections. A multidisciplinary study approach was implemented to evaluate related potential of novel viticulture technique of pre-flowering leaf removal as compared with more adopted post-flowering practices, which are now losing many of their advantages as a result of warmer vineyard conditions (global warming). Field trial was set up in two seasons with leaf removal carried out at different times in a 'Pinot Noir' (*Vitis vinifera* L.) vineyard, leading to different (monitored) microclimate scenarios in order to reveal related changes in microbial ecology. Furthermore, the effectiveness of leaf removal in suppressing *Botrytis cinerea* Pers. (known problem of Pinots) was compared with that of the fungicide application, with the scope of assessing the possibility of spraying reduction. Cluster compactness was also evaluated as an important factor in terms of sensitivity to microbial diseases. Finally, the potential for cost and energy savings was calculated, based on all the observations. The results revealed higher counts and several shifts in microbial population with veraison (late) leaf removal and for the control (without leaf removal), both also showing a higher presence of some negative microbial metabolites in the must. Earlier leaf removal treatments were thus shown to offer greater potential as an effective tool to control microbial infection. Promising results were obtained also for cluster compactness, as a looser clusters were observed in the case of pre-flowering leaf removal treatment (16-to-18% lower compactness), which was accompanied by a substantial reduction in yield (30% and 24 % in 2010 and 2011 respectively), all as compared with control. The lower yield in the case of pre-flowering defoliation can reduce the need for later cluster thinning and consequently total energy consumption, thus it can be considered a good option for more sustainable and cost effective 'Pinot Noir' vineyard management, saving 27% on costs and as much as 46% on energy.

Keywords: 'Pinot Noir' (*Vitis Vinifera* L.), pre-flowering leaf removal, *Botrytis cinerea* Pers., microbial ecology, sustainable viticulture, cost effective vineyard management.

4.4.2 Introduction

In conventional viticulture, pest and disease control is generally achieved by widespread application of pesticides, resulting in high costs and a negative impact on the environment (Ali et al., 2010). However, the public's growing concern for health and environment is leading to more sustainable approaches in all sectors of agriculture, including viticulture. One of the main goals of sustainable vineyard management is to limit the type, quantity and timing of chemical applications, by optimising viticulture strategies during the growing season.

Of the grapevine varieties, particular care should be dedicated to those with more compact clusters, since they are more susceptible to microbial infections. 'Pinot Noir' (*Vitis vinifera* L.) is known as a very tightly-clustered red grapevine cultivar, in which the mechanical pressure of adjacent berries may disrupt cuticle waxes and damage berry skins, thus making such areas liable to a higher incidence of microbial attacks (Hed, 2009). In general, the main grapevine pathogens of major economic importance are downy mildew (*Plasmopora viticola* Berg. e Curtis, Berl. & De Toni), powdery mildew (*Erysiphe necator* Schwein.) and so-called bunch rot (*Botrytis cinerea* Pers.) (Ribéreau-Gayon et al. 2005), with the latter being one of the most frequent problems in Pinot varieties. *B. cinerea* development, alone or associated with other microorganisms, can severely lowers grape quality and consequently also wine quality. Objective measurement of the sanitary state of the harvest crop is therefore of obvious interest to winegrowers (Kassemeyer and Berkelmann-Lohnertz, 2009).

With its specific characteristics, each vineyard can give rise to different microflora, with quantitative and qualitative heterogeneity of species (Renouf et al., 2005; Pretorius et al., 1999). Different yeast, bacteria and fungi cover the grape berry surface, forming a complex, large and diverse microbial community (Renouf et al., 2005; Coombe, 1992; Rosini et al., 1982). Quantitatively, mature sound grapes harbour microbial populations at levels of 10^3 to 10^5 CFU/g, consisting mostly of yeasts and various species of lactic and acetic bacteria (Fleet, 1999) with some filamentous moulds.

Alongside changes related to the cultivar (Pretorius, 1999) and the grape development stage itself (Renouf et al., 2005), several factors have been reported to have a major impact on microbial levels on grape berries, such as different environmental factors associated with vineyard geography (Fleet et al., 2002), nutritional limitations (Renouf et al., 2005; Sabate et al., 2002), application of cultivation practices such as fertilisation, irrigation and spraying with fungicides (Renouf et al., 2005; Lindow and Brandl, 2003; Sabate et al., 2002), natural climatic

stress - changes in UV radiation, temperature, rainfall regime and humidity (Barata et al., 2012), as well as the microclimate, as affected by canopy management (Pretorius, 1999). Carefully selected cultivation practices affecting the grapevine canopy microclimate may therefore play a significant role in encouraging or discouraging moulds and other microbial infections. In recent years, impacts of climate changes/global worming on agricultural crops is also present with grapes now ripen at temperatures which are approximately by 2 °C higher than 30 years ago (Vršič and Vodovnik, 2012).

Summer canopy management is thus nowadays frequently applied and leaf removal is one of the viticulture techniques that can be employed to manoeuvre the microclimate in the cluster area. However, its performance is particularly related to the phenological stages of grape berry development at which the practice is adopted (Sternad Lemut et al., 2011; Poni et al., 2006). Although this practice may have different goals, it is usually employed from fruit set to veraison on high-density canopies to improve light exposure and air circulation around the clusters, with substantial benefits also in terms of tolerance to bunch rot. The functional relationship between yield and source availability around blooming (Sabbatini and Howell, 2009; Poni et al., 2006) Gatti et al., 2012) inherently implies that a new and innovative viticulture technique, with leaf removal carried out around flowering or even before flowering, can reduce fruit set and consequently lead to looser bunches. This approach could be very useful for excessively tight clusters (as is the case in Pinot) in order to reduce berry-to-berry compression and subsequently bunch rot sensitivity. By lowering the risk of cluster diseases, the need for spraying is also reduced and a lower environmental impact is assured. However, not only environmentally-friendly but at the same time cost-effective vineyard management (Diago et al., 2012a) is also becoming very important in modern viticulture, in order to withstand competition on the global wine market.

Alongside lower cluster compactness (Poni et al., 2006; 2009), some very important improvements in grape/wine chemical composition have already been reported for a few grapevine varieties as a positive effect of implementing very early, pre-flowering leaf removal in the vineyard environment (Sternad Lemut et al., 2013; Bubola et al., 2012; Diago et al., 2012b; Poni et al., 2006). Intensive research into this new viticulture technique with different varieties and in different geo-climatic conditions is therefore undoubtedly justified from many different points of view.

A field trial was thus designed in two subsequent years in order to evaluate the effect of controlled (and up to a certain level monitored) purposely-induced microclimate alteration (achieved by leaf removal at different phenological stages of grape berry development) on microbial ecology, and particularly *B. cinerea* occurrence, by using a multidisciplinary approach. Hence the trial on the effectiveness of leaf removal in suppressing *B. cinerea* was also coupled with a trial on the effectiveness of fungicide (fludioxonil + cyprodinil) application, with the scope of evaluating the possibility of reducing spraying frequency as well as achieving energy and cost savings in the event of carefully planned canopy microclimate management in a 'Pinot Noir' vineyard.

4.4.3 Materials and Methods

4.4.3.1 Vineyard characteristics and experimental design

The experiment was carried out during 2010 and 2011 in a 'Pinot Noir' (*Vitis vinifera* L.) vineyard located in the Vipava Valley (Slovenia). The vineyard is located at an altitude 95 m a.s.l. and was planted in 2004, adopting the Guyot training system. Its plant density is 5682 plants/he (0.8 m x 2.2 m) whit the rows oriented E-W.

In 2010 a factorial experiment was set up, comparing four leaf removal treatments (LR) and verifying whether a fungicide Switch® (cyprodinil + fludioxonil, Syngenta, Basel, Switzerland) application at véraison was profitable for further reduction of grey mould (*B. cinerea*) occurrence. Thus for each LR treatment the effect of Switch®/no Switch® application at véraison was checked. The LR treatments were applied at different phenological stages of grape berry development (BBCH scale; Lorenz et al., 1995), as follows: PF (pre-flowering), LR performed before flowering at phenological stage BBCH 57; BS (berry set), LR applied at BBCH 71; VE (veraison), LR performed at BBCH 83; UN (control treatment), LR not applied (untreated vines / leaves retained). Leaf removal was performed manually, removing the basal 4-to-6 leaves of all the shoots, as normally carried out for pre-bloom treatments (Poni et al., 2009). With the "Switch®" treatment the fungicide was applied twice (2xS), at BBCH 77 and BBCH 83, while with the "no Switch® at véraison" treatment, the second application at BBCH 83 was not performed (1xS).

In the following 2011 season, three leaf removal treatments were compared: early, late and control (PF, VE and UN). Encouraged by the 2010 results, especially as regards lower cluster

compactness and the related prospective, we chose to focus in 2011 on the performance of pre-flowering leaf removal which was hence evaluated, coupled with: two Switch® applications (at BBCH 77 and BBCH 83) (PF_2S); one application of Switch® at BBCH 77 (PF_1S); no application of the fungicide (PF_0S).

4.4.3.2 Monitoring of climate conditions

The mesoclimatic characteristics of the site were followed using an IMT 300 meteorological station (Metos Instruments, New Delhi, India), collecting data on global radiation, rainfall, wind speed, leaf wetness, air temperature, relative humidity and dew point. In addition to this, further meteorological data were collected from a weather station of the National Meteorological Service of Slovenia (EARS, Slovene public information, EARS-meteo.si) located nearby, aiming to confirm and support the data downloaded from our meteorological station at the vineyard site.

Canopy microclimate conditions in the cluster area (temperature and relative humidity) were monitored during the critical - hottest period before harvest (from 14 August to 11 September 2010 and from 1 August to 1 September 2011) using i-Button sensors / mini-data loggers (SPR Hygrochron Temperature / Humidity Logger i-Button with 8KB Data-Log Memory, Maxim, CA, USA), collecting and storing the data on an hourly basis. I-button sensors were placed directly in the cluster area of all the treatments, half on the northern and half on the southern part of the canopy.

4.4.3.3 Evaluation of basic viticultural parameters and grape compactness

At harvest time, the yield/plant and cluster number were assessed for each plot and the average cluster weight was calculated. Leaf area was estimated as described in Sternad Lemut et al. (2011) by keeping the main and lateral shoots separate. The leaf area/yield was then calculated and moreover, 50 randomly selected clusters from each plot were weighed and measured (max length) at harvest, in order to calculate grape compactness ratio by rating cluster weight and length.

4.4.3.4 Visual examination of grey mould

In the week before harvest (2010) and at harvest time (2010 and 2011) all the clusters were visually examined for bunch rot, but mainly for *B. cinerea* (grey mould). Grey mould incidence and severity averages were then calculated in both years for all the plots. Since it is difficult to observe cluster infections precisely using visual inspection alone, we also carried out monitoring of the microbial population as affected by the different treatments, by direct plating on selective media (section 2.5). In addition, we monitored microbial activity by quantifying selected microbial metabolites (section 2.6) in order to obtain a clear picture of the situation from various viewpoints.

4.4.3.5 Microbial enumeration

For quick monitoring of the main grape-related microbial groups in different treatments, a week before harvest (2010) and at harvest time (2010 and 2011) a set of selective media for detection of filamentous fungi, yeasts, lactic and acetic acid bacteria was used. Microbial enumeration was performed by plating 100 μL of a dilution series of juice from 250 randomly collected berries, from all the treatments, aseptically picked, crushed in sterile bags and plated immediately. The berries (in three biological parallels) were collected from several locations (plots) in the vineyard, so that spatial fluctuations resulting from uneven microbial distribution were minimised (Barata et al., 2012). Plating was done in duplicate, on selective culture media as follows: WL (Walerstein Laboratory) nutrient agar (Fluka) supplemented with 0.03% chloramphenicol for yeast enumeration (Pallmann et al., 2001), while in 2011 WL-Differential medium (WLD; WL with added cycloheximide) was also included for easier detection of non-*Saccharomyces* yeasts (Fugelsang and Edwards, 2007); Potato dextrose agar (PDA) (Biolife) for mould enumeration; Glucose–yeast extract–carbonate medium (GYCM) (50 g L^{-1} glucose, 10 g L^{-1} yeast extract, 30 g L^{-1} $CaCO_3$, 25 g L^{-1} agar) for acetic acid bacteria and MRS medium (Biolife) supplemented with 2% tomato juice and 0.1 mg mL^{-1} cycloheximide for lactic acid bacteria enumeration (Fugelsang and Edwards, 2007). Plates were incubated at 25 °C for up to 14 days. Colonies were counted and expressed as colony forming units (CFU) per mL.

In general, bacterial and fungal identification was done at genus / species level on the basis of micro- and macromorphology. The guidelines described by Pallman et al. (2001) were also used for faster detection of yeasts on WL medium.

4.4.3.6 Determination of microbial indicator metabolites

In order to evaluate the relationship between the presence and actual activity of microbes we analysed and quantified selected microbial indicator metabolites such as gluconic acid, glycerol and volatile acids in the grape juice (must). Gluconic acid and glycerol analysis were carried out as previously described by Larcher et al. (2009). Separation and quantification was performed with the help of a high performance liquid chromatography (HPLC) Alliance 2695 (Waters Corp.; Milford, MA, USA) equipped with a mod. 540 autosampler from the same manufacturer. A RCX/10 anion exchange column (250 x 4.6 mm; Hamilton; Bonaduz, Switzerland) was used in the system. The PAD detector was a Coulochem II 5200A used in pulsed mode and equipped with a 5040 gold electrode cell (ESA Inc.; Chelmsford, MA, USA). Volatile acids were analysed with the help of a Continuous Flow Analyzer Quaatro (Bran+Luebbe, Norderstedt, Germany). The automated method uses automatic online distillation of the volatile acid medium and the subsequent reaction of the distillate with potassium iodide. After acetic acid liberates iodine, its absorbance is measured at 410 nm. Sample preparation and the analytical procedure itself were carried out according to the manufacturer instructions, and finally volatile acids were expressed as acetic acid. All analyses were done in triplicates.

4.4.3.7 Cost and energy calculations

Calculations of energy consumption were done in order to compare energy investment for all the treatment variants. Costs were also calculated based on the accounting records of the wine estate owner and manager of a vineyard involved in the experiment (cost of labour, tractor hours, diesel fuel and spraying agents) as well as from data obtained in this study and the data in the literature. These included: human labour energy: 1.96 MJ/hour (Ozkan et al., 2007); number of working-hours: data from this study, see Table 17; diesel consumption for tractor work and spraying: diesel density 0.855 kg/L (Bosch, 2007), diesel heating value: 46.9 MJ/kg (Audsley et al., 1997); diesel consumption: 7.2 L/spraying (data from this study); tractor and sprayer depreciation: tractor lifetime: 10,000 hours, sprayer lifetime: 3,000 hours (Glithero et al., 2012); energy for machinery production: 142,7 MJ/kg (Litskas et al., 2013), tractor weight 2360 kg, sprayer weight: 200 kg and number of invested working hours: data from this study, see Table 17; energy of spraying agent (Switch®): active substance content: data from producers; energy for production of active substance/kg: 288,88 MJ/kg (West et al., 2002);

Gibbs free energy of water: 4.94 kJ/kg; spraying agent consumption: 0.75 kg/ha/spraying and water consumption: 200 L/ha/spraying (data from this study).

4.4.3.8 Statistical analysis

One-way analysis of variance (ANOVA) was used to test the significance of differences in leaf removal treatments. When significant, Student-Newman-Keuls (SNK) test of multiple comparisons of mean values was performed to separate the averages ($P < 0.05$). In 2010 - only for *B. cinerea* incidence and severity - two-way analysis of variance (ANOVA) was applied to test the significance of treatments (factor 1: leaf removal; and factor 2: Switch®/no Switch® application) together with the interaction between factors. Angular arcsine transformation of calculated indexes (*B. cinerea* incidence and severity) was adopted, since percentage data distribution is binomial and the transformation makes the distribution normal.

Principal Component Analysis (PCA) was then also performed in order to ascertain whether leaf removal treatments significantly affected the microbial ecology of the berry skin surface. The data were subjected to logarithmic transformation prior to PCA analysis.

In order to perform all these tests, the STATISTICA software package (StatSoft,Tulsa, OK, USA) was used.

4.4.4 Results and Discussion

4.4.4.1 Climatic conditions

4.4.4.1.1 Macroclimate and (site) mesoclimate conditions

Climate variability, with many fluctuations in different years in terms of temperature and precipitation, significantly determines vintage-to-vintage quality differences (Jones and Hellman, 2003; Shultz, 2000). In this experiment, 2010 and 2011 were also very different. 2010 was cold and rainy, with peaks in rainfall recorded in May after budburst, but also in July and September at harvest time (Supp. Inf., Table 4_S1), creating favourable conditions for grey mould infection. On the other hand, the following year 2011 was hotter and drier than 2010, with total rainfall of 408 mm during the growing season from May till October, while in September only 68 mm were recorded (Supp. Inf., Table 4_S1). In opposite to 2010, the

weather conditions of 2011 were essentially not favourable for greater development of mould infection.

4.4.4.1.2 Microclimate conditions – temperature

In terms of average temperature, the microclimate conditions were basically the same for all the treatments during the night and in the mornings and evenings; however, there were important differences in the late morning, at mid-day and in the late afternoon (mainly between 10.30 and 17.30 hour) (Figure 18). In both years, in this period, VE treatment showed the highest temperature values in the case of south-facing (S) cluster areas of the canopy (reaching 30 °C and over in 2010; and up to a maximum of 35 °C in 2011), probably due to the smaller number of leaves in the cluster area. As leaf removal was performed later in the season, there was poor re-growth of lateral leaves and thus the sunlight could easily reach the clusters. In the northern part (N) of the canopy we also observed higher temperatures in the case of VE leaf removal in the warmer 2011 season (> 30 °C), but the differences between treatments on the N-side of the canopy were not so clear in the colder and rainier 2010 season (Figure 18). The lowest temperatures in both years were observed in the case of the untreated control (UN), while both early leaf removal treatments (PF and BS (only in 2010)) showed the canopy temperature to be somewhere between UN and VE around mid-day (≈ 30 °C and < 28 °C in 2011 and 2010 respectively).

Temperature is an important factor, which has been proven to significantly affect some plant primary and secondary metabolites. In particular, the inhibitory effect of (too) high temperature on the biosynthesis of anthocyanins has been discussed many times (Sternad Lemut et al., 2013; 2011; Tarara et al., 2008; Spayd et al., 2002; Kliewer, 1970), with the critical temperature reported by different authors varying between 30 and 35 °C.

Besides the importance for grape chemical composition, temperature also plays an important role in the sanitary state of grapes. *B. cinerea* for example, can germinate at temperatures between 2 and 37 °C, although most of the germination (98 to 100%) occurs between 10 and 25 °C (Guetsky et al., 2001), with a warmer temperature being favourable, the optimum being recorded as 20.8 °C (Nair and Hill, 1992).

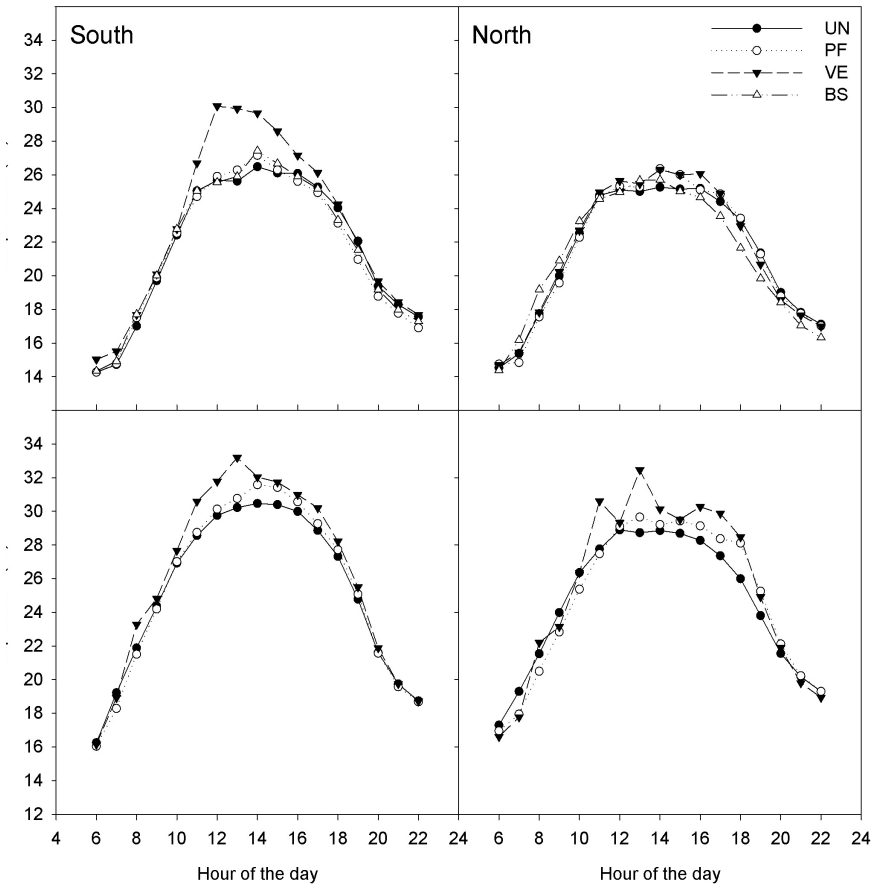


Figure 18: Temperature (°C) in the cluster area within last month of maturation in year 2010 (above) and in year 2011 (below), as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development: PF, pre-flowering leaf removal; BS, berry set leaf removal (performed only in 2010); VE, veraison leaf removal, UN=untreated control with no leaf removal performed.

4.4.4.1.3 Microclimate conditions – relative humidity

The probability of *B. cinerea* infection increases with the timing and severity of wet conditions, although it can also occur in the absence of water, if the relative humidity (RH) is very high (Kassemeyer and Berkelmann-Lohnerz, 2009).

In this experiment in both years (during August), control (UN) vines around the mid-day showed the highest relative humidity (RH) on both, the N and S sides of the canopy (Figure 19) (between 50-55% and 60-65% in 2011 and 2010 respectively), making these clusters liable to a higher risk of infection. As leaves were not removed at all from UN vines, the relative humidity in these closed canopies was considerably different (preferable for *B. cinerea*) as compared to the situation in more open canopies, as in the case of all the leaf removal treatments. The lowest RH around the mid-day was detected in the case of VE (about 10%

lower than in UN on average), as these vines were defoliated latest, thus the cluster area had the most open canopy in the measurement period. However, it should be taken into account that before late leaf removal, VE vines were exposed to the same conditions as UN vines, while BS and PF were already opened up very early in the season. Furthermore, both early leaf removal treatments (BS (2010) and PF) showed a medium RH situation in August, as they were already partly re-shadowed by new lateral leaves by that time. Nevertheless, open canopies (as in case of VE, BS and PF) also contribute to better distribution of applied fungicides (Poni et al., 2006) and consequently impact the microbial population on the grapes.

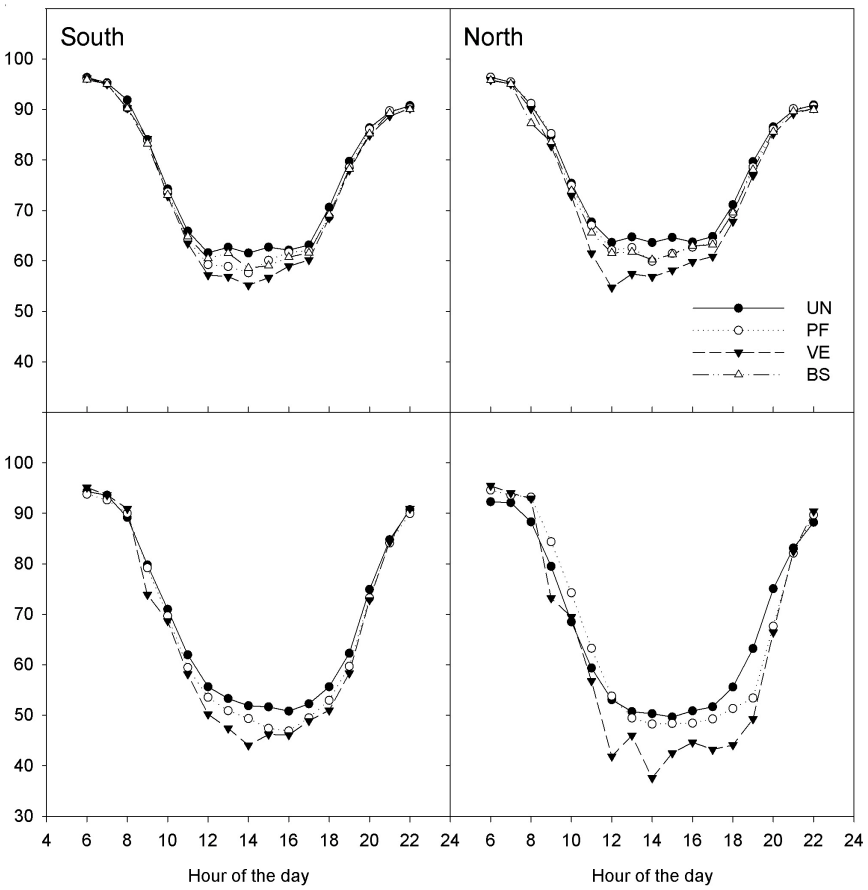


Figure 19: Relative humidity (%) in the cluster area within last month of maturation in year 2010 (above) and in year 2011 (below), as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development: PF, pre-flowering leaf removal; BS, berry set leaf removal (performed only in 2010); VE, veraison leaf removal, UN=untreated control with no leaf removal performed.

In this experiment in both years (during August), control (UN) vines around the mid-day showed the highest relative humidity (RH) on both, the N and S sides of the canopy (Figure 19) (between 50-55% and 60-65% in 2011 and 2010 respectively), making these clusters liable to a higher risk of infection. As leaves were not removed at all from UN vines, the relative

humidity in these closed canopies was considerably different (preferable for *B. cinerea*) as compared to the situation in more open canopies, as in the case of all the leaf removal treatments. The lowest RH around the mid-day was detected in the case of VE (about 10% lower than in UN on average), as these vines were defoliated latest, thus the cluster area had the most open canopy in the measurement period. However, it should be taken into account that before late leaf removal, VE vines were exposed to the same conditions as UN vines, while BS and PF were already opened up very early in the season. Furthermore, both early leaf removal treatments (BS (2010) and PF) showed a medium RH situation in August, as they were already partly re-shadowed by new lateral leaves by that time. Nevertheless, open canopies (as in case of VE, BS and PF) also contribute to better distribution of applied fungicides (Poni et al., 2006) and consequently impact the microbial population on the grapes.

4.4.4.2 Viticultural parameters and grape compactness

The timing of leaf removal can stimulate the evolution of main and lateral leaves differently, essentially because in the case of earlier defoliation the lateral leaves normally contribute more to the total leaf area (Sternad Lemut et al., 2011). However, different timing of leaf removal in our experiment did not lead to significant differences in terms of total leaf area (Table 13). Pre-flowering (PF), berry set (BS) and veraison leaf removal (VE) all reduced the amount of leaf area on the main shoots at harvest as compared with untreated vines (UN), but on the other hand, no significant triggering of lateral shoot re-growth was observed (Table 13). These results essentially disagree with the findings of Poni et al. (2006) and Diago et al. (2012), however, the same outcome was shown by Lohitnavy et al. (2010).

Average yield was significantly reduced in the case of PF as compared with UN (30% and 24% in 2010 and 2011 respectively) and when compared with VE (29% and 20% in 2010 and 2011 respectively), which is consistent with the observations of Palliotti et al. (2011), although their work was done on 'Sangiovese' (*V. vinifera* L.). BS on the other hand showed a trend towards yield reduction, but this was not significant, in contrast to PF (Table 13). In addition to yield, cluster weight was also significantly lower in the case of PF (25-26% lower when compared with UN) in both years (Table 1), providing evidence that a reduced number of cluster berries can be one of the effects of this early practice, not only in case of *V. vinifera* 'Sangiovese' and 'Trebianno' (Gatti et al., 2012; Poni et al., 2006) or *V. vinifera* 'Graciano' and 'Carignan' (Tardaguila et al. 2010), but also in case of 'Pinot Noir'. However, average berry

weight was significantly lower for both early leaf removal treatments (BS and PF) in the colder and rainier 2010 season, as already reported by Poni et al. (2006), but that was not the case in the warmer and dryer 2011 season, when the trend toward lower berry size was observed in the case of VE, probably due to berry shrinking after exposure of grapes to the sunlight in the very hot month of August (Sternad Lemut et al., 2013).

Table 13: Average values of viticulture parameters at harvest time as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development: PF=pre-flowering leaf removal; BS=berry set leaf removal (performed only in 2010); VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

Year	Treatment	Lateral shoots (m^2)	Main shoots (m^2)	Total leaf area (m^2)	Yield (kg/plant)	Cluster weight (g)	LA/yield (m^2/kg)	Berry weight (g)	Compact-ness ratio (g/cm)
2010	PF	0.75	1.27 b	2.02	1.02 b	92.1 b	2.02	1.37 b	8.9 b
	BS	0.84	1.24 b	2.08	1.29 a	117 a	1.64	1.29 b	10.7 a
	VE	0.73	1.46 b	2.18	1.43 a	119 a	1.49	1.60 a	11.1 a
	UN	0.75	1.86 a	2.61	1.45 a	123 a	1.75	1.54 a	10.9 a
	sign. F	*0.797 n.s.*	*0.009 ***	*0.062 n.s.*	*0.000 ****	*0.002 ***	*0.198 n.s.*	*0.000 ****	*0.000 ****
2011	PF	1.50	1.00 b	2.50	1.08 b	83.0 b	2.29	1.48 a	8.3
	VE	1.34	0.99 b	2.33	1.34 a	111 a	1.77	1.42 b	11.0
	UN	1.34	1.59 a	2.92	1.42 a	112 a	2.04	1.49 a	9.9
	sign. F	*0.610 n.s.*	*0.001 ****	*0.098 n.s.*	*0.016 **	*0.000 ****	*0.215 n.s.*	*0.032 **	*n.s.*

Data were processed through ANOVA and means separated using SNK test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letters are not significantly different.

Very promising results were obtained in the evaluation of grape compactness (Table 13; Figure 20). In both years PF leaf removal triggered appreciably lower cluster compactness as compared with UN (18% and 16% in 2010 and 2011 respectively), supporting both diminished fruit-set and reduced compactness, as previously reported for the 'Sangiovese' and 'Trebbiano' cultivars (Gatti et al., 2012; Poni et al., 2006). To our knowledge, looser grapes as a result of pre-flowering leaf removal have not been previously reported for 'Pinot Noir'. Although Lee and Skinkis (2013) reported on early leaf removal on 'Pinot Noir' in their work, their earliest treatment was done at 65 BBCH, while ours was implemented at 57 BBCH. Sabbatini and Howell (2010) also did an experiment on three *V. vinifera* varieties, including 'Pinot Noir', in which leaf removal was applied even earlier, at BBCH 19 (trace bloom);

however, despite the early timing (probably too early) they did not obtain significant results in terms of compactness. On the other hand, none of our post-flowering treatments showed a reduction in compactness as compared with untreated grapevines, proving that post-flowering treatments do not fundamentally affect fruit-set.

Figure 20: Cluster compactness as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development in 2011. Left: typical cluster in case of pre-flowering leaf removal; in the middle: typical cluster in case of untreated vines; right: typical cluster in case of veraison leaf removal.

If looser clusters are obtained, they result in less pressure between the berries, which also lower the risk for berry damage and consequently lowers the risk of microbial infections (Hed, 2009). Lower yield on the other hand, as observed in the case of PF, could eliminate the need for later cluster thinning in the case of high quality crop production and therefore lower production costs in the vineyard. Furthermore, Sternad Lemut et al. (2013) also reported some significant improvements, not only for grapes but also for Pinot Noir wine quality parameters in the case of early (PF and BS) leaf removal treatments.

4.4.4.3 Visual examination of grey mould

Grey mould (*B. cinerea*) growing on grapes is favoured by high rainfall before or during grape maturation period (Barata et al., 2012). Grey mould occurrence in the rainier 2010 season was thus different in the case of different treatments in several ways, essentially in favour of all leaf removal treatments (PF, BS and VE) when compared with untreated control (Table 14). A

week before harvest and at harvest, grey mould severity was significantly higher only in untreated vines (UN) (with 4.75 and 9.09% a week before and at harvest respectively) (Table 14). On the other hand, incidence at both sampling points was in favour of early leaf removal treatments (BS and PF). The relatively high values of incidence with the VE treatment as compared with early leaf removal (Table 14) are possibly related to the reduced effectiveness of the first fungicide application (BBCH 77: berries beginning to touch). It is known that application of a specific fungicide at this stage is most effective in controlling *B. cinerea* (Scannavini et al., 2007; Lavezzaro et al., 2013). If the leaves are eliminated before BBCH 77 (as in the case of PF and BS), the clusters can be reached much more easily by fungicide, thus the effectiveness of prevention is improved.

Table 14: Grey mould incidence (%) and severity (%) in 2010 as affected by: (A) microclimate manipulation through leaf removal at different phenological stages of cluster development; (B) fungicide application; (C) interaction leaf removal x fungicide application. PF=pre-flowering leaf removal; BS=berry set leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

	Factors	Treatments	13 Sep 2010		At harvest 2010	
			Incidence	Severity	Incidence	Severity
A	Leaf removal (LR)	PF	4.10 bc	0.73 b	4.84 bc	0.75 b
		BS	1.78 c	0.48 b	2.89 c	0.50 b
		VE	8.45 b	0.89 b	9.19 b	1.86 b
		UN	30.4 a	4.75 a	41.1 a	9.09 a
		Sign. F	*0.000* ***	*0.000* ***	*0.000* ***	*0.000* ***
			Incidence	Severity	Incidence	Severity
B	Fungicide application (FA)	Switch® (both applications)	8.96	1.73	10.6 b	2.44
		No Switch® at veraison	13.4	1.70	18.4 a	3.66
		Sign. F	*0.173 n.s.*	*0.490 n.s.*	*0.000* ***	*0.311 n.s.*
C	Interaction LR x FA		Incidence	Severity	Incidence	Severity
		Sign. F	*0.000* ***	*0.000* ***	*0.000* ***	*0.000* ***

Data were processed through **two-ways ANOVA (factor 1: leaf removal; factor 2: Switch®/no Switch® application at veraison)** and means separated using SNK test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter are not significantly different.

However, in the 2010 season the parallel (factorial) spraying trial showed that fungicide application itself did not show any significant reduction of grey mould severity, either a week before harvest or at harvest, while incidence was enhanced at harvest only when avoiding the second Switch® application (at veraison). Therefore leaf removal, especially if performed

between leaf removal and fungicide application revealed that only in the case of VE or UN treatments was there any significant reduction in *Botrytis cinerea* Pers. due to Switch® application, while in case of BS and PF, the effect of fungicide was negligible (data not reported).

In 2011, which was warmer and dryer as compared to 2010, and thus less favourable for *Botrytis cinerea* Pers. development, the incidence at harvest was not significant (Table 15), while severity was significantly lower in the case of PF (0.12%) as compared with UN (0.93%) and intermediate for VE (0.51%). In the parallel trial, with PF being treated with different numbers of fungicide applications (0-to-2), the fungicide did not show any significant influence on the observed pathogen, neither when applied once (BBCH 77) nor when applied twice (BBCH 77 and BBCH 83) as compared with no fungicide application. It was already reported that ecosystem toxicity of the viticultural system shows a high dependence on the varying meteorological conditions (Vázquez-Rowe et al., 2012). It seems that in the case of PF leaf removal, treatment with fungicide could be avoided in years such as 2011 and similar hot dry seasons. However, the results may be much less promising in rainier seasons. Similar trials need to be done in several seasons before avoidance of fungicide can be safely suggested to winegrowers.

Table 15: Grey mould incidence (%) and severity (%) in 2011 as affected by: (A) microclimate manipulation through leaf removal at different phenological stages of cluster development and (B) by fungicide application to PF: PF=pre-flowering leaf removal; BS=berry set leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

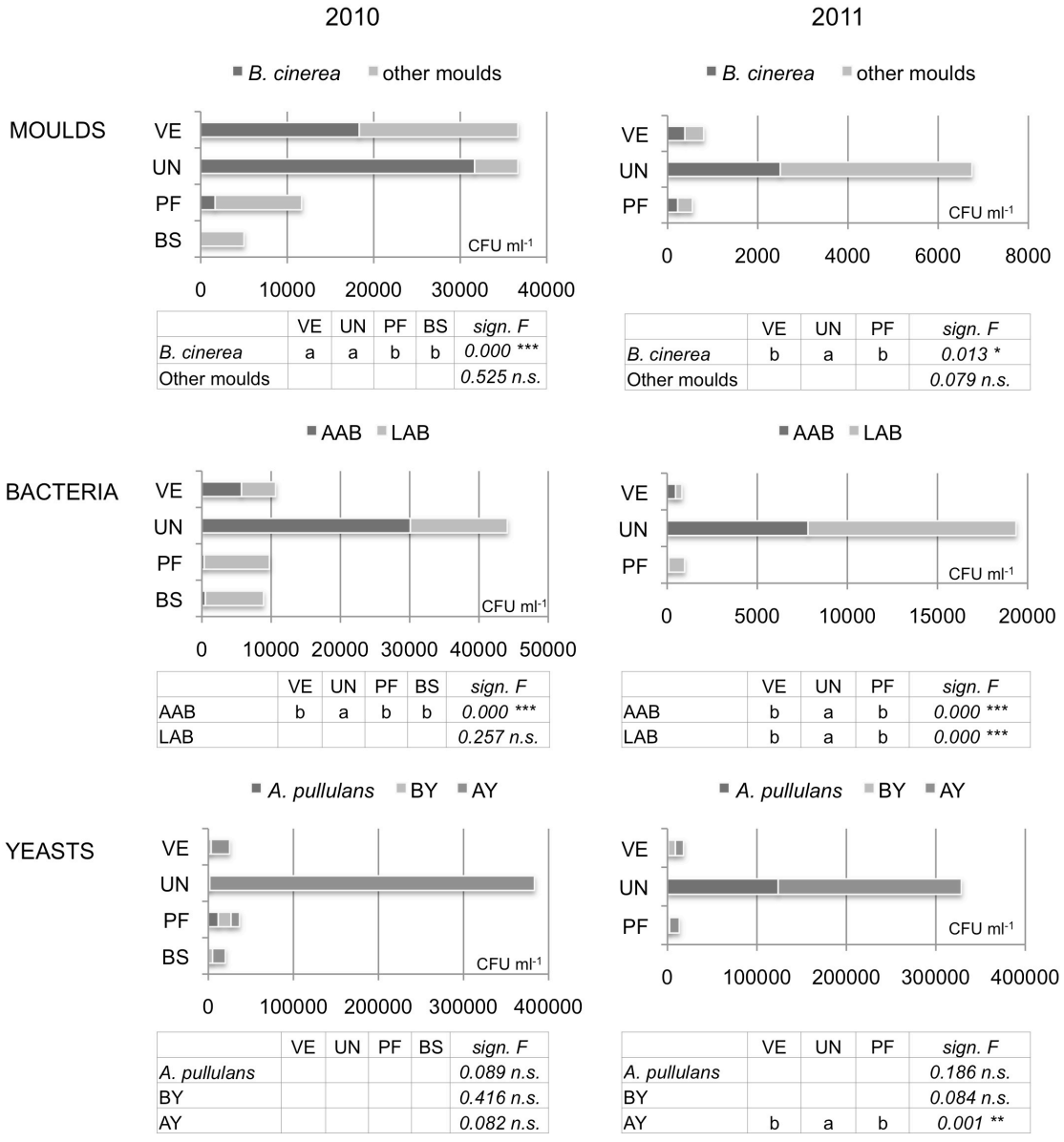
	Treatments	At harvest 2011	
A	Leaf removal (LR)	Incidence	Severity
	PF	1.50	0.12 b
	VE	1.83	0.51 ab
	UN	3.27	0.93 a
	Sign. F	*0.095 n.s.*	*0.031 **

B	Fungicide application to PF	Incidence	Severity
	Switch® (both applications)	1.50	0.12
	No Switch® at veraison	1.26	0.11
	No Switch®	1.56	0.27
	Sign. F	*0.935 n.s.*	*0.333 n.s.*

Data were processed through ANOVA and means separated using SNK test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter are not significantly different.

4.4.4.4 Microbial count and community structure

In a similar way to the visual results, the total microbial count (MC) was significantly different in both vintages as a consequence of extremely different climatic conditions. Microbial populations normally peak at harvest, when the berry surface available for adhesion is the largest and no agrochemical treatments have been applied for several weeks (Renouf et al., 2005).



	VE	UN	PF	BS	sign. F
B. cinerea	a	a	b	b	*0.000* ***
Other moulds					*0.525 n.s.*

	VE	UN	PF	sign. F
B. cinerea	b	a	b	*0.013* *
Other moulds				*0.079 n.s.*

	VE	UN	PF	BS	sign. F
AAB	b	a	b	b	*0.000* ***
LAB					*0.257 n.s.*

	VE	UN	PF	sign. F
AAB	b	a	b	*0.000* ***
LAB	b	a	b	*0.000* ***

	VE	UN	PF	BS	sign. F
A. pullulans					*0.089 n.s.*
BY					*0.416 n.s.*
AY					*0.082 n.s.*

	VE	UN	PF	sign. F
A. pullulans				*0.186 n.s.*
BY				*0.084 n.s.*
AY	b	a	b	*0.001* **

Figure 21: Mean values for microbial count (CFU ml^{-1}) on the grape samples at harvest time 2010 (left) and 2011(right) as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development: PF=pre-flowering leaf removal; BS=berry set leaf removal (performed only in 2010); VE=veraison leaf removal; UN=untreated control with no leaf removal performed. AAB=acetic acid bacteria; LAB = lactic acid bacteria; *A. pullulans* = *Aureobasidium pullulans* (yeasts-like-fungi); AY = ascomycetous yeasts; BY = basidiomycetous yeasts.

Also in this experiment, the total MC in 2010 was the highest at harvest with its maximum on the control grapes (UN) of closed, non-defoliated canopies. However, in 2011, MC on control grapes (UN) was 3.6 x 10^5 CFU ml^{-1}, while it was even 24% higher in the rainier 2010 season. Despite considerable differences in total MC, yeasts prevailed strongly within the total count at both harvests (Figure 21) and were thus also the most responsible for the differences in total count between climatically different vintages. This is in accordance with the observations of Combina et al. (2005) and Čadež et al. (2010) who reported that colder harvests with higher rainfall lead to particularly high yeast counts. However, some other authors have observed the opposite in years with very heavy rainfall (Comitini and Ciani, 2006; Rementeria et al., 2003), probably due to elution (washing-out) effects. In the literature, the total yeast population in grapes is made up roughly of between 10^2 and 10^4 cells/g (Fleet et al., 2002), but higher values have also been reported.

In the context of the goals of our trial, it was essential to include into the observation all of the randomly collected berries, regardless of their health situation. Thus the total population is relatively high, especially in case of UN, but fairly comparable with existing literature for other treatments; with total yeasts count 3.7 x 10^4 (in 2010) and 1.3 x 10^4 (in 2011) CFU ml^{-1} (representing 64% (2010) and 87% (2011) of total MC) for PF and with total yeasts count 2.5 x 10^4 (in 2010) and 2.4 x 10^4 (in 2011) (representing 35% (2010) and 92% (2011) of total MC) for VE. However, not only grape sanitary state, but also different soil, grape variety and grape growing practices may significantly influence the microbial ecosystem (Renouf et al., 2005).

With the aim of better evaluating overall microbial ecology as affected by microclimate manipulation through leaf removal, PCA analyses of microbial count projections for all the samples were prepared and shown on two-dimensional PCA plots (Figure 22). In 2010, a clear separation between UN and VE was obtained, as compared with early leaf removal treatments (PF and BS). On the other hand, in 2011, UN was separated from both leaf removal treatments (VE and PF), however the difference between VE and PF was not as evident as in 2010, again supporting the importance of carefully implemented canopy management, especially for so-called "bad vintages".

Nevertheless, microclimate conditions affected also microbial community structure. In general, during the 2010 ripening period, basidiomycetous yeasts from the *Rhodotorula* genus (and rarely *Cryptococcus*) and the *Aureobasidium pullulans* species mostly prevailed, which is in accordance with previous reports from Renouf et al. (2005). However, at harvest time in 2010

and 2011, ascomycetous yeasts from the *Hanseniaspora/Kloeckera* and *Hansenula* genera, lactic/acetic acid bacteria, *B. cinerea* and other moulds from the *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium* genera dominated (data not shown). Regarding the most dominant group - yeasts, the most marked changes were again observed in the canopies of untreated vines (UN), which had the highest *A. pullulans* count, but a relatively low count of basidiomycetous yeasts. Furthermore, as compared to control (UN) in the case of VE treatment, in 2011, a lower count was obtained for *A. pullulans*, but a higher count for basidiomycetous yeasts from the *Rhodotorula* genus, while in the rainier 2010 season a higher count was observed in VE for both (*A. pullulans* and *Rhodotorula)*. If comparing PF (2 x fungicide applications) with PF_0S and PF_1S (no fungicide and 1x fungicide application respectively), in 2011, *A. pullulans* seemed to be more susceptible to fungicide in comparison to other yeasts, however this was not confirmed in 2010 (2x fungicide application), probably due to extensive rainfall, affecting the yeast population overall.

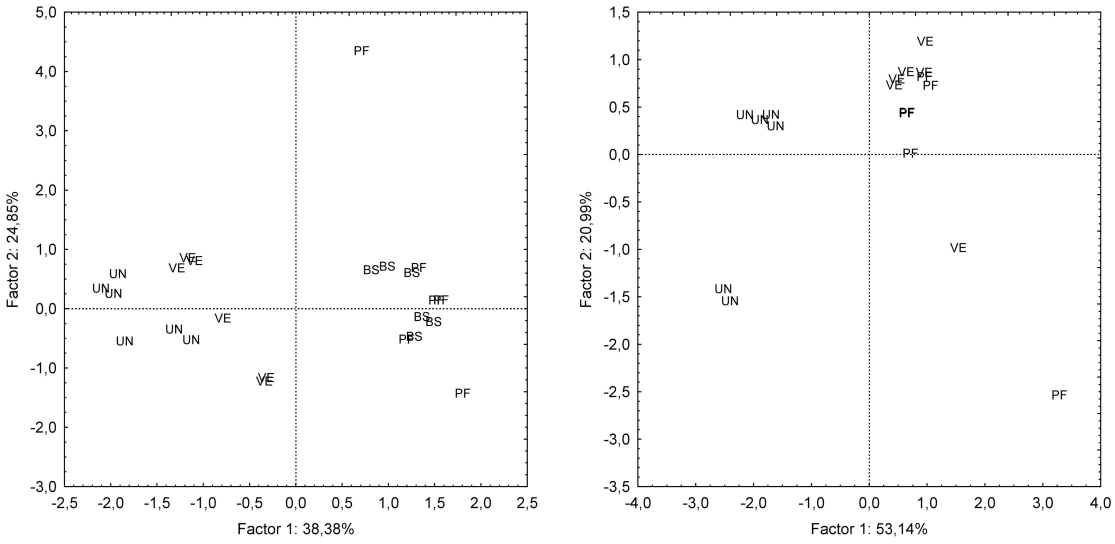


Figure 22: Principal component analyses of projection of microbial count (logarithmic transformation) of 'Pinot Noir' samples on the two dimensional PCA plots (left in 2010 and right in 2011) as affected by microclimate manipulation through leaf removal at different phonological stages of cluster development: PF=pre-flowering leaf removal; BS=berry set leaf removal (performed only in 2010); VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

Nevertheless, microclimate conditions affected also microbial community structure. In general, during the 2010 ripening period, basidiomycetous yeasts from the *Rhodotorula* genus (and rarely *Cryptococcus*) and the *Aureobasidium pullulans* species mostly prevailed, which is in accordance with previous reports from Renouf et al. (2005). However, at harvest time in 2010

and 2011, ascomycetous yeasts from the *Hanseniaspora/Kloeckera* and *Hansenula* genera, lactic/acetic acid bacteria, *B. cinerea* and other moulds from the *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium* genera dominated (data not shown). Regarding the most dominant group - yeasts, the most marked changes were again observed in the canopies of untreated vines (UN), which had the highest *A. pullulans* count, but a relatively low count of basidiomycetous yeasts. Furthermore, as compared to control (UN) in the case of VE treatment, in 2011, a lower count was obtained for *A. pullulans*, but a higher count for basidiomycetous yeasts from the *Rhodotorula* genus, while in the rainier 2010 season a higher count was observed in VE for both (*A. pullulans* and *Rhodotorula)*. If comparing PF (2 x fungicide applications) with PF_0S and PF_1S (no fungicide and 1x fungicide application respectively), in 2011, *A. pullulans* seemed to be more susceptible to fungicide in comparison to other yeasts, however this was not confirmed in 2010 (2x fungicide application), probably due to extensive rainfall, affecting the yeast population overall.

When focusing on moulds, we observed that grey mould occurrence in the colder and rainier 2010 season was significantly lower on grapes subjected to early leaf removal (BS and PF), as compared with VE and UN, while in the warmer and dryer 2011 season, both early leaf removal (PF) and late leaf removal (VE) treatments showed a significant reduction in grey mould when compared with UN (Figure 21) as also observed by visual examination (section 4.4.4.3).

Our 2011 samples, originating from all the leaf removal treatments, showed not only significantly lower counts for *B. cinerea* but also for acetic and lactic bacteria, while in 2010 the count was significantly lower in leaf removal treatments only for acetic bacteria (although with similar trends also for lactic bacteria) (Figure 21).

Any reduction in negative microbial activity is of great importance for the quality of grapes and consequently the final quality of the wines.

4.4.4.5 Microbial metabolites

In general, grapes infected with *B. cinerea* shows significant changes in their composition (e.g. a decline in sugar, titratable acids and available nitrogen; shifts in aroma compounds and the production of phytoalexins, complex polysaccharides and a powerful oxidizing enzyme called *laccase*) (La Guerche et al., 2006; Ribereau-Gayon, 1988; Jeandet et al., 1995; Zoecklein et al.,

1995). Moreover, the activity of *B. cinerea* is also associated with the formation of gluconic acid and glycerol. It is known that *B. cinerea* (and other moulds such as *Aspergillus* and *Penicillium*) can oxidise glucose into gluconic acid. *B. cinerea* also produces significant amounts of polyols, of which glycerol is quantitatively the most important, and hence Ribereau-Gayon (1988) have suggested that the ratio of glycerol to gluconic acid indicates the "quality" of the rot. Higher ratios indicate the growth of true noble rot, whereas lower ratios suggest sour rot (Ribereau-Gayon, 1988). According to the literature, about 1-5 g/L gluconic acid and 1-10 g/L glycerol are formed when noble rot occurs (Ribereau-Gayon 1988), while on the other hand gluconic acid is also significantly higher when the fruit is invaded by *Gluconobacter,* as in the case of sour bunch rot (the condition when berries swell and split due to initial grey mould infection, making them more susceptible to attack by other spoilage organisms, especially other moulds and acetic acid bacteria). Consequently, volatile acids can also be enhanced in must from infected grapes. Accumulation of those metabolites (gluconic acid, glycerol and acetic acid/volatile acidity) in the grapes (juice) can thus serve as a kind of indicators of grape deterioration. Fast screening of actual activity of the microbes presence as affected by different treatments, was thus implemented to the trial by quantifying selected microbial indicator metabolites in grape juice of all the samples.

The results revealed, that in the 2010 harvest point samples (a vintage more prone to microbial infection) gluconic acid increased significantly in the case of the untreated control (UN), followed by VE, with a significantly lower trend than UN (- 67%), but a higher (not significantly) trend than for earlier leaf removal treatments (BS and PF) - later showing the lowest average amount of gluconic acid (- 77% and - 74% for BS and PF respectively as compared with the control) (Table 16).

On the other hand, no significant differences between treatments were noted in terms of glycerol and volatile acidity (due to relatively high standard deviations); however, their concentration trends were again in agreement with other observations (Table 16).

Couto et al. (2003) imply that levels of gluconic acid up to 1-2 g/L, not accompanied by the high presence of acetic acid, suggest fungal origin or attack of sugars by acetic acid bacteria in the absence of ethanol, while on the other hand, higher levels of gluconic acid (2-3 g/L), correlated with detectable levels of acetic acid, may suggest that they originate from acetic acid bacteria and not from *B. cinerea*. This suggests in our case that fungal activity was most probably more responsible for gluconic acid enchantment then the activity of (acetic) bacteria.

After calculating ratio between glycerol and gluconic acid (glycerol/gluconic acid) we noticed that all the leaf removal treatments showed similar values, which were higher than that of untreated control (UN). According to Ribereau-Gayon (1988), the later implies that in case of UN there was higher growth of sour rot (negative) than those of noble rot (positive).

All this outcomes not only support the results obtained by visual evaluation and microbial count, but also confirm a certain relationship between the count and the actual activity of the microbes present.

Table 16: Concentration of microbial metabolites in must as affected by microclimate manipulation through leaf removal at different phenological stages of cluster development in 2010: PF=pre-flowering leaf removal; BS=berry set leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

Treatment	Gluconic acid (mg/L)	Glycerol (mg/L)	Volatile acidity as acetic acid (g/L)	Ratio glycerol / gluconic acid
PF	178.00 ± 7.55 b	2.91 ± 0.94	0.34 ± 0.16	0.0163
BS	157.33 ± 11.02 b	2.58 ± 0.74	0.39 ± 0.09	0.0164
VE	225.00 ± 24.33 b	3.81 ± 1.67	0.45 ± 0.22	0.0169
UN	687.33 ± 52.48 a	5.00 ± 2.05	0.55 ± 0.23	0.0073
Sign. F	*0.000*** *	*0.250 n.s.*	*0.558 n.s.*	

Data were processed through ANOVA and means separated using SNK test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter are not significantly different.

4.4.4.6 Costs and energy calculations

The overall results show that leaf removal, especially if performed early, could be even more effective than fungicide application (even, or particularly, in rainy and therefore more infection-prone years). In this context, we also considered very important to compare the costs and energy consumption of different viticultural practises, all of which are oriented towards higher grape quality, either from a technological point of view or in a relation to grape health (Table 17).

As the effectiveness of PF leaf removal in controlling *B. cinerea* (due to the altered microclimate as well as lower cluster compactness) was also accompanied by a considerably lower yield, we found that later practice of cluster thinning was not necessary in this case. With cluster thinning the less developed clusters and/or those that are last on the shoot would usually be removed, aiming to control the yield and consequently improve crop quality due to a better

Table 17: Comparison of costs and energy consumption in case of different combinations of viticultural practices: PF=pre-flowering leaf removal; BS=berry set leaf removal (performed only in 2010); VE=veraison leaf removal; UN=untreated control with no leaf removal performed; CT=cluster thinning; FA=fungicide application.

Combination of viticultural practices	No of practices	Manual leaf removal (needed hours/ha)	Price of manual leaf removal performance/hour (EUR)	Total cost of manual work for leaf removal performance (EUR/ha)	Number of switch® applications	Cost of (switch®) spraying per ha (EUR/ha)	Total cost of (switch®) spraying EUR/ha	Manual cluster thinning (needed hour/ha)	Price of manual leaf removal performance per hour (EUR/h)	Total cost of manual work in case of cluster thinning (EUR/ha)	TOTAL COST (EUR/ha)	Energy needed for manual leaf removal (mJ/ha)	Energy needed for (switch®) spraying (mJ/ha)	Energy needed for manual cluster thinning (mJ/ha)	TOTAL ENERGY (mJ/ha)
UN + CT + 2 x FA	4	0	6	0	2	131.05	262.10	11	6	66	328.10	0	945.09	21.56	966.65
* VE + CT + 2 x FA	4	32	6	192	2	131.05	262.10	9	6	54	508.10	62.72	945.09	17.64	1025.45
VE + CT+ 1 x FA	3	32	6	192	1	131.05	131.05	9	6	54	377.05	62.72	472.54	17.64	552.90
BS + CT + 2 x FA	4	37	6	222	2	131.05	262.10	9	6	54	538.10	72.52	945.09	17.64	1035.25
BS + CT + 1 x FA	3	37	6	222	1	131.05	131.05	9	6	54	407.05	72.52	472.54	17.64	562.70
BS + CT	2	37	6	222	0	131.05	0	9	6	54	276.00	72.52	0	17.64	90.16
PF + CT + 2 x FA	4	40	6	240	2	131.05	262.10	9	6	54	556.10	78.40	945.09	17.64	1041.13
PF + CT + 1 x FA	3	40	6	240	1	131.05	131.05	9	6	54	425.05	78.40	472.54	17.64	568.58
PF + CT	2	40	6	240	0	131.05	0	9	6	54	294.00	78.40	0	17.64	96.04
** PF + 1 x FA	2	40	6	240	1	131.05	131.05	0	6	0	371.05	78.40	472.54	0	550.94
PF	1	40	6	240	0	131.05	0	0	6	0	240.00	78.40	0	0	78.40

* Combination of viticultural practices as it is done currently.

** Combination of viticultural practices that could be implemented with the aim of more sustainable and more cost effective vineyard management.

source-to-sink ratio (Guidoni et al., 2008; 2002). The avoidance of (at least) a second application of fungicide seems to be justified according to the results of our parallel trials. So, if we compare the current combination of viticultural practices, as usually carried out in the experimental vineyard (consisting of veraison leaf removal combined with cluster thinning and two fungicide applications) (Table 17), side by side with the performance of pre-flowering leaf removal combined with only one fungicide application (as complete fungicide avoidance may not be justified in wetter years), we could already save 27% on costs and as much as 46% on energy. This shows that the implementation of a new innovative PF practice can be considered as an excellent option, aiming for more sustainable and cost effective 'Pinot Noir' vineyard management.

4.4.5 Conclusions

A simplified overview of selected results (which can be undoubtedly classified as positive or negative) as affected by leaf removal at different phenological stages of cluster development is done in order to show obtained advantages / disadvantages of all the observed leaf removal practices (Figure 23).

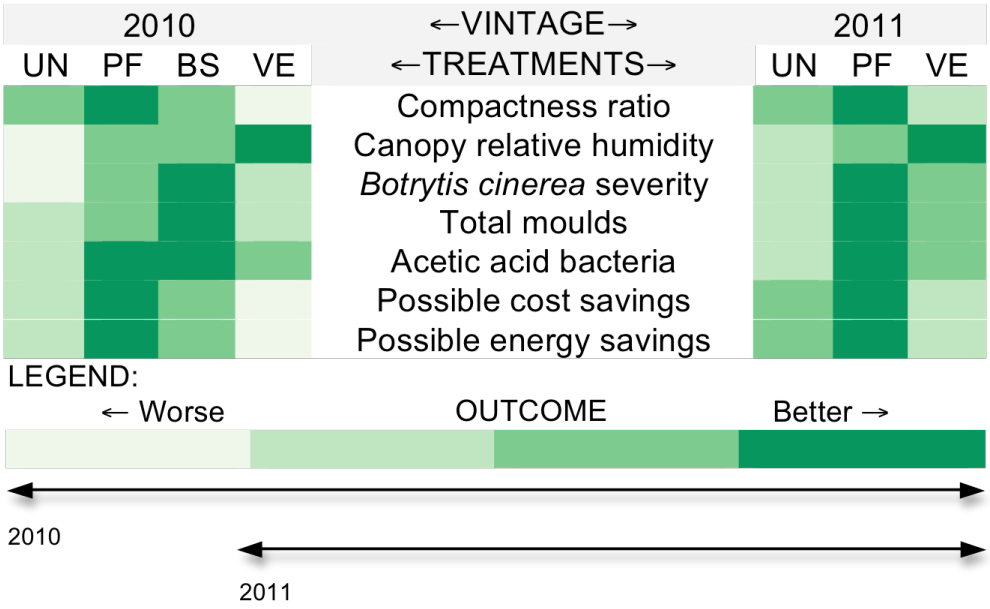


Figure 23: Simplified overview of selected results as affected by leaf removal at different phonological stages of cluster development: PF=pre-flowering leaf removal; BS=berry set leaf removal (performed only in 2010); VE=veraison leaf removal; UN=untreated control with no leaf removal performed. The results are ranked according to the average values, shown as four shades in case of 2010, but only three shades in case of 2011. In case of very similar outcomes, the same shade is used twice.

Higher microbial population counts (including potentially harmful ones such as *B. cinerea* or acetic acid bacteria) were observed with veraison (late) leaf removal and in treatments without leaf removal, probably due to minimised air circulation / canopy microclimate conditions and also lower spray penetration. Earlier leaf removal treatments were thus found to be a more effective tool for controlling microbial infections, however the performance of veraison leaf removal was better as compared to the control (grapes from untreated vines).

Furthermore, parallel trials showed that leaf removal, especially if performed early, appears to be more effective than fungicide application and that a second fungicide application in the case of early leaf removal could thus be avoided, with similar or even better results as compared to late leaf removal supplemented by two fungicide applications. In case of pre-flowering leaf removal, lower cluster compactness was also observed, contributing to lower sensitivity to microbial diseases. Moreover, the yield reduction observed in the case of pre-flowering technique can reduce the need for later cluster thinning and therefore reduce production costs, energy needs and negative environmental impact. However, further field experiments in more seasons, at other sites and with other compact-grape varieties need to be performed to ensure that pre-flowering leaf removal can be successfully applied to wider viticultural practice.

4.4.5 Acknowledgments

We would like to thank the owners of the Tilia Estate for allowing us to carry out the experimental work in their vineyards and Mia Licen and Ajda Lemut for their help with plating.

4.4.7 References

Ali, K., Maltese, F., Choi, Y.H., Verpoorte, R., 2010. Metabolic constituents of grapevine and grape- derived products. *Phytochemistry Reviews*. 9, 357-378.

Alluvione, F., Moretti, B., Sacco, D., Grignani, C., 2011. EUE (energy use efficiency) of cropping systems for a sustainable agriculture, *Energy.* 36, 4468-4481.

Audsley, E., Alber, S., Gemeinschaften, E. (Eds.), 1997. Harmonisation of environmental life cycle assessment for agriculture. European Comm., DG VI *Agriculture*, pp. 139.

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012. The microbial ecology of wine grape berries. *International Journal of Food Microbiology*. 153, 243-259.

Bosch, Automotive Handbook, 2007, 7th Edition, Bentley Publishers.

Bubola, M., Peršurić, Đ., Kovaćević Ganić., K., Karoglan, M., Kozina, B., 2012. Effect of fruit zone leaf removal on the concentration of phenolic and organic acids in Istrian Malvasia grape juice and wine. *Food Technology and biotechnology*. 50, 159-166.

Čadež, N., Zupan, J., Raspor, P., 2010. The effect of fungicides on yeast communities associated with grape berries. *FEMS Yeast Research*. 10, 619–630.

Combina, M., Mercado, L., Borgo, P., Elia, A., Jofré, V., Ganga, A., Martinez, C. and Catania, C., 2005. Yeasts associated to Malbec grape berries from Mendoza, Argentina. *Journal of Applied Microbiology*. 98, 1055–1061.

Comitini, F., Ciani, M., 2006. Survival of inoculated *Saccharomyces cerevisiae* strain on wine grapes during two vintages. *Letters in Applied Microbiology*. 42, 248-253.

Coombe, B.G., 1992. Research on development and ripening of the grape berry. *American Journal of Enology and viticulture*. 43, 101-110.

Couto, J.A., Graça, A.R., Soares-Franco J.M., Hogg, T., 2003. The use of gluconic acid level as an indicator of the actvity of acetic aced bacteria in grapes. VII Symposium International d'Œnologie, Bordeaux.

Diago, M.P., Ayestarán, B., Guadalupe, Z., Garrido, Á., Tardaguila, J., 2012. Phenolic composition of Tempranillo wines following early defoliation of the vines. *Journal of the Science of Food and Agriculture*. 92, 925-934. doi: 10.1002/jsfa.4671

Diago, M.P., Blanco, J.A., Tardaguila, J., Poni, S., 2013. Mechanical crop thinning and early defoliation as novel tools for yield management in VSP grapevines. *Acta Hort*. 978, 279-284.

EARS. ‘‘Slovene Public Information’’, EARS – meteors; http://meteo.arso.gov.si; October, 2012.

Fleet, G.H., 1999. Microorganisms in food ecosystems. *International Journal of Food Microbiology*. 50, 101-117.

Fleet, G.H., Prakitchaiwattana, C., Beh, A.L., Heard, G., 2002. The yeast ecology of wine grapes, in: Biodiversity and biotechnology of wine yeasts. Ciani (Ed.). India: Research Signpost, pp. 1-17.

Fugelsang, K.C., Edwards, C.G., 2007. Wine Microbiology, in: Practical Applications and Procedures. Springer Science+Business Media, LLC, New York (2nd edition), pp. 393.

Gatti M., Bernizzoni, F., Civardi, S., Poni, S., 2012. Effects of cluster thinning and pre-flowering leaf removal on growth and grape composition in cv. Sangiovese. *American Journal of Enology and Viticulture*. 63, 325-332.

Glithero, N.J., Ramsden, S.J., Wilson, P., 2012. Farm systems assessment of bioenergy feedstock production: Integrating bio-economic models and life cycle analysis approaches, *Agricultural Systems*. 109, 53-64.

Guetsky, R., Shtienberg, D., Dinnor, A., 2001. Combining biocontrol agents to reduce the variability of biological control. *Biological control*. 91, 621-627.

Guidoni, S., Allara, P., Schubert, A., 2002. Effect of cluster thinning on berry skin anthocyanin composition of *Vitis vinifera* Nebbiolo. *American Journal of Enology and Viticulture*. 53, 224-226.

Guidoni, S., Ferrandino, A., Novello, V., 2008. Effect of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. *American J. of Enology and Viticulture*. 59, 22-29.

(23) Hed, B., Ngugi, H.K., Travis, J.W., 2009. Relationship between cluster compactness and bunch rot in Vignoles grapes. *Plant Disease*. 93, 1195-1201.

Jeandet, P., Bessis, R., Sbaghi, M., Meunier, P., 1995. Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. *Journal of Phytopathology*. 143, 135-139.

Jones, G.V., Hellman, E., 2003. Site assessment, in: Oregon Viticulture, Hellman, E. (Ed.), 5th 1st Edition, Oregon State University Press, Corvallis, Oregon, pp. 44-50.

Kassemeyer, H-H., Berkelmann-Lohnertz, B., 2009. Fungi of Grapes, in: Biology of Microorganisms on Grapes, in Must and in Wine, Konig et al. (Eds.). Springer-Verlag Berlin Heidelberg, pp. 61-87.

Kliewer, W.M., 1970. Effect of day temperature and light intensity on coloration of *Vitis vinifera* L. grapes. *Journal of American Society of Horticulture Science*. 95, 693-697.

La Guerche, S., Dauphin, B., Pons, M., Blancard, D., Darriet, P., 2006. Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. *Journal of Agricultural and Food Chemistry*. 54, 9193-9200.

Larcher, R., Nicolini, G., Villegas, R., Bertoldi, D., Puecher, C., 2009. Determination of gluconic acid in wine using high pressure liquid chromatography with pulsed amperometric detection. *Vitis* 48, 201-204.

Lavezzaro, S., Morando, A., Ferro, S., 2013. Trattare in pre-chiusura grappolo contro la botrite della vite. *Informatore Agrario*. 69, 61-64.

Lindow, S.E., Brandl, M.T., 2003. Minireview: Microbiology of the phyllosphere. *Applied Environmental Microbiology*. 69, 1875-1883.

Litskas, V.D., Karaolis, C.S, Menexes, G.C., Mamolos, A.P., Koutsos, T.M., Kalburtji, K.L., 2013. Variation of energy flow and greenhouse gas emissions in vineyards located in Natura 2000 sites, *Ecological Indicators*. 27, 1-7.

Lohitnavy, N., Bastian, S., Collins, C., 2010. Early leaf removal increases flower abscission in *Vitis vinifera* 'Semillon'. *Vitis*. 49, 51-53.

Lorenz, D.H., Eichhorn, K.W., Bleiholder, H., Klose, R., Meier, U., Weber, E., 1995. Growth stages of the grapevine: phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. vinifera) - codes and descriptions according to the extended BBCH scale. *Australian Journal of Grape and Wine Research*. 1, 100-103.

Nair, N. G., Hill, G. K., 1992. Bunch rot of grapes caused by *Botrytis cinerea*. *Plant diseases of international importance*. 3, 147-69.

Ozkan, B., Fert, C., C. Karadeniz, C.F., 2007. Energy and cost analysis for greenhouse and open-field grape production. *Energy*. 32, 1500-1504.

Palliotti, A., Gatti, M., Poni, S., 2011. Early leaf removal to improve vineyard efficiency: gas exchange, source-to-sink balance, and reserve storage responses. *American Journal of Enology and Viticulture*. 62, 219-228.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL Medium to Profile Native Flora Fermentations. *American Journal of Enology and Viticulture*. 5, 198-203.

Poni, S, Bernizzoni, F., Civardi, S, Libelli, N., 2009. Effects of pre-bloom leaf removal on growth of berry tissues and must composition in two red *Vitis vinifera* L. cultivars. *Australian Journal of Grape and Wine Research*. 15, 185-193.

Poni, S., Casalini, L., Bernizzoni, F., Civardi, S., Intrieri, C., 2006. Effects of early defoliation on shoot photosynthesis, yield components, and grape composition. *American Journal of Enology and Viticulture*. 57, 397-407.

Pretorius, I.S., Van der Westhuizen, T.J., Augustyn O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. A review. *South African Journal of Enology and Viticulture*. 21, 92-99.

Rementeria, A., Rodriguez, J.A., Cadaval, A., Amenabar, R., Muguruza, J.R., Hernando, F.L., Sevilla, M.J., 2003. Yeast associated with spontaneous fermentations of white wines from the "Txakoli de Bizkaia" region (Basque Country, North Spain). *International Journal of Food Microbiology*. 86, 201-207.

Renouf, V., Claisse, O., Lonvaud-Funel, A., 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Australian Journal of Grape and Wine Research. 11, 316- 327.

Ribereau-Gayon, P., 1988. Botrytis: Advantages and disadvantages for producing quality wines. Proceedings of the second international cool climate viticulture and oenology symposium. Auckland, New Zealand, pp. 319-323.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2005. Handbook of Enology, Volume 1, 2nd Edition, The Microbiology of Wine and Vinifications, pp. 512.

Rosini, G., Federici, F., Martini, A., 1982. Yeast flora of grape berries during ripening. *Microbial Ecology*. 8, 83-89.

Sabate, J., Cano, J., Esteve-Zarzoso, B., Guillamon, J.M., 2002. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis ribosomal genes. *Microbiological Research*. 157, 267-274.

Sabbatini, P., Stanley Howell, G., 2010. Effects of early defoliation on yield, fruit composition, and harvest season cluster rot complex of grapevines. *HortScience*. 45, 1804-1808.

Scannavini, F., Cavazza, F., Alvisi, G., Fagioli, L., 2007. Evoluzione delle strategie contro la botrite della vite. *Informatore Agrario*. 63, 59-63.

Schultz, H.R., 2000. Climate change and viticulture: A European perspective on climatology, carbon dioxide and UV-B effects. *Australian Journal of Grape and Wine Research*. 6, 2-12.

Sivilotti, P., Butinar, L., Sternad Lemut, M., 2013. Leaf removal treatments as affecting *Botrytis Cinerea* occurance on Pinot Gris grapes from North Eastern Italy: results over four years of trials. *Ciência e técnica vitivinícola*. 28, 446-450.

Spayd, S.E., Tarara, J.M., Mee, D.L., Fergusson, J.C., 2002. Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. *American Journal of Enology and Viticulture.* 53, 171-182.

Sternad Lemut, M., Trost, K., Sivilotti, P., Arapitsas, P., Vrhovsek, U. 2013. Early versus late leaf removal strategies for 'Pinot noir' (*Vitis vinifera* L.): effect on colour-related phenolics in young wines following alcoholic fermentation. *Journal of Science of Food and Agriculture*. doi: 10.1002/jsfa.6193.

Sternad Lemut, M., Trost, K., Sivilotti, P., Vrhovsek, U., 2011. 'Pinot Noir' grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. *Journal of Food Composition and Analyses.* 24, 777-784.

Sternad Lemut, M., Sivilotti, P., Butinar, L., Laganis, J., Vrhovsek, U. 2013. Pre-flowering leaf removal in 'Pinot Noir' (*Vitis vinifera* L.): potential for successful microbial control in the

context of more sustainable, energy efficient and cost-effective vineyard management, submitted.

Tarara, J. M., Lee, J., Spayd, S. E., Scagel, C. F., 2008. Berry temperature and solar radiation alter acylation, proportion, and con- centration of anthocyanin in Merlot grapes. *American Journal of Enology Viticulture*. 59, 235-247.

Tardaguila, J., de Toda, F.M., Poni, S., Diago M.P., 2010. Impact of early leaf removal on yield and fruit and wine composition of *Vitis vinifera* L. Graciano and Carignan. *American Journal of Enology and Viticulture*. 61, 372-381.

Vázquez-Rowe, I., Villanueva-Rey, P., Moreira, M.T., Feijoo, G. 2012. Environmental analysis of Ribeiro wine from a timeline perspective: Harvest year matters when reporting environmental impacts. *Journal of Envirolmental Management* 98: 73-83.

Vršič, S., Vodovnik, T., 2012. Reactions of grape varieties to climate changes in North East Slovenia. *Plant Soil and Environment*. 58, 34-41.

West, T.O., Marland, G., 2002. A synthesis of carbon sequestration, carbon emissions, and net carbon flux in agriculture: comparing tillage practices in the United States, *Agriculture, Ecosystems & Environment.* 91, 217-232.

Zoecklein B.W., Fugelsang, K.C., Gump, B.H., Nury, F.S., 1995. Oxygen, carbon dioxide, and nitrogen, in: Wine analysis and production, Chapman & Hall, New York, pp. 216-227.

4.5 Implementation of metabolomics to the vineyard environment: secondary metabolism in the interaction with purposely-induced microclimate modifications in 'Pinot Noir' (*Vitis Vinifera* L.) vineyards from Slovenia and Italy

4.5.1 Abstract

Beyond the biological significance, grapevine metabolites are crucial in the determination of grape quality attributes. Grape berry composition can be significantly modulated by various exogenous (site, climate, soil properties) and endogenous factors (variety, clone, rootstock); however, it can also be considerably enhanced by implementing some agricultural practices in the vineyard environment. Leaf removal (defoliation) is an important canopy management technique that can lead to substantial improvements of fruit-zone microclimate, particularly if its severity and timing are carefully adjusted to each specific *terroir*. Whereas earlier research efforts to reveal the effect of canopy microclimate manipulation on the grape quality parameters were mainly focused on just a few targeted compounds, by contrast, novel analytical approaches, such as metabolomics, are offering a lot wider possibilities to study plant metabolism in the interaction with its environment. A field trial was thus designed in two 'Pinot Noir' vineyards (in Vipava Valley, Slovenia and in Trentino, Italy) to compare early (pre-flowering) *vs.* late (veraison) defoliation with untreated, control vines. The aim was to study how the induced canopy microclimate modifications can affect the plant biosynthesis of as numerous secondary metabolites as possible by implementing targeted but also untargeted metabolomics to the observation. The results of targeted metabolomics revealed a number of changes related both to the vineyard location and to the timing of leaf removal. As regards vineyard location, the occurrence of total phenolics in grape berries was 40% higher in Italian samples. Leaf removal timing, on the other hand, led to the most significant changes within the wide group of flavonols in both *terroirs*. The amount of total flavonols resulted in an increase by 216% in Slovenia and by 56% in Italy in the pre-flowering treatments, as compared with controls. Pre-flowering leaf removal also triggered significant changes in total hydroxycinnamic acids (both vineyards) and anthocyanins (in Slovenia). In comparison to phenolics, the total isoprenoids were present in more comparable amounts at both sites (with only 8% higher total content in Italy), although the total carotenoids, chlorophylls and tocopherols as separate classes revealed higher significance for Slovenian grapes, with several differences detected also among all the treatments, while mainly none or lower significance

appeared in Italy. However, when observed individually, many secondary metabolites, including numerous poorly studied up to date, showed important alterations in biosynthesis even they were frequently not significant when the total amount of group metabolites was examined. in addition, when implementing the untargeted approach, several biomarker candidates for specific treatments were indicated based on the same behaviour at both sites; however, numerous putative biomarkers remain unknown and need to be observed and confirmed in further experiments. In general, within our observation and despite geo-climatic differences between the two vineyards, grape metabolome was positively affected by both leaf removal treatments, providing interesting outcomes particularly when performed at a very early phenological stage.

Key words: canopy management, targeted metabolomics, untargeted metabolomics, canopy microclimate manipulation, leaf removal, pre-flowering leaf removal, secondary metabolism, phenolic compounds, isoprenoids, 'Pinot Noir' (*Vitis Vinifera* L.)

4.5.2 Introduction

The architecture of grapevine canopies can impact several canopy microclimate factors such as amount of light exposure, photosynthetic photon flux density (PPFD), wind speed, evaporation, air temperature and air humidity (Dry, 2000). The term canopy management includes a range of techniques that can be applied to a vineyard to alter the position or amount of leaves, shoots and fruit in space and to achieve some desired arrangements, including an improved microclimate (Smart et al., 1990). Grape berries developed in open canopy conditions (higher cluster sun exposure) can generally reach higher juice sugar concentration, improved acid balance and less incidence of unripe herbaceous fruit characters but can also show substantial changes in synthesis of grape berry phenolics (Dokoozlian & Kliever, 1996; Haselgrove et al., 2000; Berqvist et al., 2001; Main & Morris, 2004) and isoprenoids (Mendes-Pinto et al., 2005; Oliviera et al., 2004). The presence or absence of many representatives of phenolics and isoprenoids is not critical only for the plant serve functions, e.g. plant/insect and plant/pathogen interactions, preventing UV-B damage and involvement into hormone homeostasis (Carvalho et al., 2013; Kliebenstein, 2004; Wink, 2003), but it is also very important from an enological point of view, since they can largely determine the grape and consequently wine quality attributes (Verporte et al., 2002). Therefore, the efforts to achieve preferential cluster sun exposure, and accordingly also manipulate other parameters (e.g. temperature, humidity), include several viticultural practices including leaf removal

(Vasconcelos & Castagnoli, 2000; Zoecklein et al., 1992; English et al., 1990). Next to location and severity of leaf removal, its timing is probably the most important factor for the final effectiveness (Main & Morris, 2004). Widely adopted leaf removal at veraison was already reported to affect primary and secondary metabolite syntheses of many observed *V. vinifera* cultivars (Bavaresco et al., 2008). However, late (véraison) leaf removal is nowadays losing many of its advantages due to warmer vineyard conditions as a result of global warming. Winegrowers are already facing problems due to the higher temperatures and more severe UV exposure after canopy opening, leading to harmful sunburns of berry skin tissue (Chorti et al, 2010) or even to the inhibition of some technologically very important biosynthetic plant behaviour (Tarara et al., 2008).

Thus, lately, and particularly due to the climate change scenarios, the research interest in effects of canopy microclimate manipulation on the total and individual chemical compounds / secondary metabolites in grape berries is growing rapidly, aiming not only to improve the grape quality itself but essentially to improve the understanding of the biosynthesis behind it and hence enable better controlling of desired crop plant behaviour. Although many recent research efforts have already been focused into grapevine secondary metabolites, few are done in real microclimate scenarios and even fewer by implementing metabolomics into the vineyard environment. This novel analytical approach of the study of "as-many-small-metabolites-as-possible" in a system can provide a database of a much greater extent compared to those obtained by a classical analytical approach and could also reveal potential novel biomarkers, important from a biological but also a technological point of view.

A field trial was thus designed in two 'Pinot Noir' vineyards (Vipava Valley, Slovenia; and in Trentino, Italy) to observe how a controlled (monitored) microclimate manipulation within the two different vineyards can affect the biosynthetic behaviour of numerous individual phenolics and isoprenoids, including many poorly studied ones to date.

4.5.3 Material and methods

4.5.3.1 Experimental vineyards, trail design and sampling

Two 'Pinot Noir' (*Vitis vinifera* L.) vineyards, one from Slovenia and one from Italy were involved in the experiment. Vineyard Potoce is located in Vipava Valley, Primorje region (SW Slovenija) at an altitude 95 m above see level. It was planted in 2004, adopting the Guyot

training system. Its plant density is 5682 plants/he (0.8 m x 2.2 m) whit the rows oriented E-W. Vineyard Molini is located in San Michele al'Adige, Trentino region (S Italy) at an altitude 245 m above see level. It was planted in 1989, adopting the Guyot training system. Its plant density is 6178 plants/he (0.9 m x 1.8 m) whit the rows oriented 70 ° NE (E-NE: W-SW). A completely randomised experimental design with three treatments (6 plots of 5 vines/plot) was set up at both experimental sites with different leaf removal treatments applied at different phenological stages of grape berry development (BBCH scale; Lorenz et al., 1995), as follows: PF (pre-flowering), LR performed before flowering at phenological stage BBCH 57; BS (berry set), LR applied at BBCH 71; VE (veraison), LR performed at BBCH 83; UN (control treatment), LR not applied (untreated vines / leaves retained). Leaf removal was performed manually, removing the basal 4-to-6 leaves of all the shoots, as normally carried out for pre-bloom treatments (Poni et al., 2009).

At commercial harvest 500 g of ripen berries (6 biological replicates per each treatment) were randomly collected together with pedicels to avoid tissue damages and prevent oxidation. The samples were immediately cooled down and stored at -80 °C until the analyses were performed.

More details regarding vineyard characteristics and experimental design as well as sampling regime can be seen in the Chapter Experimental (3.1.1.2; 3.1.1.3)

4.5.3.2 Evaluation of climatic characteristics

The mesoclimatic characteristics of the main experimental site were followed using an IMT 300 meteorological station (Metos Instruments, New Delhi, India), collecting data for global radiation, rainfall, wind speed, leaf wetness, air temperature, relative humidity and dew point. In addition, further meteorological data was collected from a nearby weather station of the National Meteorological Service of Slovenia (EARS, Slovene public information, EARS-meteo.si), aiming to confirm and support the data downloaded from our meteo station at the vineyard site. The canopy microclimate conditions in the cluster area (temperature and relative humidity) were monitored during the season (May-August) via i-Button sensors / mini-data loggers (SPR Hygrochron Temperature / Humidity Logger i-Button with 8KB Data-Log Memory, Maxim, CA, USA), collecting and storing the data on an hourly basis (Figure 7). I-button sensors were placed directly in the cluster area of all the treatments, half of them facing north and half of them facing south. The evaluation of the incoming solar radiation in the

cluster area was computed by taking hemispherical photos (using fish-eye lens Sigma, EX DG fisheye 8mm 1:3.5) from below the grapevines and facing exactly to the north. The images were then processed with the Gap Light Analyser 2.0 software (Frazer et al., 1999) with the goal of determining the daily trend of solar radiation. The basic idea of the technique is to identify solar radiation relevant to open sky areas, and then merging them with a radiation and sun-path model in order to compute the total annual solar radiation for a grapevine (Schwalbe et al., 2009).

Comment: Similarly, the climatic characteristics were monitored also in Italian vineyard; however, these records are mainly owned by Italian colegues and are thus not included as a whole in the thesis (but will be, however, included in the common article).

4.5.3.3 Determination of basic grape quality paramethers

On a weekly basis (from veraison until harvest), the basic grape quality parameters were followed using standard procedures (Commission Regulation..., 1990) for evaluating the total soluble solids (°Brix), pH value and total titratable acidity (eq. of Tartaric Acid in g/L) in the grape juice. All analyses were performed using fresh samples and in three biological replicates.

4.5.3.4 Chemical analyses

4.5.3.4.1 Sample preparation

For the determination of secondary metabolites (phenolics and isoprenoids), the preparation of grape berry samples (6 biological lots of 500 g berries), previously stored at -80 °C, was adopted according to Theodoridis et al. (2012). First the samples were ground in liquid nitrogen using an IKA analytical mill (Staufen, Germany) in order to obtain a homogenous frozen powder. Then, in brief, 2 g of frozen powder from each sample were extracted in sealed glass vials using 5 mL of water/methanol/chloroform (20:40:40) mixture. After vortexing for 1 min, the samples were put in an orbital shaker for 15 min (at room temperature). Samples were then centrifuged at 1000g and 4 °C for 10 min, and the upper phases constituted of aqueous methanol extract were collected. Extraction was repeated by adding another 3 mL of water:methanol (1:2) to the pellet and chloroform fractions, and by shaking for another 15 min. After centrifugation, the upper (organic) phases from the two extractions were combined, brought to 10 mL in quantitative flasks, and filtered through a 0.2 μm PTFE filter prior to

analysis (Theodoridis et al., 2012; Vrhovsek et al., 2012). For the analysis of isoprenoids, the chloroform fraction was also collected and pooled as described in details by Wehrens et al. (2013).

4.5.3.4.2 Targeted analysis of phenolics and isoprenoids

For the phenolic compounds (apart for anthocyanins), a comprehensive targeted metabolomic analytical approach according to Vrhovsek et al. (2012) was applied. The method was developed with the potential to perform the qualification and quantification of 135 phenolics belonging to different chemical groups that are typically present in fruits, such as benzoates, phenylpropanoids, coumarins, stilbenes and flavonoids (flavones, isoflavones, flavanones, flavan-3-ols flavonols and dihydrochalcones). Ultra high-performance liquid chromatography (UHPLC-MS/MS) was performed using a Waters Acquity UHPLC system (Milford, MA, USA) consisting of a binary pump, online vacuum degasser, auto-sampler, and column compartment. The separation of the observed phenolic compounds was achieved using a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Samples were kept at 6 °C during the analysis. Mass spectrometry detection was performed using a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source (Vrhovsek et al., 2012). All analyses were performed in six biological replicates. Analysis (HPLC-DAD) of anthocyanins was performed as reported previously by Mattivi et al. (2006). Chromatography was carried out on a Waters 2690 HPLC system equipped with Waters 2996 DAD (Waters, Milford, MA), using a reversed-phase column Purospher RP18 250 mm x 4 mm (5 μm), with precolumn (Hewlett-Packard, CA, USA). In brief, a separation of the main (5) free anthocyanins was obtained at 40 °C, with a flow of 0.45 ms/min. Delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside were identified according to Castia et al. (1992) and quantified at 520 nm by means of calibration curve with malvidin-3-glucoside. The analysis was done in 6 biological replicates.

For the analysis of isoprenoids (carotenoids, chlorophylls, and tocopherols), the ultra-performance liquid chromatography (UPLC) was carried out on a 1290 Agilent system, equipped with an RP C30 3-μm column (250 x 2.1 mm i.d.) and coupled to a 20 x 4.6-mm C30 guard column (YMC Inc., Wilmington, NC, USA). The chromatography was performed as described by Fraser et al. (2000), with some modifications (Wehrens et al. (2013). In brief,

the mobile phases consisted of methanol (A) and tert-methyl butyl ether (B), both containing 5 % of a mixture of water/methanol (20:80) and 0.2 % (w /v) ammonium acetate. The DAD signal was acquired from 200 to 600 nm (step 1.2 nm), with a slit width of 1 nm, at a frequency of 2.5 Hz. Carotene and xanthophyll quantification was done by considering the area under each peak at a wavelength of 450 nm, whereas tocopherols were quantified by integrating the peak areas at 300 nm. The quantification of individual compounds was done by external standard calibration curves using the respective standards (Wehrens et al., 2013; Carvalho et al., 2013; Fraser et al., 2000). All analyses were performed in six biological replicates.

4.5.3.4.3 Untargeted metabolomics analysis

Analyses were performed according to the published method by Theodoridis et al. (2012) using a Waters Acquity ultra high-performance liquid chromatography (UHPLC), coupled to a Synapt High Definition Hybrid Quadrupole/Time-of-Flight Mass Spectrometer (HDMS QTOF-MS) (Waters, Manchester, UK) via an electrospray interface (ESI), operating in W-mode and controlled by MassLynx 4.1. For reversed phase chromatography (RP), an ACQUITY UPLC 1.8 μm 2.1 x 100 mm HSS T3 column (Waters, Milford, MA, USA) at 30 °C was used. Spectra were collected in positive ESI mode over a mass range of 50-3000 amu with a scan duration of 0.3 s in centroid mode. The transfer collision energy and trap collision energy were set to 6 and 4 V. External calibration with sodium formate and Lock Mass calibration with leucine enkephaline solution was applied (Theodoridis et. al., 2012). All the analyses were performed in six biological replicates.

4.5.3.5 Data processing and statistical analyses

All the data was initially processed with the help of Microsoft Excel 2008 for Mac, Version 1.2.2.3 (Microsoft Corporation, WA, USA), however, the chromatographic raw data sets were first processed using the Mass Lynx Target Lynx Application Manager (Waters, MA, USA) or with Empower Cromatography Data Software (Waters). The overall differences among treatments were evaluated by means of multivariate Principal Component Analysis (PCA) on the auto scaled data. Generalizations of simple two-variable scatter plots (biplots) were also prepared, allowing general information on both, samples and variables of a data matrix compounds, with later displayed as points, while the variables are displayed as vectors. After visually highlighting the metabolic profiles as related to all the treatments (or locations), the

data was then processed running one-way ANOVA in order to get further insight on the metabolic effects of the different leaf removal strategies. Once the differences were significant ($P < 0.05$), the Tukey's Honest Significant Difference (HSD) test was selected to search for the differences between treatments. In the case of targeted metabolomics analyses of harvest samples, together with ANOVA of single parameters, a Canonical analysis was performed in order to ascertain the multidimensional differences among the treatments by keeping the classes of different phenolic compounds separated (ellipses representing a 95% confidence interval). All these statistical tests were performed with the help of the Statistica software package (StatSoft, Tulsa, OK, USA) and/or »R« (R Core Team, 2013).

4.5.4 Results and discussion

4.5.4.1 Climatic characteristics

Physiological and phenological behaviour of grapevines can be strongly influenced by meteorological variables (Cola et al., 2009). Climate inconsistency with many fluctuations between the seasons can considerably determine grape quality between the vintages (Jones & Hellman, 2003; Shultz, 2000). The seasonal macroclimate conditions of the winegrowing region, together with the mesoclimate of a particular site, are hence very influential; however, in contrast to microclimate, winegrowers cannot manipulate them but can only adjust various cultural practices to them. As regards vintage 2011 in the vineyard Potoce, Vipava Valley (Slovenia), the season was particularly characterised by a warm and dry summer, especially if compared to vintage 2010 (Sternad Lemut et al., 2013a), with a total rainfall of 408 mm during the 2011 growing season (from May until October) and recorded average summer monthly temperatures of 21.3, 21.9 and 23.4 ºC in June, July and August respectively. In Trentino (Molini vineyard), the season was also similarly dry and warm, with a total rainfall of 438 mm during the growing season and average summer monthly temperatures of 20.3, 21.5 and 23 ºC in June, July and August respectively.

When taking into consideration a microclimate in the cluster area, this is normally a result of several environmental factors such as solar radiation, temperature, relative humidity and wind speed but also of some cluster characteristics such as size, shape, emissivity and absorbance (Zorer et al., 2013a; Cola et al., 2009). Furthermore, the orientation of the rows in the vineyard (Berquist et al., 2001), together with several applied viticultural practices, may significantly affect the bunch microclimate conditions. Within the trial in the Slovenian vineyard (SLO), the

temperature in the cluster area during the main season (May - August) was found to be the lowest in case of untreated control (UN) for both the northern and southern side of the canopy (Figure 24). With some instability, veraison (VE) treatment basically caused the highest mid-day trend (particularly around 2 p.m.) on the southern side of the canopy, whereas on the northern part of the canopy, the highest mid-day trend (from app. 3 to 5 p.m.) was recorded in the case of pre-flowering (PF) leaf removal (Figure 24). However, we should take into account that VE leaf removal was performed as late as in the beginning of August, hence the temperatures recorded before VE defoliation were principally similar to the UN situation. This indicates that there must have been considerable increase of the canopy temperature after VE leaf removal on the southern side, which is obviously masked within the average of the whole season data. Similar trends as in the Slovenian vineyard were observed also for the canopy microclimate situation in the Italian vineyard (ITA) (data not shown).

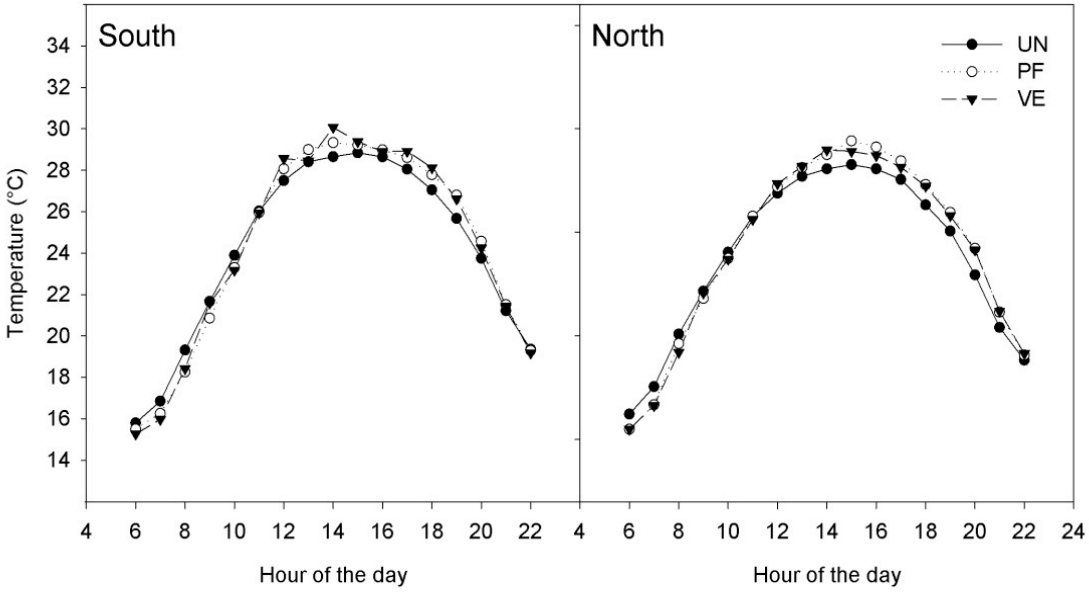


Figure 24: Average Temperature (°C) in the cluster area during the whole cluster development period (May-August), as affected by microclimate manipulation through leaf removal at different phenological stages in the Slovenian vineyard: PF, pre-flowering leaf removal; VE, veraison leaf removal; UN=untreated control with no leaf removal performed.

Any improvements in controlling the factors that affect berry surface temperature can be of great importance, since many experiments have already revealed that temperature alone can significantly affect the synthesis of several flavonoids. Whereas sufficient temperature in a synergy with sufficient light exposure is known to be essential for the synthesis of anthocyanins, overly high temperatures (> 30 - 35° C) can cause an inhibition of this most widely studied class of grapevine flavonoids (Tarara et al., 2008; Spayd et al., 2002); however,

several authors have also reported procyanidins, flavonols - e.g. quercetin (Gotto-Yamamoto et al., 2010) - and stilbenes - e.g. *cis*-piceid (Bavaresco et al., 2002) - to be affected by temperature. As the PF can cause re-shadowing of the clusters by re-growth of lateral leaves by the time of the normally hottest days (Sternad Lemut et al., 2011), and because the canopy opening at berry colouration (VE) can cause an increase of the temperature in cluster area (increasing also berry surface temperature), it is thus evident that VE is less beneficial than PF, particularly in warmer regions and/or warmer vintages, a fact that needs to be taken into account also by adaptation efforts when facing global warming consequences.

A very high temperature due to the high UV-exposure after late (veraison) defoliation can hence also lead to substantial sunburns on a berry skin tissue (Chorti et al., 2010), which may in addition to the phenolics considerably deteriorate also grape/wine aroma and related compounds) (Bubola et al., 2009), thus also isoprenoids. Nevertheless, in the future, excessive sunburns may lead to susceptible cultivars becoming unsuitable for planting in the warmer regions, especially in those terroirs where the clusters are experiencing high solar radiation during the growing season (Keller, 2010).

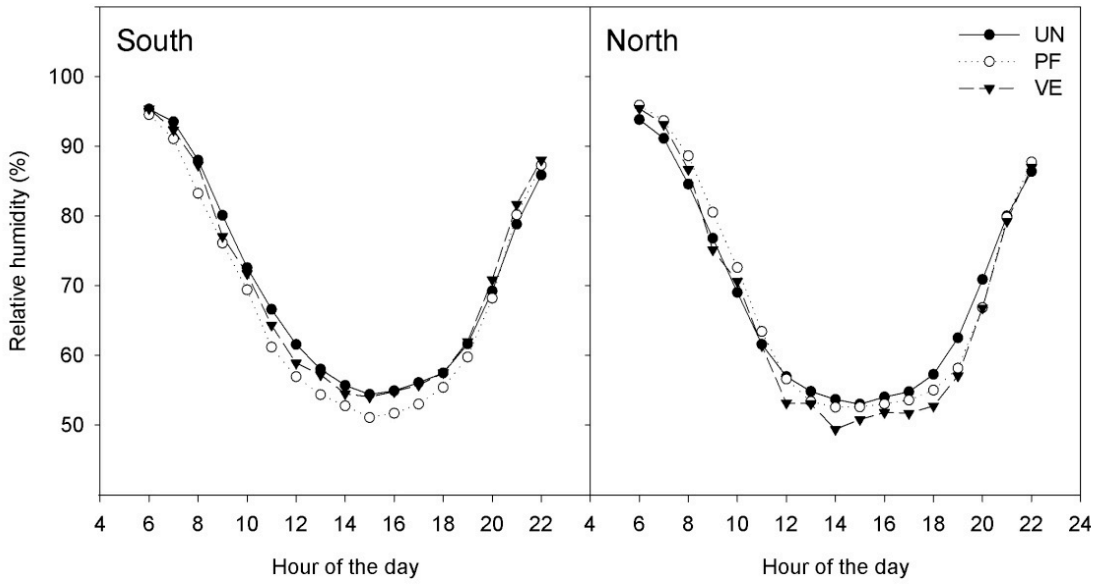


Figure 25: Average Relative humidity (RH) (%) in the cluster area during the whole cluster development period (May-August), as affected by microclimate manipulation through leaf removal at different phenological stages in the Slovenian vineyard: PF, pre-flowering leaf removal; VE, veraison leaf removal; UN=untreated control with no leaf removal performed.

Furthermore, the relative humidity (RH) in the cluster area of SLO grapevines showed the lowest (preferential) trend in the case of PF, whereas higher (similarly for both) was recorded

for VE and UN, all when observing the southern part of the canopy. Most probably, the PF treatment resulted in lower RH trend (on average over the whole season) as a result of the initially very open canopy situation early in the season and despite the fact that the re-growth of lateral leaves normally occurs later in the season. By contrast, on the northern part of the canopy, the lowest trend was observed in the case of VE, following by PF and UN, with the latter showing the highest trend of RH (Figure 25). It seems that in the northern part of the VE canopy, the second part of the season (after defoliation) was the most important for the RH situation in total over the season. However, it is known that denser canopies with lower air circulation lead to a relatively humid environment, which increases the potential for mould growth (Kassemeyer & Berkelmann-Lohnerz, 2009), thus successful controlling of RH is particularly important in order to decrease the risk for microbial diseases.

Despite the strong variability that can normally be observed in the vineyards, we tried to also derive an illustration/approximation of average canopy (sunlight) openness in each of the treatments in both vineyards by means of hemispherical photography (Zorer et al., 2013b). Sunlight exposure was previously reported to be an important factor with a known impact on several phenolic compounds (Sternad Lemut et al., 2013b), particularly flavonols (Price et al., 1995), but also on isoprenoids, particularly carotenoids (Bureau et al., 2000; 1998); however, many secondary metabolites (SMs) are still very poorly studied in relation to sunlight exposure or microclimate as a whole. Thus, in order to obtain better insight into the light environment within the canopies of different leaf removal timings, the average percentages of the sky that is free, looking at 360° upwards from the point of recovery image (Figures 27-30), as well as the fractions of global transmitted radiation were calculated. In general, in the ITA vineyard, the foliage of the PF (when approaching harvest) was found to be more closed than was the case in SLO, most probably due to lateral leaves strongly issued in the basal part of the branch and/or due to the overall lower leaf area (data not shown). PF in Italy was then followed by UN and finally by VE (Zorer et al., 2013a). In Slovenia, the differences between PF and UN seemed clearer, with later showing the closest canopy structure, as was observed also by visual observation of the trial canopies in the vineyard Potoce (SLO) (Figure 26). However, there were some problems with a levellation and consequently also the number of resalable hemispherical pictures in SLO (thus no SD is shown for this site), making the calculations of the transmitted light less trustable. New / more acquisitions should be done in future experiments in order to ascertain the results.

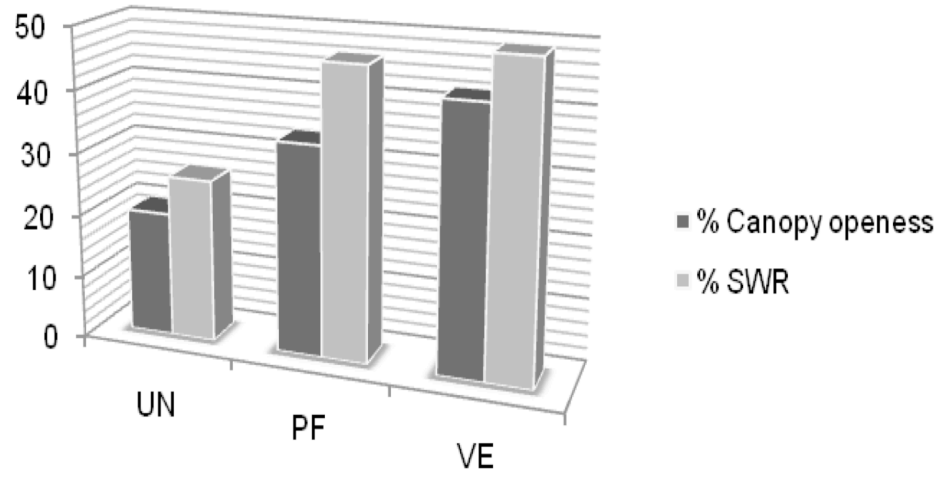


Figure 26: Canopy oppeness (%) and the transmitted global radiation (% SWR) in the Slovenian vineyard as recorded by means of hemispherical image acquisition (a week before harvest). SWR = Shortwave Radiation (energy). PF=preflowering leaf removal; VE=veraison leaf removal; UN=undefoliated control.

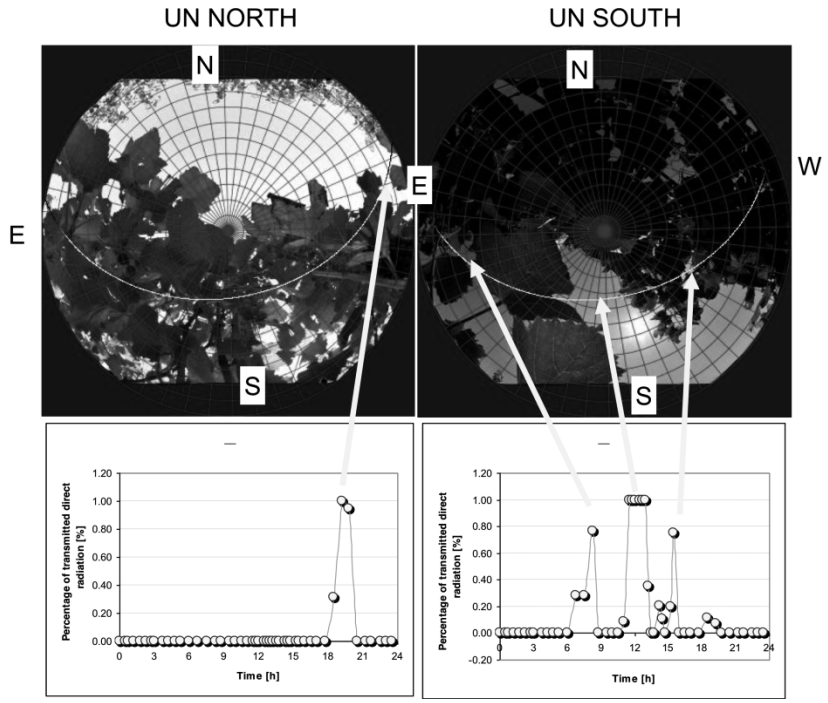


Figure 27: Example of canopy structure (N and S side) of untreated (control) vine (UN) in the **Slovenian vineyard**, obtained by hemispherical photographs, taken next to the i-button sensor location. The white curved lines indicate the path of the sun calculated on the day of image acquisition.

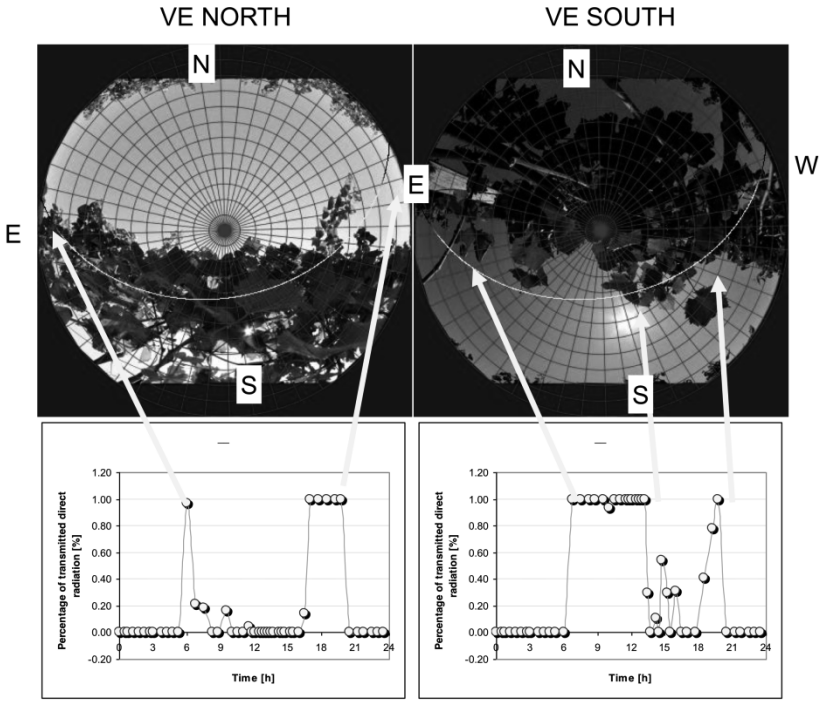


Figure 28: Example of canopy structure (N and S side) of the vine with veraison (VE) leaf removal in the Slovenian vineyard, obtained by hemispherical photographs, taken next to the i-button sensor location. The white curved lines indicate the path of the sun calculated on the day of image acquisition.

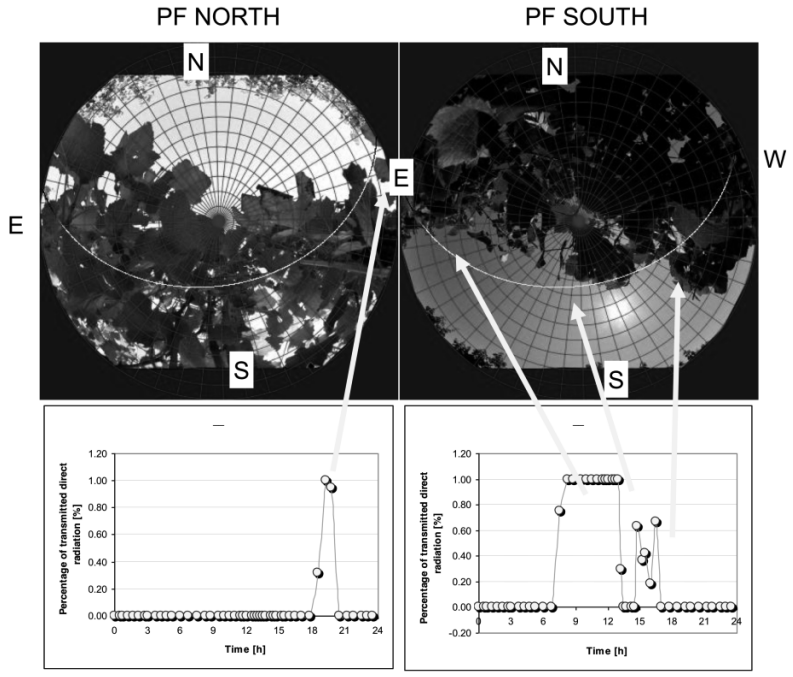


Figure 29: Example of canopy structure (N and S side) of the vine with pre-flowering (PF) leaf removal treatment in the vineyard from Slovenia, obtained by hemispherical photographs, taken next to the i-button sensor location. The white curved lines indicate the path of the sun calculated on the day of image acquisition.

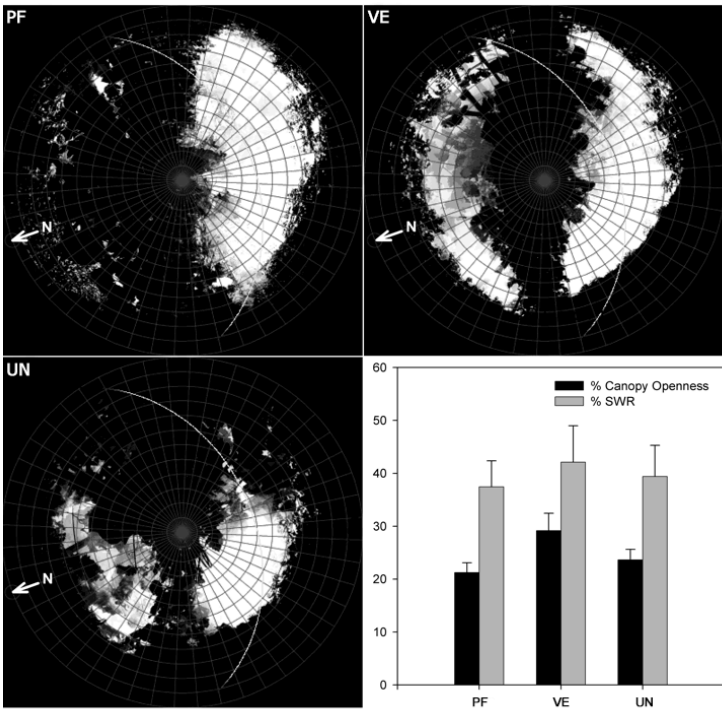


Figure 30: Hemispherical photosgraphs, characterising the canopy structure in the Italian vineyard. PF and VE are the result of overlaping 8 images, UN of 5. The white curved line indicates the path of the sun calculated on the day of images acquisition. The graph shows the mean values and standard errors of the canopy openness (%) and the percentage of global radiation transmitted (% SWR). The N-s indicate the orientation of the image (Zorer et al., 2013a).

4.5.4.2 Basic grape quality parameters

In both of the vineyards, Slovenian and Italian, late leaf removal (VE) induced slightly faster kinetics (Supp. Mat. Table 5_S1) and in general improved grape ripening parameters (namely higher sugar and lower acidity was achieved), whereas in the case of early defoliation (PF), the results were slightly different between the two locations (Supp. Mat. Table 5_S2). If a slowdown in ripening was caused by PF in the Italian Molini (ITA) vineyard, in the Slovenian Potoce (SLO) vineyard, there was an increase in sugar observed for the same treatment when compared to control (UN); however, apart from that, basically similar kinetics overall were observed at both locations. As compared with VE (and if taken into account the observations from previous vintages - presented in previous Chapters), it seems that the maturation is slightly prolonged in case of pre-flowering leaf removal despite significantly lower yield; however, this assumption needs to be further investigated.

4.5.4.3. Targeted analyses of secondary metabolites

In order to adapt to the changes in their environment, plants can produce a large variety of secondary metabolites (SMs) (Schijlen et al., 2004). Also in the present experiment, the 'Pinot Noir' grapevines showed numerous metabolic changes as a result of purposely induced microclimate modifications, which finally led to the altered chemical composition of related grape berries. By means of targeted analyses, in total 78 secondary metabolites (SMs) were identified in berry samples of different leaf removal (LR) treatments at harvest time, interestingly with all of them present at both locations (Slovenia and Italy), however with several site- and treatment-specific differences in their concentration. Detected SMs were the group representatives of phenolic compounds (64): flavonols (17), stilbenes (13), flavan-3-ols (11), benzoates (7), anthocyanins (5), hydroxycinnamates (4), flavanones (3), flavones (2), dihydrochalcones (1) and coumarins (1); and the group representatives of isoprenoids (14): carotenoids (8), chlorophylls (4) and tocopherols (2).

4.5.4.3.1 Phenolics

When focusing exclusively on the phenolic compounds, a general Principal component analysis (PCA) plot of all the dataset was first performed to highlight the general effect of leaf removal timings at both sites: Potoce vineyard, Vipava Valley, Slovenia (SLO) and Molini vineyard, Trentino, Italy (ITA). Figure 31 shows that the samples from ITA (shown in red) could be well distinguished from the samples from SLO (shown in green), especially in the case of both leaf removals, pre-flowering (PF) and veraison (VE), and it could be also seen already from this PCA that there is a clearer separation between all the treatments in the Slovenian vineyard than in the Italian one.

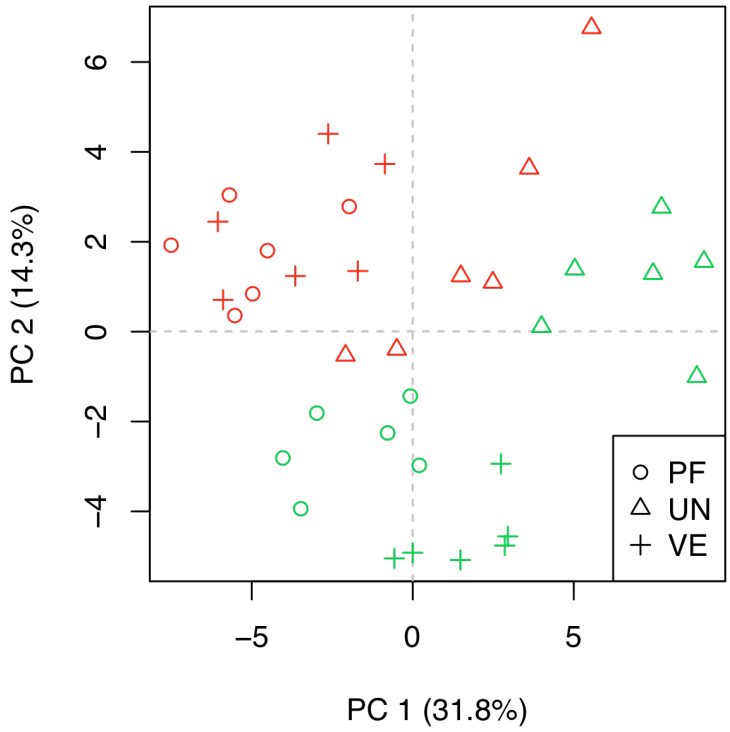


Figure 31: Principal component analyses projection of all the data sets of phenolics from both locations. Red in Italy; green in Slovenia, as affected by by leaf timing: PF=pre-flowering leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed.

Furthermore, with the aim to ascertain in detail the differences among treatments and the individual compounds responsible for them, the data of metabolic profiling were processed again throughout PCA by keeping separated the two locations. The PC1 x PC2 plane (Figure 32) revealed many differences between locations, but the distribution on several important phenolic compounds, such as the majority of flavonols and anthocyanins, was basically shown to be similar.

Moreover, a generalisation of the simple two-variable scatterplot (biplot) was performed to visualise the effects of different leaf removal strategies together with the information on chemical classes to which the observed individuals belong. Biplots thus show the contribution of the different variables to the separation. Each point is a metabolite, and they are colour coded according to chemical class. It is again evident from the SLO biplot that in Slovenia the treatments are well separated, whereas they are less distinguished in ITA, even if it is still possible to separate the LR treatments from the untreated control (Figure 33). In addition, from both biplots we can observe that the treatments seem to be highly affected particularly by flavonols but also some other classes / individuals are worth being further investigated.

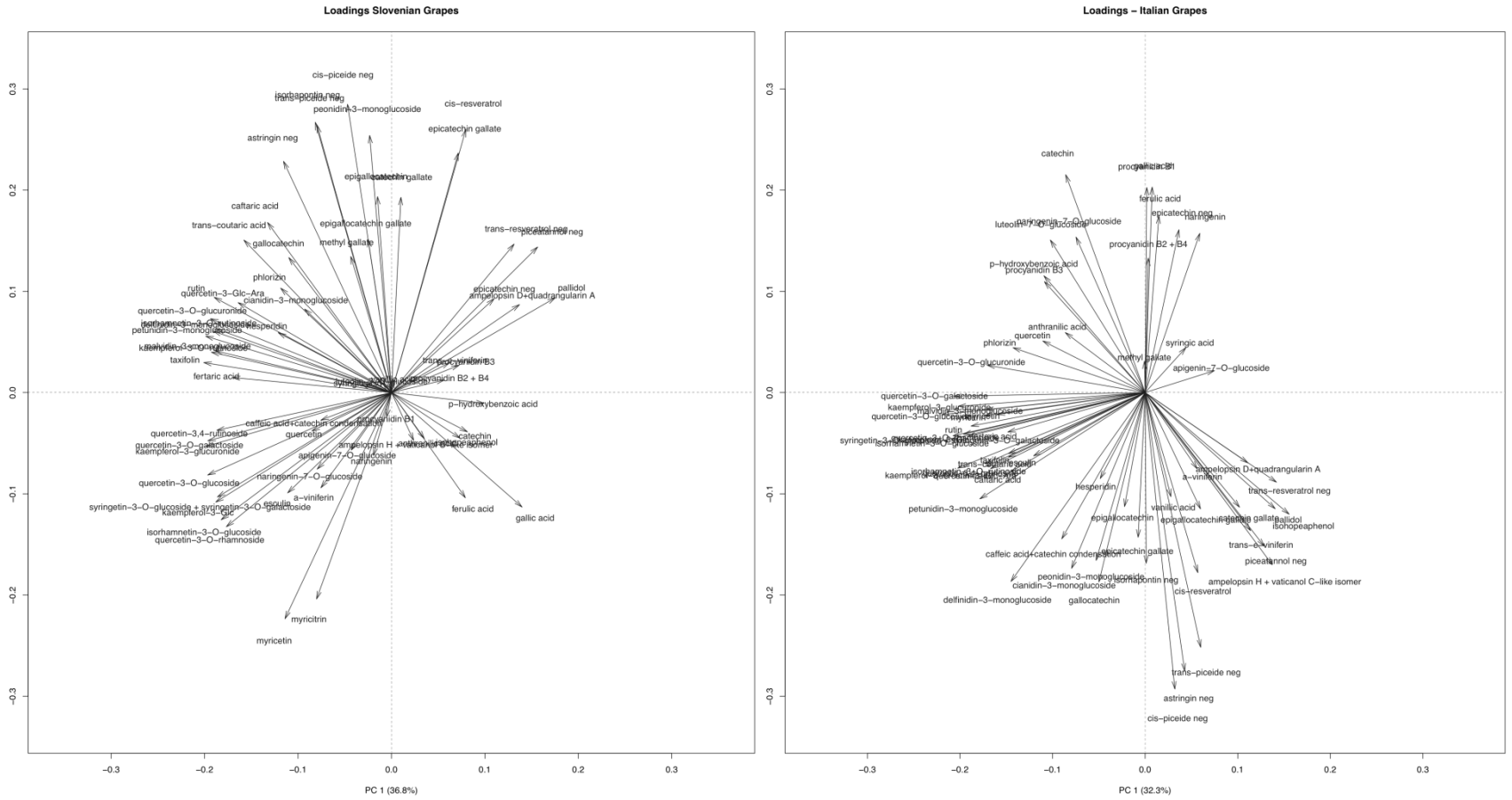


Figure: 32: Principal component analyses of the projection of phenolic metabolic constituents of 'Pinot Noir' samples (left in SLO and right in ITA) as affected by leaf removal at different phonological stages of cluster development: PF=pre-flowering leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed

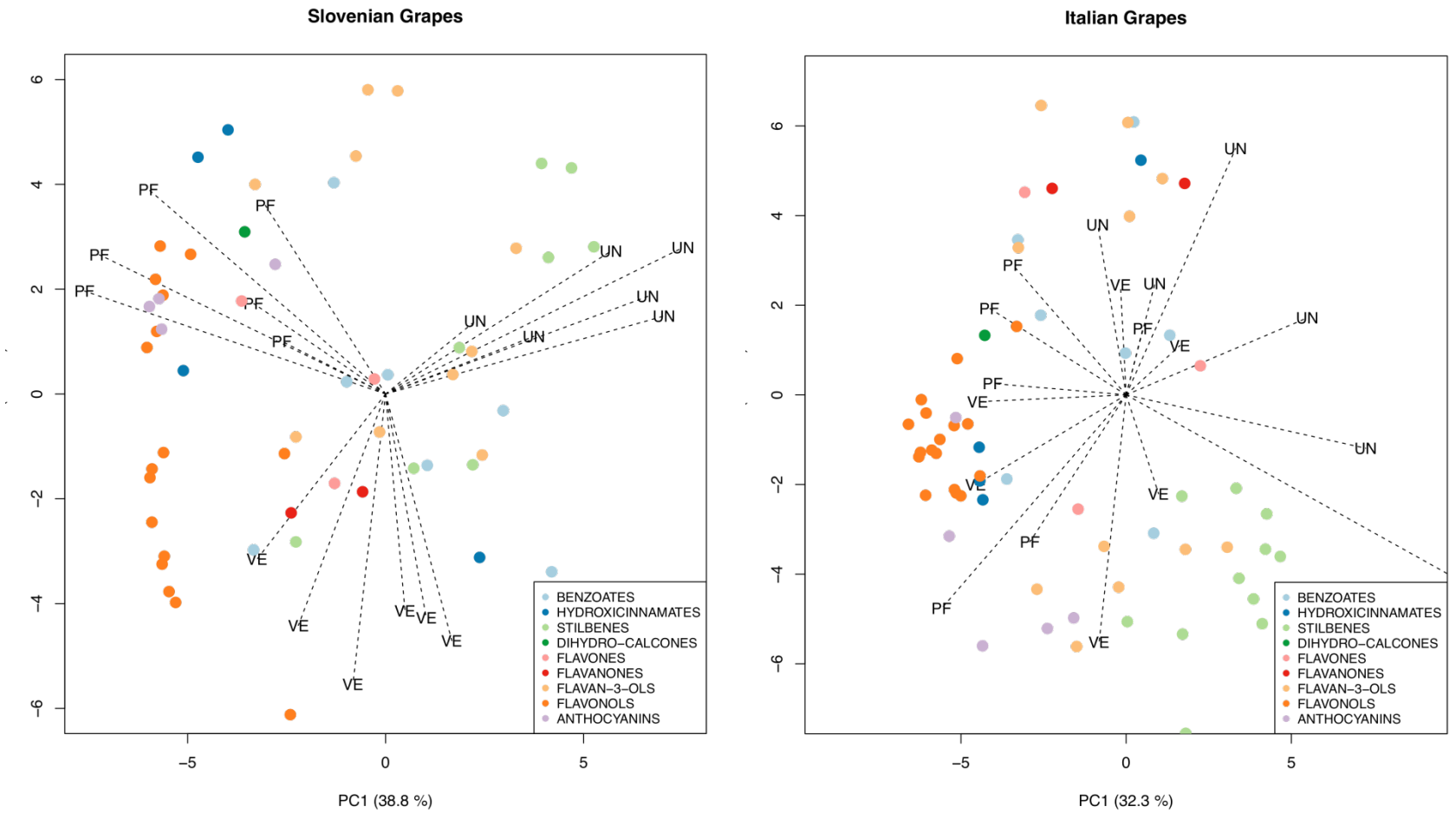
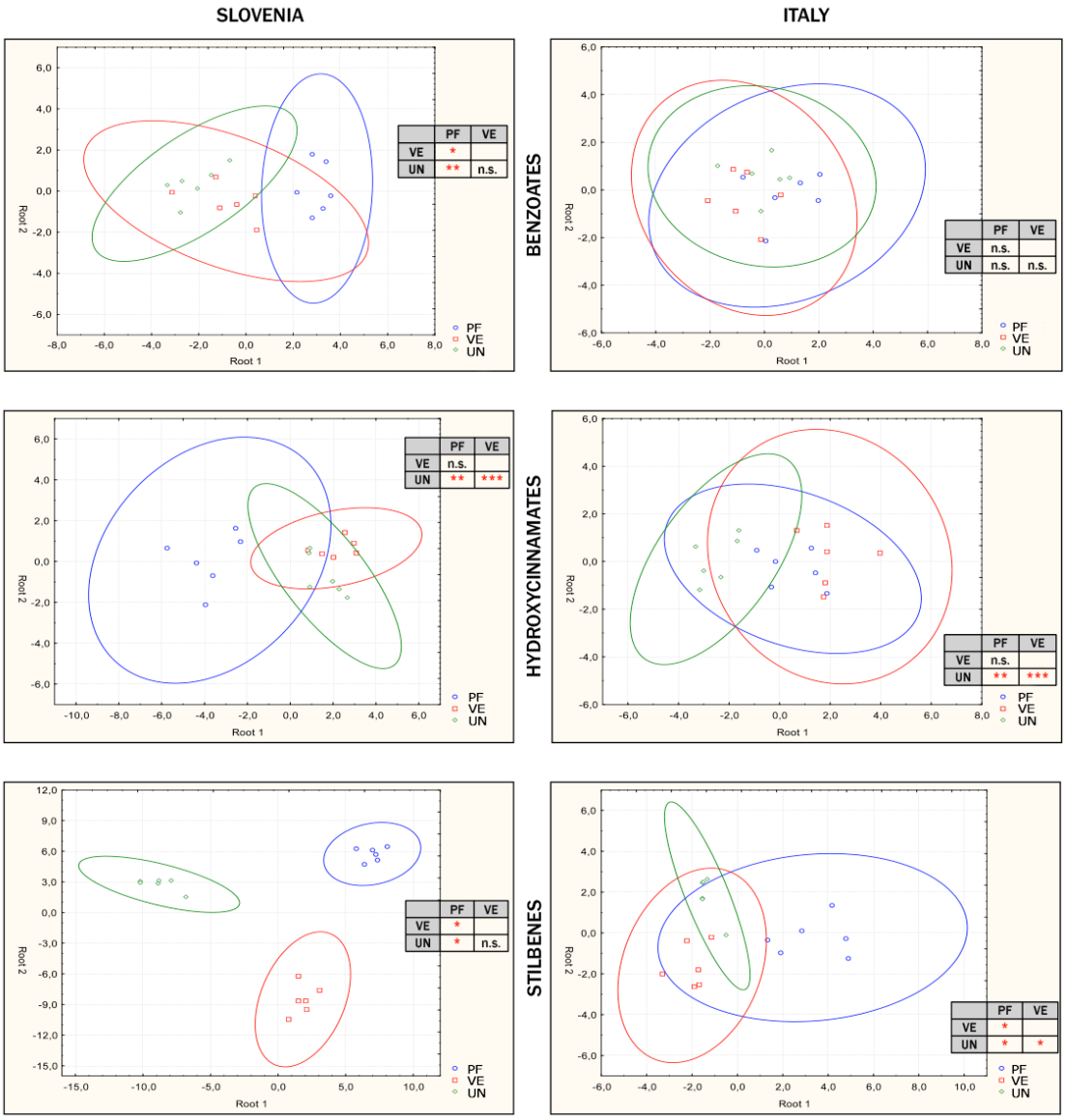


Figure 33: PCA Biplots showing the contribution of the different variables to the separation (left in SLO and right in ITA) as affected by leaf removal (vectors) at different phonological stages of cluster development: PF=pre-flowering leaf removal; VE=veraison leaf removal; UN=untreated control with no leaf removal performed. Each point is a metabolite, which is colour coded according to the chemical class.

To initially highlight the situation of various chemical classes of phenolics present in the berries of different treatments (the total amount of individual representatives of each chemical class), together with ANOVA of single parameters, a Canonical analysis was performed (separately for both locations). The aim was to ascertain the multidimensional differences among the treatments, keeping different classes of phenolic compounds separated (Figure 34).



…continuing…

…continuing…

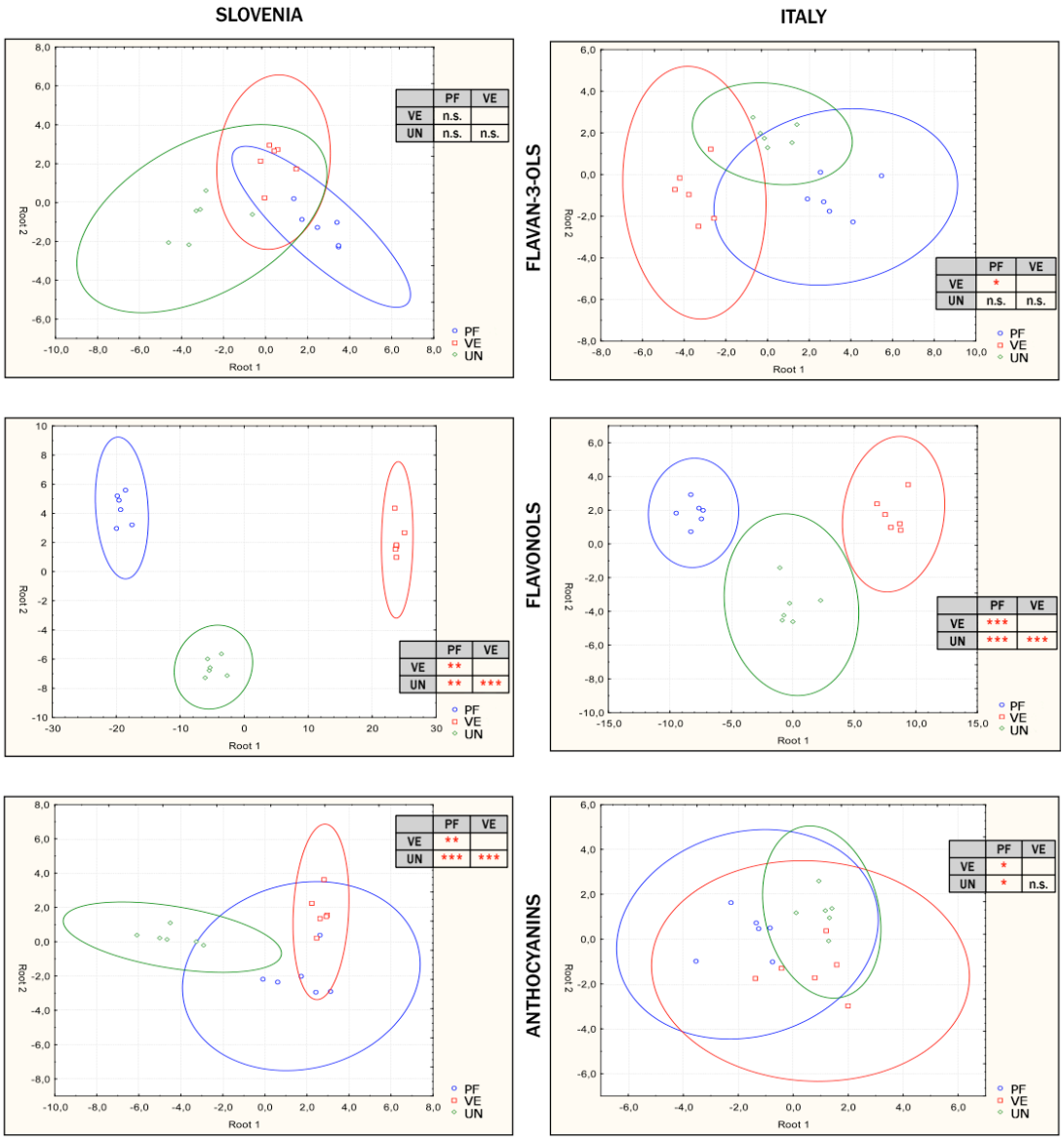


Figure 34: Canonical correlation analysis of different classes of phenolics as affected by different leaf removal timings in Slovenia (left) and Italy (right). PF= pre-flowering leaf removal (LR); VE=veraison LR; UN = untreated (control); ellipses representing a 95% confidence interval; n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001.

In general, the occurrence of total phenolics in grape berries was shown to be even 40% higher in Italian samples, probably not only due to due the geo-climatic differences between the two locations but also due to some differences in yield and leaf area (data not shown) and in the ripening status of the grapes, harvested 102 (103) days after pre-flowering leaf removal (with some adjustments to the weather conditions). Leaf removal timing, on the other hand, has caused more significant alternations of several classes of phenolic compounds in SLO compared to in ITA. Within phenolics, the flavonols were altered most significantly, resulting

in an increase by 216% and 109% in SLO and by 56% and 20% in ITA in the pre-flowering and veraison treatments respectively, as compared with the controls. Both PF and VE also affected hydroxycinnamates at both locations as compared with UN, but the differences between the LR treatments were not clearly ascertained. Total anthocyanins (with similar concentrations on average of all the treatments at both sites) revealed higher significance between the treatments for Slovenian 'Pinot Noir' grape berries, with the differences ascertained also among all the treatments in the comparison, whereas lower significance appeared in Italy. The levels of total stilbenes were 38 % higher in ITA grape berries than in SLO berries, and they also showed significant differences between the treatments; however, this significance was relatively low at both locations (Figure 33).

Aiming to get further detailed insight into the metabolic effects of the different leaf removal strategies, a separate one-way ANOVA for each individual metabolite was performed. When focusing on the individual phenolics (Table 18), the most abundant flavonols (out of 17 detected) at both sites were quercetin-3-glucuronide, quercetin-3-glucoside, taxifolin, isorhamnetin-3-glucoside, quercetin-3-galactoside and rutin, with 296, 124, 72, 31 and 29 μg/g of whole berry powder (hereinafter just μg/g) on average of all three treatments respectively in Italy; and with 217, 86, 20, 75, 18 and 16 μg/g on average of all three treatments (hereinafter a.a.t) respectively in Slovenia (Supp. Inf. Table 5_S3). As regards leaf removal, in both locations there were significant differences between the treatments and control observed for all the flavolons, except for myricetin, quercetin-3-glucoside and kaempferol-3-rutinoside (Table 18), however those three compounds were significantly changed by leaf removal in Slovenia but not also in Italy. It was previously reported that flavonols can be considered as biomarkers for the sun exposure regime, as achieved in a bunch area following canopy microclimate manipulation (Sternad Lemut et al., 2013a, 2013b), and that the earlier leaf removals can fundamentally impact the flavonols with early peak biosynthetic behaviour, such as taxifolin, kaempferol-3-rutinoside, quercetin-3-glucoside-arabinoglucoside, rutin and quercetin-3-glucuronide. On the other hand, only few individual flavonols are actually showing "two peak behaviour"; even two distinct periods of total flavonol synthesis (around flowering and veraison) were previously reported by Downey et al. (2003). In the present experiment, we observed once again a significant increase in taxifolin, rutin and quercetin-3-glucoside-arabiglucoside in early leaf removal (PF) - at both *terroirs* – as well as in kaempferol-3-rutinoside and quercetin-3-glucuronide in SLO, while in ITA, although not significant, similar trends for the latter two early-peaking flavonols were observed. Hence, those results support

the theory of early peaking flavonols being promoted only by early leaf removal, as the enhanced light environment is needed early in the season for their biosynthesis to be significantly triggered (Sternad Lemut et al., 2013b). On the other hand, at both sites, some flavonols, e.g. kaempferol-3-glucoside, quercetin-3-galactoside and syringetin-3-glucoside + syringetin-3-galactoside (co-eluting), (all previously reported as "late peaking flavonols") have shown significant alteration in case of both LR treatments (PF and VE) as compared to control, however no significance was observed between the early and late LR, as similarly reported by Sternad Lemut et al. (2013b), even when the samples arose from different vintages.

Among the five typical 'Pinot Noir' anthocyanins, as expected malvidin-3-glucoside was the most abundant at both sites with 327 and 338 μg/g a.a.t in SLO and ITA respectively; that was followed by peonidin-3-glucoside, with similar concentration (145 and 147 μg/g) for both vineyards (Supp. Inf Table 5_S3). Leaf removal, especially early defoliation (PF), enhanced several improvements in anthocyanin concentrations (Table 18). In Slovenia, only peonidin-3-glucoside was not significantly triggered by PF defoliation as compared with VE and UN, whereas in Italy such PF significance was not the case, but an improvement of LR treatments as compared to control was achieved with most of the anthocyanins also in the Italian vineyard. The results are principally in agreement with previous reports discussing light environment (Cortell et al, 2006) and also with the reports discussing synergistic effects of light and temperature (Haselgrove et al., 2000).

Flavan-3-ols: catechin, epicatechin and procyanidins (B3 and co-eluted B2 + B4), were present in much higher concentrations (560 to 5300 μg/g a.a.t) as compared to other detected flavan-3-ols, which were only present with < 12 μg/g a.a.t. (Supp. Inf. Table 5_S3). However, none of the abundantly present flavan-3-ols were significantly affected by leaf removal treatments (Table 18).

From flavones, only luteolin-7-glucoside was present in concentrations > 1 μg/g a.a.t in both of the vineyards, whereas hesperidin and apigenin-7-glucoside were detected in concentrations < 0.1 μg/g a.a.t. (Supp. Inf. 5_S3). None of them showed any significant alterations as a result of LR timing (Table 18), as was observed also for the only detected flavanone - naringenin-7-glucoside (≈0.3 μg/g a.a.t).

Table 18: Phenolic compounds as affected by different leaf removal timing in Slovenia and in Italy. PF=pre-flowering leaf removal (LR); VE=veraison LR; UN=untreated control. Green coloured: the compunds that are showing the same behaviour (significance) at both sites. Blue coloured: the compounds that are showing very similar behavior at both sites .

← SLOVENIA						ITALY →				
UN	PF	VE	p value	F	COMPOUND	F	p value	UN	PF	VE
PHENOLIC COMPOUNDS										
					Flavonols					
			0.5023	*n.s.*	quercetin	*n.s.*	0.0756			
c	a	b	0.0000	***	taxifolin	**	0.0088	b	a	ab
c	b	a	0.0000	***	myricetin	*n.s.*	0.4074			
b	a	a	0.0000	***	quercetin-3-rhamnoside	***	0.0004	c	b	a
b	a	a	0.0000	***	kaempferol-3-glucoside	*	0.0118	b	a	a
c	b	a	0.0001	***	myricitrin	***	0.0009	b	a	a
b	a	a	0.0000	***	quercetin-3-glucoside	n.s.	0.4165			
b	a	a	0.0001	***	quercetin-3-galactoside	**	0.0015	b	a	a
b	a	a	0.0000	***	isorhamnetin-3-glucoside	**	0.0020	c	a	b
b	a	a	0.0000	***	syringetin-3-glucoside+syr.-3-galactoside	**	0.0023	b	a	a
c	a	b	0.0000	***	kaempferol-3-rutinoside	n.s.	0.0565			
b	a	b	0.0002	***	quercetin-3-glucoside-arabinoglucoside	***	0.0002	c	a	b
b	a	b	0.0000	***	rutin	***	0.0008	b	a	b
c	a	b	0.0001	***	isorhamnetin-3-rutinoside	***	0.0003	b	a	b
b	a	a	0.0004	***	quercetin-3,4-rutinoside	***	0.0004	b	a	b
c	a	b	0.0000	***	quercetin-3-glucuronide	*	0.0263	b	a	a
c	a	b	0.0000	***	kaempferol-3-glucuronide	**	0.0061	b	a	b
					Anthocyanins					
b	a	b	0.0001	***	delfinidin-3-glucoside	**	0.0047	b	a	a
			0.5164	n.s.	cyanidin-3-glucoside	**	0.0017	b	a	a
c	a	b	0.0000	***	petunidin-3-glucoside	*	0.0146	b	ab	a
a	a	b	0.0002	***	peonidin-3-glucoside	*	0.0181	b	a	a
b	a	b	0.0023	**	malvidin-3-glucoside	*	0.0439	b	ab	a
					Flavan-3-ols					
			0.3291	*n.s.*	catechin	*n.s.*	0.5161			
a	b	b	0.0232	*	epicatechin	*n.s.*	0.2058			
			0.0518	*n.s.*	epigallocatechin	*n.s.*	0.4788			
b	a	b	0.0019	**	gallocatechin	**	0.0041	b	b	a
a	a	b	0.0172	*	catechin gallate	*	0.0426	b	ab	a
			0.2457	*n.s.*	epigallocatechin gallate	*n.s.*	0.9975			
a	a	b	0.0016	**	epicatechin gallate	*n.s.*	0.5556			
			0.9275	*n.s.*	procyanidin B1	*n.s.*	0.1446			
			0.6690	*n.s.*	procyanidin B2 + B4	*n.s.*	0.8325			
			0.3333	*n.s.*	procyanidin B3	*n.s.*	0.6928			
					Flavones					
			0.8351	*n.s.*	luteolin-7-glucoside	*n.s.*	0.0584			
			0.0767	*n.s.*	hesperidin	*n.s.*	0.1834			
			0.8332	*n.s.*	apigenin-7-glucoside	*n.s.*	0.3353			
					Flavanones					
			0.6317	*n.s.*	naringenin	*n.s.*	0.8539			
			0.6088	*n.s.*	naringenin-7-glucoside	*n.s.*	0.1015			

…continuing…

…continuing...

					Dihydrochalcones					
b	a	b	0.0110	*	phlorizin	*n.s.*	0.5864			
					Benzoates					
			0.7283	*n.s.*	anthranilic acid	*n.s.*	0.6234			
			0.3145	*n.s.*	*p*-hydroxybenzoic acid	*n.s.*	0.3568			
			0.9700	*n.s.*	vanillic acid	*n.s.*	0.2522			
a	b	a	0.0009	***	gallic acid	*n.s.*	0.4066			
			0.5157	*n.s.*	methyl gallate	*n.s.*	0.9237			
			0.8547	*n.s.*	syringic acid	*n.s.*	0.3121			
					Hydroxycinnamates					
b	a	a	0.0257	*	esculin	*n.s.*	0.1104			
a	b	ab	0.0345	*	ferulic acid	**	0.0036	b	a	b
b	a	b	0.0003	*	caftaric acid	*n.s.*	0.2607			
b	a	ab	0.0066	**	fertaric acid		0.0000	b	a	a
b	a	b	0.0000	***	*trans*-coutaric acid	*n.s.*	0.2678			
					Stilbenes					
a	b	b	0.0008	***	*trans*-resveratrol neg		0.0001	a	b	b
b	a	c	0.0000	***	*cis*-resveratrol	*n.s.*	0.4064			
a	b	b	0.0001	***	piceatannol	*n.s.*	0.7448			
b	a	c	0.0000	***	*trans*-piceide	*n.s.*	0.3389			
b	a	c	0.0000	***	*cis*-piceide	*n.s.*	0.3954			
b	a	c	0.0000	***	astringin	*n.s.*	0.5913			
b	a	c	0.0000	***	isorhapontin	*n.s.*	0.2844			
			0.4617	*n.s.*	*trans*-ε-viniferin	*n.s.*	0.8650			
a	b	b	0.0000	***	pallidol	*n.s.*	0.2489			
a	b	b	0.0065	**	ampelopsin D+quadrangularin A	*n.s.*	0.1335			
			0.3777	*n.s.*	α-viniferin		0.0111	a	b	a
			0.0936	*n.s.*	isohopeaphenol	*n.s.*	0.9268			
			0.6065	*n.s.*	ampelopsin H + vaticanol C-like isomer	*n.s.*	0.1549			
			0.3782	*n.s.*	caffeic acid+catechin cond.	*n.s.*	0.3326			

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly different. In addition letter »a« is used to indicate the highest concentration observed, followed by »b« for intermediate and »c« (where used) for the lowest result.

Furthermore, only phlorizin as a representative of dihydrochalcones and only esculin out of coumarins (hydroxycinnamates) were detected in the samples, both in relatively low concentrations. However, both showed slight but significant changes (only) in Slovenia, with phlorizin being the highest in PF, whereas esculin was the highest in UN.

The most abundant hydroxycinammic acids (HCAs) - caftaric (1500-1900 μg/g a.a.t) and *trans*-coutaric acid (60-80 μg/g a.a.t) (Supp. Inf. Table 5_S3) - were significantly enhanced by pre-flowering leaf removal only in Slovenia, whereas less abundant ferulic acid was promoted by early LR in Italy. In addition, in Italy, both LR treatments significantly triggered fertaric acid accumulation; it was even present in very low concentrations (< 1 μg/g a.a.t) as

compared with other class representatives. HCAs in grape berries have been previously much less studied in the relation with microclimate manipulation compared to for example anthocyanins and flavonols, even though they are probably equally essential within so-called copigmentation reactions, which are responsible for the development of more stable pigments - pyranoanthocyanins (Arapitsas et al., 2012; de Freitas et al., 2011). This is especially important for 'Pinot Noir' wines, known for their problems with colour attractiveness as well as stability (Sternad Lemut et al, 2013a). However, the few existing studies conducted for exactly this purpose show various contradictory data, with many flocculations between the vintages.

Moreover, benzoates, with gallic acid as the most plentiful representative (81 and 38 μg/g a.a.t in ITA and SLO, respectively), also did not show almost any dependence on the canopy microclimate conditions. Only gallic acid, and only in Slovenia, was significantly decreased by PF as compared to VE and UN.

Although stilbenes in their total amounts showed (small) significant differences among treatments at both sites, on the other hand, individual stilbenes in ITA basically did not show any significant changes except for *trans*-resveratrol and *α*-viniferin (in both cases a decrease was noticed for PF compared with control). By contrast, in Slovenia, most of the individual stilbenes were highly significant with *cis*- and *trans*-pieced and astringin as the most abundant representatives, being significantly increased in PF, whereas pallidol, also present in relatively high concentrations, was the highest in UN. Overall, despite less significance in the occurrence of individual compounds being shown in ITA, interestingly the total stilbenes were present in higher concentrations in Italy than Slovenia, with pieced(s), pallidol and astringin ranging from 14-71 μg/g a.a.t. (ITA) to 12-45 μg/g a.a.t (SLO). Several stilbenes were already reported to be dependent on light environment however mould infections, e.g. *Botrytis cinerea*, are also known to considerably enhance the levels of stilbenes. Hence, as the most humid environment was achieved in UN canopies, and, as the light exposure in the time of the highest occurrence of stilbenes (reported around veraison and on) (Sternad Lemut et al., 2013b; Versari et al., 2001) was the highest in VE (followed by PF with a decrease of exposure when approaching harvest), it is quite obvious that the changes are most probably triggered more by microbial infections than by light exposure, although both are apparently (synergistically) responsible for the incident.

4.5.4.3.2 Isoprenoids

It is known that not only grapevine phenolics but also isoprenoids are related to the metabolic processes of plant cells, which are furthermore dependent on both plant genetic predisposition and various environmental factors. In the present experiment, in general, total isoprenoids in grape berries were detected in similar concentrations in both of the vineyards, however the effects of leaf removal timing on their total amounts at harvest was different between the two locations. Whereas in Slovenia the PF showed significantly higher content of total isoprenoids than UN (with VE showing intermediate result), in Italy VE reached the highest result, followed by PF and finally UN with the lowest outcome.

In order to focus initially on the chemical classes of isoprenoids in the berries from different LR treatments (the total amount of individual representatives of each chemical class), a canonical correlation analysis, together with ANOVA of single parameters, was again performed. The plots and tables (Figure 35) indicate the total carotenoids to be more altered in the Slovenian berries, with significant differences among all the treatments, whereas in Italy similar results were shown for PF and VE. Also, the total chlorophylls and tochopherols revealed higher significance for Slovenian grapes, with differences among all the treatments in the comparison, while none or lower significance appeared in Italy (Figure 35).

Since highlighting the chemical groups of isoprenoids only did not provide us with any clear correspondence of metabolic behaviour as related to leaf removal timing, which would be interesting for both locations, we have continued the investigation by performing a separate one-way ANOVA for each individual metabolite. The aim was to reveal whether the differences between individuals are basically present but are masked within the observation of totals, which can easily happen if the individual representatives within one chemical class show opposite behaviour as a result of the same treatment (e.g. some increase in concentration, whereas others decrease). Hence, the results of individuals are summarised in the Table 19.

The changes in the occurrence of different grapevine carotenoids due to various climate conditions (particularly sunlight exposure) and the dynamics of carotenoids synthesis during grape berry development have already been studied (Oliviera et al., 2004; Bureau et al., 1998; Razungles et al., 1996; Marais et al., 1991). Namely, α-

carotene and some xanthophylls (neoxanthin, flavoxanthin and lutein) were previously reported to be abundant before veraison, but to decrease thereafter dramatically, whereas three other xanthophylls: violaxanthin, luteoxanthin, and 5,6-

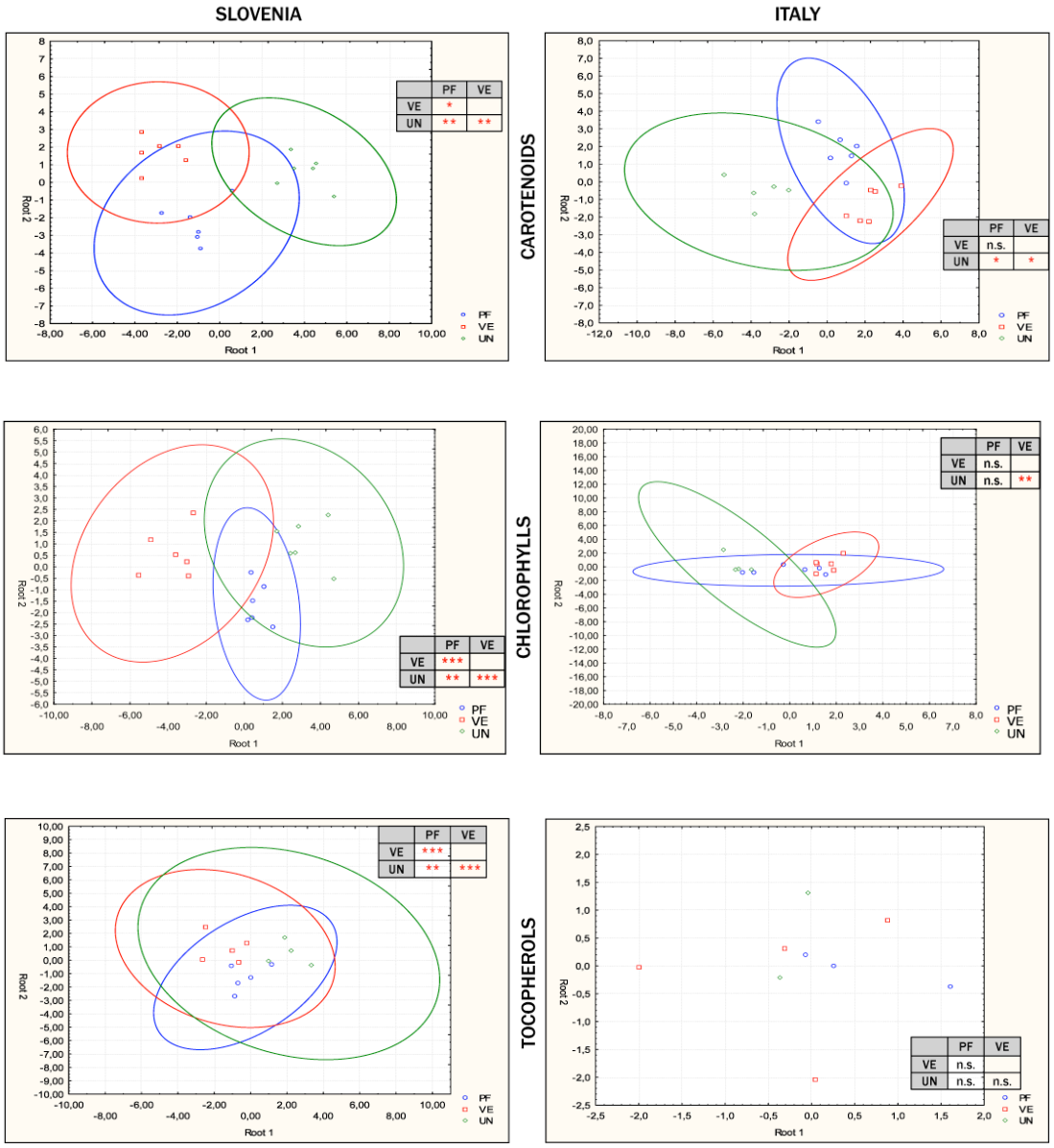


Figure 35: Canonical correlation analysis of different classes of isoprenoids as affected by different leaf removal timings in Slovenia (left) and Italy (right). PF= pre-flowering leaf removal (LR); VE=veraison LR; UN = untreated (control); ellipses representing a 95% confidence interval; n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001.

epoxylutein, on the other hand, reportedly appear only after veraison (Razungles et al., 1996; 1988). Nevertheless, according to the literature (Mendes-Pinto et al., 2010; Oliviera et al., 2004), at maturity the grapes exposed to the sunlight seem to have lower total carotenoids concentrations than shaded grapes. On the other hand, due to

the very few varieties under observation in relation to microclimate manipulation, potential cultivar-specifics are not yet clear up to now. In the present experiment, in 'Pinot Noir' berries, eight carotenoids in total were identified at harvest, although only antheraxanthin showed the same behaviour at both sites; however, in contrast to the existing reports, the most shaded UN berries showed the lowest results (Table 19). Also, zeaxanthin and violaxanthin at harvest were significantly increased in the case of (sunlight exposed) LR treatments as compared to control in Slovenia, but with no significant changes at all in Italy. Those results indicate some other cultivar- and/or site-specifics in addition to light exposure / microclimate only. Lutein 5,6-epoxide was actually the only carotenoid that decreased due to late sunlight exposure (VE), as was previously reported, although that was again the case only in Slovenia. Finally, low or no significant alternations, with several inconsistencies between the two locations, were achieved by microclimate manipulation in the case of β-carotene, 9Z-β-carotene and lutein, while α-carotene was not detected at all, hence it is difficult to come to any reliable conclusions based on microclimate related synthesis of those compounds. As the carotenoids are not only important as the pigments (co)responsible for the wine colour but are also known (together with their non-aromatic intermediates) as the precursors of aroma-active norisoprenoids (Winterhalter & Rouseff, 2002), any additional knowledge of how they can be enhanced/controlled would be very useful for the viticulture practice.

Two types of chlorophyll are normally found in plants: chlorophyll a and chlorophyll b. As the chlorophylls are very important for the plant photosynthetic functioning, they are widely studied mainly in the grapevine leaves (or green parts in general) but less in a ripe grape berry. However, it is known that similarly to carotenoids, chlorophylls also decrease on a per berry basis from veraison to harvest (Mendes-Pinto et al., 2010; Olivera et al., 2004), although those observations were mainly done for white cultivars. Even 'Pinot Noir' ripe berries contain twice as much chlorophylls in ITA compared to SLO; their content in reality was very low (with 2 and 0.4 μg/g a.a.t for chlorophylls a and a' respectively). Both chlorophyll b and b' were on the other hand present in similar concentrations at both sites and with slightly higher concentrations (around 2.5 for chlorophylls b and around 0.7 μg/g a.a.t for chlorophylls b'). As a result of canopy microclimate manipulation, chlorophylls a and a' were significantly triggered at both sites, however in Slovenia

they were present in highest concentrations for PF, whereas in Italy for VE, with UN always showing the lowest content. Hence, it seems that chlorophylls a/a' can be promoted by leaf removal (regardless of its timing), most likely due to the better sun exposure after canopy openness (as the closed canopy of UN showed consistently lower results); however, as always when the observations are done in the field, the synergistic effect of multiple microclimate parameters is to be considered as a possible reason also. On the other side, 'Pinot Noir' berries from SLO showed significantly the lowest content of chlorophylls b and b' in the case of VE as compared to UN and PF, whereas the significance was not shown for any of the treatments in Italy (even the trend was lowest for PF). Although difficult to come to any reliable conclusion, it seems that LR is basically not promoting b/b' chlorophylls in ripe berries**.**

Table 19: Isoprenoids as affected by different leaf removal timing in Slovenia and in Italy. PF=pre-flowering leaf removal (LR); VE=veraison LR; UN=untreated control. Green coloured: the compounds that are showing the same behaviour (significance) at both sites. Blue coloured: the compounds that are showing very similar behavior at both sites.

← SLOVENIA						ITALY →				
UN	PF	VE	p value	F	COMPOUND	F	p value	UN	PF	VE
					ISOPRENOID COMPOUNDS					
					Carotenoids					
c	a	b	0.000	***	violaxanthin	n.s.	0.125			
a	a	b	0.002	**	lutein 5,6-epoxide	n.s.	0.116			
			0.169	n.s.	neoxanthin	n.s.	0.049			
b	a	a	0.004	**	antheraxanthin	*	0.018	b	a	a
			0.233	n.s.	lutein	*	0.016	a	b	ab
c	b	a	0.000	***	zeaxanthin	n.s.	0.053			
			0.493	n.s.	β-carotene	*	0.013	a	b	a
b	a	a	0.017	*	9Z-β-carotene	*	0.043	a	b	a
					Tocopherols					
b	a	a	0.000	***	α-tocopherol	n.s.	0.455			
			0.467	n.s.	ϒ-tocopherol	n.s.	0.539			
					Chlorophylls					
a	a	b	0.001	***	chlorophyll b	n.s.	0.129			
a	b	c	0.000	***	chlorophyll b'	n.s.	0.255			
b	a	b	0.001	***	chlorophyll a	***	0.000	c	b	a
ab	a	b	0.009	**	chlorophyll a'	***	0.000	c	b	a

Data were processed through ANOVA and means separated using Student- Newman-Keuls test (P<0.05) (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). Means followed by the same letter or no letters are not significantly different. In addition the letter »a« is used to indicate the highest concentration observed, followed by »b« for intermediate and »c« (where used) for the lowest result.

Finally, only two tocopherols were identified in 'Pinot Noir' samples: α- and γ-tocopherol. The α-tocopherol was present in about 5-times higher amounts than γ

tocopherols, with the latter detected in about 1 μg/g a.a.t in the samples of both vineyards. Furthermore, in Slovenia only α-tocopherols was significantly enhanced by both leaf removals as compared to control, whereas γ-tocopherol did not show any significance for any of the treatments. Tocopherols (together with tocotrienols) are known under the generic name vitamin E and are hence under research interest not only due to plant benefits but also human (consumer) potential benefits. Although confirmed also in the grape berries, they are frequently studied in the leaves but often also in the seeds and seed oils. Especially for the seeds, it is known that tocopherol levels decrease with seed maturation or age (Horvath et al., 2006). As our samples were prepared from the whole berry, including the seeds, it is impossible to know from which tissue and to what extent (proportion) the tocopherols arose. In future, a separate "seed metabolome" should be studied to ascertain the origin of tocopherols but also many other metabolites known (or not yet known) to be present also in the seeds, though not exclusively.

4.5.4.4 Untargeted metabolomics

In order to cluster a much wider range of information about grape berry metabolome as affected by induced microclimate modifications within grapevine cluster-zone, we have supplemented the targeted analyses with untargeted metabolomics analyses. This hypothesis-free and totally holistic approach moves towards the detection of as many groups of metabolites as possible to obtain the patterns or fingerprints without necessarily identifying or quantifying a specific compound(s) (Cevallos-Cevallos et al., 2009). In our case, it represents a way to study plant metabolism as a whole without any burden of existing theories, thus is a very useful tool to upgrade but also to re-define (wherever needed) the existing knowledge of plant biochemical responses to canopy microclimate manipulation. In addition, the goal was also to define potential novel biomarkers involved in the plant bioprocess under induced and (up to certain level) monitored climate conditions.

Out of several thousands of features detected (in a negative and positive mode), only those that showed very significant results ($p < 0.01$) when subjected to one-way ANOVA analysis (and furthermore to Tukey's HSD test) will be discussed in this chapter.

Comment: Despite plentiful results that were obtained in this last part of the thesis research

work, up to date they can only be presented as preliminary results, since much further time and efforts are needed for appropriate data processing (initially scanning and filtering the adducts and fragments) and for gaining sufficient knowledge and "data libraries" that would enable researchers to proceed with the identifications of numerous up to date unknown compounds/features, with also up to date unknown relations to environmental factors; however, most probably many years will be needed to develop enough skills to fulfil at least the majority of the gaps within current knowledge of plant biosynthetic behaviour.

4.5.4.4.1 Biomarkers

A biomarker is defined by the FDA (United States Food and Drug Administration) as ''a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention'' (Chakravarty, 2003). The biomarker hence generally refers to measured/evaluated characteristics that may be used to indicate biologically based responses to factors of interest. It can also be used to indicate exposure to various environmental factors as in our experiment.

However, to avoid misunderstandings or even misleading information in the case of studies that are still under development, researchers are often using "safer" terms such as "putative biomarker" and "potential biomarker" or "biomarker candidate", whereas the combined term "potential biomarker candidate" (PBC) was also recreantly proposed (Martins-de-Souza, 2010).

In the following, the lists of such PBCs are presented (Tables 21 to 24). The compounds/features that showed high significance as a result of (*i)* treatment (microclimate manipulation), (*ii)* leaf removal (both), (*iii)* pre-flowering leaf removal and (*iv*) veraison leaf removal are listed for the Slovenian vineyard. In addition, the compounds/features that showed the same behaviour also in the Italian vineyard are marked with a green shading, indicating higher probability for the compounds to have a potential as biomarker – as they are confirmed in two different geo-climatic conditions.

Although many known compounds were indicated as PBCs already by means of targeted metabolomics (and were frequently confirmed by the untargeted approach, along with the fact that the solving of "fragments-issue" will probably improve the

coverage of results), there are in addition numerous other unknown compounds indicated as PBCs.

For a better understanding of what is a possible cause behind the high significance in either increase or decrease in abundance of PBCs, a brief summary (based on the observations of three and not only actual vintage) of our microclimate observations is shown in Table 20. In addition, a summarised figure of different microclimate scenarios is presented in Chapter 3 (Figure 4).

Table 20: A simplified summary of microclimate observations as affected by different leaf removal timings: PF = pre-flowering leaf removal (LR); VE =veraison LR; UN = untreated (control); * = the lowest; ** = intermediate; *** = the highest.

Treatment →	UN	PF	VE
Cluster area microclimate parameter ↓	Before/after veraison	Before/after veraison	Before/after veraison
Temperature	*/*	***/**	*/***
Relative humidity	***/***	*/**	***/*
Sunlight exposure	*/*	***/**	*/***

Looking to preliminary results also from this perspective, we have initially observed 58 PBCs in Slovenia for the treatment itself; however, none of them showed completely the same behaviour also in Italy, making it very difficult for us to decide on which compounds we should focus in the future. By contrast, when observing PBCs for leaf removal (primarily increased sunlight exposure, regardless of the timing), we noted 313 PBCs in Slovenia, but in this case 26 of them responded the same at both sites - in Slovenia as well as in Italy. Out of those 26, only 2 were undoubtedly identified (kampferol-3-rutinoside and syringetin-3-glucoside), whereas the others remain unknown. Interesting outcomes were achieved also when observing the novel viticulture technique of pre-flowering leaf removal – the treatment that caused very early exposure to the sunlight, which was decreasing later due to the appearance of the new lateral leaves; that was consequently characterised by higher cluster area temperature early in the season but was later more beneficial when the air temperature was the highest in late season; and the one that basically showed the lowest humidity after defoliation and then kept the intermediate situation until the harvest. This PF treatment showed 131 PBCs, but up to this stage of data processing 4 of them (2 unknown, rutin and isorhamnetin-3-

glucoside) can be treated as the most promising as they were same-behaved in both of the vineyards. Rutin in particular might be very interesting for further observations due to at least two additional reasons: firstly, it is very abundant, and secondly, it was previously indicated as the early peaking flavonol that can most probably be enhanced only by early leaf removal intervention (Sternad Lemut et al., 2013b). Finally, PBCs for late – veraison leaf removal were followed. In general, the clusters of VE treatment are subjected to the same conditions as UN before the defoliation, whereas after that middle-hot-summer intervention, a fast change in sunlight exposure, canopy temperature (both increased) as well as humidity (decrease) is achieved. In case of such conditions during the whole season, 58 VE PBCs show significant alterations at harvest in Slovenia, but none of them have repeated this behaviour also in Italy. However, that does not necessarily mean that there was no important triggering of plant biosynthetic behaviour after performing the veraison leaf removal but only that (if present) PBCs seem not to be retained until the harvest in significant amounts. At least in the Italian vineyard, it seems that the maturation was ahead of the maturation in Slovenia and was potentially already approaching the phase of so-called phenolic decline. Further intensive research is needed to derive more and better conclusions in this type of multidisciplinary study, also in the relation to the detailed observations of biosynthesis during grape berry development. And, as discussed previously (Martins-de-Souza, 2010; Mischak et al., 2007) by implementing various parameters and techniques, by studying various sets of samples (in various vintages and *terroirs*) and last but not least by considering as many potential confounding factors as possible.

Comment: All the following tables are continuing over more pages.

Table 21: PUTATIVE BIOMARKERS FOR TREATMENTS: compounds/features (fragments) that are different in their concentration (with high significance) for the grapes of all the observed treatments (PF, VE, UN) in SLOVENIA. RT, retension time; pos, positive; neg, negative; PF, preflowering leaf removal; VE, veraison leaf removal; UN, untreated (without leaf removal).

No.	RT	Mass	Mode	Compound/Feature	PF	VE	UN	p-value	sign F.
1	28.91	135.0449	pos	*unknown*	a	c	b	0.0000	***
2	2.83	136.0603	pos	*unknown*	c	a	b	0.0003	***
3	27.4	153.0195	pos	*unknown*	a	b	c	0.0000	***
4	5.18	175.0289	pos	*unknown*	c	a	b	0.0001	***
5	28.92	229.0854	pos	*trans* resveratrol	a	c	b	0.0000	***
6	28.92	241.0865	pos	*unknown*	a	c	b	0.0000	***
7	28.39	259.0180	pos	*unknown*	a	b	c	0.0000	***

8	22.8	259.0607	pos	*unknown*	a	b	c	0.0021	**
9	25.7	259.0977	pos	*unknown*	a	c	b	0.0000	***
10	1.43	264.1091	pos	*unknown*	b	a	c	0.0000	***
11	28.91	271.0974	pos	*unknown*	a	c	b	0.0000	***
12	1.03	282.0656	pos	*unknown*	c	b	a	0.0001	***
13	21.69	291.0871	pos	epicatechin	c	b	a	0.0000	***
14	28.4	303.0501	pos	quercetin-3-glucuronide	a	b	c	0.0000	***
15	27.4	305.0652	pos	*unknown*	a	b	c	0.0001	***
16	25.62	321.0605	pos	*unknown*	a	b	c	0.0000	***
17	22.54	335.0768	pos	*unknown*	a	b	c	0.0003	***
18	28.92	391.1387	pos	*unknown*	a	c	b	0.0000	***
19	28.4	401.0217	pos	*unknown*	a	b	c	0.0000	***
20	28.38	410.0371	pos	*unknown*	a	b	c	0.0000	***
21	28.91	410.1089	pos	*unknown*	a	c	b	0.0000	***
22	28.93	414.1229	pos	*unknown*	a	c	b	0.0000	***
23	12.5	417.0329	pos	*unknown*	b	c	a	0.0000	***
24	29.78	435.1284	pos	*unknown*	a	b	c	0.0000	***
25	28.39	457.0076	pos	*unknown*	a	b	c	0.0000	***
26	28.7	465.1020	pos	*unknown*	a	b	c	0.0000	***
27	27.39	470.0939	pos	*unknown*	a	b	c	0.0002	***
28	28.39	479.0810	pos	petunidin-3-glucoside	a	b	c	0.0000	***
29	11.49	483.0207	pos	*unknown*	b	a	c	0.0000	***
30	28.39	498.0519	pos	*unknown*	a	b	c	0.0000	***
31	28.39	501.0622	pos	*unknown*	a	b	c	0.0000	***
32	15.2	505.0852	pos	*unknown*	a	b	c	0.0000	***
33	31.09	513.0942	pos	*unknown*	a	b	c	0.0001	***
34	28.39	517.0315	pos	*unknown*	a	b	c	0.0000	***
35	28.71	557.1019	pos	*unknown*	a	b	c	0.0000	***
36	28.91	605.1723	pos	*unknown*	a	c	b	0.0000	***
37	28.71	611.1602	pos	*unknown*	a	b	c	0.0000	***
38	15.19	667.1174	pos	*unknown*	a	b	c	0.0000	***
39	28.7	789.1424	pos	*unknown*	a	b	c	0.0002	***
40	16.78	197.0449	neg	*unknown*	b	a	c	0.0000	***
41	28.36	299.0197	neg	*unknown*	a	b	c	0.0000	***
42	28.36	301.0346	neg	*unknown*	a	b	c	0.0000	***
43	21.98	403.1022	neg	*unknown*	b	c	a	0.0002	***
44	24.25	435.0922	neg	*unknown*	a	b	c	0.0000	***
45	29.07	449.1080	neg	*unknown*	a	b	c	0.0000	***
46	20.2	463.0874	neg	*unknown*	a	b	c	0.0002	***
47	28.35	477.0658	neg	quercetin-3-glucuronide	a	b	c	0.0000	***
48	22.52	495.1133	neg	*unknown*	a	b	c	0.0000	***
49	29.39	505.0966	neg	*unknown*	a	b	c	0.0000	***
50	21.37	549.2553	neg	*unknown*	b	a	c	0.0000	***
51	30.81	593.1527	neg	kaempferol-3-rutinoside	a	b	c	0.0000	***
52	25.4	639.1202	neg	*unknown*	a	b	c	0.0001	***
53	32.66	737.1365	neg	*unknown*	b	a	c	0.0000	***
54	28.89	779.2558	neg	*unknown*	a	c	b	0.0000	***
55	27.38	899.2252	neg	*unknown*	a	b	c	0.0000	***
56	28.64	941.1637	neg	*unknown*	a	b	c	0.0000	***
57	28.39	941.1654	neg	*unknown*	a	b	c	0.0000	***
58	28.68	1073.2461	neg	*unknown*	a	b	c	0.0000	***

Data were processed through ANOVA and means separated using HSD test (P<0.01) (* = p<0.05; ** = p<0.01; *** = p<0.001). However, only features showing high significance (* = p<0.05 excluded) and clear significance (cases with intermediates like: “a, ab, b” excluded) are listed in this table.

Table 22: PUTATIVE BIOMARKERS FOR LEAF REMOVAL: compounds/features (fragments) that are different (with high significance) in concentration for the grapes from vines with leaf removal (PF and VE) and from untreated vines (UN) in SLOVENIA. RT, retension time; pos, positive; neg, negative; PF, preflowering leaf removal; VE, veraison leaf removal; UN, untreated (whitout leaf removal).

No.	RT	Mass	Mode	Compound/Feature	PF	VE	UN	p-value	sign F.
1	2.40	84.0803	pos	*unknown*	b	b	a	0.0000	***
2	1.04	108.9629	pos	*unknown*	b	b	a	0.0085	**
3	18.21	123.0443	pos	*unknown*	b	b	a	0.0045	**
4	21.70	123.0447	pos	*unknown*	b	b	a	0.0002	***
5	27.40	129.0556	pos	*unknown*	a	a	b	0.0004	***
6	15.21	137.0607	pos	*unknown*	a	a	b	0.0000	***
7	21.69	139.0391	pos	*unknown*	b	b	a	0.0000	***
8	17.45	140.0459	pos	*unknown*	a	a	b	0.0000	***
9	16.77	140.0459	pos	*unknown*	a	a	b	0.0001	***
10	21.70	147.0453	pos	procyanidin B4	b	b	a	0.0037	**
11	18.47	149.0246	pos	*unknown*	a	a	b	0.0001	***
12	18.21	151.0400	pos	*unknown*	b	b	a	0.0020	**
13	21.70	151.0400	pos	*unknown*	b	b	a	0.0004	***
14	22.81	153.0194	pos	*unknown*	a	a	b	0.0019	**
15	29.77	153.0202	pos	*unknown*	a	a	b	0.0005	***
16	17.45	155.0713	pos	*unknown*	a	a	b	0.0058	**
17	16.76	155.0714	pos	*unknown*	a	a	b	0.0028	**
18	21.70	165.0552	pos	*unknown*	b	b	a	0.0000	***
19	11.49	167.0357	pos	*unknown*	a	a	b	0.0000	***
20	21.69	169.0509	pos	*unknown*	b	b	a	0.0002	***
21	15.21	178.0391	pos	*unknown*	a	a	b	0.0004	***
23	28.39	196.9781	pos	*unknown*	a	a	b	0.0005	***
24	20.35	199.0765	pos	*unknown*	b	b	a	0.0000	***
25	18.47	204.9820	pos	*unknown*	a	a	b	0.0014	**
26	22.09	207.1405	pos	*unknown*	a	a	b	0.0001	***
27	19.35	223.0593	pos	*unknown*	a	a	b	0.0001	***
28	16.73	224.0249	pos	*unknown*	b	b	a	0.0001	***
30	1.42	234.0976	pos	*unknown*	a	a	b	0.0004	***
31	29.77	243.0659	pos	*unknown*	a	a	b	0.0014	**
32	30.74	244.0314	pos	*unknown*	a	a	b	0.0000	***
33	28.69	252.0279	pos	*unknown*	a	a	b	0.0000	***
34	30.74	253.0355	pos	*unknown*	a	a	b	0.0031	**
35	22.81	253.0359	pos	*unknown*	a	a	b	0.0006	***
36	31.02	259.0363	pos	*unknown*	a	a	b	0.0000	***
37	27.40	259.0607	pos	*unknown*	a	a	b	0.0001	***
38	24.26	259.0611	pos	*unknown*	a	a	b	0.0012	**
39	15.21	259.0646	pos	*unknown*	a	a	b	0.0001	***
40	26.38	260.0262	pos	*unknown*	a	a	b	0.0000	***
41	28.69	261.0314	pos	*unknown*	a	a	b	0.0001	***
42	28.39	268.0210	pos	*unknown*	a	a	b	0.0024	**
43	31.02	268.0393	pos	*unknown*	a	a	b	0.0000	***
44	26.38	269.0286	pos	*unknown*	a	a	b	0.0001	***
45	29.78	271.0597	pos	*unknown*	a	a	b	0.0006	***
46	21.69	273.0760	pos	*unknown*	b	b	a	0.0007	***
47	27.65	273.0761	pos	*unknown*	a	a	b	0.0006	***
48	31.11	274.0419	pos	*unknown*	a	a	b	0.0061	**
49	23.85	275.0530	pos	*unknown*	a	a	b	0.0000	***
50	29.11	287.0541	pos	*unknown*	a	a	b	0.0003	***
51	30.74	287.0549	pos	kampferol-3-rutinoside	a	a	b	0.0000	***
52	27.40	287.0555	pos	*unknown*	a	a	b	0.0002	***
53	20.19	291.0392	pos	*unknown*	b	b	a	0.0006	***
54	21.37	295.1110	pos	*unknown*	a	a	b	0.0001	***

55	28.69	301.0197	pos	*unknown*	a	a	b	0.0074	**
56	28.69	303.0500	pos	quercetin-3-glucuronide	a	a	b	0.0000	***
57	30.63	303.0501	pos	quercetin-3-glucoside or	a	a	b	0.0000	***
58	22.81	305.0663	pos	*unknown*	a	a	b	0.0004	***
59	31.02	308.0243	pos	*unknown*	a	a	b	0.0021	**
60	13.83	308.0593	pos	*unknown*	a	a	b	0.0020	**
61	25.33	309.0517	pos	*unknown*	b	b	a	0.0009	***
62	21.70	310.0583	pos	*unknown*	b	b	a	0.0063	**
63	17.24	312.0496	pos	*unknown*	b	b	a	0.0054	**
64	21.69	313.0687	pos	epicatechin	b	b	a	0.0002	***
65	31.02	317.0650	pos	*unknown*	a	a	b	0.0000	***
66	25.98	319.0444	pos	*unknown*	a	a	b	0.0012	**
67	26.38	319.0447	pos	*unknown*	a	a	b	0.0000	***
68	23.86	321.0606	pos	*unknown*	a	a	b	0.0001	***
69	30.74	325.0023	pos	*unknown*	a	a	b	0.0008	***
70	29.77	327.0183	pos	*unknown*	a	a	b	0.0010	***
71	29.03	333.0602	pos	*unknown*	a	a	b	0.0000	***
72	15.21	336.0940	pos	*unknown*	a	a	b	0.0002	***
73	13.95	339.1035	pos	*unknown*	a	a	b	0.0021	**
74	15.22	339.1035	pos	*unknown*	a	a	b	0.0002	***
75	28.69	340.9980	pos	*unknown*	a	a	b	0.0006	***
76	30.63	340.9984	pos	*unknown*	a	a	b	0.0000	***
77	31.11	347.0763	pos	syringetin-3-galactoside	a	a	b	0.0000	***
78	20.35	349.1070	pos	*unknown*	b	b	a	0.0000	***
79	16.13	353.0846	pos	*unknown*	a	a	b	0.0003	***
80	13.56	355.0337	pos	caftaric acid	b	b	a	0.0055	**
81	12.49	355.0421	pos	*unknown*	a	a	b	0.0014	**
82	15.21	355.0723	pos	*unknown*	a	a	b	0.0004	
83	28.68	358.5683	pos	*unknown*	a	a	b	0.0017	**
84	23.85	359.0073	pos	*unknown*	a	a	b	0.0029	**
85	11.51	359.0409	pos	*unknown*	a	a	b	0.0053	**
86	20.34	361.1067	pos	*unknown*	b	b	a	0.0037	**
87	25.85	361.1079	pos	*unknown*	b	b	a	0.0000	***
88	31.01	365.5769	pos	*unknown*	a	a	b	0.0001	***
89	21.68	366.0957	pos	*unknown*	b	b	a	0.0045	**
90	15.58	369.1134	pos	*unknown*	a	a	b	0.0076	**
91	15.21	370.0333	pos	*unknown*	a	a	b	0.0007	***
92	18.46	371.0089	pos	*unknown*	a	a	b	0.0005	***
93	15.22	371.0413	pos	*unknown*	a	a	b	0.0015	**
94	17.94	371.0453	pos	*unknown*	b	b	a	0.0004	***
95	17.07	377.0579	pos	*unknown*	b	b	a	0.0001	***
96	20.34	379.0999	pos	*unknown*	b	b	a	0.0002	***
97	19.97	381.1132	pos	*unknown*	a	a	b	0.0083	**
98	17.45	383.0936	pos	*unknown*	a	a	b	0.0000	***
99	16.76	383.0940	pos	*unknown*	a	a	b	0.0009	***
100	18.24	383.1307	pos	*unknown*	a	a	b	0.0017	**
101	11.49	385.0448	pos	*unknown*	a	a	b	0.0001	***
102	20.74	385.0564	pos	*unknown*	b	b	a	0.0004	***
103	13.55	385.0625	pos	*unknown*	b	b	a	0.0011	**
104	15.21	385.1010	pos	*unknown*	a	a	b	0.0002	***
105	27.40	387.0704	pos	*unknown*	a	a	b	0.0008	***
106	32.69	389.0501	pos	*unknown*	a	a	b	0.0000	***
107	13.83	389.0846	pos	*unknown*	a	a	b	0.0042	**
108	28.92	391.1387	pos	*unknown*	a	c	b	0.0000	***
111	20.73	394.0597	pos	*unknown*	b	b	a	0.0005	***
112	17.45	399.0651	pos	*unknown*	a	a	b	0.0021	**
113	16.75	399.0689	pos	*unknown*	a	a	b	0.0037	**
114	13.56	401.0377	pos	*unknown*	a	a	b	0.0015	**
115	15.22	401.0750	pos	*unknown*	a	a	b	0.0003	***
116	28.69	403.0473	pos	*unknown*	a	a	b	0.0019	**
117	28.43	403.0477	pos	*unknown*	a	a	b	0.0000	***

118	13.83	408.0631	pos	*unknown*	a	a	b	0.0061	**
119	20.19	422.1295	pos	*unknown*	b	b	a	0.0005	***
120	21.13	422.1302	pos	*unknown*	b	b	a	0.0017	**
121	16.00	425.0868	pos	*unknown*	b	b	a	0.0054	**
122	21.13	425.1401	pos	*unknown*	b	b	a	0.0018	**
123	20.20	425.1403	pos	*unknown*	b	b	a	0.0000	***
124	27.40	433.1128	pos	quercetin-3-sulfate	a	a	b	0.0002	***
125	53.83	435.3692	pos	*unknown*	b	b	a	0.0036	**
126	15.21	437.0679	pos	*unknown*	a	a	b	0.0005	***
127	21.14	441.1099	pos	*unknown*	b	b	a	0.0004	***
128	20.20	441.1109	pos	*unknown*	b	b	a	0.0000	***
129	16.76	445.0654	pos	*unknown*	a	a	b	0.0000	***
130	30.74	449.1087	pos	*unknown*	a	a	b	0.0010	***
131	13.56	453.0122	pos	*unknown*	b	b	a	0.0003	***
132	15.22	453.0458	pos	*unknown*	a	a	b	0.0006	***
134	20.99	453.0805	pos	*unknown*	b	b	a	0.0000	***
135	17.07	453.0808	pos	*unknown*	b	b	a	0.0000	***
136	29.77	454.0998	pos	*unknown*	a	a	b	0.0000	***
137	21.69	455.0959	pos	*unknown*	b	b	a	0.0003	***
138	25.85	455.1495	pos	pallidol or ampelopsin D	b	b	a	0.0000	***
139	35.95	455.1496	pos	*trans*-ω-viniferin	b	b	a	0.0000	***
140	20.35	455.1497	pos	*unknown*	b	b	a	0.0000	***
141	29.77	457.1099	pos	*unknown*	a	a	b	0.0001	***
143	14.95	467.0252	pos	*unknown*	a	a	b	0.0019	**
144	30.73	468.0784	pos	*unknown*	a	a	b	0.0000	***
145	30.74	471.0891	pos	syringetin-3-glucoside	a	a	b	0.0000	***
146	30.16	471.0894	pos	*unknown*	a	a	b	0.0001	***
147	29.11	473.1048	pos	*unknown*	a	a	b	0.0014	**
148	31.02	479.1181	pos	*unknown*	a	a	b	0.0000	***
149	26.37	481.0974	pos	*unknown*	a	a	b	0.0000	***
150	28.69	484.0724	pos	*unknown*	a	a	b	0.0000	***
151	23.86	486.0892	pos	*unknown*	a	a	b	0.0006	***
152	30.73	487.0622	pos	*unknown*	a	a	b	0.0005	***
153	27.89	487.0699	pos	*unknown*	b	b	a	0.0013	**
154	28.69	487.0835	pos	*unknown*	a	a	b	0.0000	***
155	20.19	487.1138	pos	*unknown*	b	b	a	0.0002	***
156	23.86	489.0991	pos	*unknown*	a	a	b	0.0006	***
157	16.75	489.1010	pos	*unknown*	a	a	b	0.0002	***
158	25.98	495.0773	pos	*unknown*	a	a	b	0.0000	***
159	16.76	497.0373	pos	*unknown*	a	a	b	0.0009	***
160	31.02	498.0895	pos	*unknown*	a	a	b	0.0000	***
161	26.38	500.0679	pos	*unknown*	a	a	b	0.0001	***
162	31.02	501.1000	pos	*unknown*	a	a	b	0.0000	***
163	28.69	503.0536	pos	*unknown*	a	a	b	0.0000	***
164	26.38	503.0787	pos	*unknown*	a	a	b	0.0047	**
167	29.02	514.0827	pos	*unknown*	a	a	b	0.0001	***
168	31.01	517.0705	pos	*unknown*	a	a	b	0.0001	***
169	29.03	517.0955	pos	*unknown*	a	a	b	0.0000	***
170	29.77	519.0813	pos	*unknown*	a	a	b	0.0003	***
171	16.90	521.0890	pos	*unknown*	b	b	a	0.0030	**
172	23.11	529.0862	pos	*unknown*	b	b	a	0.0001	***
173	20.35	529.0866	pos	*unknown*	b	b	a	0.0017	**
174	26.38	529.0877	pos	*unknown*	b	b	a	0.0013	**
176	31.11	531.1096	pos	*unknown*	a	a	b	0.0002	***
177	30.73	533.0577	pos	*unknown*	a	a	b	0.0000	***
178	20.20	539.0827	pos	*unknown*	b	b	a	0.0016	**
179	28.69	549.0563	pos	*unknown*	a	a	b	0.0000	***
180	31.01	563.0696	pos	*unknown*	a	a	b	0.0000	***
182	26.38	565.0490	pos	*unknown*	a	a	b	0.0005	***
184	32.70	577.0993	pos	*unknown*	a	a	b	0.0000	***
186	25.33	579.1513	pos	*unknown*	b	b	a	0.0012	**

187	30.73	585.0325	pos	*unknown*	a	a	b	0.0012	**
188	21.69	600.1319	pos	*unknown*	b	b	a	0.0007	***
189	28.69	601.0260	pos	*unknown*	a	a	b	0.0001	***
190	26.38	617.0210	pos	*unknown*	a	a	b	0.0000	***
191	49.23	625.3941	pos	*unknown*	b	b	a	0.0008	***
192	21.47	627.1584	pos	*unknown*	a	a	b	0.0019	**
194	28.68	716.1205	pos	*unknown*	a	a	b	0.0000	***
195	28.67	805.0912	pos	*unknown*	a	a	b	0.0074	**
196	28.69	951.1803	pos	*unknown*	a	a	b	0.0001	***
197	1.34	75.0077	neg	*unknown*	b	b	a	0.0070	**
198	18.43	121.0290	neg	*unknown*	a	a	b	0.0007	***
199	21.69	137.0240	neg	*unknown*	b	b	a	0.0038	**
200	17.14	149.0087	neg	*unknown*	a	a	b	0.0075	**
201	15.23	153.0550	neg	*unknown*	a	a	b	0.0007	***
202	1.04	158.9779	neg	*unknown*	b	b	a	0.0045	**
203	17.14	163.0394	neg	*unknown*	a	a	b	0.0024	**
204	18.42	165.0189	neg	*unknown*	a	a	b	0.0013	**
205	21.69	179.0346	neg	caffeic acid	b	b	a	0.0007	***
206	21.69	205.0501	neg	*unknown*	b	b	a	0.0018	**
207	18.21	205.0503	neg	*unknown*	b	b	a	0.0038	**
208	24.16	241.0500	neg	*unknown*	b	b	a	0.0010	**
209	54.44	255.2310	neg	palmitic acid	b	b	a	0.0006	***
210	29.34	273.0758	neg	*unknown*	a	a	b	0.0047	**
211	27.38	285.0396	neg	*unknown*	a	a	b	0.0001	***
212	21.70	289.0708	neg	(-)-epicatechin	b	b	a	0.0006	***
213	18.84	289.0714	neg	*unknown*	a	a	b	0.0031	**
214	23.84	301.0352	neg	*unknown*	a	a	b	0.0001	***
215	29.07	303.0499	neg	*unknown*	a	a	b	0.0097	**
216	27.38	303.0502	neg	*unknown*	a	a	b	0.0006	***
217	13.54	315.0707	neg	*unknown*	b	b	a	0.0021	**
218	16.78	315.0708	neg	*unknown*	a	a	b	0.0000	***
219	15.77	315.0710	neg	*unknown*	b	b	a	0.0000	***
220	15.23	315.1078	neg	*unknown*	a	a	b	0.0004	***
221	1.33	317.0535	neg	*unknown*	b	b	a	0.0015	**
222	23.84	339.0711	neg	*unknown*	a	a	b	0.0042	**
223	1.51	341.1066	neg	*unknown*	b	b	a	0.0024	**
224	15.77	345.0821	neg	*unknown*	a	a	b	0.0014	**
225	51.56	353.1993	neg	*unknown*	b	b	a	0.0056	**
226	20.35	355.1018	neg	*unknown*	b	b	a	0.0004	***
227	17.45	359.0971	neg	*unknown*	a	a	b	0.0000	***
228	16.78	359.0976	neg	*unknown*	a	a	b	0.0012	**
229	55.93	369.3005	neg	*unknown*	b	b	a	0.0039	**
230	1.03	386.9382	neg	*unknown*	b	b	a	0.0046	**
231	57.18	397.3316	neg	*unknown*	b	b	a	0.0006	***
232	21.12	401.1436	neg	*unknown*	b	b	a	0.0063	**
233	20.18	401.1438	neg	*unknown*	b	b	a	0.0001	***
234	24.16	403.1024	neg	*unknown*	b	b	a	0.0003	***
235	21.39	406.1645	neg	*unknown*	b	b	a	0.0028	**
236	31.46	417.0811	neg	*unknown*	a	a	b	0.0058	**
237	26.84	419.0981	neg	*unknown*	a	a	b	0.0002	***
239	25.28	425.0860	neg	*unknown*	b	b	a	0.0017	**
240	28.89	425.1001	neg	*unknown*	a	a	b	0.0039	**
241	27.85	429.1030	neg	*unknown*	b	b	a	0.0014	**
242	32.87	431.0985	neg	*unknown*	b	b	a	0.0004	***
243	21.42	431.1908	neg	*unknown*	a	a	b	0.0003	***
244	29.13	433.0767	neg	*unknown*	a	a	b	0.0000	***
245	29.74	433.1119	neg	*unknown*	a	a	b	0.0000	***
246	31.53	433.1128	neg	*unknown*	a	a	b	0.0000	***
247	30.05	453.1315	neg	*unknown*	b	b	a	0.0000	***
248	25.83	453.1329	neg	pallidol or ampelopsin D	b	b	a	0.0000	***
249	35.93	453.1330	neg	*trans*-ω-viniferin	b	b	a	0.0000	***

250	30.55	461.0709	neg	kaempferol-3-	a	a	b	0.0000	***
251	27.77	463.0871	neg	myricitin	a	a	b	0.0000	***
252	28.65	463.0880	neg	quercetin-3-galactoside	a	a	b	0.0000	***
253	23.83	465.1020	neg	*unknown*	a	a	b	0.0001	***
254	22.79	465.1025	neg	*unknown*	a	a	b	0.0001	***
255	29.80	465.1182	neg	*unknown*	b	b	a	0.0019	**
256	19.88	471.1434	neg	*unknown*	b	b	a	0.0021	**
257	20.36	471.1437	neg	*unknown*	b	b	a	0.0000	***
259	30.99	477.1018	neg	isorhamnetin-3-glucoside	a	a	b	0.0000	***
260	26.36	479.0816	neg	*unknown*	a	a	b	0.0000	***
261	15.79	484.1030	neg	*unknown*	b	b	a	0.0016	**
262	31.16	491.0820	neg	*unknown*	a	a	b	0.0000	***
263	25.97	493.0608	neg	*unknown*	b	b	a	0.0000	***
264	29.01	493.0977	neg	*unknown*	a	a	b	0.0000	***
265	29.01	507.0778	neg	*unknown*	a	a	b	0.0004	***
266	31.06	507.1129	neg	syringetin-3-galactoside	a	a	b	0.0001	***
267	24.47	507.1134	neg	*unknown*	a	a	b	0.0082	**
268	1.04	516.8963	neg	*unknown*	b	b	a	0.0050	**
269	28.65	547.0419	neg	*unknown*	a	a	b	0.0097	**
271	21.38	561.1392	neg	*unknown*	b	b	a	0.0029	**
273	19.87	569.1228	neg	*unknown*	b	b	a	0.0062	**
274	16.01	575.1194	neg	*unknown*	b	b	a	0.0068	**
275	16.91	576.1230	neg	*unknown*	b	b	a	0.0024	**
276	21.18	576.1233	neg	*unknown*	b	b	a	0.0004	***
277	30.68	577.0506	neg	*unknown*	a	a	b	0.0079	**
278	25.28	577.1336	neg	*unknown*	b	b	a	0.0016	**
279	21.22	577.1339	neg	*unknown*	b	b	a	0.0006	***
280	19.38	577.1350	neg	procyanidin B2	b	b	a	0.0003	***
281	21.68	579.1501	neg	*unknown*	b	b	a	0.0017	**
282	13.52	593.1294	neg	*unknown*	a	a	b	0.0015	**
283	30.98	607.0615	neg	*unknown*	a	a	b	0.0020	**
284	19.71	611.1601	neg	*unknown*	a	a	b	0.0031	**
285	21.44	625.1398	neg	*unknown*	a	a	b	0.0000	***
286	25.58	627.1561	neg	*unknown*	a	a	b	0.0001	***
287	20.35	633.1979	neg	*unknown*	b	b	a	0.0000	***
288	26.56	641.1363	neg	*unknown*	a	a	b	0.0001	***
289	22.81	652.1266	neg	*unknown*	b	b	a	0.0028	**
290	21.46	652.1280	neg	*unknown*	b	b	a	0.0003	***
291	26.31	655.1182	neg	*unknown*	a	a	b	0.0001	***
292	17.57	720.1546	neg	*unknown*	b	b	a	0.0049	**
293	21.58	728.1333	neg	*unknown*	b	b	a	0.0004	***
294	20.73	729.1457	neg	*unknown*	b	b	a	0.0001	***
295	26.92	729.1457	neg	*unknown*	b	b	a	0.0073	**
296	36.10	737.1367	neg	*unknown*	a	a	b	0.0003	***
297	32.75	745.1254	neg	*unknown*	a	a	b	0.0000	***
298	22.63	753.1682	neg	*unknown*	a	a	b	0.0004	***
299	34.16	759.1058	neg	*unknown*	a	a	b	0.0037	**
300	21.70	775.1371	neg	*unknown*	a	a	b	0.0001	***
301	22.26	775.1385	neg	*unknown*	a	a	b	0.0001	***
302	20.99	865.1988	neg	*unknown*	b	b	a	0.0008	***
303	28.65	927.1846	neg	*unknown*	a	a	b	0.0000	***
304	23.82	931.2186	neg	*unknown*	a	a	b	0.0006	***
305	30.99	955.2178	neg	*unknown*	a	a	b	0.0002	***
306	23.83	957.2328	neg	*unknown*	a	a	b	0.0008	***
307	23.84	975.2427	neg	*unknown*	a	a	b	0.0001	***
308	31.05	985.2280	neg	*unknown*	a	a	b	0.0014	**
309	23.64	1015.2754	neg	*unknown*	a	a	b	0.0038	**
310	20.35	1017.2111	neg	*unknown*	b	b	a	0.0003	***
311	23.10	1017.2122	neg	*unknown*	b	b	a	0.0015	**
312	25.66	1019.2685	neg	*unknown*	a	a	b	0.0005	***
313	23.61	1033.2854	neg	*unknown*	a	a	b	0.0022	**

Data were processed through ANOVA and means separated using HSD test (P<0.01) (* = p<0.05; ** = p<0.01; *** = p<0.001). However, only features showing high significance (* = p<0.05 excluded) and clear significance (cases with intermediates like: "a, ab, b" excluded) are listed in this table.

Table 23: PUTATIVE BIOMARKERS FOR PRE-FLOWERING LEAF REMOVAL: compounds/features (fragments) with highly significant difference in berry concentration between PF and other two treatments (VE and UN) in SLOVENIA. RT, retension time; pos, positive; neg, negative; PF, preflowering leaf removal; VE, veraison leaf removal; UN, untreated control (without leaf removal).

No.	RT	Mass	Mode	Compound/Feature	PF	VE	UN	p-value	sign F.
1	1.42	69.0342	pos	*unknown*	b	a	a	0.0065	**
2	15.11	89.0397	pos	*unknown*	a	b	b	0.0046	**
3	1.42	97.0288	pos	*unknown*	b	a	a	0.0025	**
4	15.12	117.0343	pos	*unknown*	a	b	b	0.0032	**
5	24.86	135.0446	pos	*unknown*	a	b	b	0.0009	***
7	10.47	136.0621	pos	*unknown*	b	a	a	0.0084	**
8	15.12	145.0295	pos	*unknown*	a	b	b	0.0045	**
9	15.12	163.0395	pos	*unknown*	a	b	b	0.0005	***
10	22.54	167.0348	pos	*unknown*	a	b	b	0.0006	***
11	15.63	188.0688	pos	*unknown*	b	a	a	0.0024	**
12	29.26	214.0398	pos	*unknown*	a	b	b	0.0009	***
13	24.87	215.0464	pos	*unknown*	a	b	b	0.0002	***
14	29.26	227.0709	pos	*unknown*	a	b	b	0.0000	***
15	24.87	229.0864	pos	*unknown*	a	b	b	0.0000	***
16	61.01	230.8897	pos	*unknown*	b	a	a	0.0042	**
17	60.82	238.8850	pos	*unknown*	b	a	a	0.0074	**
18	22.27	245.0817	pos	*unknown*	a	b	b	0.0000	***
20	60.80	281.1805	pos	*unknown*	b	a	a	0.0014	**
21	21.14	285.0974	pos	*unknown*	a	b	b	0.0035	**
22	19.38	300.0467	pos	*unknown*	b	a	a	0.0020	**
23	28.39	301.0336	pos	*unknown*	a	b	b	0.0074	**
25	17.82	305.0663	pos	*unknown*	a	b	b	0.0082	**
26	32.35	317.1025	pos	*unknown*	b	a	a	0.0025	**
27	28.93	317.1025	pos	*unknown*	b	a	a	0.0026	**
28	18.98	321.0609	pos	*unknown*	a	b	b	0.0047	**
29	28.72	325.0560	pos	*unknown*	a	b	b	0.0000	***
30	17.36	332.0784	pos	*unknown*	a	b	b	0.0014	**
31	15.12	338.9881	pos	*unknown*	a	b	b	0.0011	**
32	14.35	355.0998	pos	*unknown*	a	b	b	0.0043	**
33	17.07	368.0529	pos	*unknown*	b	a	a	0.0004	***
34	14.35	371.0736	pos	*unknown*	a	b	b	0.0006	***
35	27.40	397.0694	pos	*unknown*	a	b	b	0.0032	**
36	29.25	408.0941	pos	*unknown*	a	b	b	0.0000	***
37	24.87	410.1098	pos	*unknown*	a	b	b	0.0000	***
38	29.26	411.1031	pos	*unknown*	a	b	b	0.0006	***
39	24.87	413.1199	pos	*trans* piceide	a	b	b	0.0000	***
40	27.40	415.1018	pos	*unknown*	a	b	b	0.0002	***
41	29.26	427.0728	pos	*unknown*	a	b	b	0.0000	***
42	28.39	429.0129	pos	*unknown*	a	b	b	0.0036	**
43	29.04	440.1011	pos	*unknown*	a	b	b	0.0002	***
44	51.79	446.2833	pos	*unknown*	b	a	a	0.0015	**
45	28.88	447.0901	pos	*unknown*	a	b	b	0.0001	***
46	24.86	455.1071	pos	*unknown*	a	b	b	0.0000	***
47	24.26	459.0881	pos	*unknown*	a	b	b	0.0018	**
48	29.01	462.0981	pos	*unknown*	a	b	b	0.0000	***
49	28.43	465.1019	pos	*unknown*	a	b	b	0.0002	***
50	19.07	467.1841	pos	*unknown*	b	a	a	0.0058	**

51	14.35	469.0425	pos	*unknown*	a	b	b	0.0046	**
52	29.26	473.0785	pos	*unknown*	a	b	b	0.0014	**
53	27.40	473.1054	pos	*unknown*	a	b	b	0.0039	**
54	24.87	475.0911	pos	*unknown*	a	b	b	0.0000	***
55	25.69	485.1173	pos	delphinidin-3-glucoside	a	b	b	0.0010	**
56	15.11	488.0503	pos	*unknown*	a	b	b	0.0001	***
57	27.40	489.0777	pos	*unknown*	a	b	b	0.0017	**
58	22.27	491.0870	pos	*unknown*	a	b	b	0.0002	***
59	15.12	501.0195	pos	*unknown*	a	b	b	0.0017	**
60	28.87	520.1213	pos	*unknown*	a	b	b	0.0000	***
61	3.40	520.9871	pos	*unknown*	a	b	b	0.0014	**
62	14.04	521.0441	pos	*unknown*	a	b	b	0.0001	***
63	29.26	525.0458	pos	*unknown*	a	b	b	0.0021	**
64	24.87	527.0623	pos	*unknown*	a	b	b	0.0008	***
65	28.39	563.0340	pos	*unknown*	a	b	b	0.0009	***
67	28.92	607.1840	pos	*unknown*	b	a	a	0.0017	**
68	19.38	617.1002	pos	*unknown*	b	a	a	0.0025	**
69	28.71	633.1406	pos	*unknown*	a	b	b	0.0000	***
70	15.12	663.0508	pos	*unknown*	a	b	b	0.0001	***
71	27.39	695.1519	pos	*unknown*	a	b	b	0.0001	***
72	27.40	793.1308	pos	*unknown*	a	b	b	0.0008	***
73	27.40	923.2202	pos	*unknown*	a	b	b	0.0007	***
74	28.38	979.1376	pos	*unknown*	a	b	b	0.0000	***
75	28.39	995.1040	pos	*unknown*	a	b	b	0.0000	***
76	17.81	119.0498	neg	*unknown*	a	b	b	0.0037	**
77	15.22	135.0446	neg	*unknown*	a	b	b	0.0076	**
78	15.22	149.0082	neg	*unknown*	a	b	b	0.0000	***
79	1.30	159.0295	neg	*unknown*	b	a	a	0.0002	***
80	17.81	163.0394	neg	*unknown*	a	b	b	0.0000	***
81	15.22	179.0340	neg	*unknown*	a	b	b	0.0001	***
82	29.23	225.0549	neg	*unknown*	a	b	b	0.0001	***
83	24.84	227.0704	neg	*unknown*	a	b	b	0.0000	***
84	56.05	283.2624	neg	methyl heptadecanoate or methyl	b	a	a	0.0015	**
85	17.81	295.0451	neg	*trans* coutaric acid	a	b	b	0.0000	***
86	1.86	295.0662	neg	*unknown*	b	a	a	0.0005	***
87	13.88	305.0664	neg	*unknown*	a	b	b	0.0025	**
88	15.23	311.0397	neg	caftaric acid	a	b	b	0.0000	***
89	61.00	320.8860	neg	*unknown*	b	a	a	0.0005	***
92	56.06	351.2505	neg	*unknown*	b	a	a	0.0075	**
93	21.07	359.0973	neg	*unknown*	b	a	a	0.0010	***
94	16.59	359.9566	neg	*unknown*	b	a	a	0.0050	**
95	20.26	379.1591	neg	*unknown*	b	a	a	0.0052	**
96	23.24	381.1765	neg	*unknown*	b	a	a	0.0000	***
97	13.23	383.1548	neg	*unknown*	b	a	a	0.0048	**
98	29.23	387.1081	neg	*unknown*	a	b	b	0.0004	***
99	24.84	389.1233	neg	*trans* piceide	a	b	b	0.0000	***
100	22.27	405.1177	neg	astringin	a	b	b	0.0000	***
101	22.81	429.2114	neg	*unknown*	b	a	a	0.0001	***
102	22.07	431.1908	neg	*unknown*	a	b	b	0.0003	***
103	21.14	435.1281	neg	*unknown*	a	b	b	0.0046	**
104	25.34	441.0821	neg	*unknown*	b	a	a	0.0016	**
106	20.63	453.1179	neg	*unknown*	a	b	b	0.0010	**
107	14.13	457.0977	neg	*unknown*	a	b	b	0.0001	***
108	29.22	471.0617	neg	*unknown*	a	b	b	0.0026	**
109	14.14	473.0934	neg	*unknown*	a	b	b	0.0001	***
110	18.94	481.0981	neg	*unknown*	a	b	b	0.0000	***
111	21.58	597.1612	neg	*unknown*	a	b	b	0.0001	***
112	23.17	605.1653	neg	*unknown*	b	a	a	0.0018	**
114	25.40	605.1661	neg	*unknown*	b	a	a	0.0046	**
115	30.75	605.1672	neg	*unknown*	b	a	a	0.0010	***
116	33.79	605.1684	neg	*unknown*	b	a	a	0.0045	**

117	28.69	609.1452	neg	rutin	b	a	a	0.0000	***
118	15.22	623.0886	neg	*unknown*	a	b	b	0.0001	***
119	31.27	623.1612	neg	isorhamnetin-3-rutinoside	a	b	b	0.0001	***
120	19.63	641.1340	neg	*unknown*	a	b	b	0.0002	***
122	28.71	707.1241	neg	*unknown*	a	b	b	0.0003	***
123	19.48	729.1470	neg	*unknown*	b	a	a	0.0024	**
124	29.23	775.2283	neg	*unknown*	a	b	b	0.0000	***
126	22.89	876.2592	neg	*unknown*	a	b	b	0.0009	***
127	28.33	953.1297	neg	*unknown*	a	b	b	0.0011	**
128	28.34	955.1437	neg	*unknown*	a	b	b	0.0000	***
129	15.21	973.0867	neg	*unknown*	a	b	b	0.0003	***
130	28.36	977.1269	neg	*unknown*	a	b	b	0.0004	***
131	23.83	989.2615	neg	*unknown*	a	b	b	0.0001	***

Data were processed through ANOVA and means separated using HSD test (P<0.01) (* = p<0.05; ** = p<0.01; *** = p<0.001). However, only features showing high significance (* = p<0.05 excluded) and clear significance (cases with intermediates like: "a, ab, b" excluded) are listed in this table.

Table 24: PUTATIVE BIOMARKERS FOR VERAISON LEAF REMOVAL: compounds/features (fragments) with highly significant difference in berry concentration between VE and other two treatments (PF and UN) in SLOVENIA. RT, retension time; pos, positive; neg, negative; PF, preflowering leaf removal; VE, veraison leaf removal; UN, untreated control (without leaf removal).

No.	RT	Mass	Mode	Compound/Feature	PF	VE	UN	p-value	sign F.
1	20.35	145.0296	pos	*unknown*	a	b	a	0.0011	**
2	24.87	151.0401	pos	*unknown*	a	b	a	0.0070	**
3	12.51	153.0194	pos	*unknown*	a	b	a	0.0001	***
4	20.35	177.0556	pos	*unknown*	a	b	a	0.0006	***
5	61.02	194.9005	pos	*unknown*	b	a	b	0.0026	**
6	28.92	201.0077	pos	*unknown*	a	b	a	0.0001	***
7	24.87	201.0081	pos	*unknown*	a	b	a	0.0003	***
9	28.92	215.0458	pos	*unknown*	a	b	a	0.0002	***
10	24.19	243.0660	pos	*unknown*	a	b	a	0.0003	***
11	1.46	248.1146	pos	*unknown*	b	a	b	0.0010	**
12	28.91	253.0855	pos	*unknown*	a	b	a	0.0000	***
13	29.48	259.0978	pos	*unknown*	a	b	a	0.0009	***
14	28.93	267.0335	pos	*unknown*	a	b	a	0.0001	***
15	15.99	273.0414	pos	*unknown*	a	b	a	0.0004	***
16	1.44	284.0809	pos	*unknown*	b	a	b	0.0014	**
17	28.92	295.0943	pos	*unknown*	a	b	a	0.0004	***
18	24.87	319.0809	pos	*unknown*	a	b	a	0.0049	**
19	1.29	319.1618	pos	*unknown*	b	a	b	0.0001	***
20	19.47	329.0332	pos	*unknown*	a	b	a	0.0097	**
21	29.48	329.0338	pos	*unknown*	a	b	a	0.0026	**
22	18.85	329.0345	pos	*unknown*	a	b	a	0.0019	**
23	28.91	355.1114	pos	*unknown*	a	b	a	0.0004	***
24	11.50	369.0739	pos	*unknown*	b	a	b	0.0005	***
25	16.74	413.0767	pos	*unknown*	b	a	b	0.0000	***
26	28.91	413.1193	pos	*unknown*	a	b	a	0.0000	***
27	28.92	429.0875	pos	quercetin-sulphate	a	b	a	0.0000	***
30	28.92	445.0583	pos	*unknown*	a	b	a	0.0001	***
31	29.48	459.1055	pos	*unknown*	a	b	a	0.0055	**
32	28.92	459.1120	pos	*unknown*	a	b	a	0.0002	***
33	16.13	467.0265	pos	*unknown*	b	a	b	0.0015	**
34	12.51	469.0053	pos	*unknown*	a	b	a	0.0022	**
35	28.91	475.0903	pos	*unknown*	a	b	a	0.0000	***
36	24.87	503.1158	pos	*unknown*	a	b	a	0.0016	**
37	28.92	527.0617	pos	*unknown*	a	b	a	0.0091	**

38	18.85	537.0916	pos	*unknown*	a	b	a	0.0090	**
39	16.36	715.0757	pos	*unknown*	a	b	a	0.0018	**
40	14.98	167.0345	neg	*unknown*	b	a	b	0.0022	**
41	20.36	193.0501	neg	*unknown*	a	b	a	0.0021	**
42	13.82	206.0460	neg	*unknown*	b	a	b	0.0002	***
43	14.07	218.1028	neg	*unknown*	b	a	b	0.0036	**
44	28.89	227.0699	neg	*trans*-resveratrol	a	b	a	0.0000	***
45	25.68	257.0812	neg	*unknown*	a	b	a	0.0000	***
46	2.45	323.0974	neg	*unknown*	b	a	b	0.0002	***
47	23.50	323.1241	neg	*unknown*	b	a	b	0.0009	***
48	14.97	329.0874	neg	*unknown*	b	a	b	0.0067	**
49	19.26	367.1610	neg	*unknown*	b	a	b	0.0003	***
50	28.89	389.1220	neg	*cis*-piceide	a	b	a	0.0000	***
51	28.53	395.1911	neg	*unknown*	b	a	b	0.0000	***
52	25.68	419.1327	neg	isorhapontin	a	b	a	0.0004	***
53	29.46	419.1328	neg	*unknown*	a	b	a	0.0000	***
54	27.51	441.1960	neg	*unknown*	b	a	b	0.0000	***
55	24.85	479.1174	neg	*unknown*	a	b	a	0.0013	**
56	28.60	535.1826	neg	*unknown*	a	b	a	0.0000	***
58	27.37	813.1888	neg	*unknown*	b	a	b	0.0002	***

Data were processed through ANOVA and means separated using HSD test (P<0.01) (* = p<0.05; ** = p<0.01; *** = p<0.001). However, only features showing high significance (* = p<0.05 excluded) and clear significance (cases with intermediates like: “a, ab, b” excluded) are listed in this table.

Briefly summarising the outcomes of the present study, the timing of leaf removal resulted in many changes in canopy microclimate, namely different light exposure, different temperature as well as different relative humidity in the cluster area, as confirmed in both vineyards under observation. By means of targeted metabolomics, the results revealed many classes of secondary metabolites to be affected by canopy microclimate manipulation, whereas in general, the chemical classes of phenolics (when observed as totals) showed more significant alterations than isoprenoids. Even if the trends highlighted in the Slovenian vineyard were basically similar to the trends detected in the Italian samples, in general there were much less significant results at harvest in Italy than in Slovenia, which was the case again when focusing further on each of the individual secondary metabolites. Many representatives of flavonols, anthocyanins (at both sites) and stilbenes, hydroxycinnamic acids, carotenoids and chlorophylls (mainly in Slovenia) responded significantly, whereas only a few individuals out of flavan-3-ols, benzoates and tocopherols were significantly affected and finally none of the (few detected) flavones/flavanones were triggered by any of the treatments, at least not the way, that the difference would stay until harvest. Many unique responses were observed for individuals also within the same chemical group, which confirms the importance of defining climate related modifications of each compound separately in order to obtain improved knowledge together with the potential to upgrade the understanding of plant behaviour. Furthermore, by

implementing untargeted metabolomics to the observation, the preliminary results indicated many potential biomarker candidates for different canopy management techniques - with only a few identified to date, while an extensive number of the others remain unknown. However, intensive further efforts are needed to be done in the future to ascertain which of those candidates can actually reach the definition of biomarker and which compound they basically represent. On the other hand, the existing preliminary results can sufficiently serve as an early sense of direction in which the detail focuses in future work might deliver the most fruitful results.

4.5.5 References

Bavaresco M., Gatti S., Pezzutto M., Fregoni M., Mattivi F. Effect of leaf removal on grape yield, berry composition, and stilbene concentration. *American Journal of Enology and Viticulture* 59, 3 (2008) 292-298

Bavaresco L., Fregoni M., Trevisan M., Mattivi F., Vrhovsek U., Falchetti R. The occurrence of the stilbene piceatannol in grapes. *Vitis* 41, 3 (2002) 133-136

Berqvist J., Dokoozlian N., Ebisuda N. Sunlight exposure and temperature effects on berry growth and composition of Cabernet Sauvignon and Grenache in the Central San Joaquin Valley of California. *American Journal of Enology and Viticulture* 52, 1 (2001) 1-6

Bubola M., Peršuric Đ., Kovačević G., Cossetto M. 2009. Influence of timing and intensity of basal leaf removal on aromatic composition of cv. Istrian Malvasia wines. In *Abstracts* Book III International Symposium "Malvasias", Canary Islands, Spain, pp. 64-65

Bureau S.M., Baumes R.L., Razungles A.J. Effects of vine or bunch shading on the glycosylated flavor precursors in grapes of *Vitis vinifera* L. Cv. Syrah. *Journal of Agricultural and Food Chemistry* 48, 4 (2000) 1290-1297

Bureau S., Razungles A., Baumes R., Bayonove C. Effect of vine or bunch shading on the carotenoid composition in *Vitis Vinifera* L. berries. I. Syrah grapes. Wine-Wiessenschaft 53, 2 (1998) 64-71

Carvalho E., Fraser P.D., Martens S. Carotenoids and tocopherols in yellow and red raspberries. *Food Chemistry* 15, 139 (2013) 744-52

Castia T., Franco M.A., Mattivi F., Muggiolu G., Sferlazzo G., Versini G. Characterization of grapes cultivated in Sardinia : chemometric methods applied to the anthocyanic fraction = Caractérisation de cultivar en Sardaigne : méthode chémométriques appliquées à l'analyse fractionnée des anthocyanes. *Science des Aliments* 12, 2 (1992) 239-255

Cevallos-Cevallos J.M., Reyes-De-Corcuera J.I., Etxeberriaa E., Danyluk M.D., Rodrick G.E. Metabolomic analyses in food science. *Trends in Food Science & Technology* 20, 11-12 (2009) 557-566

Chakravarty A. 2003. Surrogate markers: their role in regulatory decision process. Food and Drug Administration, http://www.fda.gov/cder/Offices/Biostatistics/Chakravarty_376/sld016.htm

Chorti E., Guidoni S., Ferrandino A., Novello V. Effect of different cluster sunlight exposure levels on ripening and anthocyanin accumulation in Nebbiolo grapes. *American Journal of Enology and Viticulture* 61, 1 (2010) 23-30

Cola G., Failla O., Mariani L. BerryTone-A simulation model for the daily course of grape berry temperature. *Agricultural and Forest Meteorology* 149, 8 (2009) 1215-1228

Cortell J.M., Kennedy J.A. Effect of Shading on Accumulation of Flavonoid Compounds in (*Vitis vinifera* L.) Pinot Noir Fruit and Extraction in a Model System. *Journal of Agricultural and Food Chemistry* 54, 22 (2006) 8510-8520

De Freitas V., Mateus N. Formation of pyranoanthocyanins in red wines: a new and diverse class of anthocyanin derivatives: a review. *Analls of Bioanalytical Chemistry* 401, 5 (2011) 1467–1477

Dokoozlian N.K., Kliewer W.M. Influence of light on grape berry growth and composition varies during fruit development. *Journal of the American Society for Horticultural Science* 121, 5 (1996) 869-874

Downey M.O., Harvey J.S., Robinson S.P. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Australian Journal of Grape and Wine Research 9 (2003) 15-27

Dry P. Canopy management for fruitfulness. *Australian Journal of Grape and Wine Research* 6, 2 (2000) 109-115

EARS. Slovene Public Information, http://meteo.arso.gov.si; October 2012.

English T., Bledsoe A.M., Marois J.J., Kliewer W.M. Influence of grapevine canopy - management on evaporative potential in the fruit zone . *American Journal of Enology and Viticulture* 41, 4 (1990) 137-141

Fraser et al. (2000) Fraser P.D., Pinto M.E., Holloway D.E., Bramley P.M. Technical advance: Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *The Plant Journal: For Cell and Molecular Biology* 24, 4 (2000) 551–558

Frazer G.W., Canham C.D., Lertzman K.P. 1999. Gap Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation. Copyright © 1999: Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York.

Goto-Yamamoto N., Mori K., Numata M., Kazuya Koyama K., Kitayama M. Effects of temperature and water regime on flavonoid content and composition in the skin of red-wine grapes. *Journal International des Sciences de la Vigne et du Vin*, special issue Macrowine (2010) 75-80 E

Haselgrove L., Botting D., Van Heeswijck R., Hoj P.B., Dry C., Ford C., Iland P.G. Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of *Vitis vinifera* L. Cv. Shiraz grape berries. *Australian Journal of Grape and Wine Research* 6, 2 (2000) 141-149

Horvath G., Wessjohann L., Bigirimana J., Monica H., Jansen M., Guisez Y., Caubergs R., Horemans N. Accumulation of tocopherols and tocotrienols during seed development of grape (*Vitis vinifera* L. cv. Albert Lavallée). *Plant Physiology and Biochemistry* 44, 11–12 (2006) 724-731

Jones G.V., Hellman E. 2003. Site assessment, in Oregon Viticulture (5th 1st edn), Hellman E. (Ed.) Oregon State University Press, Corvallis, OR, pp. 44–50

Kassemeyer H-H., Berkelmann-Lohnertz B. 2009. Fungi of Grapes. In: Biology of Microorganisms on Grapes, in Must and in Wine, Konig et al. (Ed.). Springer-Verlag Berlin Heidelberg: 61-88

Keller M. 2010. The science of Grapevines: Anatomy and Physiology. Keller M. (Ed.) Elsevier Academic Press Inc.: pp. 377

Kliebenstein D.J. Secondary metabolites and plant/environment interactions: a view through Arabidopsis thaliana tinged glasses. *Plant, Cell & Environment* 27, 6 (2004) 675-684

Lorenz D.H., Eichhorn K.W., Bleiholder H., Klose R., Meier, U., Weber, E. Growth stages of the grapevine: phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. vinifera) − codes and descriptions according to the extended BBCH scale. *Australian Journal of Grape and Wine Research* 1, 2 (1995) 100−103

Main G.L., Morris J.R. Leaf-removal effects on Cynthiana yield, juice composition and wine composition. *American Journal of Enology and Viticulture* 55, 2 (2004) 147-152

Marais J., van Wyk C., Rapp A. Carotenoid levels in maturing grapes as affected by climatic regions, sunlight and shade. *South African Journal for Enology and Viticulture* 12, 2 (1991) 64-69

Martins-de-Souza D. Is the word 'biomarker' being properly used by proteomics research in neuroscience. *European Archives of Psychiatry and Clinical Neuroscience* 260, 7 (2010) 561–562

Mattivi F., Guzzon R., Vrhovsek, U., Stefanini M., Velesco R. Metabolite profiling of grape: flavonols and anthocyanins. *Journal of Agricultural and Food Chemistry* 54, 20 (2006) 7692−7702

Mendes-Pinto M.M., Silva Ferreira A.C., Caris-Veyrat V., Guedes de Pinho P. Carotenoid, chlorophyll, and chlorophyll-derived compounds in grapes and Port wines. *Journal of Agricultural and Food Chemistry* 53, 26 (2005) 10034-10041

Mischak H., Apweiler R., Banks R.E. Clinical proteomics: a need to define the field and to begin to set adequate standards. *Proteomics Clinical Applications* 1, 2 (2007) 148-156

Oliveira, C., Silva Ferreira A. C., Costa, P., Guerra, J., Guedes de Pinho, P. Effect of some viticultural parameters on the grape carotenoid profile. *Journal of Agricultural and Food Chemistry* 52, 13 (2004) 4178-4184.

Panagiotis A., Perenzoni D., Nicolini G., Mattivi F. Study of Sangiovese wines pigment profile by UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry* 60*,* 42 (2002) 10461-10471

Poni S., Bernizzoni F., Civardi S., Libelli N. Effects of pre-bloom leaf removal on growth of berry tissues and must composition in two red *Vitis vinifera* L. cultivars. *Australian Journal of Grape and Wine Research* 15, 2 (2009) 185-193

Price S.F., Breen P.J., Valladao M., Watson B.T. Cluster sun exposure and quercetin in Pinot noir grapes and wine. *American Journal of Enology and Viticulture* 46, 2 (1995) 187-194

R Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria, 2013; http://www.R-project.org

Razungles A., Babic, I., Sapis J., Bayonove C. Particular behavior of epoxy xanthophylls during veraison and maturation of grape. *Journal of Agriculture and Food Chemistry* 44, 12 (1996) 3821-3825

Razungles A., Bayonove C., Cordonnier R., Sapis J. Grape carotenoids: changes during the maturation period and localization in mature berries. *American Journal of Enology and Viticulture* 39, 1 (1988) 44-48

Schijlen E.G.W.M., de Vos C.H.R.,van Tunen, A.J., Bovy A.G. Modification of flavonoid biosynthesis in crop plants. *Phyochemistry* 65, 19 (2004) 2631−2648

Schultz H.R. Climate change and viticulture: a European perspective on climatology, carbon dioxide and UV-B effects. *Australian Journal of Grape and Wine Research* 6 (2000) 2–12

Schwalbe E., Mass H., Kenter M., Wagner S. Hemispheric image modeling and analysis techniques for solar radiation determination in forest ecosystems. *Photogrammetric Engineering and Remote Sensing* 75, 4 (2009) 375-384

Smart R.E., Dick J.K., Gravett I.M., Fisher B.M. Canopy management to improve grape yield and wine quality - principles and practices. *South African Journal of Enology and Viticulture* 11, 1 (1990) 3-17

Spayd S.E., Tarara J.M., Mee D.L., Fergusson J.C. Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. *American Journal of Enology and Viticulture* 53, 3 (2002) 171-182

Sternad Lemut M., Sivilotti P., Franceschi P., Wehrens R., Vrhovsek U. Use of metabolic profiling to study grape skin polyphenol behavior as a result of canopy microclimate manipulation in a 'Pinot Noir' vineyard. *Journal of Agricultural and Food Chemistry* 61, 37 (2013b) 8976-8986

Sternad Lemut M., Trost K., Sivilotti P., Arapitsas P., Vrhovsek U. Early *versus* late leaf removal strategies for 'Pinot Noir' (*Vitis vinifera* L.): effect on colour-related phenolics in young wines following alcoholic fermentation. *Journal of the Science of Food and Agriculture* (2013a) DOI: 10.1002/jsfa.6193

Sternad Lemut M., Trost K., Sivilotti P., Vrhovsek U. Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. *Journal of Food Composition and Analyses* 24, 6 (2011) 777-784

Tarara J.M., Lee J., Spayd S.E., Scagel C.F. Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in Merlot grapes. *American Journal of Enology Viticulture* 59, 3 (2008) 235-247

Theodoridis G., Gika H., Franceschi P., Caputi L., Arapitsas P., Scholz M., Masuero D., Wehrens R., Vrhovsek U., Mattivi F. LC-MS based global metabolite profiling of grapes: solvent extraction protocol optimization *Metabolomics* 8, 2 (2012) 175-185

Vasconcelos M.C., Castagnoli S. Leaf canopy structure and vine performance. *American Journal of Enology and Viticulture* 51, 4 (2000) 390-396

Verpoorte R., Contin A., Memelink J. Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews* 1,1 (2002) 13–25

Versari A., Parpinello G.P., Tornielli G.B., Ferrarini R., Giulivo C. Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. *Journal of Agriculture and Food Chemistry* 49, 11 (2001) 5531–5536

Vrhovsek U., Masuero D., Gasperotti M., Franceschi P., Caputi L., Viola R., Mattivi F. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *Journal of Agricultural and Food Chemistry* 60, 36 (2012) 8831−8840

Wehrens R., Carvalho E., Masuero D., de Juan A., Martens S. High-throughput carotenoid profiling using multivariate curve resolution. *Analytical and Bioanalytical Chemistry* 405, 15 (2013) 5075-5086

Wink M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Review. *Phytochemistry* 64, 1 (2003) 3-19

Winterhalter P., Rouseff R. Carotenoid-Derived Aroma Compounds: An Introduction. ACS Symposium Series 802, 1 (2002) 1-17

Zoecklein B.W., Wolf T.K., Duncan N.W., Judge J.M., Cook M.K. Effects of fruit zone leaf removal on yield, fruit composition, and fruit rot incidence of Chardonnay and white Riesling (*Vitis vinifera* L.) grapes. *American Journal of Enology and Viticulture* 43, 2 (1992) 139-148

Zorer R., Moffat T., Sternad Lemut M., Vrhovsek U., Sivilotti P. (2013a) Effetto del'epoca di defogliazione sul microclima del grappolo e sulla qualita delle uve. IV Convegno Nazionale di Viticoltura, Conavi, Asti, in press.

Zorer R., Moffat T., Strever A., Hunter J.J. 2013b. Hourly simulation of grape bunch light microclimate using hemispherical photography. 18th International Symposium G*i*ESCO, Porto, Portugal, 7-11 July

4.5.6 Acknowledgement

We would like to thank the owners of the Tilia Estate and of Molini vineyard (FEM IASMA, Italy) for allowing us to carry out the experimental work in their vineyards.

4.5.7 Supplementary informations

Appendix G / Table 5_S1: Basic quality paramethers of grape juice from Pinot Noir vines with different leaf removal treatments - vintage 2011 at two vineyards: Potoce and Molini

Appendix G / Table 5_S2: Maturation kinetics of grapes from Pinot Noir vines with different leaf removal treatments - vintage 2011 at two vineyards: Potoce and Molini (Trentino, Italy).

Appendix H / Table 5_S3: Concentrations of detected individual secondary metabolites, sorted according to theit chemical classes and according to their abudance as detected in 'Pinot Noir' grape berries at two experimental vineyrds (Potoce, Vipava Valley, Slovenia; Molini, Trentino, Italy). The presented amouts are the averages of all three treatments (a.a.t) at one location (avUN + avPF + avVE/3). PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal).

5 CONCLUSIONS

Our results, obtained by means of classical targeted analyses (season 2009), have initially indicated a good potential of canopy microclimate manipulation through leaf removal for the improved accumulation of total as well as several individual flavonols, anthocyanins and hydroxycinnamic acids in the 'Pinot Noir' berry skins. Those biologically as well as technologically important classes of polyphenols were thus affected by leaf removal timing but with differing intensities and with some minor variations between the two experimental locations (both in the Vipava Valley). In general, the flavonols were found to respond most significantly to the leaf removal treatments, anthocyanins were affected to an intermediate extent and hydroxycinnamic acids (present in skins) reacted only slightly, the most noticeable at earlier stages of the maturation period.

After adding a novel viticulture technique of pre-flowering leaf removal to the observations (in vintage 2010) and despite substantial differences between the two successive seasons, we confirmed several important shifts in biosynthesis of the observed phenolic compounds as a result of canopy microclimate manipulation. Consequently, although to a leaser extent, several positive alterations were proven also in the young wines of both vintages. As a result of improved yielding of some wine-colour related phenolics in the grapes, a promoted formation of more stable pigments, vitisin A-like pyranonathocyanins, was detected during fermentation of 2010 musts if leaf removal was performed at any time.

To improve understanding of the results obtained in different purposely-induced microclimate scenarios, the monitoring of several climatic conditions was performed and has confirmed that leaf removal at different timings can cause substantial deviations in the canopy microclimate conditions. In general, earlier leaf removal treatments (at berry set and at pre-flowering) were characterised by increase of cluster light exposure early in the season (June, July), which was later decreasing due to the re-growth of lateral leaves, causing a partial re-shadowing of the clusters. In parallel, the temperature in the cluster area was initially higher, whereas humidity was lower as compared with the other treatments, but again the results were mitigated when approaching the harvest. On the other hand, the canopy microclimate of the late, veraison treatment was basically the same as the untreated control until the defoliation at berry coloration (beginning of August) was performed. Thereafter, the light exposure, berry surface temperature and air temperature in the cluster area increased (probably too much in warmer vintages), whereas relative humidity decreased, all as compared to the untreated control vines.

By taking into account the monitored microclimate alterations, the more complex, multi-method skin metabolite profiling of 2010 samples (targeted metabolomics) facilitated us to reveal microclimate related differences in the accumulation trends of numerous metabolites during grape berry development interval. The alterations were observed not only between multiple classes of skin phenolics but interestingly also between many individual class representatives, including numerous very poorly studied ones to date. Although many significant changes in metabolites concentration were observed early in the season, these were often less significant by the end of maturation. However, at harvest, nearly half (43%) of the 72 detected individual phenolics still showed significant variation in their occurance for at least one of the three leaf removal approaches if compared to the control.

The total as well as 22 detected individual flavonols were clearly related to the modifications of light environment in the canopy. By observing their biosynthetic behaviour, we noticed that most of them showed the greatest synthesis later in the season (late peaking behaviour), whereas others showed the greatest synthesis early in the season (early peaking behaviour). Even though there were fewer peaking earlier, those same flavonols occurred in much higher concentrations then late peaking flavonols. We could also observe that earlier leaf removal treatments fundamentally affected the occurrence of flavonols with early peaking behaviour, and that the related improvement in total flavonol content was retained at harvest, which was not the case for late, veraison leaf removal. This has clearly indicated that early peaking flavonols could only be promoted by early leaf removal interventions.

In contrast, the highest synthesis of (late peaking) anthocyanins was generally detected in the case of late leaf removal. As 2010 was rather cold and rainy in the Vipava Valley, the results differed slightly from the observations from 2009, which was a warmer and drier year, when earlier leaf removal at berry set led to a higher total anthocyanin content than veraison treatment. This was probably due to the synthesis inhibition resulting from too high berry surface temperatures after veraison leaf removal exposed the grapes directly to the sun in the hot August of 2009, whereas earlier treated grapes had been partially re-shadowed by that time.

Based on those results and despite the fact that flavonols and anthocyanins are on the same biosynthetic pathway, it seems possible to positively affect both classes by taking into account their (different) peaking behaviour as well as their dependence on specific microclimate conditions.

Furthermore, a majority of the detected 18 individual stilbenes were the highest in content in the samples of control treatment, which was in our case probably more of a consequence of preferential conditions for moulds such as *Botrytis cinerea* Pers. (higher humidity) than of UV exposure. Within benzoates, syringic acid was the only representative showing significant changes that were at harvest in favour of early leaf removal, while many representatives of other classes of skin polyphenols, such as flavones, flavan-3-ols, flavanones and hydroxycinnamic acids, essentially reduced their concentration from the time of veraison, with different trends between the treatments often shown only in the first stages of maturation.

When focusing on the whole berry metabolome as obtained in the harvest of 2011, the targeted metabolomics analytical approach was implemented in two vineyard environments in order to evaluate the existing and new outcomes also in different geoclimatic conditions. The results revealed many classes of grape berry secondary metabolites to be significantly affected by leaf removal timing, whereas in general the chemical classes of phenolics (when observed as totals) showed more considerable alterations than isoprenoids, with later added to the observation just the last year of survey. Even if the trends highlighted in the main experimental (Slovenian) vineyard were basically similar to the trends detected in the Italian (Trentino) vineyard, in general there were much less significantly enhanced outcomes at harvest in Italy than in Slovenia, which was the case again when we separately monitored each of the individual secondary metabolites. Many representatives of grape berry flavonols, anthocyanins (at both sites) and stilbenes, hydroxycinnamic acids, carotenoids and chlorophylls (mainly in Slovenia) responded to the shifts in microclimate with changes in synthesis that remained significantly evident until the harvest, whereas only few individuals out of flavan-3-ols, benzoates and tocopherols were altered to an extent that the difference was significant also at the end of maturation, and finally none of (few detected) flavones/flavanones were triggered by any of the treatments in a manner that the changes would still be important at harvest. However, many unique responses were observed again for the individuals originating from the same chemical groups, supporting once more the importance of evaluating the climate related modifications of each compound separately in order to understand their biosynthetic behaviour.

After implementing untargeted metabolomics to the observation of 2011 samples, the preliminary results indicated many potential biomarker candidates for different canopy management techniques - with only a few having been identified to date, while an extensive number of others remain unknown. However, intensive further effort is needed to ascertain

which of those candidates can actually reach the definition of biomarker and which compound is behind it. On the other hand, the existing preliminary results can sufficiently serve as an early sense of direction in which detailed focus in future work might deliver the most fruitful results.

Finally, the results of the microbial ecology survey generally showed a higher microbial population count (including potentially harmful ones such as *B. cinerea* Pers. or acetic acid bacteria) for late leaf removal and the untreated control, most probably due to the minimised air circulation and lower spray penetration. Earlier leaf removal treatments were thus found to show greater potential as an effective tool for controlling microbial infections. Parallel trials also indicated that leaf removal, especially if performed early, appears to be more effective than fungicide application in suppression of *Botrytis cinerea* and similar moulds, and that a second fungicide application in the case of early leaf removal could be avoided, reaching similar or even better results as compared to late leaf removal supplemented by two fungicide applications. In case of pre-flowering leaf removal, lower cluster compactness was ascertained, which can additionally contribute to lower sensitivity to microbial diseases. Moreover, the yield reduction observed in the case of the pre-flowering technique can reduce the need for later cluster thinning and therefore reduce production costs and energy needs and thus altogether a reduced negative environmental impact can be achieved.

In summary and to our best knowledge: Canopy microclimate manipulation through leaf removal performances was evaluated for the first time using specific targeted and untargeted metabolomic studies, thus the biosynthetic behaviour of several poorly studied secondary metabolites was revealed and knowledge about several previously well-explored representatives was upgraded; Terroir microbial ecology - an ecological survey in relation to the different leaf removal treatments (microclimate scenarios) was performed for the first time in the Vipava Valley vineyard and on the grape berries of the 'Pinot Noir' cultivar as well as most certainly for the first time ever by taking into consideration also novel and innovative viticulture techniques; The possibilities for pesticide use reduction through carefully selected leaf removal were explored and scientifically confirmed for the first time in the specific conditions of the Vipava Valley; and finally: Pre-flowering leaf removal performance was observed and evaluated for the first time in the growing conditions specific to the Vipava Valley, Slovenia and in Trentino, Italy and, as far as we know, for the first time on the cultivar 'Pinot Noir' at phenological stage BBCH 57. Pre-flowering leaf removal has shown to be a very good potential to become a more environmentally-friendly as well as quality-promoting alternative to the more widely adopted veraison application, which has lately lost many of its

advantages due to the higher vineyard temperatures resulting from global warming (climate change) effects.

6 SUMMARY

In conventional viticulture, pest and disease control is generally achieved through widespread application of pesticides, resulting in high costs and a negative impact on the environment. One of the main goals of sustainable vineyard management is to limit the type, quantity and timing of chemical applications, although the grape quality should not be compromised too much or not at all. Of the grapevine varieties, particular care should be dedicated to those that are more susceptible to microbial diseases. 'Pinot Noir' (*Vitis vinifera* L.) is an early ripening red grapevine variety that achieved promising results soon after its introduction to the Vipava Valley. However, it is known for its lower genetic potential for phenolics, particularly anthocyanins content as well as for its high disease sensitivity due to typical compact clusters and relatively low natural protection of phenolics - tending to be made worse by global warming - thus it is one of the varieties most urgently needing rapid solutions in the frame of predicted climate change. When exposed to environmental abiotic or biotic stresses, a plant normally reacts by producing various protective secondary metabolites, although their synthesis can also be inhibited in some specific conditions. Those compounds are however not only important for plant adaptation and survival, but their occurrence and chemical diversity can also account for the quality of many agricultural crops. Apart from variety specifics, the occurrence of secondary metabolites in grape berries is largely determined by geo-climatic conditions (*terroir*) in which the vine is grown. Although regional macroclimate or site mesoclimate cannot be influenced, leaf removal is one of the techniques that can be employed to manoeuvre the microclimate in the cluster area and consequently affect gene expression responsible for the synthesis of desirable metabolites. However, within the context of climate change, many winegrowers are already facing the problem of loss of effectiveness of the widely adopted late (veraison) leaf removal, due to the higher temperatures and severe UV exposure after canopy opening. On the other hand, much less studied earlier leaf removal alternatives, particularly the novel and innovative technique of pre-flowering leaf removal, need to be well explored before their adaptation to any grapevine variety and/or geoclimatic conditions can be justified.

In the presented research work, a field trial was thus designed in three consecutive seasons to compare different leaf removal timings (at berry set, veraison, pre-flowering - later as a completely novel approach in the region) with untreated (control) grapevines. The aim was to study how the purposely-induced microclimate modifications could affect Bunch Rot

occurrence and the profiles of secondary metabolites in grape berries during maturation, at harvest and during the vinification and how better understanding and controlling of sunlight, temperature and humidity exposure can contribute to future, more sustainable regional approaches. Basic grape quality and viticultural parameters were initially evaluated. Classical targeted chemical analyses (HPLC-VIS; HPLC-DAD) of polyphenols were then supplemented with novel multi-method metabolomic analytical approaches (UHPLC-QqQ-MS/MS; HDMS QTOF-MS), offering a lot wider possibilities to study plant secondary metabolism in the interaction with its environment. To better support the observed biosynthetic responses of plentiful metabolites as altered by microclimate manipulation, the berry surface temperature, air temperature and relative humidity as well as cluster light exposure were monitored. Moreover, a microbial ecology survey was performed to reveal the influence of environmental parameters and especially the practice of leaf removal on species composition and population dynamics of the microbial community inhabiting the 'Pinot Noir' variety. The effectiveness of early leaf removal performances comparing to fungicide application was finally evaluated in the induced microclimate scenarios.

Despite substantial differences between the observed successive seasons, several important shifts in phenolics biosynthesis as a result of microclimate manipulation were consistently observed. Consequently, although to a leaser extent, several positive alterations were proven in related young wines. The more complex, multi-method skin metabolite profiling furthermore revealed microclimate related accumulation trends of numerous metabolites during the berry development interval. The alterations were observed not only between multiple classes of skin phenolics but interestingly also among plentiful individual class representatives. The flavonols were consistently related to the modifications of light environment in the canopy but have also showed significantly different biosynthetic behaviour between their individual representatives, with some of them being mainly synthetised early in the seasons, while others only later in the season. Taking that into account, we could observe that earlier leaf removal treatments fundamentally affected the occurrence of flavonols with early peaking behaviour and that the related improvement in total flavonols was retained at harvest. This has clearly indicated that early peaking flavonols, present in relatively high concentrations, could only be promoted by early leaf removal interventions. In contrast, anthocyanins synthesis was found to be more season dependent. In colder vintage, the highest content was detected in the case of late leaf removal, whereas in the warmer vintages, anthocyanins were better promoted by early leaf removal, indicating that further global warming would definitely worsen the situation, but early

defoliation may offer one of the solutions for more successful adaptation. Furthermore, a majority of detected stilbenes were the highest in their concentration for closed control canopies, which was in our case probably more of a consequence of promoting moulds than of UV exposure. Within benzoates, only syringic acid showed significant changes in favour of early leaf removal at harvest, while altered trends of many flavones, flavan-3-ols, flavanones and hydroxycinnamic acids were often shown only in the first stages of maturation. Questions for future research were thus raised: Is the higher concentration obtained with early leaf removal at the beginning of maturation nevertheless still important within the complex pattern of physiological changes of the berry and finally for overall grape quality at harvest, and if so how could the early peaking affect late peaking compounds in different canopy microclimate scenarios? By focusing on the whole berry metabolome, it was revealed that in general the phenolics were altered to a greater extent and more consistently than isoprenoids, especially if comparing the main Slovenian experimental vineyard with the comparative Italian vineyard. However, many unique and interesting responses were observed again for individuals originating from same chemical groups. This new information regarding their behaviour needs to be taken into consideration as many compounds of higher technological interest are often on the same biosynthetic route, and the improvement of one might easily mean the loss of some others. Moreover, the preliminary results of the untargeted metabolomics approach indicated many potential biomarker candidates for different canopy management techniques - with only a few identified to date, while an extensive number of others remain unknown. Finally, the results of the microbial ecology survey showed a higher microbial population count for late leaf removal and control, whereas parallel trials indicated that leaf removal, especially if performed early, appears to be more effective than fungicide application in suppression of *Botrytis cinerea* Pers. Additionally, in the case of pre-flowering leaf removal, a lower cluster compactness and substantial yield reduction were ascertained, with the former reducing the risk for diseases and the latter the need for cluster thinning. Thus, in overall, the lower production costs (botrycid fludioxonil + cyprodimil (switch ®)) and energy needs and a reduced negative environmental impact accompanied with several quality improvements of "Pinot Noir' grape berries could be achieved by implementing pre-flowering leaf removal to the viticulture practice of Vipava Valley or to the vinegrowing regions with similar conditions.

Key words: 'Pinot Noir' (*Vitis vinifera* L.), canopy microclimate manipulation, leaf removal, pre-flowering leaf removal, metabolite profiling, metabolomics, secondary metabolites, phenolics, isoprenoids, microbial ecology, *Botrytis cinerea* Pers., fungicides, sustainable

viticulture, Vipava Valley.

6 POVZETEK

V konvencionalnem vinogradništvu se proti škodljivcem in boleznim borimo s pomočjo široke uporabe pesticidov, kar je povezano z visokimi stroški kot tudi z negativnimi vplivi na okolje. Eden glavnih ciljev trajnostnega vinogradništva je omejiti raznolikost, količino in pogostost uporabe kemičnih sredstev, hkrati pa se ob tem čim manj ali celo povsem izogniti zmanjšanju kakovosti grozdja. Najbolj intenzivno se je potrebno posvetiti tistim sortam vinske trte, ki so dovzetnejše za različna mikrobna obolenja. Modri pinot (*Vitis vinifera* L.) je zgodaj dozorevajoča rdeča sorta vinske trte, ki je dosegla obetavne rezultate kmalu po vpeljavi v Vipavsko dolino. Vendar je sorta znana po genetsko pogojeni manjši vsebnosti fenolov, predvsem antocianov, kot tudi po večji občutljivosti na bolezni zaradi svojih tipično zbitih grozdov in relativno nizke naravne fenolne zaščite, kar se na račun višanja temperatur zaradi globalnega segrevanja lahko še poslabša. V okviru napovedanih podnebnih sprememb zato še posebej nujno potrebuje hitro ukrepanje. Kadar je rastlina izpostavljena okoljskemu abiotskemu ali biotskem stresu, se nanj praviloma odzove s sintezo večjih količin zaščitnih sekundarnih metabolitov, določeni pogoji pa lahko to sintezo tudi inhibirajo. Poleg sortno povezanih dejavnikov, je pojavnost sekundarnih metabolitov v grozdni jagodi v veliki meri odvisna tudi od geoklimatskih pogojev, v katerih trta uspeva. Sekundarni metaboliti pa niso pomembni le za uspešno prilagoditev in preživetje rastline, temveč lahko njihova prisotnost in raznolikost pomembno vplivata na kakovost pridelka. Čeprav na regionalno makroklimo ali lokalno mezoklimo ne moremo vplivati, pa je odstranjevanje listov v predelu grozdov (defoliacija), eden od ukrepov, s katerim lahko bistveno vplivamo na mikroklimo v grmu vinske trte in s tem posledično na izraženost genov, odgovornih za sintezo željenih metabolitov. Zaradi klimatskih sprememb pa se mnogi vinogradniki že soočajo s problemom, ko uveljavljeno, pozno odstranjevanje listov (v fazi obarvanja jagod), izgublja na svoji učinkovitosti zaradi višjih temperatur v vinogradu in močnejše izpostavljenosti grozdov UV-žarkom takoj po posegu v vročih avgustovskih dneh. Po drugi strani so manj razširjene alternativne, predvsem nova in inovativna vinogradniška tehnika odstranjevnja listov že pred cvetenjem vinske trte, še premalo raziskane ter preizkušene na različnih sortah in v različnih geoklimatskih pogojih, da bi jih lahko upravičeno vpeljevali v širšo vinogradniško prakso.

V okviru raziskave smo tako izvedli večletni terenski poskus s ciljem preučevanja različnih časovnih izvedb defoliacije (ob tvorbi jagod, v času obarvanja jagod; in pred cvetenjem, slednje kot popolnoma nov pristop na Primorskem). Obravnavane trte smo primerjali med

seboj in z nerazlistanimi trtami. Zanimalo nas je, kako lahko načrtno sprožene spremembe v mikroklimi vplivajo na pojavnost bolezni ter na profile sekundarnih metabolitov v grozdnih jagodah med zorenjem, ob trgatvi in med fermentacijo moštov oziroma kako bi lahko z boljšim razumevanjem in kontroliranjem teh pogojev prispevali k bolj trajnostnim pristopom vinogradništva v prihodnosti. Poleg meritev osnovnih vinogradniških in kakovostnih parametrov grozdja, smo klasične, tarčno usmerjene kemijske analize polifenolov (HPLC-VIS; HPLC-DAD) nadgradili z novimi multi-metodnimi metabolomskimi analitskimi pristopi (UHPLC-QqQ-MS/MS; HDMS QTOF-MS), kar nam je omogočilo veliko bolj obsežno preučevanje sekundarnega metabolizma rastline v interakciji z njenim okoljem. Da bi lažje razumeli in podkrepili izsledke, vezane na spremenjene biosintetske odzive rastline v odvisnosti od upravljanja z mikroklimo, smo pri vseh obravnavanjih vzporedno spremljali temperaturo in relativno vlago v predelu grozdov ter izpostavljenost grozdov sončni svetlobi. Dodatno smo se posvetili še mikrobni ekologiji in sicer z namenom, da bi ugotovili vpliv okoljskih parametrov in predvsem vpliv časa odstranjevanja listov na številčnost in sestavo mikrobne populacije na grozdnih jagodah preučevane sorte. V okviru vzporednega poskusa smo nato v doseženih mikroklimatskih pogojih ocenili še učinkovitost zelo zgodnjega odstranjevanja listov v primerjavi z uporabo fungicidnega sredstva.

Kljub precejšnjim razlikam med opazovanimi zaporednimi letniki smo opazili več pomembnih sprememb v biosintezi fenolov, nastalih kot rezultat upravljanja z mikroklimo. Posledično, čeprav v manjši meri, smo potrdili pozitivne spremembe tudi v sestavi mladih vin. Kompleksnejše multi-metodno profiliranje metabolitov v kožici je dodatno razkrilo akumulacijske trende številnih, nekaterih do danes zelo slabo raziskanih metabolitov tekom razvoja grozdne jagode. Zaznali smo spremembe ne le v pojavnosti različnih skupin fenolov v kožici, temveč, zanimivo, tudi med mnogimi posameznimi predstavniki skupine. Vsebnost flavonolov je bila sicer dosledno povezana s spremembami osvetljenosti, a smo hkrati zaznali značilno različno biosintetsko obnašanje med njihovimi posameznimi predstavniki, pri čemer je sinteza nekaterih potekala bolj zgodaj v sezoni, drugih pa le pozno v sezoni. Ob upoštevanju teh ugotovitev smo potrdili, da je zgodnejša odstranitev listov bistveno vplivala na pojav tistih flavonolov, ki že zgodaj dosežejo maksimalno koncentracijo, in da se je povezano izboljšanje skupne vsebnosti flavonolov ohranilo vse do trgatve. To je jasno nakazalo, da je povečan izplen zgodaj akumuliranih flavonolov, praviloma prisotnih v relativno visokih koncentracijah, možno doseči le z zgodnjim posegom. Nasprotno, je bila najintenzivnejša sinteza antocianov v povezavi s časom odstranjevanja listov bolj odvisna od samega letnika. V hladnejšem letu je

bila najvišja vsebnost določena v primeru poznega posega, v toplejših letih pa je na sintezo antocianinov bolje vplivala zgodnja odstranitev listov, kar dodatno nakazuje, da bi nadaljnje globalno segrevanje še poslabšalo učinkovitost pozne defoliacije, vendar lahko zgodnejši poseg predstavlja eno izmed možnosti za uspešnejšo adaptacijo na nove razmere. Večina stilbenov je dosegla najvišje koncentracije pri kontrolnih trtah; v našem primeru verjetno bolj kot posledica ugodnejših pogojev za razvoj plesni kot pa na račun različnih izpostavljenosti UV-žarkom. Kar zadeva benzoate, smo bistveno prednost zgodnje odstranitve listov opazili le pri siringični kislini ob trgatvi, medtem ko so bile spremembe trendov tvorjenja mnogih flavonov, flavan-3-olov, flavanonov in hidroksicinamičnih kislin pogosto izražene le v zgodnjih stadijih razvoja grozdne jagode. Na podlagi teh ugotovitev so se pojavila pomembna vprašanja za nadaljne raziskave: Ali je višja koncentracija določenih metabolitov, pridobljena v začetku sezone, še vedno pomembna v okviru kompleksnega vzorca fizioloških sprememb v grozdnih jagodah in ali vpliva na končno kakovost grozdja ob trgatvi kljub temu, da se te zgodnje razlike pogosto zabrišejo do trgatve? Če je temu tako, kako lahko metaboliti, katerih maksimalna sinteza poteka že zgodaj v sezoni, v nadaljnem celokupnem biosintetskem delovanju rastline vplivajo na spojine, ki se bodo sintetizirale kasneje v sezoni? V nadaljevanju je osredotočenost na celoten metabolom grozdne jagode razkril, da so se polifenoli na splošno odzvali v večji meri in bolj ponovljivo kot izoprenoidi, predvsem ko smo osrednji slovenski eksperimentalni vinograd primerjali z dodatno opazovanim italijanskim. Vsekakor pa smo ponovno opazili mnogo zanimivih in edinstvenih odzivov med posameznimi predstavniki spojin, ki izhajajo iz istih kemičnih skupin. Te nove informacije bo potrebno skrbno upoštevati zlasti zato, ker nekatere tehnološko zanimive spojine pogosto nastajajo po isti biosintetični poti in ker izboljšan izplen ene izmed njih lahko hkrati povzroči izgubo nekaterih drugih. Preliminarni rezultati pristopa z netarčno metabolomiko so dodatno nakazali mnogo potencialnih biomarkerjev različnega upravljanja z mikroklimo, vendar je do sedaj uspešno identificiranih le nekaj kandidatov, velika večina pa zaenkrat ostaja neznanih. Rezultati raziskave mikrobne ekologije so pokazali številčnejšo mikrobno populacijo na jagodah trt s poznim odstranjevanjem listov in pri kontrolni skupini, medtem ko so vzporedni poskusi nakazali, da je odstranitev listov, predvsem če je opravljena zgodaj, lahko uspešna alternativa uporabi fungicidov pri zatiranju *Botrytis cineree*. Pri odstranjevanju listov pred cvetenjem smo potrdili tudi manjšo zbitost grozdov in znatno znižanje pridelka, pri čemer lahko manjša zbitost zniža tveganje za bolezni, znižanje pridelka pa potrebo po kasnejšem redčenju grozdov. Rezultati tako v celoti nakazujejo, da bi lahko v Vipavski dolini ali v klimatsko primerljivih vinorodnih deželah z uvedbo ampelotehnike odstranjevanja listov pred

cvetenjem vinske trte pomembno znižali pridelovalne stroške (botricid fludioksonil + ciprodimil (switch ®)) in energijske potrebe ter vzporedno zmanjšali negativne vplive na okolje ob hkratnem izboljšanju nekaterih kakovostnih parametrov grozdja sorte ′Modri pinot′.

Ključne besede: Modri pinot (*Vitis vinifera* L.), manipulacija mikroklime v grmu vinske trte, odstranjevanje listov, odstranjevanje listov pred cvetenjem, profiliranje metabolitov, metabolomika, sekundarni metaboliti, polifenoli, izoprenoidi, mikrobna ekologija, *Botrytis cinerea* Pers., fungicidi, trajnostno vinogradništvo, Vipavska dolina.

Doktorski študij je delno sofinancirala Evropska unija in sicer iz Evropskega socialnega sklada. Sofinanciranje se izvaja v okviru Operativnega programa razvoja človeških virov za obdobje 2007-2013, 1. razvojne prioritete: Spodbujanje podjetništva in prilagodljivosti; *prednostne usmeritve 1.3: Štipendijske sheme*.

This work was partially performed within European Union through the European Social Fund of Operational Programme for Human Resources Development for the period 2007− 2013, first development priorities: Promoting entrepreneurship and adaptability, policy priorities 1.3: Scholarship Schemes …

… and it was partially performed within the Creative Core programme (AHA-MOMENT) with contract no. 3330-13-500031, co-supported by RS-MIZS and European Regional Development Fund Research.

7 ACKNOWLEDGEMENT

I would first like to express my deepest appreciation to all my friends and colleagues from Slovenia (University of Nova Gorica) and Italy (IASMA Research and Innovation Centre, Edmund Mach Foundation) – to all of you that have worked with me, helped me, supported me and inspired me, allowed me or simply accepted having me around while I was working on this thesis. Thank you very much for all your support and above all for your invaluable sharing of knowledge and skills from extremely varied backgrounds. It enabled me to see the problems from many interesting perspectives and hence always filled me with further motivation.

In Slovenia, special thanks go to my colleagues: dr. Paolo Sivilotti, dr. Lorena Butinar, dr. Kajetan Trošt, dr. Jana Laganis and to Mia Ličen. Thank you also to all the other members and co-operators of our Wine Research Centre as well as to my big bosses for their understanding and support. In Italy I would especially like to thank Domenico Masuero, Mattia Gasperotti, Danielle Perenzoni, Cesare Lotti, Luca Zulini, dr. Roberto Zorer, dr. Panagiotis Arapitsas, dr. Pietro Franceschi, dr. Ron Wehrens and dr. Fulvio Mattivi. However, thank you also to all the others that are unfortunately just too numerous to mention.

And above all, the most special thank you to my dear tutor dr. Urška Vrhovšek. You were an absolutely perfect mentor to me at every step of the way. Thank you so much, Urška, for guiding me exactly as you did, for passing on all your valuable experience so altruistically, as well as for all your support and friendship when I was becoming too concerned. Thank you as well to my other tutor, dr. Klemen Lisjak. Thank you, Klemen, for always being there for me and particularly for introducing me to the research with all your unique and contagious enthusiasm.

I also want to express my sincere appreciation to the members of my Committee for the thesis evaluation: doc. dr. Andreja Vanzo, prof. Enrico Peterlunger and prof. dr. Stanko Vršič. Thank you very much for following my work throughout its progress and for appreciating my efforts to do my best.

Last but not least – big thanks to my beloved family – to Matjaž and to my wonderful children: Nika, Gal and Ajda, as well as to my dear parents. Thank you very much for all your patience, encouragement and understanding, even though it was definitely not easy. I also appreciate all your help very much, whenever and for whatever I really needed it.

Thank you!

8 REFERENCES (CHAPTERS 1-3)

Adams D.O. Phenolics and ripening in grape berries. *American Journal of Enology and Viticulture* 57, 3 (2006) 249-256

Adrian M., Jeandet P., Breuil A. C., Levite D., Debord S., Bessis R. Assay of resveratrol and derivative stilbenes in wines by direct injection high performance liquid chromatography. *American Journal of Enology and Viticulture* 51, 1 (2000) 37-41

Adrian M., Jeandet P., Douillet-Breuil A.C., Tesson L., Bessis R. Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. *Journal of Agricultural Food Chemistry* 48, 12 (2000) 6103-6105

Aherne S.A., O'Brien N.M. Dietary flavonols: chemistry, food content, and metabolism. Review article. *Nutrition* 18, 1 (2002) 75-81

Ali K., Maltese F., Choi Y.H., Verpoorte R. Metabolic constituents of grapevine and grape- derived products. *Phytochemistry Reviews* 9 (2010) 357-378

Ali K., Maltese F., Fortes A.M., Pais M.S., Choi Y.H., Verpoorte, R. Monitoring biochemical changes during grape berry development in Portuguese cultivars by NMR spectroscopy. *Food Chemistry*, 124, 4 (2011) 760-1769

Alluvione F., Moretti B., Sacco D., Grignani C. EUE (energy use efficiency) of cropping systems for a sustainable agriculture, *Energy,* 36 (2011) 4468-4481

Altieri M.A. 1995. Agroecology: the science of sustainable agriculture. 2nd edition. Westview Press, Boulder, Colorado, USA: 995 pp + 433 pp

Amati A., Piva A., Castellari M., Arfelli G. Preliminary studies on the effect of *Oidium tuckeri* on the phenolic composition of grapes and wines. *Vitis* 35, 3 (1996) 149-150

Anil Shrestha A., Fidelibus M. Grapevine row orientation affects light environment, growth, and development of black nightshade. *Weed Science* 53, 6 (2005) 802-812

Arapitsas P., Perenzoni D., Nicolini G., Mattivi F. (2012) Study of Sangiovese wines pigment profile by UHPLC-MS/MS. *Journal of agricultural and food chemistry*, 60, 42 (2012) 10461-10471

Arapitsas P., Scholz M., Vrhovsek U., Di Blasi S., Bartolini A.B., Masuero, D., Mattivi, F. A metabolomic approach to the study of wine micro-oxygenation. *PloS one*, 7(5) (2012) e37783.

Asen S., Stewart R.N., Norris K.H. Co-pigmentation of anthocyanins in plant tissue and its effect on color. *Phytochemistry* 11 (1972) 1139-1144

Audsley E., Alber S., Gemeinschaften E. (Eds.) (1997) Harmonisation of environmental life cycle assessment for agriculture. European Comm., DG VI *Agriculture*, pp. 139.

Aziz A., Poinssot B., Daire X., Adrian M., Bézier A., Lambert B., Joubert J.M., Pugin A. Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Molecular Plant-Microbe Interactions* 16, 12 (2003) 1118-1128

Bahar E., Alain Carbonneau A., Korkutal I. The effect of extreme water stress on leaf drying limits and possibilities of recovering in three grapevine (*Vitis vinifera* L.) cultivars. *African Journal of Agricultural Research* 6, 5 (2011) 1151-1160

Barata A., Malfeito-Ferreira M., Loureiro V. The microbial ecology of wine grape berries. *International Journal of Food Microbiology*, 153 (2012) 243-259

Bavaresco L., Fregoni M., Trevisan M., Mattivi F., Vrhovsek U., Falchetti R. The occurrence of the stilbene piceatannol in grapes. *Vitis* 41, 3 (2002) 133-136

Bavaresco L., Petegolli D., Cantu E., Fregoni M., Chiusa G., Trevisan M. Elicitation and accumulation of stilbene phytoalex- ins in grapevine berries infected by *B. cinerea*. *Vitis* 36, (1997) 77-83

Bavaresco L., Pezzuto S., Gatti M., Mattivi F. Role of the variety and some environmental factors on grape stilbenes. *Vitis* 46, 2 (2007) 57-61

Bavaresco M., Gatti S., Pezzutto M., Fregoni M., Mattivi F. Effect of leaf removal on grape yield, berry composition, and stilbene concentration. *American J. of Enology and Viticulture* 59,3 (2008) 292-298

Beever E.R., Weeds P.L. 2007. Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In: *Botrytis*: Biology, Pathology and Control. Y. Elad (Ed.). Springer: 29-52

Bell S-J.. Robson A. Effect of nitrogen fertilization on growth, canopy density and yield of *Vitis vin,fera* L. cv. Cabernet Sauvignon. *American Journal of Enology and Viticulture* 50, 3 (1999) 351-358

Belvini P., Pascarella G., Dalla Costa L., Sivilotti P. Con interventi al verde su vite aumenta l'efficienza delle foglie. *Informatore Agrario* 66, 17 (2010) 46-50

Benjamini, Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B. Met.,* 57 1995) 289–300

Bennett, R.N. Wallsgrove R.M Tansley Review No. 72. Secondary metabolites in plant defence mechanisms. *New Phytologist* (1994) 617-633

Bergqvist J., Dokoozlian N., Ebisuda N. Sunlight exposure and temperature effects on berry growth and composition of Cabernet Sauvignon and Grenache in the Central San Joaquin Valley of California. *American Journal of Enology and Viticulture* 52, 1 (2001) 1-6

Bertelli A., lberto A., Dipak K. Grapes, wines, resveratrol and heart health. Invited review article. *Journal of Cardiovascular Pharmacology* 54, 6 (2009) 468-476

Bigot G., Ostan M., Sandra M., Degano F., Chiavoni A., Paladin M., Fabbro A., Malossini G., Colussi G., Sivilotti P. Controllo della *Botrytis* cinerea su Pinot Grigio: effetto della sfogliatura e dell'acido gibberellico. *Atti Giornate Fitopatologiche* 2 (2008) 353-359

Bino R.J., Hall R.D., Fiehn O., Kopka J., Saito K., Draper J. Potential of metabolomics as a functional genomics tool. *Trends in Plant Science* 9 (2004) 418-425

Bledsoe A. M., Kliewer W. M., Marois J. J. Effects of timing and severity of leaf removal on yield and fruit composition of Sauvignon Blanc grapevines. *American Journal of Enology and Viticulture* 39 (1988) 49-54

Bonnardot V., Planchon O., Carey V.A. Diurnal wind relative humidity and temperature variation in the Stellenbosch-Groot Drakenstein wine growing area. *South African Journal of Enology and Viticulture* 23, 2 (2002) 62-71

Boss P.K., Davies C., Robinson S.P. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for the pathway regulation. *Plant Phisiology* 111 (1996) 1059-1066

Boulton R. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture* 52, 2 (2001) 67-87

Bourgaud F., Gravot A., Milesi S., Gontier E. Production of plant secondary metabolites: a historical perspective. Review. *Plant Science* 161, 5 (2001) 839-851

Braidot E., Zancani M., Petrussa E., Peresson C., Bertolini A., Patui S., Macrì F., Vianello A. Transport and accumulation of flavonoids in grapevine (*Vitis vinifera* L.). Rewiev. *Plant Signaling Behavior* 3, 9 (2008) 626-632

Broome J.C., English J.T., Marois J.J., Lattore B.A., Aviles J.C. Development of an infection model for *Botrytis* bunch rot of grape based on wetness duration and temperature. *Phytopathology* 85 (1995) 97-102

Bulit J., Dubos B. *Botrytis* bunch rot and blight. 1989. In: Compendium of Grape Diseases.. American Phytopathological Society: pp 13-14

Bundy J.G., Davey M.P., Viant M.R. Environmental metabolomics: a critical review and future. *Metabolomics* 5 (2009) 3-21

Candolfi-Vasconcelos M. C., Koblet W., Howell G. S., Zweifel W. Influence of defoliation, rootstock, training system, and leaf position on gas exchange of Pinot Noir grapevines. *American Journal of Enology and Viticulture* 45, 2 (1994) 173-180

Cantos E., Espin J.C., Tomas-Barberan F.A. Postharvest induction modeling method using UV irradiation pulses for obtaining resveratrol-enriched table grapes: A new "functional" fruit? *Journal of Agricultural and Food Chemistry* 49 ,10 (2001) 5052-5058

Carbonneau A. (2001) Concepts 'terroir'. In: Groupe Europe´ en d'E´ tude des Syste` mes de Conduite de la Vigne. (GESCO) (Ed.) Proc. XIIe`mes Journe´es du GESCO 3 – 7 July, Montpellier, France, 2: pp 669

Carey V., Barbeau G., Cadot Y., Conradie W.J., Vermeulen A. 2004. The implication of terroir for development of *Botrytis cinerea* into Gray Rot or Noble Rot on grapes of *V. Vinifera* L. cv. Chenin Blanc. OIV Congress, Vienna: 4-10

Castañeda-Ovando A., Pacheco-Hernández L., Páez-Hernández E., Rodríguez J.A., Galán-Vidal C.A. Chemical studies of anthocyanins: A review. *Food Chemistry* 113, 4 (2009) 859-871

Castellarin S.D., Matthews M.A., Gaspero G.D., Gambetta G. A. Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. *Planta* 227, 1 (2007) 101-112

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

Castillo-Muoz N., Fernndez-Gonzlez M., Gmez-Alonso S., Garca-Romero E., Hermosn-Gutirrez I. Red-color related phenolic composition of Garnacha Tintorera (*Vitis vinifera* L.) grapes and red wines. *Journal of Agricultural and Food Chemistry* 57, 17 (2009) 7883-7891

Cevallos-Cevallos J.M., Reyes-De-Corcuera J.I., Etxeberriaa E., Danyluk M.D., Rodrick G.E. Metabolomic analyses in food science. *Trends in Food Science & Technology* 20 (2009) 557-566

Chapman D.M., Matthews M.A., Guinard J.X. Sensory attributes of Cabernet Sauvignon wines made from vines with different crop yields. *American Journal of Enology and Viticulture* 55, 4 (2004) 325-334

Cheynier V. Polyphenols in foods are more complex than often thought. *American Journal of Clinical Nutrition*, 81 (2005) 223-229

Cheynier V., Dueñas-Paton M., Salas E., Maury C., Souquet J.M., Sarni-Manchado P., Fulcrand H. Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture* 57 (2006) 298-305

Cheynier V., Rigaud J. HPLC separation and characterization of flavonols in the skins of *Vitis vinifera* var. Cinsault. *American Journal of Enology and Viticulture* 37 (1996) 248-252.

Chorti E., Guidoni S., Ferrandino A., Novello V. Effect of Different Cluster Sunlight Exposure Levels on Ripening and Anthocyanin Accumulation in Nebbiolo Grapes. *American Journal of Enology and Viticulture* 61, 1 (2010) 23-30

CIE, Colorimetry (1986) Publication No. 15.2 (2nd ed.), Central Bureau of the CIE, Vienna, Austria

Coertze S., Holz G., Sadie A. Germination and establishment of infection on grape berries by single airborne conidia of *Botrytis cinerea*. *Plant Disease* 85 (2001) 668-677

Cohen S., Tarara J.M., Kennedy J.A. Assessing the impact of temperature on grape phenolic metabolism. *Analitica Chimica Acta* 621 (2008) 57-67

Commission Regulation Determining Community Methods for the Analysis of Wines (Reg. 2676/ 90). EEC (1990) Brussels: Commission of the European Communities.

Conde C., Silva P., Fontes N., Dias A.C.P., Tavares R.M., Sousa M.J., Agasse A., Delrot S., Gerós H. 2007. Biochemical changes throughout grape berry development and fruit and wine quality In: "Food". Global Science Books: 1-22

Coombe B. G. Influence of temperature on composition and quality of grapes. *Acta Horticulturae* 206 (1987) 23-35

Coombe B.G. Research on development and ripening of the grape berry. *American Journal of Enology and viticulture* 43, 1 (1992) 101-110

Coombe B.G. The grape berry as a sink. *Acta Horticulturae* 239 (1989) 149-158

Coombe B.G., Hale C.R. The hormone content of ripening grape berries and the effects of growth substance treatments. *Plant Physiology* 51 (1973) 629-634

Corino L., Calo A. Sustainable viticulture: current practices and future developments. Review paper. *Agriculturae Conspectus Scientificus* 66, 1 (2001) 3-11

Cortell J.M., Halbleib M., Gallagher A.V., Righetti T.L., Kennedy J.A. Influence of vine vigor on grape (*Vitis vinifera* L. cv. Pinot Noir) anthocyanins. 1. Anthocyanin concentration and composition in fruits. *Journal of Agricultural and Food Chemistry* 55,16 (2007) 6575-6584

Cortell J.M., Kennedy J.A. Effect of shading on accumulation of flavonoid compounds in (*Vitis vinifera* L.) Pinot Noir fruit and extraction in a model system. *Journal of Agricultural and Food Chemistry* 54, (2006) 8510-8520

Creasy G.L., Creasy L.L. 2009. Grapes (Crop production and Science in Horticulture series 16). MPG Books Group: 29-64

Croteau R., Kutchan T.M., Lewis N.G. 2000. Natural Products (Secondary Metabolites). American Society of Plant Physiologists. In: Biochemistry & Molecular Biology of Plants. Buchanan B., Gruissern W., Jones R. (Ed.) ASPB: 1250-1318

Crozier A., Jaganath I.B., Clifford M.N. 2006. Phenols, polyphenols and tannins. In: Plant secondary metabolites: occurance, structure and role in the human diet. Crozier A., Clifford M.N., Ashihara H. (Ed.). Blackwell Publishing Ltd.: 02-18

Dan D.H., Lee C.H. The effects of GA3, CPPU and ABA applications on the quality of Kyoho (*Vitis vinifera* L. x *lambrusca* L.) grape. *Acta Horticoltura* 653 (2004) 193-197

de Andrés-de Prado R., Yuste-Rojas M., Sort X., Andrés-Lacueva C., Torres M., Lamuela-Raventós R.M. Effect of soil type on wines produced from *Vitis vinifera* L. cv. Grenache in commercial vineyards. *Journal of Agricultural and Food Chemistry* 55, 3 (2007) 779-786

Deikman J., Hammer P.E. Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiology* 108 (1995) 47-57

Delgado R., Martín P., del Álamo M., González M-R. Changes in the phenolic composition of grape berries during ripening in relation to vineyard nitrogen and potassium fertilisation rates. *Journal of the Science of Food and Agriculture* 84, 7 (2004) 623-630

Deloire A., Vaudou E., Carey V., Bonnapart V., van Leeuwen C. Grapevine responses to terroir, a global approach. *Journal International des Sciences de la Vigne et du Vin* 39, 4 (2005) 149-162

Dettmer K., Hammock B.D. Metabolomics - a new exciting field within the "omics" sciences. *Environmental Health Perspectives* 112, 7 (2004) A396-A397

Dewick, P.M. The biosynthesis of C5–C25 terpenoid compounds. *Natural product reports* 19, 2 (2002) 181-222

Di Stefano R., Cravero M.C., Gentilini N. 1989. Methods for the study of wine polyphenols. L'Enotecnico, 5: 83-89

Diago M.P., Ayestara B., Guadalupe Z., Garrido A., Tardaguila J. Phenolic composition of Tempranillo wines following early defoliation of the vines. *Journal of the science of food and agriculture* 92 (2012) 925–934

Diago M.P., Vilanova M., Blanco J. A., Tardaguila J. Effects of mechanical thinning on fruit composition and sensory attributes of Grenache and Tempranillo varieties (*Vitis vinifera* L.). *Australian Journal of Grape and Wine Research*, 16, 2 (2010a) 314-326.

Diago M.P., Vilanova M., Tardaguila J. Effects of timing of manual and mechanical early defoliation on the aroma of *Vitis vinifera* L. Tempranillo wine. *American Journal of Enology and Viticulture* 61, 3 (2010b) 382-391

Dick R.P. A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. *Agriculture, Ecosystems & Environment* 40, 1-4 (1992) 25-36

Dick W. A., Blevins R. L., Frye W. W., Peters S. E., Christenson D. R., Pierce F. J., Vitosh M. L. Impacts of agricultural management practices on C sequestration in forest-derived soils of the eastern Corn Belt. *Soil and Tillage Research* 47, 3-4 (1998) 235-244

Dixon R.A. Phytochemistry in the genomic and post-genomic eras. *Phytochemistry* 57 (2001) 145-148

Dokoozlian N.K., Kliewer W.M. Influence of light on grape berry growth and composition varies during fruit development. *J. of the American Society for Horticultural Science* 121, 5 (1996) 869-874

Downey M.O., Dokoozlian N.K., Krstic M.P. Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: A review of recent research. *American Journal of Enology and Viticulture* 57, 3 (2006) 257-268

Downey M.O., Harvey J.S. Robinson S.P. 2004a Flavonol accumulation and expression of a gene encoding flavonol synthase demonstrates light sensitivity of flavonol biosynthesis in grapevines. In: Proceedings of the XXII International Conference on Polyphenols. University of Helsinki: Helsinki: 59-60

Downey M.O., Harvey J.S., Robinson S.P. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Australian Journal of Grape and Wine Research* 9 (2003a) 15-27

Downey M.O., Harvey J.S., Robinson S.P. Synthesis of flavonols and expression of flavonol synthase genes in developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Australian Journal of Grape and Wine Research* 9 (2003b) 110-121

Downey M.O., Harvey J.S., Robinson S.P. The effect of bunch shading on berry development and flavonol accumulation in Shiraz grapes. *Australian Journal of Grape and Wine Research* 10, 1 (2004b) 55-73

Dry P. 2009. Bunch exposure managemnet. In: Technical booklet, Grape and wine research and development coorporation, Adelaide (GWDC): 2-45

Dry P. Canopy management for fruitfulness. *Australian Journal of Grape and Wine Research* 6, 2 (2000) 109-115

Du Preez I. F., 2002. Infection pathways of *Botrytis cinerea* on selected wine grape cultivars. MscAgric thesis, Stellenbosch University, South Africa.

Dunn W. B., Bailey N. J., Johnson H. E. Measuring the metabolome: current analytical technologies. *Analyst* 130 (2005) 606-625

Easterwood G.W. Calcium's role in plant nutrition. *Fluid Journal*, 10, 4 (2002) 1-3

Edelmann A., Lendl B. Toward the optical tongue: flow-through sensing of tannin−protein interactions based on FTIR spectroscopy. *Journal of the American Chemical Society* 124, 49 (2002) 14741-14747

Eiro M.J., Heinonen M. Anthocyanin color behavior and stability during Storage: Effect of intermolecular copigmentation. *Journal of Agricultural and Food Chemistry* 50, 25 (2002) 7461-7466

Elad Y., Stewart A. 2004. Microbial control of *Botrytis spp*. In: *Botrytis*: Biology, Pathology and Control.

Elad Y., Williamson B., Tudzynski P., Delan N., (Eds.).Dordrecht, The Netherlands: Kluwer Academic 223-241

Ellis M., Doohan D., Bordelon B., Welty C., Williams R., Funt R., Brown M. 2004. Midwest small fruit pest management handbook. Ohio State University Extension: 133-134

Elmer P. A. G., Reglinski T. Biosuppression of *Botrytis cinerea* in grapes. Rewiev. *Plant Pathology* 55 (2006) 1-9

Elmer P.A.G., Michailides T.M. 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. In: Elad Y, Williamson B, Tudzynski P., Delan N. (Eds.) *Botrytis*: Biology, Pathology and Control. Dordrecht, The Netherlands: Kluwer Academic: 243-272

Emmett R.W., Harris A. R., Taylor, R. H., McGechan, J. K. 1992. Grape diseases and vineyard protection. In Viticulture Vol. 2. Practices Coombe B.G. and Dry P.R. (Eds.). Adelaide: Winetitles Australia: 232-278

English J. T., Thomas C. S., Marois J. J., Gubler W. D. Microclimates of grapevine canopies associated with leaf removal and control of *Botrytis* bunch rot. *Phytopathology* 79 (1989) 395-401

English T., Bledsoe A.M., Marois J.J., Kliewer W.M. Influence of grapevine canopy - management on evaporative potential in the fruit zone . American Journal of Enology and Viticulture 41, 4 (1990) 137-141

Escribano-Bailón M.T., Guerra M.T., Rivas-Gonzalo J.C., Santos-Buelga C. Proanthocyanidins in skins from different grape varieties. *Zeitschrift fur Lebensmittel- Untersuchung und -Forschung* 200, 3 (1995) 221-224

Esteban M.A., Villanueva M.J., Lissarrague J.R. Effect of irrigation on changes in the anthocyanin composition of the skin of cv Tempranillo (*Vitis vinifera* L) grape berries during ripening. *Journal of the Science of Food and Agriculture* 81, 4 (2001) 409-420

Failla O. 2010. The agronomic challenges of sustainable viticulture Enovitis in campo: environment protection and multi-functionality the new drivers that lead innovation in the vineyard In: Enovitis Conference Proceedings: 1-5

Fiehn O. Metabolomics – the link between genotypes and phenotypes. *Plant Molecular Biology* 48 (2002) 155-171

Fleet G.H. 1998. Microbiology of alcoholic beverages. In: Microbiology of fermented foods, Vol. 1 ed. Wood B.J. London: Blackie Academic & Professional: 217-262

Fleet G.H. 2001. Wine. In: Food microbiology fundamentals and frontiers. Doyle M.P., Beuchat L.R.,Montville T.J. (Ed.), Washington DC, ASM press: 747-772

Fleet G.H. Microorganisms in food ecosystems. *International Journal of Food Microbiology* 50 (1999) 101-117

Fleet G.H. Prakitchaiwattana C. Beh, A.L., Heard G. 2002. The yeast ecology of wine grapes. In Biodiversity and biotechnology of wine yeasts. Ciani, M (Ed.). India: Research Signpost: 1-17

Fleet G.H. Yeast interaction and wine flavour. *International Journal of Food Microbiology* 86 (2003) 11-22

Fleet G.H., Heard G.M. 1993. Yeast-Growth during fermentation. In: Wine microbiology and biotechnology. Fleet, G.H. (Ed.) Chur, Switzerland: Harwood Academic Publisher: 27-54

Flint S.D., Jordan P.W., Caldwell M.M. Plant protective response to enhanced UV-B radiation under field conditions: leaf optical properties and photosynthesis. *Photochemistry and Photobiology* 41 (1986) 95-99

Frazer G.W., Canham C.D., Lertzman K.P. 1999. Gap Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation. Copyright © 1999: Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York.

Freeman B. M., Kliewer W. M., Stern P. Influence of Windbreaks and Climatic Region on Diurnal Fluctuation of Leaf Water Potential, Stomatal Conductance, and Leaf Temperature of Grapevines. *American Journal of Enology and Viticulure* 33, 4 (1982) 233-236

Fugelsang K.C. 1997. Wine Microbiology. Fuglesang K.C. (Ed.) Springer, New York: pp 245

Fugelsang K.C., Edwards C.G. (2007) Wine Microbiology, in: Practical Applications and Procedures. Springer Science+Business Media, LLC, New York (2nd edition), pp. 393.

Gabler F. M., Fassel R., Mercier J., Smilanick J. L. Influence of temperature, inoculation interval, and dosage on biofumigation with Muscodor albus to control postharvest gray mold on grapes. *Plant Disease* 90, 8 (2006) 1019-1025

Gatti M., Bernizzoni F., Civardi S., Poni, S. Effects of cluster thinning and preflowering leaf removal on growth and grape composition in cv. Sangiovese. *American Journal of Enology and Viticulture*, 63, 3 (2012) 325-332

Gatto P., Vrhovsek U., Muth J., Segala C., Romualdi C., Fontana P., Pruefer D., Stefanini M., Moser C., Mattivi F., Velasco R. Ripening and Genotype Control Stilbene Accumulation in Healthy Grapes. *Journal of Agricultural and Food Chemistry* 56, 24 (2008) 11773-11785

Gladstone J. 1992. Viticulture and Environment. Adelaide Weintitles: pp 310

Gliessman S.R. 1998. Agroecology: ecological processes in sustainable agriculture. Ann Arbor Press, Chelsea, Michigan, USA: pp 357

Glithero N.J., Ramsden S.J., Wilson P. Farm systems assessment of bioenergy feedstock production: Integrating bio-economic models and life cycle analysis approaches, *Agricultural Systems*, 109 (2012) 53-64

Gonzales-Neves G., Gil G., Ferrer M. Effect of different vineyard treatments on the phenolic contents in Tannat (*Vitis vinifera* L.) grapes and their respective wines. *Food Science and Technology International* 8 (2002) 315-321

Goodacre R., York E. V., Heald J. K., Scott I. M. Chemometric discrimination of unfractionated plant extracts analyzed by electrospray mass spectrometry. *Phytochemistry* 62 (2003) 859-863

Goto-Yamamoto N., Mori K., Numata M., Kazuya Koyama K., Kitayama M. Effects of temperature and water regime on flavonoid content and composition in the skin of red-wine grapes. Journal *International des Sciences de la Vigne et du Vin, special issue Macrowine* (2010) 75-80 E

Gould K.S., Lister C. 2006. Flavonoid functions in plants. In: Flavonoids: chemistry, biochemistry and applications. Andresen M., Markham K.R. (Eds.). CRC Taylor & Francis: Boca Raton: 397-441

Gubler W. D., Bettiga L. J., Heil D. Comparisons of hand and machine leaf removal for the control of *Botrytis* bunch rot. *American Journal of Enology and Viticulture* 42 (1991) 22-36

Gubler W. D., Marois J. J., Bledsoe A. M., Bettiga L. J. Control of *Botrytis* bunch rot of grape with canopy management. *Plant Disease* 71 (1987) 599-601

Guetsky R., Shtienberg D., Dinnor A. Combining biocontrol agents to reduce the variability of biological control. *Biological control* 91, 7 (2001) 621-627

Guidoni S., Allara P., Schubert A. Effect of cluster thinning on berry skin anthocyanin composition of *Vitis vinifera* cv. Nebbiolo. *American Journal of Enology and Viticulture* 53, 3 (2002) 224-226

Guidoni S., Ferrandino A., Novello V. Effect of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. *American Journal of Enology and Viticulture* 59 (2008) 22-29

Guy C., Kaplan F., Kopka J., Selbig J., Hincha D. K. Metabolomics of temperature stress. *Physiologia Plantarum* 132 (2008) 220-235

Hall R., Beale M., Fiehn O., Hardy N., Sumner L., Bino R. Plant Metabolomics: The missing link in functional genomics strategies. *Plant Cell* 14 (2002) 1437-1440

Halliwell B., Rafter J., Jenner A. Dietary polyphenols and health: Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *American Journal of Clinical Nutrition* 81,1 (2005) 268-276

Harbertson J.F., Picciotto E.A., Douglas O. Measurement of polymeric pigments in grape berry extract sand wines using a protein precipitation assay combined with bisulfite bleaching. *American Journal of Enology and Viticulture* 2003 54, 4 (2003) 301-306

Harbertson J.F., Spayd S. Measuring phenolics in the winery. *American Journal of Enology and Viticulture* 57, 3 (2006) 280-288

Haselgrove L., Botting D., Van Heeswijck R., Hoj P.B., Dry C., Ford C., Iland P.G. Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of *Vitis vinifera* L. Cv. Shiraz grape berries. *Australian Journal of Grape and Wine Research* 6, (2000) 141-149

Hed B., Ngugi H. K., Travis J. W. Relationship between cluster compactness and bunch rot in Vignoles grapes. *Plant Disease* 93 (2009) 1195-1201

Hennebert G.L. 1973. *Botrytis* and *Botrytis* like genera. Persoonia 7, 183-204

Howell G. S. Sustainable grape productivity and the growth-yield relationship: A Review. *American Journal of Enology and Viticulture* 52, 3 (2001) 165-174

Ingels C. Sustainable agriculture and grape production. *American Journal of Enology and Viticulture* 43, 3 (1992) 296-298

Intrieri C., Filippetti G., Allegro M., Centinari M., Poni S. Early defoliation (hand vs mechanical) for improved crop control and grape composition in Sangiovese (*Vitis vinifera* L.). *Australian Journal of Grape and Wine Research* 14, (2008) 25-32

Intrieri C., Silvestroni O., Rebucci B., Poni S., Filippetti I. 1996. The effects of row orientation on growth yield, quality and dry matter partitioning in Chardonnay vines trained to simple curtain and spur-pruned cordon. In: Proceedings of the 4th International Cool Climate Viticulture & Enology Symposium. Rochester, NY, USA: 10-15

Iriti M., Faoro F. Grape phytochemicals: a bouquet of old and new nutraceuticals for human health. *Medical Hypotheses* 67 (2006) 833-838

Iwashina, T. The structure and distribution of the flavonoids in plants. *Journal of Plant Research*, 113 (2000) 287−299.

Jackson D. I., Lombard P. B. Environmental and management practices affecting grape composition and wine quality - A Review. *American Journal of Enology and Viticulture* 44, 4 (1993) 409-430

Jackson R.S. 1994. Grape species and varieties. In Wine science: Principles and applications. Taylor S.L. (Ed.) Academic Press. USA: 13-41

Jeandet P., Bessis R. and Gautheron B. The production of resveratrol (3,5,4'- trihydroxystilbene) by grape berries in different developmental stages. *American Journal of Enology and Viticulture* 42, 1 (1991) 41-46

Jeandet P., Douill-Breuil A.C., Bessis R., Debord S., Sbaghi M., Adrian M. Phytoalexins from the vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and mabolism. *Journal of Agricultural and Food Chemistry* 50,10 (2002) 2731-2741

Jeffery D.W., Parker M., Smith P.A. Flavonol composition of Australian red and white wines determined by high-performance liquid chromatography. *Australian Journal of Grape and Wine Research* 14, 3 (2008) 153–161

Jeong S. T., Goto-Yamamoto N., Kobayashi S., Esaka, M. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Science* 167, 2 (2004) 247-252

Jeong S. T., Yamamoto N. G., Hashizume K., Esaka M. Expression of multi-copy flavonoid pathway genes coincides with anthocyanin, flavonol and flavan-3-ol accumulation of grapevine. *Vitis* 47, 3 (2008) 135-140

Jones G.V., White M.A., Cooper O.R., Storchmann K. Climate change and global wine quality. *Climatic Change* 73 (2005) 319-343

Kassemeyer H-H., Berkelmann-Lohnertz B. 2009. Fungi of Grapes. In: Biology of Microorganisms on Grapes, in Must and in Wine, Konig et al. (Ed.). Springer-Verlag Berlin Heidelberg: 61-88

Keller M. 2010. The science of Grapevines: Anatomy and Physiology. Elsevier Academic Press Inc.: pp. 377

Keller M., Mills L.J., Wample R.L., Spayd S.E. Cluster thinning effects on three deficitirrigated *Vitis vinifera* cultivars. *American Journal of Enology and Viticulture* 56, 2 (2005) 91-103

Kennedy J.A. Matthews M.A., Waterhouse A.L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry* 55, 1 (2000) 77-85

Kennedy J.A., Saucier C., Glories Y. Grape and wine phenolics: History and perspective. *American Journal of Enology and Viticulture* 57, 3 (2006) 239-248

Kitano H. Systems biology: a brief overview. *Science* 295 (2002) 1662-1664

Kliebenstein D.J. Secondary metabolites and plant/environment interactions: a view through Arabidopsis thaliana tinged glasses. *Plant, Cell & Environment* 27, 6 (2004) 675-684

Kliebenstein D.J., Rowe H.C., Denby K.J. Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant Journal* 44, 1 (2005) 25-36

Kliewer W.M. Effect of day temperature and light intensity on coloration of *Vitis vinifera* L. grapes. *Journal of American Society of Horticulture Science* 95 (1970) 693-697

Kliewer W.M., Torres R.E. Effect of controlled day and night temperatures on grape coloration. *American Journal of Enology and Viticulture* 23, 1 (1972) 71-76

Koblet W., Candolfi-Vasconcelos M.C., Zweifel W., Howell S. Influence of leaf removal on rootstock and training system on yield and fruit composition of Pinot noir grapevines. *American Journal of Enology and Viticulture* 45, 2 (1994) 181-187

Koblet, W. "Translocation of photosynthate in vine shoots and influence of leaf area on quantity and quality of the grapes." *Wein-Wiss*. 24 (1969) 277-319

Kobriger J. M., Kliewer W. M., Lagier S. T. Effects of wind on water relations of several grapevine cultivars. *American Journal of Enology and Viticulture* 35, 3 (1984) 164-169

Komárek M., Cadková E., Chrastný V., Bordas F., Bollinger J.C. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environment International* 36, 1 (2010) 138-151

Kopka J., Schauer N., Krueger S., Birkemeyer C., Usadel B., Bergmuller E. GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics* 21 (2005) 1635-1638

Koundouras S., Hatzidimitriou E., Karamolegkou M., Dimopoulou E., Kallithraka S., Tsialtas J.T., Zioziou E., Nikolaou N., Kotseridis Y. Irrigation and rootstock effects on the phenolic concentration and aroma potential of *Vitis vinifera* L. cv. Cabernet Sauvignon grapes. *Journal of Agricultural and Food Chemistry* 57, 17 (2009) 7805-7813

Koundouras S., Tsialtas J.T., Zioziou E., Nikolaou N. Rootstock effects on the adaptive strategies of grapevine (*Vitis vinifera* L. cv. Cabernet Sauvignon) under contrasting water status: Leaf physiological and structural responses. *Agriculture, Ecosystems and Environment* 128 (2008) 86-96

Kriedemann P.E., Kliewer W.M., Harris J.M. "Leaf age and photosynthesis in *Vitis vinifera* L." *Vitis* 9 (1970) 97-104

Kula H., Kokta C. Side effects of selected pesticides on earthworms under laboratory and field conditions. *Soil Biology and Biochemistry* 24, 12 (1992) 1711-1714

Kunkee R.E., Bisson L.F. 1993. Wine-making yeasts. In: The yeasts: 69-127

Kurooka H., Fukunaga S. "Effect of carbon dioxide enrichment on vine growth and berry quality of Kyoho grapes." *Journal of Japanese Society for Horticultural Science* 59 (1990) 463-470

Landers A.J. 2008 Sustainable plant protection within sustainable fruit growing - an American experience. Agricultural Engineering International: the CIGR Ejournal. Manuscript ALNARP 08 008. Vol. X. May, 2008:10

Landrault N., Larronde F., Delaunay J-C., Castagnino C., Vercauteren J., Merillon J-M., Gasc F., Cros G., Teissedre P-L. Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development. *Journal of Agricultural and Food Chemistry* 50,7 (2002) 2046-2052

Larcher R., Nicolini G., Villegas R., Bertoldi,D., Puecher C.. Determination of gluconic acid in wine using high pressure liquid chromatography with pulsed amperometric detection. *Vitis,* 48 (2009) 201-204

Laville P.1993. Le terroir, un concept indispensable al'elaboration et á la protection des appellations d'origine comme a la gestation des vignobles: le cas de la France In: Le Bulletin de l'OIV 2009

Lee C.Y., Jaworski A.W. Identification of some phenolics in white grapes. *American Journal of Enology and Viticulture* 41, 1 (1990) 87-89

Lee J., Schreiner R.P. Free amino acid profiles from 'Pinot noir' grapes are influenced by vine N-status and sample preparation method. *Food Chemistry* 119 (2010) 484–489

Lee, J-E., Hwang G-S., Van Den Berg F., , Cherl-Ho Lee C-H., Hong Y-S. Evidence of vintage effects on grape wines using 1H NMR-based metabolomic study. *Analytica Chimica Acta* 648, 1 (2009) 71-76

Leroux P., Gredt M., Leroch M., Walker A-S. Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Applied Environmental Microbiology* 76 (2010) 6615-6630

Lindow S.E., Brandl M.T. Minireview: Microbiology of the phyllosphere. *Applied Environmental Microbiology* 69, 4 (2003) 1875-1883

Longo J., Cangado J., Agrelo D., Villa T.G. Effect of climatic conditions on yeast diversity in grape must from north west Spain. *American Journal of Enology and Viticulture* 42, 2 (1991) 141-144

López-Rituerto E., Savorani F., Avenoza A., Busto J. H., Peregrina J. M., Engelsen S.B. Investigations of La Rioja terroir for wine production using 1H NMR metabolomics. *Journal of agricultural and food chemistry*, 60, 13 (2012) 3452-3461

Lorenz D.H., Eichhorn K.W., Bleiholder H., Klose R., Meier U., Weber E. Growth stages of the grapevine: phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. vinifera) - codes and descriptions according to the extended BBCH scale. *Australian. Journal of Grape and Wine Research,* 1 (1995) 100–103.

Lu Y., Foo L. The polyphenol constituents of grape pomace. *Food Chemistry* 65, 1 (1999) 1-8

Luis Cabrita L., Fossen T., Andersen Q.M. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry* 68 (2000) 101-107

Luthria D. L., Lin L. Z., Robbins R. J., Finley J. W., Banuelos G. S., Harnly, J. M. Discriminating between cultivars and treat- ments of broccoli using mass spectral fingerprinting and analysis of variance-principal component analysis. *Journal of Agricultural and Food Chemistry* 56, 21 (2008) 9819-9827

Macheix J.J., Fleuriet A., Billot J. 1990. Fruit Phenolics, CRC Press Inc., Boca Raton, FL: 16-33

Magali A., Grant D., Grant L.E. Eco-labeling strategies and price-premium: the wine industry puzzle. Business Society (2010) doi:10.1177/0007650310362254

Magarey P.A., Magarey R.D., Emmett R.W. 2000. Principles for managing the foliage diseases of grapevine with low input of pesticides. In: Proceedings 6th international congress on organic viticulture. Willer H., Meier U. (Eds.): 140-148

Main G.L., Morris J.R. Leaf-removal effects on Cynthiana yield, juice composition and wine composition. *American Journal of Enology and Viticulture* 55, 2 (2004) 147-152

Makris D.P., Kallithraka S., Kefalas P. Flavonols in grapes, grape products and wines: Burden, profile and influential parameters. *Journal of Food Composition and Analysis* 19 (2006) 396-404

Mamelink J. Tailoring the plant metabolome without a loose stitch. *Trends in plant science* 7 (2004) 305-307

Mapelli V., Olsson L., Nielsen J. Metabolic footprinting in microbiology: methods and applications in functional genomics and biotechnology. Review. *Trends in Biotechnology* 26, 9 (2008) 490-497

Marais J., Hunter J.J., Haasbrock P.D. Effect of canopy microclimate, season and region on Sauvignon Blanc grape composition and wine quality. *South African Journal of Enology and Viticulture* 20, 1 (1999) 19-30

Martens S., Mithöfer, A. Flavones and flavone synthases. *Phytochemistry* 66 (2005) 2399–2407

Mateus N., de Freitas V. Anhocyanins as food colorants. Biomedical and Life Sciences. (2009), doi 10.1007/978-0-387-77335-3_9: 284-304

Mateus N., Marques S., Gonçalves A.C., Machado J.M., de Freitas V. Proanthocyanidin composition of red Vitis vinifera varieties from the Duoro valley during ripening: Influence of cultivation altitude. *American Journal of Enology and Viticulure* 52 (2001) 115-121

Mattivi F., Guzzon R., Vrhovsek U., Stefanini M., Velesco R. Metabolite profiling of grape: flavonols and anthocyanins. *Journal of Agricultural and Food Chemistry* 54, (2006) 7692-7702

Mattivi F., Reniero F., Korhammer S. Isolation, Characterization, and evolution in red wine vinification of resveratrol monomers. *Journal of Agricultural and Food Chemistry* 43 , 7 (1995) 1820-1823

Mattivi, F., Nicolini, G. Analysis of polyphenols and resveratrol in Italian wines. *BioFactors*, 6 (1997) 445 - 448

Mazza G. Anthocyanins in grapes and grape products. Critical Review. *Food Science and Nutrition* 35 (1995) 341-371

Mazza G., Fukumoto L., Delaquis P., Girard B., Ewert B. Anthocyanins, phenolics, and volor of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *Journal of Agricultural and Food Chemistry* 47, 10 (1999) 4009-4017

McDougall G., Martinussen I., Stewart D. Towards fruitful metabolomics: high throughput analyses of polyphenol composi- tion in berries using direct infusion mass spectrometry. *Journal of Chromatography and Analytical Technologies in the Biomedical and Life Sciences* 871 (2008) 362-369

Memelink Johan. The use of genetics to dissect plant secondary pathways. *Current opinion in plant biology,* 8,3 (2005) 230-235

Moco S., Bino R.J., De Vos R.C.H., Vervoort J. Metabolomics technologies and metabolite identification. *Trends in Analytical Chemistry* 26, 9 (2007) 855-866

Moco S., Forshed J., De Vos R. C. H., Bino R. J., Vervoort J. Intra- and inter-metabolite correlation spectroscopy of tomato metabolomics data obtained by liquid chromatography mass spectrometry and nuclear magnetic resonance. *Metabolomics* 4, 3 (2008) 202-215

Monagas M., Bartolomé B., Gómez-Cordovés C. Updated knowledge about the presence of phenolic compounds in wine. Critical Review. *Food Science and Nutrition* 45, 2 (2005) 85-118

Monagas M., Bartolomé, B. 2009. Anthocyanins and anthocyanin-derived compounds. In: Wine Chemistry and Biochemistry Eds. Moreno-Arribas., Polo. (Eds.). Springer, NY, USA: 437-462

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

Mori K., Sugaya S., Gemma H. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. Scientia Horticulturae 105, 3 (2005) 319-330

Mori K., Sugaya S., Gemma H. Regulatory mechanism of anthocyanin biosynthesis in 'Kyoho' berries grown under different temperature conditions. *Environmental Control in Biology* 42 (2004) 21-30

Morlat R., Bodin F. Characterization of viticultural terroirs using a simple field model based on soil depth – II. validation of the grape yield and berry quality in the Anjou vineyard (France). *Plant and Soil* 281, 1-2 (2001) 55-69

Morrissey J.P., Osbourn A.E. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiology and molecular biology Reviews. *American Society for Microbiology* (1999) 708-724

Mozetič B., Trebše P., Hribar J. Determination and quantitation of anthocyanins and hydroxycinnamic acids in different cultivars of sweet cherries (*Prunus avium* L.) from Nova Gorica region (Slovenia). *Food Technology and Biotechnology* 40, 3 (2006) 207-212

Mullins M.G., Bouquet A., Williams L.E. 1994. In: Biology of horticultural crops: Biology of the grape vine. Mullins M.G. (Ed.), Cambridge University Press: 189-201

Nair N.G., Allen R.N. Infection of grape flowers and berries by Botrytis cinerea as a function of time and temperature. *Mycological Research* 97, 8 (1993) 1012-1014

Nair N.G., Emmett R.W., Parker F. E. Some factors predisposing grape berries to infection by Botrytis cinerea. N. Z. J. *Experimental Agriculture* 16 (1988) 257-263

Nardini M., Forte M., Vrhovsek U., Mattivi F., Viola R., Scaccini C. White wine phenolics are absorbed and extensively metabolized in humans. *Journal of Agricultural and Food Chemistry* 57: (2009) 2711-2718

Nawaz H., Shi J., Gauri S. Mittal G.S., Kakuda Y. Extraction of polyphenols from grape seeds and concentration by ultrafiltration. *Separation and Purification Technology* 48, 2 (2006) 176-181

Nielsen J., Jewett M.C. 2007. The role of metabolomics in systems biology. In: Metabolomics. A powerful Tool in System Biology. Nielsen J., Jewett M.C (Eds), Springer, XVI, 284 pp

O.I.V – International Organisation of Vine and Wine (2008) Definition – Resolution CST 1-2004, In: Framework and Challenges for Organic wine production in the EU

Oikawa A., Matsuda F., Kusano M., Okazaki Y., Saito K. *Rice Metabolomics*. Rice 1 (2008) 63-71

Ojeda H., Andary C., Kraeva E., Carbonneau A., Deloire A. Influence of pre- and post-veraison water deficit on synthesis and concentration of skin phenolic compounds during berry growth of *Vitis vinifera* cv. Shiraz. American Journal of Enology and Viticulture 53, 4 (2002) 261-267

Oliver S.G. Systematic functional analysis of the yeast genome. *Trends in Biotechnology* 16 (1998) 373-378

Ollat N., Gaudillere J.P. The effect of limiting leaf area during stage I of berry growth on development and composition of berries of *Vitis vinifera* L. cv. Cabernet Sauvignon. *American Journal of Enology and Viticulture*, 49, 3 (1998) 251-258.

Ozden M., Vardin H., Simsek M., Karaaslan M. Effects of rootstocks and irrigation levels on grape quality of *Vitis vinifera* L. cv. Shiraz. *African Journal of Biotechnology* 9, 25 (2010) 3801-3807

Ozkan B., Fert C., Karadeniz C.F. Energy and cost analysis for greenhouse and open-field grape production. *Energy*, 32 (2007) 1500-1504

Pallmann C.L., Brown J.A., Olineka T.L., Cocolin L., Mills D.A., Bisson L.F. 2001. Use of WL Medium to Profile Native Flora Fermentations. *American Journal of Enology and Viticulture*, 5 (2001) 198-203

Pastor del Rio J., Kennedy J. A. Development of proanthocyanidins in Vitis vinifera L. cv. Pinot noir grapes and extraction into wine. *American J of Enology and Viticulure* 57 (2006) 125-131

Percival D.C., Fisher K.H., Sullivan J.A. Use of Fruit Zone Leaf Removal With Vitis vinifera L. cv. Riesling Grapevines. II. Effect on Fruit Composition, Yield, and Occurrence of Bunch Rot (*Botrytis cinerea* Pers.:Fr.). *American Journal of Enology and Viticulture* 45 (1994) 133-139

Pereira G.E., Gaudillere J.P., Pieri P., Hilbert G., Maucourt M., Deborde C., Moing, A., Rolin D. Microclimate influence on mineral and metabolic profiles of grape berries. *Journal of Agricultural and Food Chemistry* 54, 18 (2006) 6765-6775

Perez-Magariaeo S., Gonzalez-San Jose M.S. Effect of ripening stage of grapes on the low molecular weight phenolic compounds of red wines. *European Food Research and Technology* 220, 5-6 (2005) 597-606

Pezet R., Cuenat P. Resveratrol in wine: extraction from skin during fermentation and post-fermentation standing of must from Gamay grapes. *American Journal of Enology and Viticulture* 47, 3 (1996) 287-290

Pichersky E., Gang D. Genetics and biochemistry of secondary metabolites: an evolutionary perspective. *Trends in Plant Science* 5 (2000) 439-445

Pimente D. Environmental and economic costs of the application of pesticides primarily in the Unitad States? *Environment, Development and Sustainability* 7, 1 (2005) 229-252

Poni S., Bernizzoni F., Civardi S., Libelli N. Effects of pre-bloom leaf removal on growth of berry tissues and must composition in two red *Vitis vinifera* L. cultivars. *Australian Journal of Grape and Wine Research* 15, 2 (2009) 185-193

Poni S., Casalini L., Bernizzoni F., Civardi S., Intrieri C. Effects of early defoliation on shoot photosynthesis, yield components and grape composition. *American Journal of Enology and Viticulture* 57, 4 (2006) 397-407

Pretorius I.S. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16 (2000) 675-729

Pretorius I.S., Van der Westhuizen T.J., Augustyn O.P.H. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. A review. *South African Journal of Enology and Viticulture* 21, 2 (1999) 92–99

Price S.F., Breen P.J., Valladao M., Watson B.T. Cluster sun exposure and quercetin in Pinot noir grapes and wine. *American Journal of Enology and Viticulture* 46, (1995) 187-194

R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing. Vienna, Austria, 2013, http://www.R-project.org

Ramachandra Rao S., Ravishankar G. A. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology advances* 20, 2 (2002) 101-153

Ramautar R., Demirci A., Jong G. J. Capillary electrophoresis in metabolomics. *Trends in Analytical Chemistry* 25, 5(2006) 5-12

Ramos R., Andrade P. B., Seabra R. M., Pereira C., Ferreira M. A., Faia M. A. A preliminary study of non-coloured phenolics in wines of varietal white grapes (códega, gouveio and malvasia fina): effects of grape variety, grape maturation and technology of winemaking. *Food Chemistry* 67, 1 (1999)

Rapp A, Mandery H. Wine aroma. *Experientia* 42 (1986) 873-880

Reeve J. R., Carpenter-Boggs L., Reganold J. P., York A. L., McGourty G., McCloskey L.P. Soil and winegrape quality in biodynamically and organically managed vineyards. *American Journal of Enology and Viticulture* 56, 4 (2005) 367-376

Renouf V., Claisse O., Lonvaud-Funel A. Inventory and monitoring of wine microbial consortia. *Applied Microbiology and Biotechnology* 75 (2007) 149-164

Renouf V., Claisse O., Lonvaud-Funel A. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Australian Journal of Grape and Wine Research* 11, 3 (2005) 316-327

Renouf V., Strehaiano P., Lonvaud-Funel A. Yeast and bacteria analysis of grape, wine and cellar equipments by PCR-DGGE. *International Journal of Vine and Wine Sciences* 41, 1 (2007) 51-61

Renwick A.G. Pesticide residue analysis and its relationship to hazard characterisation (ADI/ARfD) and intake estimations (NEDI/NESTI) *Pest Management Science* 58, 10 (2002) 1073-1082

Revilla E., Garcia-Beneytez E., Cabello F. Anthocyanin fingerprint of clones of Tempranillo grapes and wines made with them. *Australian Journal of Grape and Wine Research,* 15,1 (2009) 70-78

Revilla, E., García-Beneytez E., López J.F., Cabello, F. Anthocyanin pattern of several red grape cultivars and single-cultivar young wines. *Acta Alimentaria,* 42,1 (2013) 23-35

Reynolds A.G., Schlosser J., Sorokowsky D., Roberts R., Willwerth J., De Savigny C. Magnitude of viticultural and enological effects. II. Relative impacts of cluster thinning and yeast strain on Composition and Sensory Attributes of Chardonnay Musqué. *American Journal of Enology and Viticulture* 58, 1 (2007) 25-41

Reynolds A.G., Senchuk I.V., van der Reest C., de Savigny C. Use of GPS and GIS for elucidation of the basis for terroir: spatial variation in an ontario Riesling vineyard. *American Journal of Enology and Viticulture* 58 (2007) 145-162

Reynolds A.G., Wardle D.A., Hall J.W., Dever M. Fruit maturation of four *Vitis vinifera* cultivars in response to vineyard location and basal leaf removal. *American Journal of Enology and Viticulture* 46 (1995) 542-558

Ribereau-Gayon P., Glories Y., Maujean A., Dubourdieu,D. 2006. Phenolic compounds. In: Handbook of enology, Vol 2: The chemistry of wine stabilization and treatments. Ribéreau-Gayon P. (Ed.). John Wiley & Sons Ltd. Chichester: 141-204

Rigo A., Vianello F., Clementi G., Rossetto M., Scarpa M., Vrhovsek U., Mattivi F. Contribution of the proanthocyanidins to the peroxy radical scavenging capacity of some Italian red wines. *Journal of Agricultural and Food Chemistry* 48, (2000) 1996-2002

Ristic R., Downey M., Iland P., Bindon K., Francis L., Herderich M., Robinson S. Exclusion of sunlight from Shiraz grapes alters wine color, tannin and sensory properties. *Australian Journal of Grape and Wine Research* 13 (2007) 10-39

Robbins R.J. Phenolic acids in foods: An overview of analytical methodology. *Journal of Agriculture and Food Chemistry* 51, 10 (2003) 2866-2887

Robins R.J., Bean, S.R. Development of a quantitative high-performance liquid chromatography–photodiode array detection measurement system for phenolic acids. *Journal of Chromatography A* 1038 (2004) 97-105

Roby G., Harbertson J. F., Adams D. A., Matthews M. A. Berry size and vine water deficits as factor in wine grape composition: anthocyanins and tannins. *Australian Journal of Grape and Wine Research* 10, 2 (2004) 100-107.

Rochfort S., Ezernieks V., Bastian S.E., Downey M.O. Sensory attributes of wine influenced by variety and berry shading discriminated by NMR metabolomics. *Food Chemistry*, 121, 4 (2010) 1296-1304

Rodriguez Montealegre R., Romero Peces R., Chacòn Vozmediano J.L., Martinez Guascueña J., Garcìa Romero E. Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis* 19 (2006) 687-693

Roessner U., Jairus Bowne J. What is metabolomics all about? *BioTechniques* 46 (2009) 363-365

Rosini G., Federici F., Martini A. Yeast flora of grape berries during ripening. *Microbial Ecology* 8, 1 (1982) 83-89

Rossetto M., Vanzani P., Zennaro L., Mattivi F., Vrhovsek U., Scarpa M, Rigo A., Stable free radicals and peroxyl radical trapping capacity in red wines. *Journal of Agricultural and Food Chemistry*, 52 (2004) 6151-6155

Rosslenbroich H.J., Stuebler D. *Botrytis cinerea* - history of chemical control and novel fungicides for its management. *Crop Protection* 19 (2000) 557-561

Rossouw D., Nas T., Bauer F. F. Linking gene regulation and the exo-metabolome: a comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. BMC *Genomics* 9, (2008) 530-545

Rothwell J.A., Urpi-Sarda M., Boto-Ordoñez M., Knox C., Llorach R., Eisner R., Cruz J., Neveu V., Wishart D., Manach C., Andres-Lacueva C., Scalbert A. (2012) Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. Database, doi: 10.1093/database/bas031.

Rudell D. R., Mattheis J. P., Curry F. A. Prestorage ultraviolet-white light irradation alters apple peel metabolome. *Journal of Agricultural and Food Chemistry* 56, 3 (2008) 1138-1147

Ryan D., Robards K. Metabolomics: The Greatest Omics of Them All? *Analytical Chemistry* 78 (2006) 7954-7958

Ryan M., Wilson D., Hepperly P., Travis J., Halbrendy N., Wise A. Compost tea potential is still brewing. *Biocycle* 46 (2005) 30-32

Sabate J., Cano J., Esteve-Zarzoso B., Guillamon J.M. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis ribosomal genes. Microbiological Research 157, 45 (2002) 267-274

Satisha J., Somkuwar R.G., Sharma J., Upadhyay A.K., Adsule P.G. Influence of rootstocks on growth yield and fruit composition of thompson seedless grapes grown in the pune region of India. *South African Journal of Enology and Viticulture* 31 1 (2010) 76-82

Saunders C. The anti-proliferative effect of different tomato varieties on the human colon adenocarcinoma cells. *Bioscience Horizont* 2, 2 (2009) 172-179

Savage S.D., Sall M.A. *Botrytis* bunch rot of grapes: Influence of trellis type and canopy microclimate. *Ecology and Epidemiology* 74, 1 (1984) 65-70

Schwalbe E., Mass H., Kenter M., Wagner S. Hemispheric image modeling and analysis techniques for solar radiation determination in forest ecosystems. *Photogrammetric Engineering and Remote Sensing* 75, 4 (2009) 375-384

Serra R., Peterson S.W. *Penicillium astrolabium* and *Penicillium neocrassum*, two new species isolated from grapes and their phylogenetic placement in the *P. olsonii and P. brevicompactum* clade. *Mycologia,* 99,1 (2007) 78-87

Shaulis N.J., Amberg H., Crowe D. Response of concord grapes to light exposure and Geneva Double Curtain training. *Proceedings of the American Society for Horticultural Science*, 89 (1966) 268-280

Siegfried W., Viret O., Huber B., Wohlhauser R. Dosage of plant protection products adapted to leaf area index in viticulture. *Crop Protection* 26, 2 (2007) 73-82

Singleton V.L. 1980. Grape and wine phenolics: background and prospects, in: Proceedings of Grape and Wine Cent. Symp, University of California, Davis.: 215-227

Singleton V.L. 1982. Grape and wine phenolics; background and prospects. In: Webb AD (Ed.) Proceedings of the University of California, Davis, Grape and Wine Centennial Symposium. University of California Press, Davis: 215-227

Singleton V.L., Timberlake Z.F., Lea A.G.H. The phenolic cinnamates of white grapes and wines. *Journal of the Sciences of Food of Agriculture* 29 (1978) 403-410

Singleton V.L., Trousdale E.K. Anthocyanin-Tannin Interactions Explaining Differences in Polymeric Phenols Between White and Red Wines. *American Journal of Enology and Viticulture* 43 (1992) 63-70

Sivilotti P., Bonetto C., Paladin M., Peterlunger E. Effect of soil moisture availability on Merlot: From leaf water potential to grape composition. *American Journal of Enology and Viticulture* 56 (2005) 9-18

Sivilotti P., Butinar, L., Sternad Lemut M. Leaf removal treatments as affecting *Botrytis Cinerea* occurance on Pinot Gris grapes from North Eastern Italy : results over four years of trials. *Ciênc. téc. vitiviníc.*, 28,1 (2013) 446-450

Smart R.E. Principles of grapevine canopy microclimate manipulation with implications for yield and quality: A review. *American Journal of Enology and Viticulture* 36, (1985) 230-239

Smart R.E., Dick J.K., Gravett I.M., Fisher B.M. Canopy management to improve grape yield and wine quality - principles and practices. *South African Journal of Enology and Viticulture* 11, 1 (1990) 3-17

Son H. S., Kim K. M., Van den Berg F., Hwang G. S., Park W. M., Lee C. H. H-1 nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. *Journal of Agricultural and Food Chemistry* 56, 17 (2008) 8007-8016

Son H.S., Hwang G.S., Park W.M., Hong Y.S., Lee C.H. Metabolomic characterization of malolactic fermentation and fermentative behaviors of wine yeasts in grape wine. *Journal of agricultural and food chemistry*, 75, 1 (2009) 4801-4809.

Souquet J.M., Labarbe B., Le Guernevé C., Cheynier V., Moutounet M. Phenolic composition of grape stems. *Journal of Agricultural and Food Chemistry* 48, 4 (2000), 1076-1080

Spadaro D., Gullino M.L. State of the art and future prospects of the biological control of postharvest fruit diseases. *International Journal of Food Microbiology* 91, 1 (2004) 185-194

Spayd S.E., Tarara J.M., Mee D.L., Fergusson J.C. Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries. *American Journal of Enology and Viticulture* 53, 3 (2002) 171-182

Sternad Lemut, M., Trost, K., Sivilotti, P., Arapitsas, P., Vrhovsek, U., 2013. Early versus late leaf removal strategies for 'Pinot noir' (*Vitis vinifera* L.): effect on colour-related phenolics in young wines following alcoholic fermentation. J. Sci. Food Agric. doi: 10.1002/jsfa.6193

Sternad Lemut, M., Trost, K., Sivilotti, P., Vrhovsek, U., 2011. Pinot Noir grape colour related phenolics as affected by leaf removal treatments in the Vipava Valley. *Journal of Food Composition and Analyses* 24, 777-784

Sternad Lemut, Sivilotti, P., Franceschi P., Wehrens R., Vrhovsek U. The use of metabolic profiling to study grape skin polyphenol behaviour as a result of canopy microclimate manipulation in a Pinot noir vineyard. *Journal of Agricultural and Food Chemistry* 61 (2013), 8976-8986

Su C.T., Singleton V.L. Identification of three flavan-3-ols from grapes. *Phytochemistry* 8, 8 (1969) 1553-1558

Sudha G., Ravishankar G.A. Involvement and interaction of various signaling compounds on the plant metabolic events during defence response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. *Plant Cell, Tissue and Organ Culture* 71, 3 (2002) 181-212

Sumner L.W., Mandes P., Dixon R.A. Plant metabolomics: large-scale phytochemistry in the functional genomics area. *Phytochemistry* 63 (2003) 817-836

Sun B., Spranger M.I. Quantitative extraction and analyses of grape and wine proanthocyanidins and stilbenes. Rewiev. *Ciência e Técnica Vitivinícola*. 20, 2 (2005) 59-89

Sun B.S., Ribes A., Leandro M.C., Belchior A.P., Spranger M.I. Stilbenes: quantitative extraction from grape skins, contribution of grape solids to wine and variation during wine maturation. *Analytica Chimica Acta* 563 (2006) 382–390

Szolnoki G. A cross-national comparison of sustainability in the wine industry, *Journal of Cleaner Production*. 53, 15 (2013) 243-251

Tarara J. M., Lee J., Spayd S. E., Scagel C. F. Berry temperature and solar radiation alter acylation, proportion, and con- centration of anthocyanin in Merlot grapes. *American Journal of Enology Viticulture 59*, 3 (2008) 235-247

Tardaguila J., Diago M.P., Martinez de Toda F., Poni S., Vilanova M. Effects of timing of leaf removal on yield, berry maturity, wine composition and sensory properties of cv. Grenache grown under non irrigated conditions. *Journal Internation de la Science de la Vigne et du Vin* 42, 4 (2008) 221-229

Tardaguila J., Martinez de Toda F., Poni S., Diago M.P. Impact of early leaf removal on yield and fruit and wine composition of *Vitis vinifera* L. Graciano and Carignan. *American Journal of Enology and Viticulture* 61, 3 (2010) 372-381

Tarr P.T., Dreyer, M.L., Athanas, M. Shahgholi M., Saarloos K. Second, T.P.A metabolomics based approach for understanding the influence of terroir in *Vitis vinifera* L. *Metabolomics,* 9 (2013) 170−177

Tassie E., Boag S,. Hooper R.L. Sustainable viticulture: what is the potential? *Australian and New Zealand Wine Industry Journal* 5, 2 (1990) 126-130

Theodoridis G., Gika H., Franceschi P., Caputi L., Arapitsas P., Scholz M., Masuero D., Wehrens R., Vrhovsek U., Mattivi F. LC-MS based global metabolite profiling of grapes: solvent extraction protocol optimization *Metabolomics* 8, 2 (2012) 175-185

Thomas C.S., Marois J.J., English J.T. The effect of wind speed, temperature and relative humidity on development of aerrial mycelium and Conidia og *Botrytis cinerea* on grape. *Phytopathology* 78 (1988) 260-265

Tian R-R., Pan Q-H., Zhan J-C., Li J-M., Wan S-B., Zhang Q-H., Wei-Dong Huang W-D. Comparison of phenolic acids and flavan-3-ols during wine fermentation of grapes with different harvest times. *Molecules* 14, 2 (2009), 827-838

Tixier M-S., Kreiter S., Auger P., Weber M. Colonization of Languedoc vineyards by phytoseiid mites (Acari: Phytoseiidae): influence of wind and crop environment. *Experimental and Applied Acarology* 22, 9 (1998) 523-542

Toffali K., Zamboni A., Anesi A., Stocchero M., Pezzotti M., Levi M., Guzzo F. Novel aspects of grape berry ripening and post-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis. *Metabolomics*, 7, 3 (2011) 424-436

Tomana T., Utsunomiya N., Kataoka I. The effect of environmental temperatures on fruit ripening on the tree. II. The effect of temperatures around whole vines and clusters on the col- oration of 'Kyoho' Grapes. Journal of Japanese Society of Horticultural Science 48 (1979) 261-266

Tomera J.F. Current knowledge of the health benefits and disadvantages of wine consumption. *Trends in Food Science & Technology* 10, 4-5 (1999) 129-138

Trost K., Golc-Wondra A., Prosek M., Milivojevic L. Anthocyanin degradation of blueberry-aronia nectar in glass compared with carton during storage. *Journal of Food Science* 73,8 (2008) 405-411

Trousdale E.K., Singleton V.L. Astilbin and engeletin in grapes and wines. *Phytochemistry* 22, 26 (1983) 619-620

Ubalde J.M., Sort X., Zayas A., Poch R.M. Effects of soil and climatic conditions on grape ripening and wine quality of Cabernet Sauvignon. *Journal of Wine Research* 21, 1 (2010) 1-17

Ursem R., Tikunov Y., Bovy A., van Berloo R., van Eeuwijk F. A correlation network approach to metabolic data analysis for tomato fruits. *Euphytica* 161, 1-2 (2008) 181-193

van Leeuwen C., Friant P., Chone X., Tregoat O., Koundouras S., Dubourdieu D. Influence of climate, soil, and cultivar on terroir. *American Journal of Enology and Viticulture* 55, 3 (2004) 207-217

van Leeuwen C., Seguin G.The concept of terroir in viticulture. *J. of Wine Research* 17, 1 (2006) 1-10

Vasconcelos M.C., Castagnoli S. Leaf canopy structure and vine performance. *American Journal of Enology and Viticulture* 51, 4 (2000) 390-396

Vaudour E. The Quality of grapes and wine in relation to geography: Notions of terroir at various scales. *Journal of Wine Research* 13, 2 (2002) 117-141

Verpoorte R., Contin A., Memelink J. Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews* 1 (2002) 13–25

Versari A., Parpinello G.P., Tornielli G.B., Ferrarini R., Giulivo C. Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. *Journal of Agriculture and Food Chemistry* 49, 11 (2001) 5531–5536

Vian M.A., Tomao V., Coulomb P.O., Lacombe J.M., Dangles O. Comparison of the anthocyanin composition during ripening of Syrah grapes grown using organic or conventional agricultural practices. *Journal of Agricultural and Food Chemistry* 54, 15 (2006) 5230–5235

Villas-Boas S.G., Nielsen J., Smedsgaard J., Hansen M.A.E., Roessner-Tunali U. 2007 In: Metabolome analysis: An Introduction. Wiley-Interscience, John Wiley & sons Inc: pp 311

Vitrac X., Castagnino C., Waffo-Téguo P., Delaunay J-C., Vercauteren J., Monti J-P., Gérard Deffieux G., Mérillon J-M. Polyphenols newly extracted in red wine from southwestern France by centrifugal partition chromatography. *Journal of Agricultural and Food Chemistry* 49,12 (2001) 5934-5938

Vogt T., Pollak P., Taryln N., Taylor L.P. Pollination or wound-induced kaempferol accumulation in petunia stigmas enhances seed production. *Plant Cell* 6, 1 (1994) 11-23

Vrhovsek U. Extraction of Hydroxycinnamoyltartaric Acids from Berries of Different Grape Varieties. *Journal of Agricultural and Food Chemistry.* 46,10 (1998) 4203-4208.

Vrhovsek U., Malacarne G., Masuero D., Zulini L., Guella G., Stefanini M., Velasco R., Mattivi F. Profiling and accurate quantification of trans-resveratrol, trans-piceid, trans-pterostilbene and eleven viniferins induced by *Plasmopara viticola* in partially resistant grapevine leaves. *Australian Journal of grape and wine research* 18, 1 (2012) 11-19

Vrhovsek U., Masuero D., Gasperotti M., Franceschi P., Caputi L., Viola R., Mattivi F. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *Journal of Agricultural and Food Chemistry* 60, 36 (2012) 8831−8840

Vrhovsek U., Wendelin S., Eder R. Effects of Various Vinification Techniques on the Concentration of cis- and trans-Resveratrol and Resveratrol Glucoside Isomers in Wine. *American Journal of Enology and Viticulture* 48, 2 (1997) 214-219

Vršič S., Šuštar V., Pulko B., Šumenjak. T.K. Trends in climate parameters affecting winegrape ripening in northeastern Slovenia. *Climate Research*, 58 (2014) 257-266.

Vršič S., Ivančič A., Pulko B., Valdhuber J. Effect of soil management systems on erosion and nutrition loss in vineyards on steep slopes. *Journal of Environmental Biology* 32, 3 (2011) 289-294

Wang M., Lamers R-J.A.N., Henrie A. A. J., Korthout H.A.J., van Nesselrooij J.H.J., Renger F. Witkamp R.F., van der Heijden R., Voshol P.J., Louis M., Havekes L.M., Verpoorte R., van der Greef J. Metabolomics in the context of systems biology: bridging traditional Chinese medicine and molecular pharmacology. Review article. *Phytotherapy Research* 19 (2005) 173–182

Webb L.B., Whetton P.H., Barlow E.W.R. (2008) Modelling the relationship between climate, winegrape price and winegrape quality in Australia. *Climate Research*, 36 (2008) 89-98

Weckwerth W. Metabolomics in systems biology. *Annual Review of Plant Biology* 54 (2003) 669-689

West T.O., Marland G. A synthesis of carbon sequestration, carbon emissions, and net carbon flux in agriculture: comparing tillage practices in the United States, *Agriculture, Ecosystems & Environment.* 91 (2002) 217-232

Wink M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Review. *Phytochemistry* 64, 1 (2003) 3-19

Winkler A.J., Cook J.A., Kliewer W.M., Lider L.A. 1974. General Viticulture. University of California press Ltd.: 141-146

Wishart D. S., Querengesser L. M. M., Lefebvre B. A., Epstein N. A., Greiner R., Newton J. B. Magnetic resonance diagnostics: a new technology for high-throughput clinical diagnostics. *Clinical Chemistry* 47 (2001) 1918-1921

Wishart D. S., Tzur D., Knox C., Eisner R., Guo A. C., Young N. HMDB: the human metabolome database. *Nucleic Acids Research* 35 (2007) 521-526

Wishart D.S. Metabolomics: applications to food science and nutrition research. *Trends in Food Science & Technology* 19 (2008) 482-493

Wolf T. K., Baudoin A. B. A. M., Mariti- nez-Ochoa N. Effect of floral debris removal from fruit clusters on *Botrytis* bunch rot of Chardonnay grapes. *Vitis* 36 (1997) 27-33

Xu C., Zhang Y., Zhu L., Huang Y., Lu J. Influence of Growing Season on Phenolic Compounds and Antioxidant Properties of Grape Berries from Vines Grown in Subtropical Climate. *Journal of Agricultural and Food Chemistry* 59, 4 (2011) 1078-1086

Zahavi T., Cohen L., Weiss B., Schena L., Daus A., Kaplunov T., Zutkhi J., Ben-Arie R., Droby S. Biological control of *Botrytis*, Aspergillus and Rhizopus rots on table and wine grapes in Israel. *Postharvest Biology and Technology* 20, 2 (2000) 115-124

Zamboni A., Vrhovsek U., Kassemeyer H-H., Mattivi F., Velasco R. Elicitor-induced reservatrol production in cell cultures of different grape genotypes (*Vitis spp*.). *Vitis* 45,2 (2006) 63-68

Zhao J., Davis L.C., Verpoorte R. Elicitor signal transduction leading to production of plant secondary metabolites. Research review paper. *Biotechnology Advances* 23, 4 (2005) 283-333

Zoecklein B.W., Fugelsang K. C., Gump B. H., Nury F. S. 1995. Phenolic compounds and wine color. In: Wine analysis and production (Ed.). Chapman & Hall, New York:115-151

Zoecklein B.W., Wolf T.K., Duncan N.W., Judge J.M., Cook M.K. Effects of fruit zone leaf removal on yield, fruit composition, and fruit rot incidence of Chardonnay and white Riesling (Vitis vinifera L.) grapes. *American Journal of Enology and Viticulture* 43, 2 (1992) 139-148

Zoecklein B.W., Wolf T.K., Pelanne L., Miller M. K., Birkenmaier S.S. Effect of vertical shoot-positioned, smart-dyson, and Geneva double-curtain training systems on viognier grape and wine composition. *American Journal of Enology and Viticulture* 59, 1 (2008) 11-2

9 APPENDICES

APPENDIX A

Table 1_S1: Hydroxycinnamic acid profiles (mg/kg) as affected by leaf removal at the Podraga vineyard. Means were separated using Student-Newmann-Keuls test (P<0,05).

	Sampling dates				
trans-caftaric acid	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	54,4 b	50,8 b	20,6 b	20,9 b	12,2 b
Berryset leaf removal (BS)	81,3 a	61,5 a	33,4 a	39,0 a	19,4 a
Veraison leaf removal (VE)	53,9 b	43,4 b	15,7 b	33,5 a	13,1 b
F	*47,6*	*9,19*	*6,97*	*16,3*	*5,26*
sign F	*0,000* ***	*0,015* *	*0,027* *	*0,004* **	*0,048* *
cis-coutaric acid	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	7,97 b	5,38 b	1,95 ab	1,76 b	0,78
Berryset leaf removal (BS)	12,4 a	7,61 a	2,92 a	3,51 a	1,31
Veraison leaf removal (VE)	7,63 b	5,89 b	1,14 b	2,98 a	0,66
F	*47,5*	*7,15*	*8,24*	*10,2*	*3,34*
sign F	*0,000* ***	*0,026* *	*0,019* *	*0,012* *	*0,106 n.s.*
trans-coutaric acid	1-Aug-09	8-Aug-09	16-Aug-09	23-Aug-09	25-Aug-09
Untreated (UN)	47,0 b	34,4 b	10,1 ab	11,0 b	2,35
Berryset leaf removal (BS)	71,5 a	47,7 a	14,3 a	26,1 a	7,25
Veraison leaf removal (VE)	43,8 b	37,1 b	6,73 b	22,2 a	4,60
F	*41,3*	*11,5*	*7,22*	*17,1*	*3,61*
sign F	*0,000* ***	*0,009* **	*0,025* *	*0,003* **	*0,093 n.s.*

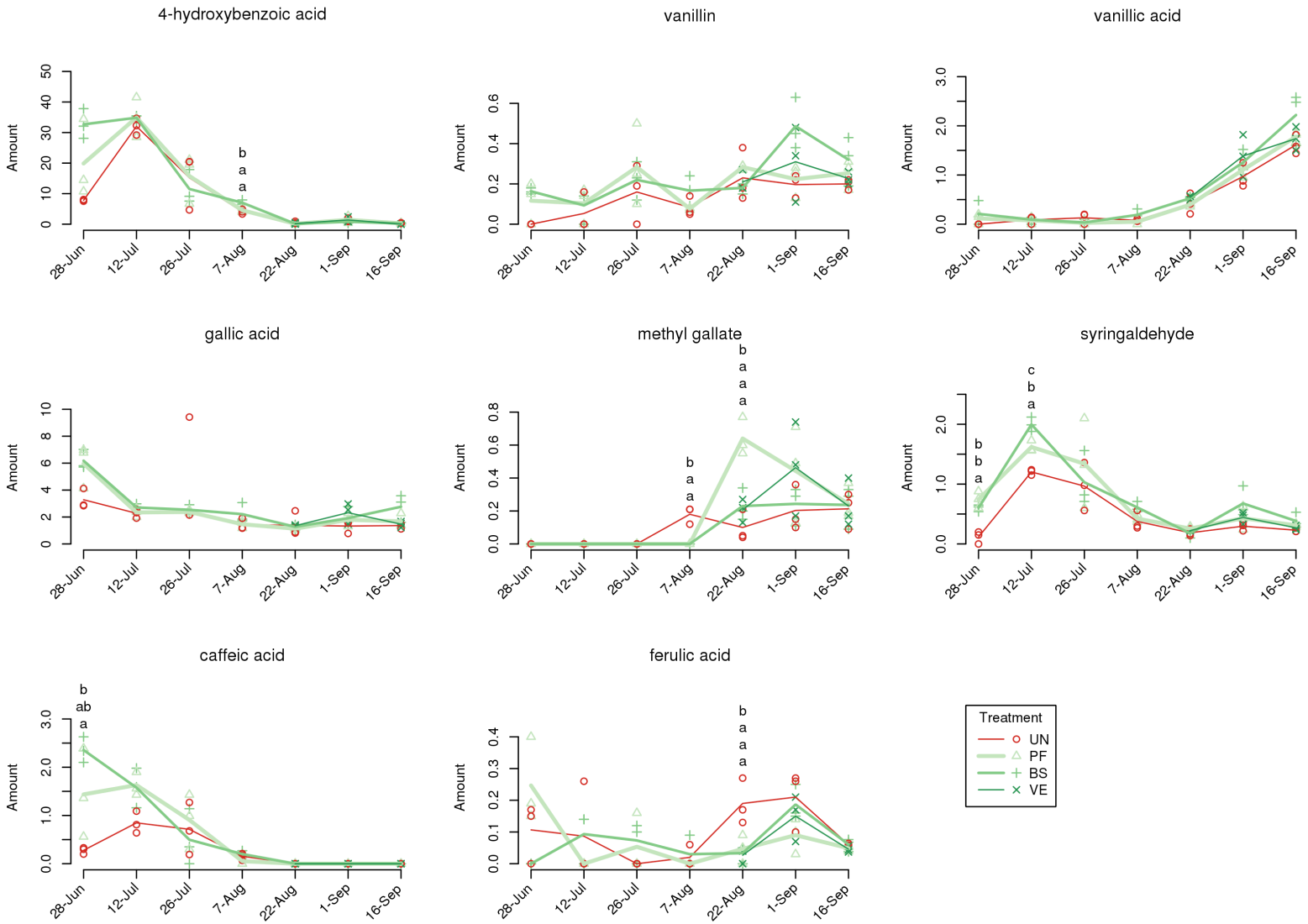
APPENDIX B

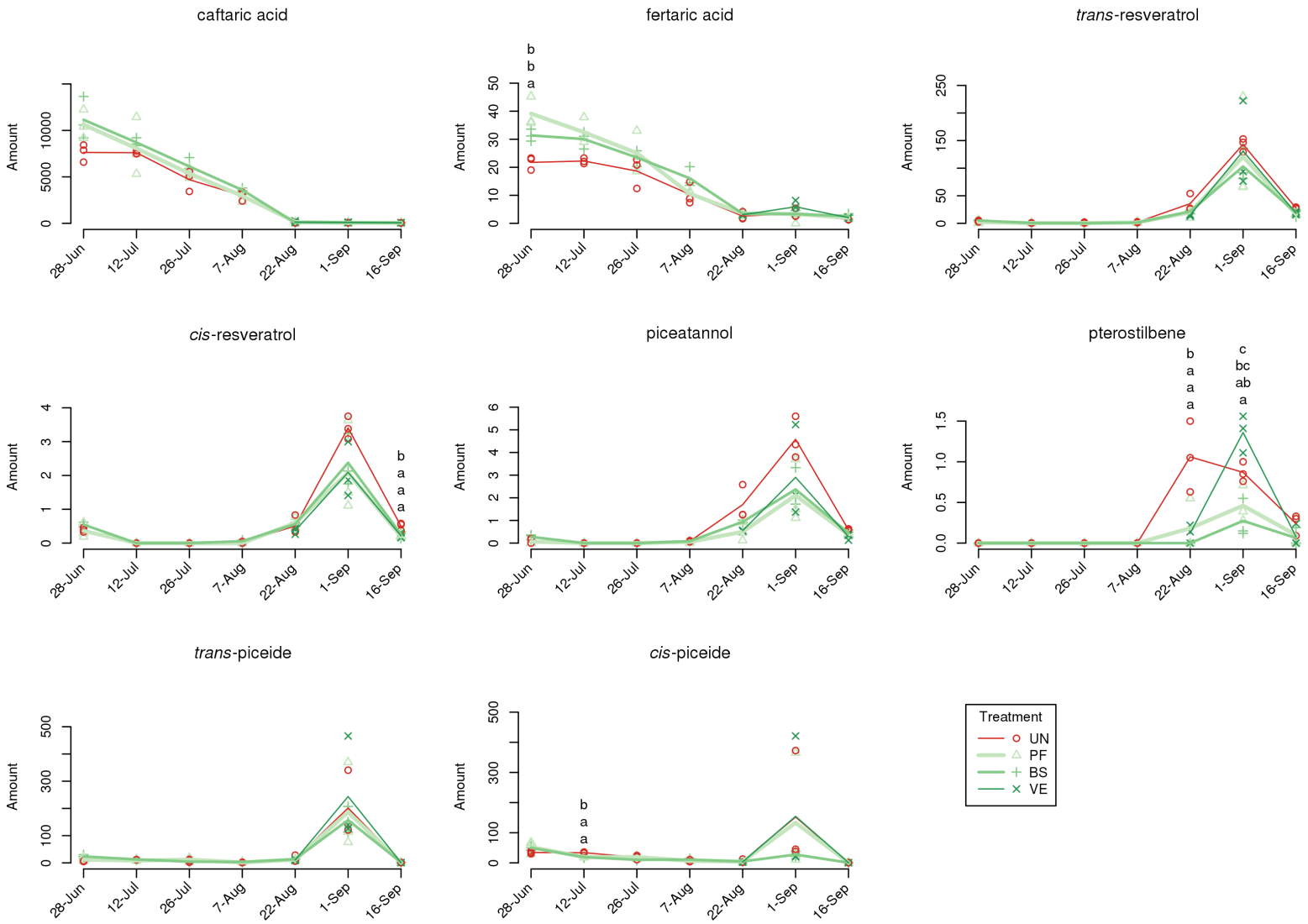
Table 1_S2: Hydroxycinnamic acid profiles (mg/kg) as affected by leaf removal at the Potoce vineyard. Means were separated using Student-Newmann-Keuls test (P<0,05).

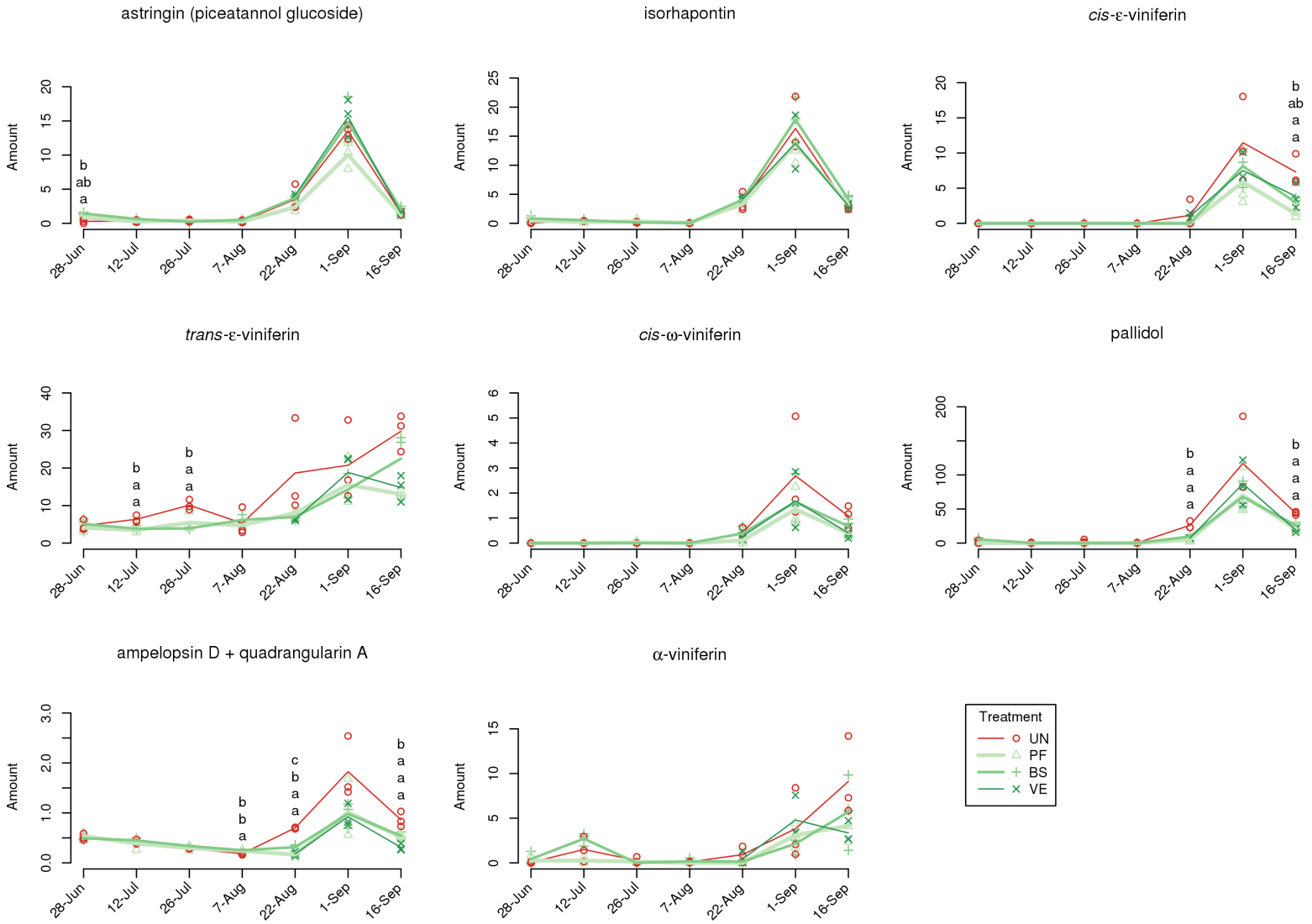
	Sampling dates				
trans-caftaric acid	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	52,8 b	39,0 ab	23,8	32,3	7,72 b
Berryset leaf removal (BS)	67,8 a	43,8 a	34,5	31,1	9,87 a
Veraison leaf removal (VE)	49,7 b	30,8 b	27,5	40,1	7,69 b
F	*12,5*	*5,87*	*1,38*	*4,00*	*5,24*
sign F	*0,007* **	*0,039* **	*0,321 n.s.*	*0,079 n.s.*	*0,048* *
cis-coutaric acid	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	9,12 b	5,16	2,91	4,36	0,95
Berryset leaf removal (BS)	13,0 a	5,82	3,77	4,06	1,02
Veraison leaf removal (VE)	8,10 b	3,97	3,79	5,02	0,95
F	*31,7*	*5,12*	*0,452*	*2,38*	*0,152*
sign F	*0,001* ***	*0,050 n.s.*	*0,656 n.s.*	*0,174 n.s.*	*0,862 n.s.*
trans-coutaric acid	8-Aug-09	16-Aug-09	24-Aug-09	5-sep-09	7-sep-09
Untreated (UN)	43,2 b	23,0 ab	17,6	28,1	4,91
Berryset leaf removal (BS)	61,9 a	30,3 a	19,7	33,0	5,26
Veraison leaf removal (VE)	42,5 b	16,8 b	23,3	34,9	5,68
F	*27,5*	*8,80*	*0,414*	*2,42*	*0,262*
sign F	*0,001* **	*0,016* *	*0,679 n.s.*	*0,170 n.s.*	*0,778 n.s.*

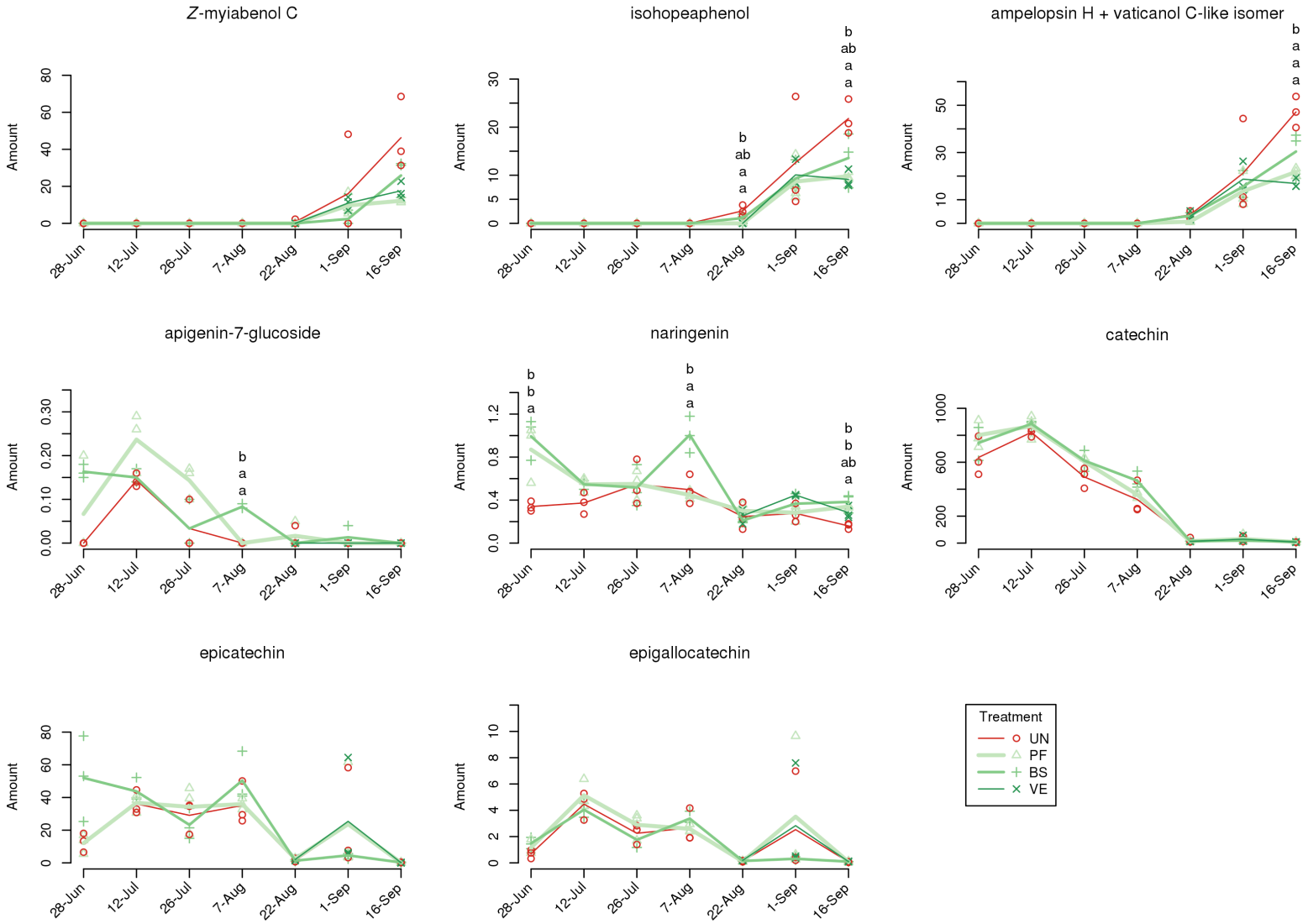
APPENDIX C

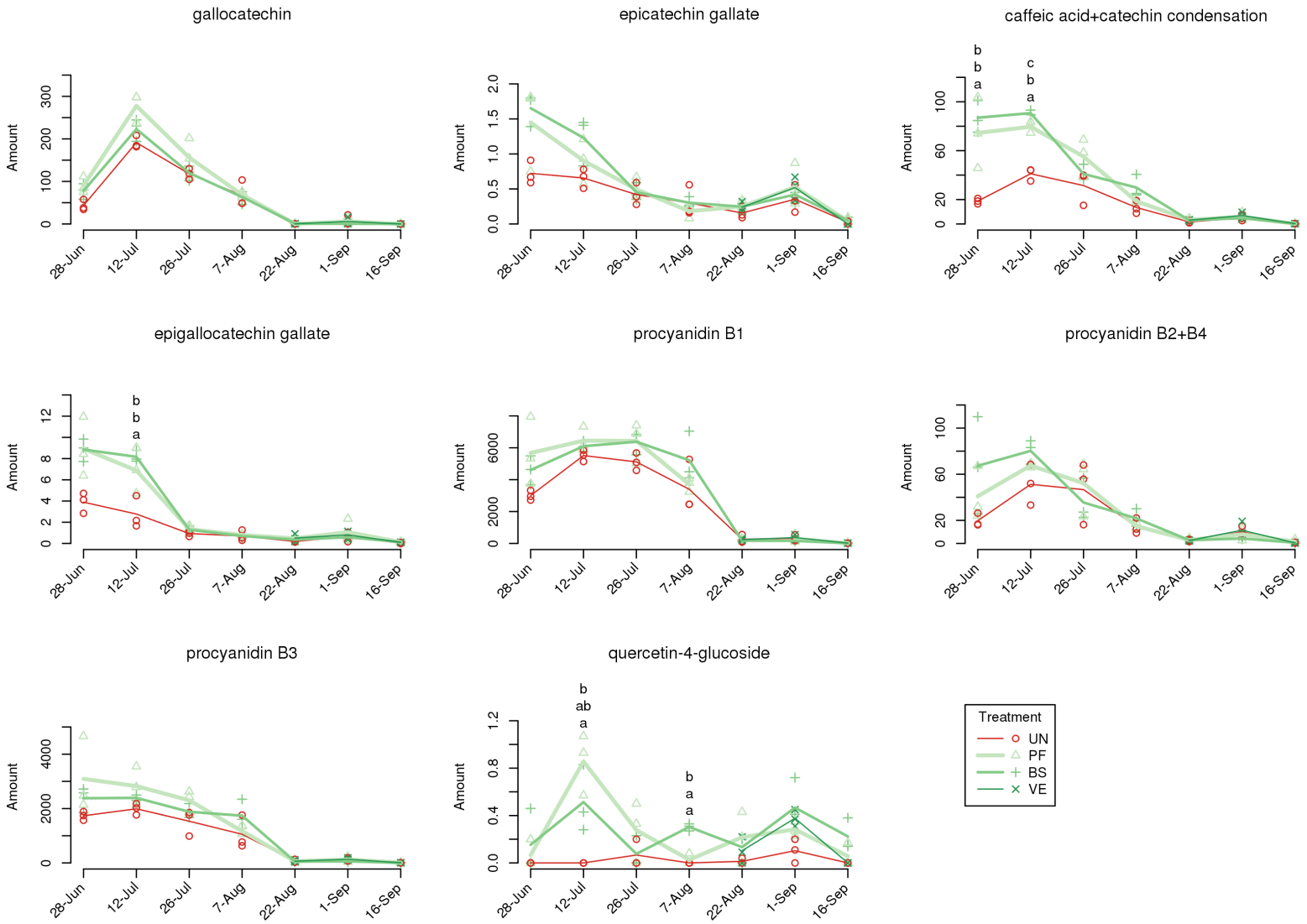
Figure **Group** 3_S1(8 Pages-Figures: C1-C8): Accumulation dynamics (μg/g skins) of 64 (not shown in the article) individual representatives from different phenolic groups as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal).

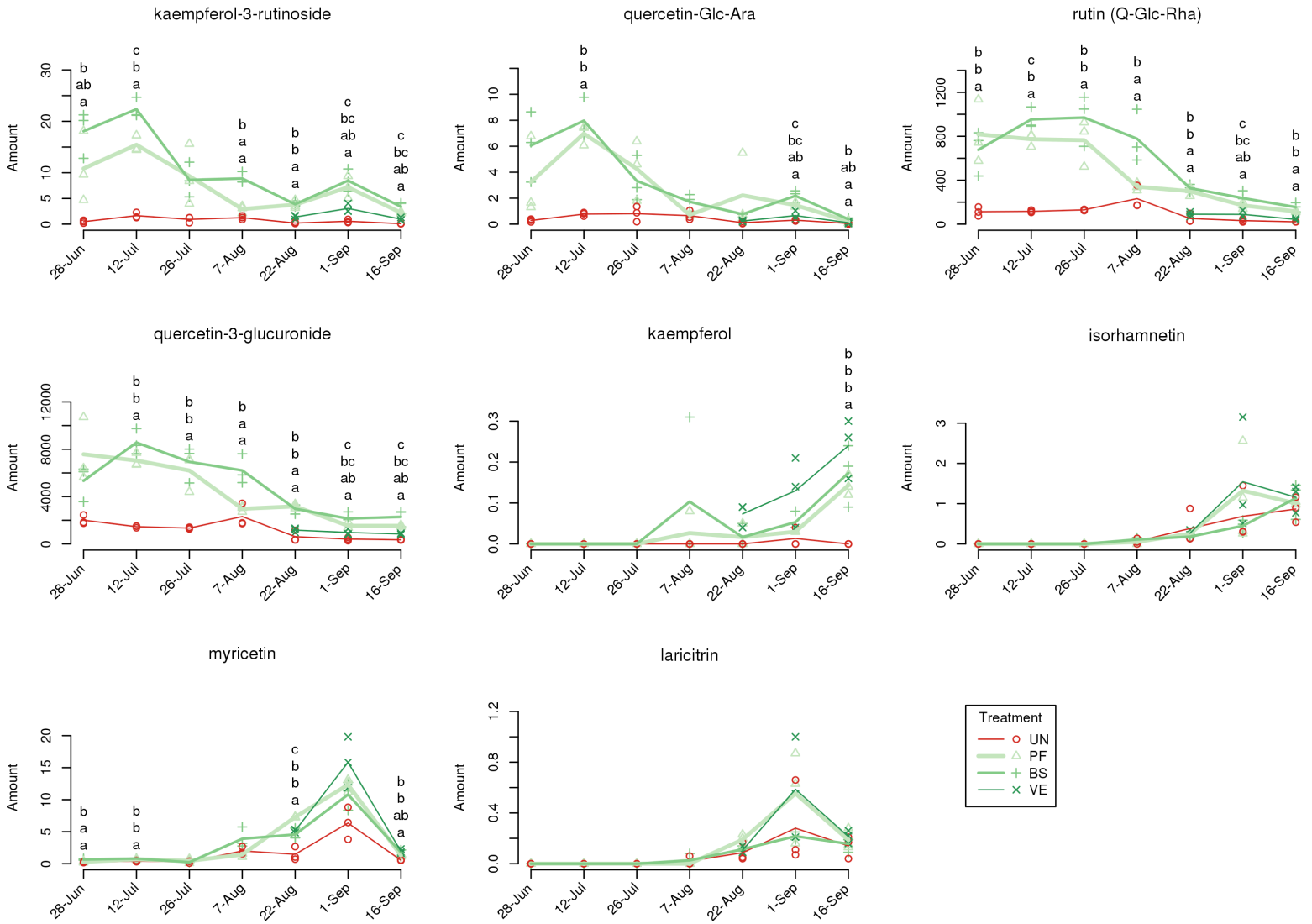

4-hydroxybenzoic acid
vanillin
vanillic acid
gallic acid
methyl gallate
syringaldehyde
caffeic acid
ferulic acid
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE

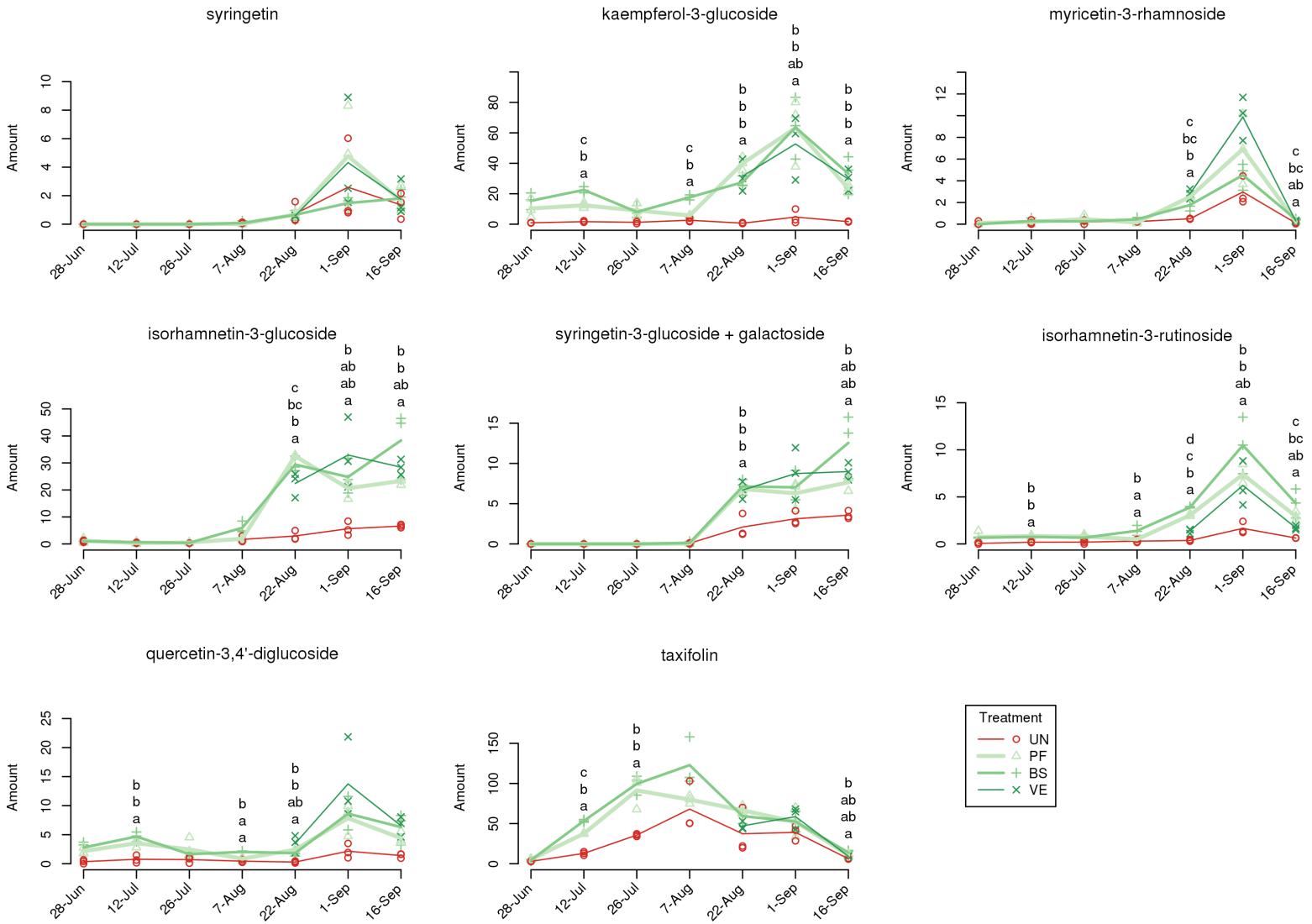

caftaric acid
fertaric acid
trans-resveratrol
cis-resveratrol
piceatannol
pterostilbene
trans-piceide
cis-piceide
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
b b a
b a a a
b a a a a
c bc ab a
b a a
Treatment
UN
PF
BS
VE

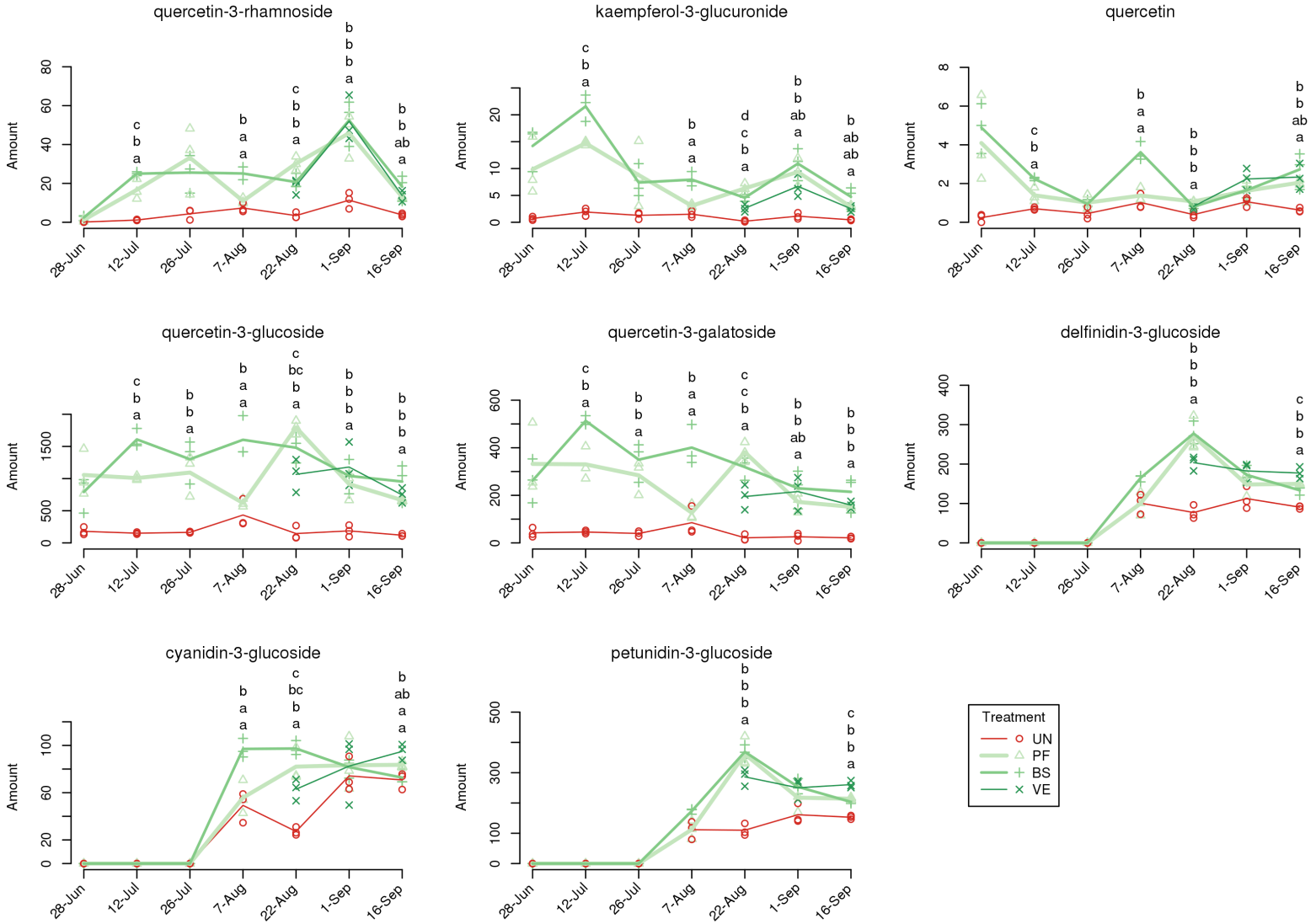

astringin (piceatannol glucoside)
isorhapontin
cis-ε-viniferin
trans-ε-viniferin
cis-ω-viniferin
pallidol
ampelopsin D + quadrangularin A
α-viniferin
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE


Z-myiabenol C
isohopeaphenol
ampelopsin H + vaticanol C-like isomer
apigenin-7-glucoside
naringenin
catechin
epicatechin
epigallocatechin
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE


gallocatechin
epicatechin gallate
caffeic acid+catechin condensation
epigallocatechin gallate
procyanidin B1
procyanidin B2+B4
procyanidin B3
quercetin-4-glucoside
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
b
b
a
c
b
a
b
ab
a
Treatment
UN
PF
BS
VE


kaempferol-3-rutinoside
quercetin-Glc-Ara
rutin (Q-Glc-Rha)
quercetin-3-glucuronide
kaempferol
isorhamnetin
myricetin
laricitrin
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE


syringetin
kaempferol-3-glucoside
myricetin-3-rhamnoside
isorhamnetin-3-glucoside
syringetin-3-glucoside + galactoside
isorhamnetin-3-rutinoside
quercetin-3,4'-diglucoside
taxifolin
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE


quercetin-3-rhamnoside
kaempferol-3-glucuronide
quercetin
quercetin-3-glucoside
quercetin-3-galatoside
delfinidin-3-glucoside
cyanidin-3-glucoside
petunidin-3-glucoside
Amount
28-Jun
12-Jul
26-Jul
7-Aug
22-Aug
1-Sep
16-Sep
Treatment
UN
PF
BS
VE

APPENDIX D

Table 3_S1: Basic seasonal (monthly) characteristics of the observed vintage (2010) (EARS. "Slovene Public Information", http://meteo.arso.gov.si; October 2012)

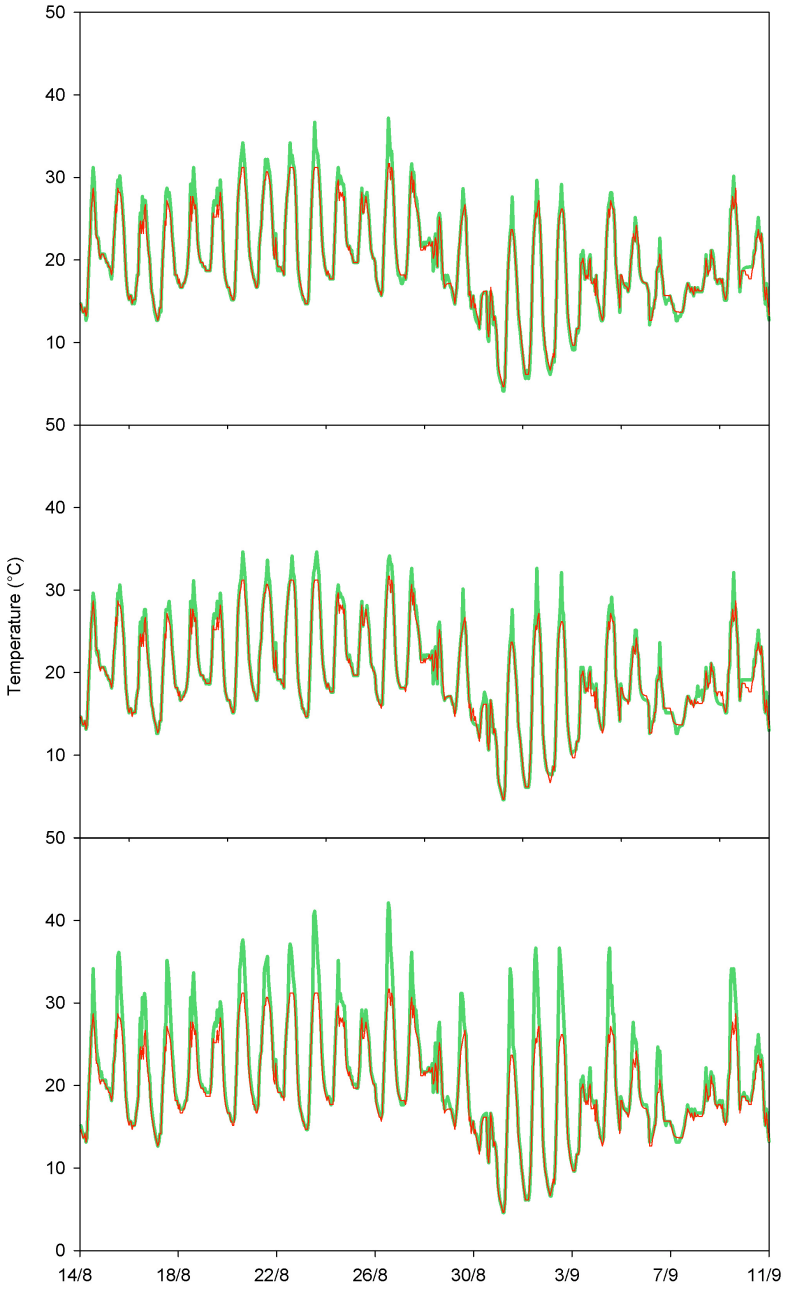
	Cloudiness (%)	Sun hours	Rainfall (mm)	Average T (°C)
May	64	166	258	16.0
June	48	272	108	20.7
July	33	319	200	23.5
August	43	283	75	21.1
September	51	190	367	16.7

Table 3_S2: Basic viticultural parameters as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal) (relative values).

	Total leaf area (m^2)	Yield (kg/plant)	Average cluster weight (g)	LA/yield (m^2/kg)
PF / UN	77,1%	70,3%	74,9%	115,2%
BS / UN	79,5%	89,0%	95,1%	93,8%
VE / UN	83,6%	98,6%	96,7%	85,4%

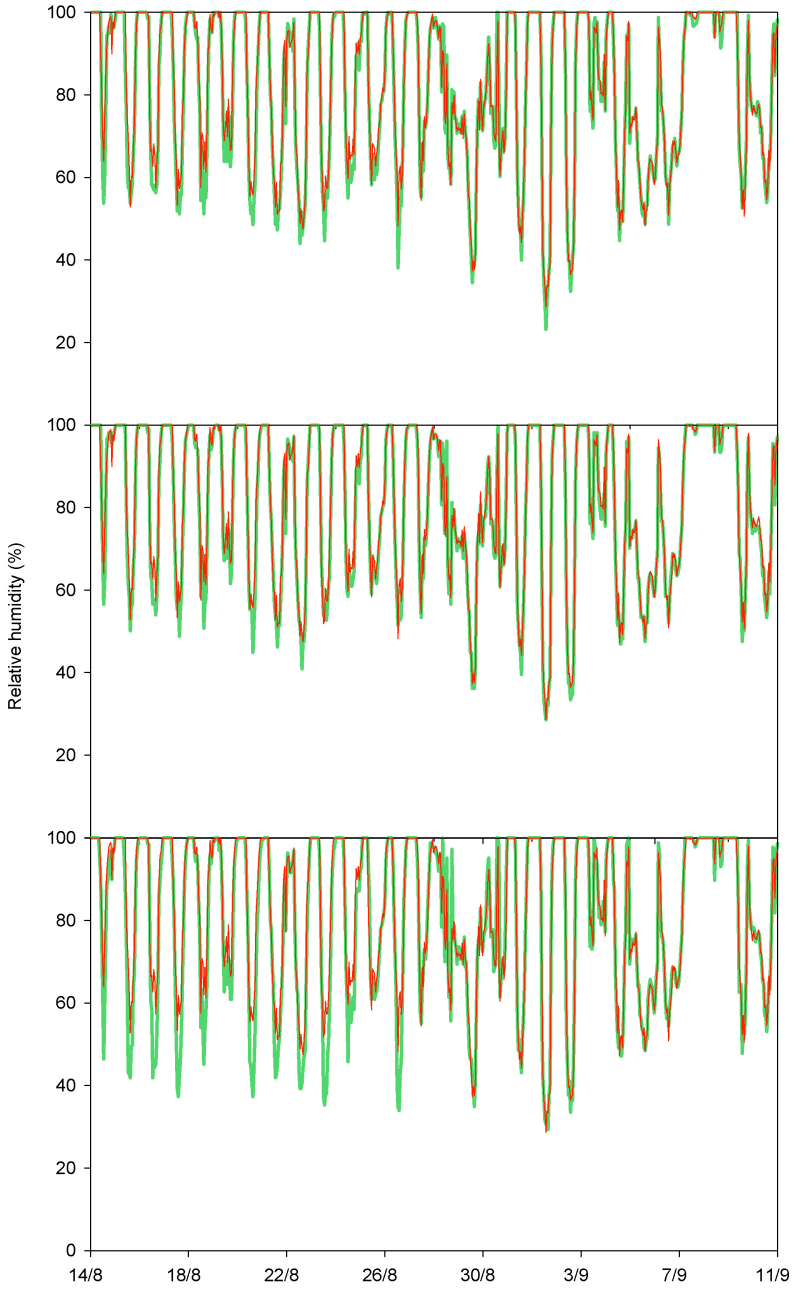
APPENDIX E

Figure 3_S2: Hourly temperatures (°C) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Upper figure shows the comparison between control (UN) and pre-flowering leaf removal (PF, green line). Middle figure shows the comparison between control (UN) and berry set leaf removal (BS, green line). Lowest figure shows the comparison between control (UN) and veraison leaf removal (VE, green line).



APPENDIX F

Figure 3_S3: Hourly relative humidity (%) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Upper figure shows the comparison between control (UN) and pre-flowering leaf removal (PF, green line). Middle figure shows the comparison between control (UN) and berry set leaf removal (BS, green line). Lowest figure shows the comparison between control (UN) and veraison leaf removal (VE, green line).



APPENDIX G

Table 5_S1: Basic quality parameters of grape juice from 'Pinot Noir' vines with different leaf removal treatments - vintage 2011 at two vineyards: Potoce (Vipava Valley, Slovenia) and Molini (Trentino, Italy) (mean values and standard errors of 3 biological replicates).

Vineyard-Treatment	Sugars [°Brix]	pH	Titratable acidity [g L^{-1}]
SLO Potoce– UN	21.6 ± 0.1	3.30 ± 0.01	7.36 ± 0.10
SLO Potoce – VE	21.5 ± 0.1	3.35 ± 0.03	6.77 ± 0.05
SLO Potoce – PF	22.0 ± 0.1	3.30 ± 0.01	7.33 ± 0.12
ITA Molini – VE	22.7 ± 0.7	3.26 ± 0.01	7.47 ± 0.12
ITA Molini – UN	22.5 ± 0.6	3.22 ± 0.02	8.03 ± 0.12
ITA Molini – PF	21.7 ± 0.6	3.21 ± 0.02	7.70 ± 0.28
Zorer et al., 2013			

Table 5_S2: Maturation kinetics of grapes from 'Pinot Noir' vines with different leaf removal treatments - vintage 2011 at two vineyards: Potoce (Vipava Valley, Slovenia) and Molini (Trentino, Italy).

Vineyard-Treatment	Sugar accomulation [°Brix/GDD_{10}]	pH increasing rate [pH/GDD_{10}]	Degradation of acidity [ln[AcTot]/ GDD_{10}]
SLO Potoce – PF	0.0206	0.0012	-0.0021
SLO Potoce – VE	0.0216	0.0014	-0.0024
SLO Potoce– UN	0.0205	0.0015	-0.0023
ITA Molini – PF	0.0189	0.0011	-0.0022
ITA Molini – VE	0.0231	0.0014	-0.0025
ITA Molini – UN	0.0225	0.0013	-0.0023
Zorer et al., 2013			

Legend:
PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal). pH increasing rate

APPENDIX H

Table 5_S3: Concentrations of detected individual secondary metabolites, sorted according to their chemical classes and according to their abundance as detected in 'Pinot Noir' grape berries at two experimental vineyards (Potoce, Vipava Valley, Slovenia; Molini, Trentino, Italy). The presented amounts are the averages of all three treatments (a.a.t) at one location (avUN + avPF + avVE/3). PF=pre-flowering leaf removal; BS=berry-set leaf removal; VE=veraison leaf removal; UN=control with no leaf removal).

	SLOVENIA		ITALY
Compound BENZOATES	Average (a.a.t) (µg/g powder)	Compound BENZOATES	Average (a.a.t) (µg/g powder)
gallic acid	37.75	gallic acid	81.26
p-hydroxybenzoic acid	20.53	p-hydroxybenzoic acid	22.46
syringic acid	0.41	syringic acid	0.43
vanillic acid	0.30	vanillic acid	0.30
methyl gallate	0.11	methyl gallate	0.13
anthranilic acid	0.02	anthranilic acid	0.06
Compound HCAs	Average (a.a.t) (µg/g powder)	Compound HCAs	Average (a.a.t) (µg/g powder)
Compound HCAs	Average (a.a.t) (µg/g powder)	Compound HCAs	Average (a.a.t) (µg/g powder)
caftaric acid	1526.51	caftaric acid	1985.04
trans-coutaric acid	61.03	trans-coutaric acid	80.98
fertaric acid	12.138	fertaric acid	14.16
ferulic acid	0.06	ferulic acid	0.06
Compound STILBENES	Average (a.a.t) (µg/g powder)	Compound STILBENES	Average (a.a.t) (µg/g powder)
cis-piceide	44.91	cis-piceide	70.61
trans-piceide	20.05	pallidol	25.24
pallidol	16.95	trans-piceide	20.43
astringin	11.55	astringin	13.69
isorhapontin	6.32	isorhapontin	6.98
trans-resveratrol	4.24	trans-ε-viniferin	4.51
trans-ε-viniferin	3.95	isohopeaphenol	3.23
isohopeaphenol	3.00	trans-resveratrol neg	2.89
(ampelopsin H + vaticanol C-like isomer)	2.65	(ampelopsin H + vaticanol C-like isomer)	2.77
piceatannol	1.47	piceatannol	1.20
α-viniferin	1.36	(ampelopsin D+quadrangularin	1.18
(ampelopsin	0.70	α-viniferin	0.82
cis-resveratrol	0.26	cis-resveratrol	0.12

Table continuing...

...

...		Table continuing...	
Compound FLAVONES	Average (a.a.t) (µg/g powder)	Compound FLAVONES	Average (a.a.t) (µg/g powder)
luteolin-7–glucoside	1.09	luteolin-7-glucoside	1.21
hesperidin	0.08	hesperidin	0.11
apigenin-7-glucoside	0.04	apigenin-7-glucoside	0.04
Compound FLAVANONES	Average (a.a.t) (µg/g powder)	Compound FLAVANONES	Average (a.a.t) (µg/g powder)
naringenin-7-glucoside	0.30	naringenin-7-glucoside	0.36
naringenin	0.03	naringenin	0.03
Compound FLAVAN-3-OLS	Average (a.a.t) (µg/g powder)	Compound FLAVAN-3-OLS	Average (a.a.t) (µg/g powder)
catechin	4078.00	catechin	5303.60
epicatechin	1352.34	procyanidin B3	1715.54
procyanidin B3	736.63	epicatechin	1666.26
procyanidin B1	699.63	procyanidin B1	1026.37
procyanidin B2 + B4	557.58	procyanidin B2 + B4	631.19
catechin gallate	9.30	catechin gallate	11.72
epicatechin gallate	6.90	epicatechin gallate	6.54
gallocatechin	4.41	gallocatechin	5.96
epigallocatechin	0.89	epigallocatechin	1.61
epigallocatechin gallate	0.25	epigallocatechin gallate	0.48
Compound FLAVONOLS	Average (a.a.t) (µg/g powder)	Compound FLAVONOLS	Average (a.a.t) (µg/g powder)
quercetin-3-glucuronide	216.87	quercetin-3-glucuronide	295.68
quercetin-3-glucoside	85.56	quercetin-3-glucoside	124.02
taxifolin	75.19	taxifolin	71.50
isorhamnetin-3-glucoside	20.17	quercetin-3-galactoside	31.23
quercetin-3-galactoside	18.08	rutin	29.27
rutin	16.39	isorhamnetin-3-glucoside	22.44
kaempferol-3-glucoside	11.54	kaempferol-3-glucoside	19.30
syringetin-3-glc+syringetin-3-gal	9.54	syringetin-3-glc+syringetin-3-gal	12.05
quercetin-3-rhamneside	7.24	quercetin-3-rhamneside	9.31
quercetin-3,4-rutinoside	2.15	kaempferol-3-rutinoside	2.15
myricitrin	2.07	myricitrin	2.12
kaempferol-3-glucuronide	1.33	kaempferol-3-glucuronide	2.10
myricetin	1.16	quercetin-3,4-rutinoside	2.04
isorhamnetin-3-rutinoside	1.09	isorhamnetin-3-rutinoside	1.37
kaempferol-3-rutinoside	0.76	myricetin	1.16
quercetin	0.31	quercetin-3-Glc-Ara	0.42
quercetin-3-Glc-Ara	0.27	quercetin	0.34