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Effects and modes of action of canopy management practices on vine physiology and berry composition in organically-cultivated cv. Sangiovese (*Vitis vinifera* L.)

Presentata da: **Paola Tessarin**

Coordinatore Dottorato **Relatore**

Prof. Giovanni Dinelli **Prof. Adamo Domenico Rombolà**

Esame finale anno 2016

To Rosanna, Antonio

and Mattia

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List of pubblications:

The PhD Research Activity produced three Papers submitted in International Journals with Peer review:

- Tessarin, P., Versari, A. and Rombolà A.D. Physiological and enological implications of post-veraison trimming in an organically managed vineyard (*Vitis vinifera* L, cv. Sangiovese). (*Submitted*).
- Tessarin, P., Ricci, A., Parpinello G.P., Rombolà A.D.Effects of bunch- zone late defoliations on berry composition and wine quality in organically-cultivated cv. Sangiovese (*Vitis vinifera* L.) . (*Submitted*).

Tessarin, P., Lugli, A., Conde, A., Martins, V., Bregoli, A.M., Rombolà, A.D. ABA-mediated transcriptional reprogramming of secondary metabolism in cultured cells from Sangiovese mature grapes. (*In litteris*).The PhD Research Activity provided several additional experimental data that will be included in other Papers.

During the PhD activity I have also participated in the writing of different Papers submitted to IF Journals:

- Tessarin P., Chinnici F., Donnini S., Liquori E., Riponi C., Rombolà A.D. Implications of canopy-applied chitosan on berry and wine composition in two different *Vitis vinifera* red varieties under organic management. (*Under Revision*).

- Tessarin, P., Boliani, A. C., Botelho, R. V., Rusin, C., Versari A., Parpinello G. P., Rombolà A.D. (2014) Effects of late defoliation on chemical and sensory characteristics in wine of the late maturing variety Uva Longanesi. Journal of Soil Science and Plant Nutrition, 14 (4), 1021- 1038.
- Bondada, B., Covarrubias, J.I., Tessarin, P., Boliani, A.C., Marodin, G. and Rombolà A.D. (2016). Post-veraison shoot trimming reduces cluster compactness without compromising fruit quality attributes in organically-grown Sangiovese grapevines. American Journal of Enology and Viticulture, 67 (2), 206-211.
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- Bombai G., Pasini F., Verardo V., Sevindik O., Di Foggia M., Tessarin P., Bregoli A.M., Taddei P., Caboni M.F., Rombolà A.D. Monitoring of compositional changes during berry ripening in grape seed extracts from organically cultivated cv. Sangiovese vines (*Vitis vinifera* L.). (*Submitted*).

During the PhD I spent abroad a 4-months period:

- 1 Month: June 09th, 2013- July 10th, 2013, "Department of Biology, Campus de Gualtar, University of Minho (Braga, Portugal). The purpose of this period abroad was to develop Research work on the study of the functional role of the vacuole of grape berry (*Vitis vinifera*) cells. In the scope of the staying, a protocol for protoplast isolation and vacuole purification from grape cells actively accumulating anthocyanins was optimized and knowledge for initiating grape cell cultures from the exocarp of red grape berry varieties was acquired.

- 3 Months: October 23rd, 2013, January 1st, 2014, "Department of Biology, Campus de Gualtar, University of Minho (Braga, Portugal) in the scope of Marco Polo Project.

The purpose of this period abroad was to develop experimental work aiming to elucidate the implications of specific viticulture practices on grape berry composition, particularly on anthocyanin and sugar concentration. Also, several molecular biology techniques were optimized - including the extraction of RNA from grape samples, primer design and real-time PCR, aiming at the study of the mechanisms underlying the transcriptional regulation of the proteins involved in anthocyanin biosynthesis and intracellular transport.

The Professor in charge of the exchange at the University of Minho was Hernâni Varanda Gerós.

Introduction

The increasing demand for healthy products has favored the diffusion of alternative cultivation methods, such as organic and biodynamic farming (Ponzio et al., 2013, Willer and Lernoud, 2014), which can provide numerous environmental, agronomic and social benefits (Reganold et al., 1993, Mader et al., 2002, Gattinger et al., 2012). This trend is particularly evident in viticulture (Willer and Lernoud, 2014, Rombolà et al., 2014, Rombolà et al., 2015, Rombolà, 2015a, Parpinello et al., 2015, Botelho et al., 2016) mainly because of the prominent cultural and social role of wine and to the peculiar attention that has always been devoted to both grape cultivation and winemaking process.

According to FIBL and IFOAM surveys, over 280,000 ha of organic grapes were grown in 2014, (Willer and Lernoud, 2014). In Europe, 240,000 ha, which constitute 6% of the harvested grape area, are under organic management; the European countries with the largest organic grape area, including more than 55,000 ha of organic grapes, are Spain, France and Italy (Willer and Lernoud, 2014). Worldwide, approximately 147,000 ha are managed according to Demeter biodynamic standards; in particular, there are 520 Demeter wineries in the world, with 8000 ha of vineyards; biodynamic viticulture is spreading the fastest in Argentina, Chile and France (Demeter International, 2012, Botelho et al., 2016). Noteworthy, growers adopting the biodynamic cultivation method require prior organic certification.

Organic and biodynamic cultivation methods can become highly sustainable integrated systems of agricultural practices, characterized by a site-specific application. In these

systems, peculiar attention is paid to the efficient use of natural resources that are preserved by reducing or even suspending the supply of irrigation water and fertilizers (Rombolà et al., 2014, Rombolà et al., 2015). Higly sustainable agricultural systems provide tangible benefits on environment, soil fertility, biodiversity, productivity, incomes and capability to meet the needs of future generations (Rombolà, 2015b).

Noteworthy, in organically and biodynamically managed vineyards, also the implementation of waste management (e.g. seed extracts) may become an attractive opportunity for growers (Bombai et al., 2016) by conferring to residues economical value and increasing the sustainability of the whole production process.

Organic growers adopt agricultural practices and processing methods, which in many countries are regulated by specific laws (e.g. Reg. EC 2012, AQIS, 2013). For instance, the European Community recently enacted a Regulation (EC, 2012) stating that "organic wines" must originate only from organic grapes (EC, 2007). On the other hand, for biodynamic viticulture and winemaking there is still no official European Regulation. This stems from the fact that biodynamic farming is considered as a holistic approach for the employment of the natural resources (Lotter, 2003), an ongoing path of knowledge, rather than a simple assemblage of methods and techniques (Ponzio et al., 2013).

For these reasons, biodynamic viticultural practices can vary, depending on the grower's beliefs and rates of adoption. However, biodynamic growers often refer to protocols proposed by private organizations. For example, those following the Demeter Protocol adopt specific soil (e.g. tillage without turning over soil) and canopy (prohibition of shoot trimming) management strategies in the vineyards. In the winery,

spontaneous fermentation is practiced instead of using commercial yeasts. However, several growers adopt biodynamic practices without following specific protocols.

Regardless of certification procedures, biodynamic agriculture differs from organic management in the use of specific fermented preparations (e.g. horn manure 500, horn silica 501, Fladen, etc.), applied on crops or soil in very small amounts (Koepf et al., 2001). Various explanations regarding the mode of action of biodynamic preparations on plants have been provided, including hormonal stimulation, and enhancement of plant growth, particularly at the root level (Fritz and Köpke, 2005). Biodynamic preparations are not expected to act as fertilizers (Botelho et al., 2016), rather to harmonize environmental processes that naturally occur, improving the vegetative-reproductive balance of plants (Koepf et al., 2001).

The diffusion of organic and biodynamic viticulture will have a prominent environmental and social role. For instance, the Italian Association for Organic Agriculture (AIAB) promoted the development of 13 Bio-districts on the whole National surface (www.biodistretto.net), such as that of Greve in Chianti (Firenze, Italy), constituted, in 2012, in a famous viticultural area whose enological tradition is known worldwide (Figure 1).

Figure 1. Vineyards and Olive Trees in the Conca d'Oro of Panzano in Chianti (Bio-District of Greve in Chianti, Firenze, Toscana, Italy).

The possibility to create Bio-Districts is of paramount importance to overcome the current fractionation of organic agricultural areas and for emphasizing the real meaning of the word *terroir*, with all its peculiarities and all its complexity. Bio-districts are geographical area displaying natural vocation for organic farming, where growers, citizens, tour operators, associations and public administration forge a pact for a sustainable management of natural resources, starting from the organic model of production and consumption (e.g. short chain, buying groups, public canteens providing organic food and beverages) (www.biodistretto.net). In this way the promotion of organic products is indissolubly connected with the promotion of a specific *terroir* with the aim of achieving the full development of economic, social and cultural potential.

Although organic and biodynamic cultivation methods actually represent attractive alternative, not only for growers, some factors (e.g. agronomic, economical, institutional, commercial and social) may discourage their implementation in vineyards.

For instance, both organic and biodynamic farming are characterized by lower inputs of pesticides and fertilizers that imply the need of alternative strategies for what concerns plant nutrition and protection from pathogens. This calls for a specific approach to the vineyard-system requiring deep knowledge of grapevine physiology, soil and climatic conditions, constant monitoring, physical presence in the field and the ability to adopt flexible agronomic strategies according to seasonal weather conditions.

In organic and biodynamic vineyards, the control of the main grape pathogens such as powdery, downy mildew and *Botrytis* cluster rot can be troublesome due to few plant protection products (e.g. sulfur and copper) and because of the restriction on the amount of copper to a maximum of 6 kg/ha/year regardless of cultivation method (EC Regulation 473/2002) (Botelho et al., 2016).

In addition, there are limitations on the amount of sulfites that can be added to the must during wine-making (EC, 2012) (e.g. 100 mg/L for organic red wine); therefore grapes, must arrive to the winery in a good sanitary status, even in rainy seasons, when fighting pathogens is though, for the above mentioned reasons (Botelho et al., 2016).

Moreover, the EC Regulation (EC 2012) for the production of organic wines provides a series of restrictions and prohibitions in the use of determinate enological practices (e.g. dealcohlization), highlighting the indispensability of preventive action in the field to safeguard the quality of the grapes and derived wines.

For these reasons, in organic and biodynamic viticulture, all field strategies, including canopy and soil management practices, should be addressed to preserve and enhance plant health and resilience capability, with the aim of improving the effectiveness of plant protection products and producing healthy grapes (Rombolà et al., 2014, Rombolà et al., 2015, Bondada et al., 2016).

Beyond the constraints imposed by Regulations or peculiar private specification, one of the main desires of winegrowers adopting these cultivation methods is leading the vineyard to the auto-regulation that means minimizing all the interventions in the field (e.g. use of plant protection products, fertilizers, mechanical and manual interventions).

An intriguing and ambitious challenge of organic and biodynamic viticulture is to increase plant resistance, in a system as the monoculture, in which vine tends to be very sensitive, also due to the breaking of most of the links with environmental biodiversity.

Recently, it has been reported that biodynamic preparations (500, 500K, 501 and Fladen) led to an increase in leaf enzymatic activities of chitinase, and β-1,3-glucanase, which are typically correlated with plant biotic and abiotic stresses and associated with induced plant resistance (Botelho et al., 2016). Moreover, it has been shown that the systemic acquired resistance (SAR) (Gozzo, 2003) can be induced by microbe-associated molecular patterns (MAMPs) that trigger plant defence mechanisms (Iriti and Faoro, 2007). Among MAMPs, chitosan (β-1,4-D-glucosamine), a polysaccharide obtained after partial deacetylation of chitin (from 15% to 80%), deserves peculiar attention. The implications of canopy-applied chitosan on berry and wine composition in two different *Vitis vinifera* red varieties (cvs. Sangiovese and Cabernet Sauvignon) under organic management have been recently investigated (Tessarin et al., 2016). Differences in phenolic acids amounts in berries and wines were detected in cv. Cabernet Sauvignon but not in cv. Sangiovese. Moreover, a considerable increase in γ-aminobutyric acid (GABA) was observed in the berry flesh of cv. Cabernet Sauvignon. The increase in phenolic acids and nitrogenous compounds, especially GABA, in the pulp of Cabernet Sauvignon grapes suggests changes in stress response. The defense responses elicited by chitosan in plants include different mechanism such as the raising

of cytosolic Ca^{2+}, activation of mitogen-activated protein-kinases (MAP-K), callose apposition, oxidative burst, synthesis of abscissic acid (ABA) and jasmonate (Iriti and Faoro, 2009). In addition, Yin et al., (2010) found that chitosan-treated plants increased the production of reactive oxygen species (ROS), implicated in various plant/pathogen interactions. Iriti et al., (2010) demonstrated that chitosan-induced resistance in plants was associated with enhanced concentration of polyphenolic phytoalexins, due to the stimulation of the phenylpropanoid pathway. In particular, elicitation of this biosynthetic route by chitosan was correlated with the increase of both activity and transcript level of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), two key enzymes of the phenylpropanoid pathway (Nandeeshkumar et al., 2008, Falcón-Rodríguez et al., 2009). Moreover, it has been reported for chitosan a preventive effect on bunch rot (Romanazzi, 2010).

Another important aspect that is carefully considered by growers adopting the organic and biodynamic cultivation methods for improving plant conditions in the vineyard is soil management. This agricultural practice is one of the most delicate choices in the conduction of a sustainable cultural system, since it can influence the soil physical-chemical, biological properties, water balance, nutrients, presence of adventitious flora, enthomophages, pathogens, biological diversity within the agro-ecosystem and, therefore, the vegetative-productive balance of the grapevine (Sevindik et al., 2014, Rombolà et al., 2014, Rombolà, 2015a, Rombolà et al., 2015).

Noteworthy, in organic and biodynamic cultivation methods the use of herbicides, such as glyphosate, whose level of dangerousness is currently at the center of a global debate, is forbidden. In a recent study, the implications of glyphosate on berry phenolic composition of non-target plants (cv. Ancellotta grapevines) were evaluated, showing a

decrease in some of the detected anthocyanins (Donnini et al., 2016). The herbicidal effect of glyphosate can be ascribed to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), an enzyme of the shikimate pathway, blocking the biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan (Siehl, 1997). The blockage of the shikimate pathway results in substantial accumulation of shikimate in plant tissues provoking a decrease of flavonoid and lignin concentrations (Franz et al., 1997, Olesen and Cedergreen, 2010). The possibility of establishing Bio-Districts can lead to abandon the use of products, such as glyphosate, whose impact on human health and environment is questionable, by raising awareness in public administration for not to employ it for extra-agricultural aims, such as weeding the margins of the streets crossing interconnected organic areas (e.g. Toscana Region).

On the other hand, controlled intercropping in the vineyard provides most of the evident benefits of organic cultivation methods. In fact, this agricultural practice enhance the organic matter contents in the soil, improve the chemical-physical properties, limits the risk of erosion, increases soil bearing capacity, and contributes to the diversification of the agro-ecosystem (Rombolà et al., 2014, Rombolà, 2015a, Rombolà et al., 2015).

Recent Research conducted in organic vineyards located in Tebano, Faenza (RA) by the Research Group in Plant Stress Physiology - Development and Management of Highly Sustainable Agricultural Systems, coordinated by Prof. Rombolà, demonstrated the possibility to obtain vines with satisfactory vegetative-productive balance, yields higher than those imposed by the Protected Designation of Origin (PDO) for "Romagna Sangiovese", healthy bunches of high quality in vineyards intercropped with different herbaceous species (Rombolà et al., 2014, Sevindik et al., 2014, Rombolà et al., 2015, Rombolà, 2015a). In these organic vineyards, herbaceous species (e.g. barley, fava

bean, *Phacelia tanacetifolia*, *Trifolium subterraneum*) did not compete with the vine for the absorption of water; conversely they allowed, to effectively contain the vigor of vine shoots before late mowing imposition (end of spring) (Rombolà, 2015). Following late mowing, the biomass was maintained on the soil surface creating a natural mulching, which allowed to reduce evaporation losses from the soil; furthermore, intercropping, characterized by the presence of several legumes and grasses species, provided nitrogen uptake and enhanced the availability of nutrients (Rombolà et al., 2014, Sevindik et al., 2014, Rombolà et al., 2015, Rombolà, 2015a).

In vineyards under organic and biodynamic cultivation methods, canopy management helps to prevent fungal attack. Especially in vegetative seasons characterized by frequent rainfall during spring and summer, the effective control of the main fungal diseases of the vine can be difficult, due to limitations in the use of plant protection products. Moreover, an excessive use of sulfur-containing compounds (Rauhut, 2009) to combat powdery mildew may cause adverse effects on the sensorial properties of wine (e.g. off-flavours). Because of the need to adhere to the principles of organic viticulture, organic grape growers seek out a wide repertoire of summer pruning practices (e.g. shoot thinning, topping/trimming, leaf removal, shoot positioning) in order to maintain healthy vines and preserve grape and wine quality. These operations may influence berry quality, through both direct (e.g. changes of source-sink relations, hormonal status) and indirect (e.g. modified microenvironment) effects.

Trimming is a widespread, fully mechanizable summer pruning practice, commonly adopted to reduce vine vigour, improve grape quality, enhance the efficiency of plant protection products, facilitate harvest and the transitability of agricultural machineries in the vineyard. The consequences of trimming depend on its severity and on the

phenological stage of application (Peterson and Smart, 1975, Kliewer and Bledsoe, 1987, Reynolds and Wardle, 1989, Solari et al., 1988, Poni et al., 2003, Rombolà et al., 2011, Satta et al., 2012, De Pau et al., 2012, Martinez de Toda et al., 2013, Filippetti et al., 2015, Bondada et al., 2016). In the past, several studies concerning the effects of different timings of shoot trimming on vine growth and grape quality showed preference for an intervention applied once early in the season, thus enabling a rather large number of laterals to reach the physiological maturity around veraison (Reynolds and Wardle 1989, Poni and Intrieri 1996, Keller et al., 1999) and a compensation for the temporary loss in functional leaf area (Poni and Giacchino, 2000). When performed before fruit set, trimming can increase the fruit set percentage, promotes the allocation of carbohydrates to the clusters and the production of more compact bunches (Baldini and Intrieri, 1984, Baldini and Intrieri, 2004, Poni et al., 2003). However, in the contest of climate change, the effects of this practice must be carefully considered together with other factors (e.g. cultivar, global warming, law-enforced yield constraints), in order to avoid an excessive and rapid total soluble solids accumulation in the grapes not coupled by adequate levels of phenolic maturity at harvest. In fact, excessive sugar concentrations in grapes, occurring in particularly warm seasons, leads to wines with high alcohol levels (Palliotti et al., 2014). The limitation of alcohol strength can be achieved by costly interventions in the winery aimed at dealcoholizing the wine (e.g. supercritical fluid extraction, vacuum distillation, inverse osmosis etc.). However, by the Regulation EC 606/2009, the reduction of alcohol strength must not be higher than 2% and, in the case of Regulation EC 203/2012 this enological practices is even prohibited.

Recent papers showed that post-veraison trimming, decreasing leaf area/yield ratio during the last phase of berry ripening, can slow down total soluble solids accumulation without altering berry skin anthocyanins concentration at harvest (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016).

However, in the case of trimming, a lively debate animates winegrowers. In fact, nowadays, many growers, particularly those adopting the organic and biodynamic cultivation method, avoid this practice (Mazzilli and Braccini, 2010, Rombolà, 2015). Especially in warm seasons, renouncing to trimming represents a solution for providing some shade to the clusters ("accucciatura") (Figure 2). In the case of biodynamic cultivation method, trimming is not practiced in order to preserve the shoot apex and the long shoots are twisted on the top wires ("accapannatura") (Figure 3).

Figure 2. Grapevine with long shoots (“accucciatura”) in an organic vineyard.

Figure 3. Long shoots twisted on the top wires ("accapannatura") in a biodynamic vineyard.

The consequences of these practices on vine performance and berry quality are scarcely been investigated. Moreover, bundling of vegetation in the upper part of the canopy may favor the occurrence of fungal infections during the vegetative season and, in areas with high winds, the consequent heaviness of the plant trellis system can produce serious structural damages to the vineyard.

Defoliation is another frequently adopted summer pruning practice (Poni et al., 2006, Intrieri et al., 2008, Matus et al., 2008, Diago et al., 2012, Palliotti et al., 2013, Pastore et al., 2013, Poni et al., 2013, Risco et al., 2014, Gatti et al., 2015, Rombolà et al., 2014, Tessarin et al., 2014, Rombolà et al., 2015, Baiano et al., 2015), enabling to establish a microclimate contrasting the development of fungal diseases, thus allowing healthy bunches to be maintained on the vine up to the beginning of autumn. The effects on quality parameters seem to be related to the cultivar and timing of treatment imposition (Bledsoe et al., 1988, Poni et al., 2006, Intrieri et al., 2008, Poni et al., 2009, Diago et al., 2012, Pastore et al., 2013, Poni et al., 2013, Palliotti et al., 2013, Tessarin et al.,

2014, Baiano et al., 2015) which may influence the source–sink balance and to the modifications of microclimate around the bunches. Several studies reported the impact of early (pre-bloom-fruit set) defoliation on grape quality parameters (Poni et al., 2006, Guidoni et al., 2008, Intrieri et al., 2008, Diago et al., 2010, Pastore et al., 2013) and vineyard efficiency in terms of gas exchange, source-to-sink balance and reserve storage responses (Palliotti et al., 2011). As regard to grapes, early leaf removal has been shown to produce a reduction in bunch weight, promote the loosening of clusters and improve berry quality (Poni et al., 2006, Intrieri et al., 2008). However, recentPapers suggest that defoliation just before anthesis should be applied with caution and probably be limited to specific seasons, due to a possible cumulative negative effect on vine bud fertility (Risco et al., 2014). Furthermore, it is still unclear to what extent this practice may preserve grapes from ripening disorders as sunburn (Gatti et al., 2015, Pastore et al., 2013) and the effects of a long time berry exposure to light radiation on epicuticular waxes. Moreover, it has been reported that early defoliation may decrease must titratable acidity, increase must pH (Diago et al., 2012, Risco et al., 2014) and enhance malic acid degradation (Gatti et al., 2015) with negative consequences on wine quality. Late defoliation (imposed at the onset of veraison or later) is a frequently adopted practice, consisting in removing a variable number basal leaves of the shoot and/or laterals to allow air circulation, light radiation and plant protection products penetration in the bunch zone. The effects of this late intervention on vine performance and berry quality vary in relation to the cultivar and modalities of treatment imposition (Pastore et al., 2013, Poni et al., 2013, Palliotti et al., 2013, Tessarin et al., 2014, Baiano et al., 2015). For instance, leaf removal imposed at veraison, in the bunch zone, may exert a negative effect on anthocyanin concentration, as for cvs. Sangiovese (Pastore et al.,

2013), Cabernet Sauvignon (Matus et al., 2009) and Uva Longanesi (Tessarin et al., 2015); however, the impact of this practice on total soluble solids is highly dependent to the concerned variety.

In the case of *Botrytis cluster* rot, defoliation, may have a crucial role, in preserving the grapes quality and sanitary status, also due to the absence of effective products against this pathogen (Rombolà et al., 2014, Rombolà et al., 2015). However, there is still scant information on the effects of this practice in organic vineyards. This gap in scientific knowledge should be filled, due to the importance of this practice in sustainable viticultural systems.

The increasing development of organic farming requires a strong and continuous interaction between the World of Research and Agriculture. In fact, despite the diffusion of organic viticulture, detailed scientific information providing evidence and elucidation on the effects of specific agricultural practices (e.g. soil and canopy management) in organic vineyards is still scarce.

The PhD Thesis has been conducted on cv. Sangiovese, one of the most widespread grape varieties in Italy, covering around 70,300 ha (Unione Italiana Vini). This cultivar displays a relevant agronomic and economic importance since it is used in the production of different famous wines, such as Brunello di Montalcino, Chianti Classico, Romagna Sangiovese (Bergamini et al., 2012). Sangiovese is characterized by a wide number of clones, high reactivity and variability of response to environmental and cultural factors (Rustioni et al., 2013) and by a delicate anthocyanin profile (Mattivi et al., 2006, Castellarin et al., 2012). Especially in warm season, this variety is subjected to uncoupling between technological and phenolic maturity (Rombolà et al., 2011, Palliotti et al., 2013, Poni et al., 2013, Rombolà et al., 2014, Rombolà et al., 2015, Bondada et

al., 2016), a phenomenon that can seriously compromise the overall quality of the final product (Palliotti et al., 2014). In addition, a key reproductive feature of cv. Sangiovese is that, regardless of how it is cultivated, it produces tight clusters, classified as moderately compact, semi-compact, and compact (Nelson-Kluk 2006). This morphology may cause losses in cuticular barrier properties at the contact surfaces and hence bunches become victims of a host of fungal diseases, mostly *Botrytis* cluster rot during ripening, especially in grapevines cultivated with organic method. Other concerns of cv. Sangiovese include its tendency to overcrop deriving from the high fruitfulness of its shoots regardless of origin (primary or secondary buds, basal buds) and a vigorous procumbent growth habit leading to dense canopies, that can produce negative effects on fruit quality, wood maturity, and vine size maintenance (Poni et al., 2006).

In the context of climate change, several agronomic approaches, such as canopy management practices (Rombolà et al., 2011, Palliotti et al., 2013, Poni et al., 2013, Rombolà et al., 2015, Bondada et al., 2016), have been implemented to solve these issues and improve cv. Sangiovese berry and wine quality. However, only few recent Research provides scientific information on the implications of specific summer pruning practices (e.g. trimming) in organically cultivated cv. Sangiovese vineyards (Rombolà et al., 2011, Bondada et al., 2016).

The main objectives of the PhD Thesis are:

- providing scientific knowledge on the implications of different canopy management practices, imposed since post-veraison (trimming, defoliation, shoot positioning) on vine physiology, grape and wine quality of organically cultivated cv. Sangiovese;

- identifying agricultural techniques for preserving and improving the chemical properties and sanitary status of organic cv. Sangiovese grapes, in the context of climate change;
- contributing to couple phenolic and technological maturity in organic cv. Sangiovese grapes;
- investigating the modes of actions through which canopy management practices may influence flavonoids accumulation in cv. Sangiovese berry cells.

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CHAPTER 1: Physiological and enological implications of post-veraison trimming in an organically managed vineyard (*Vitis vinifera* L, cv. Sangiovese).

Paola Tessarin[1], Andrea Versari[2], Adamo Domenico Rombolà[1*]

[1]Department of Agricultural Sciences, School of Agriculture and Veterinary Medicine, Alma Mater Studiorum, University of Bologna, Viale G. Fanin 44, 40127 Bologna, Italy.

[2]Department of Agricultural and Food Sciences, School of Agriculture and Veterinary Medicine, Alma Mater Studiorum, University of Bologna, Piazza Goidanich 60, 47521, Cesena, Italy.

[*]Corresponding author: e-mail: adamo.rombola@unibo.it

Abstract

Backgrounds and aims: For the first time post-veraison trimmed plants were compared with untrimmed controls, assessing the effects on vine physiology, grape and wine quality.

Methods and Results: In 2013 and 2014, post-veraison late trimming (LT) imposed at 15 Brix was compared to untrimmed control (CK) and early (pea-size) trimming (ET). Late trimming increased berry skin anthocyanin and phenolic concentration without modifying berry soluble solids, pH and titratable acidity, reduced malic acid levels at harvest, contained plant productivity, loosened clusters and limited the severity of *Botrytis* cluster rot. Improved berry color and decreased malic acid levels were observed in ET vines only in 2013. Bunches from ET plants showed higher compactness and discoloration compared to those of LT plants. Trimming did not modify the main chemical characteristics of wine such as, alcohol strength, dry matter, total and volatile acidity and pH. However, in both years, a sensible increase in color components and tannins was found in LT wines and with formation of more stable components which can enhance wine quality.

Conclusions: Trimming in post-veraison is an effective practice for improving berry color, without modifying technological parameters and enhancing wine quality in contrasting climatic conditions.

Significance of the study: The Research strongly highlights the need to consider additional physiological mechanisms other than source-sink relationship. The enological results are a contribution to the enhancement of the wine quality.

Key words: anthocyanin, organic viticulture and wine, phenols, topping.

Introduction

Increased consumer awareness of environmental pollution in agriculture and the importance of food quality in relation to human health encouraged the adoption of alternative agronomical strategies, such as organic and biodynamic farming (Parpinello et al., 2015, Bondada et al., 2016, Botelho et al., 2016).

According to the FIBL, IFOAM surveys (Willer and Lernoud, 2014), over 280'000 hectares of organic grapes were grown in 2014, which constitutes 4% of the world's grape growing area (7 million hectares in 2011, according to the FAOSTAT). Organic farming adopts peculiar management practices and processing methods which in many countries are regulated by specific laws (Reg. EC 2012, AQIS, 2013).

In organic vineyards all field strategies, including canopy and soil management, should be addressed to preserve and enhance plant health and resilience capability, with the aim of improving the effectiveness of plant protection products and producing high quality and healthy grapes. Therefore, with regard to canopy management, some interventions (e.g. early shoot thinning, removal of suckers) become compulsory. It is important to stress that grape berry primary and secondary metabolites are sensitive to cultural practices and environmental effects. In particular, climate change can modify the composition of the berry and consequently wine quality, by reducing anthocyanins and organic acids concentration (Barnuud et al., 2014), but also modifying aroma profiles (Keller et al., 2010). As a consequence, wine production and tipicity, in the long term, will be threatened in several viticultural areas (Schultz and Jones, 2010).

Over the last two decades a trend toward overly fast grape ripening with excessive total soluble solids accumulation and reduced color in the fruit and high alcohol in the resulting wine emerged in several countries (Duchene and Schneider, 2005, Godden and

Gishen, 2005, Dokoozlian, 2009, Chaves et al., 2010, De Orduna, 2010, Keller, 2010, Rombolà et al., 2011, Jones, 2012, Palliotti et al., 2014, Herrera et al., 2015). Decoupling between sugars and anthocyanins accumulation is particularly evident in some red varieties, such as Sangiovese (Rombolà et al., 2011, Palliotti et al., 2013, Poni et al., 2013, Filippetti et al., 2015, Bondada et al., 2016) and changes its intensity depending on the terroir and climate.

Trimming is a widespread, fully mechanizable canopy management practice, commonly adopted to contain canopy volume, improve grape quality, enhance the efficiency of plant protection products, facilitate harvest and the transitability in the vineyard. The consequences of trimming depend on its severity and on the phenological stage of application (Peterson and Smart, 1975, Kliewer and Bledsoe, 1987, Reynolds and Wardle, 1989, Cartechini et al.,, 2000, Solari et al.,, 1988, Poni and Giacchino, 2000, Rombolà et al., 2011, Satta et al., 2012, De Pau et al., 2012, Martinez de Toda et al., 2013, Filippetti et al., 2015, Herrera et al., 2015, Bondada et al., 2016). In cv. Sangiovese, post-veraison trimming, decreasing leaf area/yield ratio during the last phase of berry ripening, can slow-down total soluble solids accumulation in the berry without altering skin anthocyanins (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016) and tannins concentration (Filippetti et al., 2015) and determine a reduction in clusters weight and yield and consequently of cluster compactness (Rombolà et al., 2011, Bondada et al., 2016). In cv. Merlot, severe trimming imposed at late veraison could reduce sugars without affecting anthocyanins concentration (Herrera et al., 2015).

Previous papers aimed to counteract the effects of climate change, focused on agronomic approaches for delaying sugars accumulation through post-veraison

trimming, without producing adverse effects on the berry poliphenols, particularly anthocyanins and tannins (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016).

To our knowledge, there is no information derived from the comparison between plants subjected to post-veraison trimming and not trimmed control vines. This lack of knowledge is surprising considering that many growers, particularly those adopting the organic and biodynamic cultivation method do not perform trimming. Therefore we were stimulated to establish an experiment for investigating the effects of post-veraison trimming on organically cultivated cv. Sangiovese, on vine physiology and grape and wine composition.

Material and Methods

Plant material and experimental layout

The experiment was conducted in 2013-2014, in a mature vineyard planted in 2003 with cv. Sangiovese grapes (clone FEDIT 30 ESAVE), *Vitis vinifera* L., grafted onto Kober 5BB, trained to cordon de Royat training system (VSP). The vineyard was located in Tebano (Faenza, RA), Italy (44°17′7″ N, 11°52′59′E, 117 m a.s.l.), on a medium slope, with southeast/northwest and downhill-oriented rows. Vines were spaced 2.8 m within the row and 3.0 m between rows, 3,571 plants/ha. Starting in 2007, the vineyard was managed as organic in accordance with Reg. EC 834/2007 (EC, 2007).

Beginning in 2007, no irrigation water was applied and the vineyard was not fertilized. The loamy clay alkaline soils of the vineyard presented medium organic matter (2.2 %) and nitrogen (1.5 ‰) concentration, high levels of carbonates (total carbonates 14.7 %; active lime: 6.7 %), medium-high concentration of assimilable phosphorus (P: 10 ug/g)

and potassium ions (K: 188 ug/g), as well as assimilable iron (23 ug/g) and manganese (11.07 ug/g). Available copper in the soil showed high values, around 19 µg/g. Annually, at the end of each vegetative season, herbaceous species were sowed in alternate planting rows, such as fava bean (*Vicia faba*), barley (*Hordeum vulgare*) and *Trifolium subterraneum*. Soil was managed by mowing the vegetation during late spring, which maintained biomass on the soil surface. In October 2014, subterranean clover has been sowed also along the row strip.

The vineyard was treated against diseases and pests, using products allowed by the EC Regulation (EC, 2002). Treatments consisted mainly of copper (an average of 6 kg/ha /year) and sulfur (an average of 70 kg/ha/year), enabling control of fungal pathogens (*Plasmopara viticola*, *Uncinula necator* and *Botrytis cinerea*). At the end of February vines were spur-pruned to two count nodes/buds equating to 12-14 buds per vine. The noncount shoots (shoots arising from base buds of the spur) were removed at the beginning of the season by leaving 12 shoots with uniformed distribution per meter of cordon. The cluster number was adjusted by cluster thinning at veraison, in order to mantain a maximum number of 16 bunches per plant.

The experimental design included 3 treatments: a control (CK), non-treated vines; two timings of trimming: early trimming (ET), performed at fruit set, late trimming (LT), imposed in post-veraison. Each treatment was replicated three times in a completely randomized experimental design, with 30 monitored vines per treatment, for a total of 90 plants. The control treatment (CK) presented long shoots (24 nodes), falling from both sides of the canopy, without obstructing the passage of agricultural machineries. Early trimming (ET) was imposed at 19 DAF (days after beginning of flowering: BBCH 61: 10% of flowerhoods fallen), 2013 and 27 DAF in 2014, up to 140 cm from the

cordon (18 nodes per main shoot) at BBHC 75 (berries pea-sized, bunches hang). Late trimming was imposed when berry juice reached 15 Brix, 11 days after veraison (recorded when 50% of the berries began to develop variety-specific color), 86 DAF in 2013; 21 days after veraison 92 DAF, in 2014 by keeping 14 nodes per main shoot. The trimmed parts of the shoots were immediately removed from the experimental plots in order to avoid release of nutrients in the soil and subsequent uptake from the vines.

Leaf macro and micronutrients and SPAD index

At veraison (75 DAF, 2013; 71 DAF, 2014) 20 mature, exposed and completely expanded leaves per experimental plot (60 leaf per treatments), inserted at the 4th node above the first bunch were collected, in order to monitor the leaf nutritional status. Leaves were sampled from shoots originating from true buds, bearing at least one bunch. The leaf chlorophyll index was detected with a Minolta SPAD-502 chlorophyll meter (Minolta, Osaka, Japan) performing five measurements per leaf blade.

Leaf blades, deprived of petioles, were washed in a detergent solution (HCl 0.1 N + Tween 20 0.1 %) to remove nutrients that may be present on the leaf (Alvarez-Fernandez et al., 2001), rinsed with distilled water, dried at 65 °C until constant weight, than weighed and finally milled.

Leaf blades were dried at 70 °C, and ground (sieve < 0.5 mm). Total nitrogen was determined by the Kjeldhal method and phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and boron (B) were analysed as decribed in García-Escudero et al., (2013).

Leaf gas exchange

Measurement of leaf gas exchange was determined on two plants per experimental plot (6 plants per treatment) using an infrared gas analyser (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-Cor, Inc., Lincoln, NE, USA). Measurements were performed in the morning between 9:00 AM and 10:30 AM and in the afternoon between 3:00 PM and 4:30 PM, on a leaf inserted in the middle third of a fruiting shoot (12^{th} node), originating from true buds. Leaves were illuminated by the LI-COR 6400 LED light source providing a photosynthetic photon flux density ca.1200 $\mu mol/(m^2 \cdot s)$. The level of CO_2 was fixed at 380 ppm within the leaf chamber. Net photosynthesis was measured when foliar CO_2 uptake was steady. The measurements were taken at 3 dates during ripening: at 75, 113 and 127 DAF, in 2013 and 70, 109 and 118 DAF, in 2014.

Stem water potential

Stem water potential (Mpa) was measured at midday during berry ripening (75 DAF, 2013; 109 DAF and 118 DAF, 2014) on 2 plants per experimental plot (6 plants per treatment), by using the Scholander pressure chamber method (Scholander et al., 1965). From each vine, one mature, completely expanded, exposed, healthy non transpiring leaf, inserted at the 12^{th} node of a fruiting shoot was selected. About 60 minutes before midday measurement, leaves for determination of $\psi stem$ were enclosed in plastic bags covered with aluminum foil.

Leaf and bunch temperature

An assay of leaf and bunch temperature values was performed through an infrared thermometer (Raytek Raynger^{TM} ST, Santa Cruz, CA, USA). The monitored leaves (6

per treatment) were inserted at the 12th node of fruiting shoots originating from true buds. The temperature of the basal cluster, inserted on the same shoot, was detected on the sunlight exposed surface and on the shaded portion of 6 bunches per treatment. The measurements of leaf and bunch temperatures were repeated 5 times per days (at 8:00 AM, 9.00 AM, 10:00 AM, 11:00 AM, 2:00 PM, and 3:00 PM) and taken at 5 (2013) and 4 dates (2014) during ripening.

Vegetative-productive parameters

The vines phenological phases were monitored. After one (April 2014) and two years (April 2015) of treatment imposition, the percentage of bud burst of count nodes (number of shoots from (SCN)/ CN) and the fruitfulness of count nodes (number of inflorescences (INF)/ SCN) were detected on 30 plants per treatment, when inflorescences were clearly visible (BBCH 53).

The contribution of primary and lateral leaves was measured separately per position through a LI-3000A leaf area meter (Li-Cor Biosciences, Lincoln, NE, USA) on 9 representative fruiting shoots per treatment, at the end of the vegetative growth, sampled from three additional vines. After the shoot sampling these vines were not used for any additional sampling or measurements. The total leaf area per vine (TLA), the total main shoots leaf area (SLA) and the total laterals leaf area (LLA) per plant were estimated by multiplying the average leaf area per shoot by the number of shoots per vine. The number of laterals per shoot, the length and the number of nodes and the average leaf area of each lateral were also determined. At harvest (127 DAF, 2013; 125 DAF, 2014), productive parameters, such as number of clusters per plant, productivity per vine (kg) and bunch weight (g) (Wunder Digital Dynamometer, Wunder SA-Bi S.r.l, Milan,

Italy), leaf area (LA/yield) ratio were detected on 10 plants per experimental plot (30 vines per treatment).

After leaves abscission, the pruning wood weight (kg) was determined and the Ravaz Index (yield/ pruning wood ratio) calculated on 10 plants per experimental plot (30 vines per treatment).

Berry growth and technological parameters

Berry weight, expressed as g per berry (technical balance Gibertini Elettronica S.r.l., Milan, Italy), total soluble solids (TSS; Brix; Electronic Refractometer Maselli Misure S.P.A., Parma, Italy), titratable acidity (TA; expressed as g/L of tartaric acid) and pH (Crison Compact Titrator, Crison Instrument SA, Barcelona, Spain) were periodically determined by collecting 50 berries per replicate since veraison until harvest.

Organic acids

A sample of 10 berries was grinded under liquid nitrogen, then an aliquot of 1 gram (FW) of berry powder was extracted in 5 mL of bidistilled water and left for 30 min at room temperature as reported by Mikulic-Petkovsek and others (2007).

After the extraction, the homogenate was centrifuged, by slightly adapting the above mentioned method, at 24000 *g* for 7 min at 10 °C. The supernatant was filtered through a 0.45 μm cellulose ester filter (GVS Filter Technology, Zola Predosa, BO), transferred into a vial, and 10 μL of the sample was used for the analysis.

Organic acids were quantified as described by Neumann (2006) using an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a PDA detector and a reversed-phase column RP18 250 × 4.6 mm (5-µm particle size) (Phenomenex, Castel Maggiore, BO,

Italy). High-performance liquid chromatography elution buffer was 18 mmol/L KH_2PO_4, pH 2.10 adjusted with H_3PO_4. Chromatograms were run for 40 min using a detection wavelength of 210 nm. During the analysis, two organic acids were identified and quantified (tartaric and malic acid).

A calibration curve was established with tartaric and malic acid standards (Sigma-Aldrich) and the concentrations were expressed as mg/g of FW.

Berry skin anthocyanin and total phenolics analysis

Additional berry samples (20 berries per experimental plot) were collected during ripening for determining total anthocyanins and phenolics. The extraction was performed on berry skins in methanol according to Mattivi et al., (2006).

An aliquot of the berry skin extract was acidified in order to contain 0.1% HCl (v/v) at pH 1 and incubated at room temperature (about 25°C) for 24 h in the dark with occasional shaking. The anthocyanins concentration of the extract was determined by measuring the absorbance at 520 nm at pH 1 using an extinction coefficient (molar absorbance value) of 28000 and molecular mass of 529 (typical of malvidin-3-glucoside).

Total phenolics were measured by adapting the procedure reported in Palliotti et al., (2013): to each 0.1-mL acidified skin extract sample, 1.9 mL of distilled water were added, followed by 5 mL of 10% aqueous Folin-Ciocalteau reagent (Labochimica S.r.l., Padova, Italy) and 4 mL of 7.5% (w/v) aqueous Na_2CO_3 (J.T. Baker). The mixture was maintained at 24°C. The absorbance at 750 nm was read after 2 h, and compared with a gallic acid standard curve. Anthocyanins and total phenolics were expressed as mg/g of berry skin.

Bunch qualitative parameters

At harvest (127 DAF, 2013; 125 DAF, 2014) the qualitative parameters of bunches were measured. For each bunch, the index of cluster compactness (according to the 1983 OIV classification) and the surface area affected by discoloration (%) were detected. The incidence (number affected cluster per vine) and severity (number of affected berries per cluster) of bunch rot were monitored on 10 plants per experimental plot (30 plants per treatment).

One bunch per plant (30 per treatments) was sampled for determining bunch weight, length and width, the number of berries per cluster, the rachis weight (g), length and width.

Wine chemical analysis

In both vintages, grapes collected at optimum technological maturity were processed according to organic winemaking protocol proposed by the Italian Association for Organic Farming (AIAB, Italy) in accordance to the dispositions of Reg. CE N. 203/2012 and Reg CE n. 834/2007. Nine vinifications (CK, ET, LT repeated for 3 different vine blocks) of 20 kg of grapes were carried out: briefly, after destemming and crushing, skin and must were placed in stainless steel tanks and treated with sulfur dioxide (as potassium metabisulphite: 10 g/hl, AEB, Italy), complex nutrients (30 g/hl, Nutristart, Lafford, France) and inoculated with OGM-free yeasts (20 g/hl *Saccharomyces cerevisiae*, F15, Laffort, France). Sugar consumption was monitored over time by means of a Babo densimeter throughout fermentation. The tank content was homogenised every day to dissolve the cap into the wine. At zero degree Babo,

both free run wine and wine extracted from marc with a piston press (2 bar) were collected and pooled. After the final racking, carried out one month from the end of alcoholic fermentation, the wines were cold-stabilized, then bottled and stored at 10 °C prior to chemical analyses. Wines were analysed for alcohol strength (AS, %), dry matter (DM, g/L), pH (U), total acidity (TA, g/L), volatile acidity (VA, g/L according to European official methods (EC, 1990). Moreover, analyses for total (TS, mg/L) and free (FS, mg/L) sulphur dioxide (Ripper and Schmitt, 1896) and reducing substances (RS, g/L) (Lane and Eynon, 1923) were carried out. Wines were also analysed for the following color and phenolics related parameters: total anthocyanins (ANT, AU), total red color (TCO, AU), co-pigmentation (COP, AU) (Boulton, 2001), large polymeric pigments (LLP, AU), small polymeric pigments (SPP, AU), tannins (TNN, mg/L) and non-tannin total iron-reactive phenolics (IRP, mg/L) (Harbertson et al., 2003) carried out by spectrophotometric assay (UV–Vis 1240 mini, Shimadzu, Milano, Italy). All the listed analyses were carried out at the end of alcoholic fermentation; moreover, in order to monitor the change of wine composition over time, the analyses of color and phenolic components were repeated in both wines after 15 months, and for 2014 wines after 27 months, from the end of the fermentation. Data are presented as mean values obtained from two replicated analyses of each vinification.

Statistical analysis on berries and vegetative-productive parameters data

Analysis of variance and comparison of means of parametric data were performed using SAS 6.04 software (SAS INSTITUTE, CARY, NC, USA) and Student-Newman-Keuls test ($P=0.05$). Non parametric data were subjected to Kruskall Wallis test, followed by Dunn's comparison test ($P=0.05$).

Analysis of variance for mean separation and Fisher's LSD as post hoc were carried out with XLSTAT version 2011.1.05. For sensory analysis the results were evaluated using statistical tables of binomial distribution. All statistics were performed with significance at $P = 0.05$.

Principal components analysis (PCA) was used as an unsupervised multivariate data tool to find hidden structure among the observations XLSTAT version 2011.1.05 (Addinsoft, Anglesey, UK).

Results

Climatic conditions

In the 2 years of the research, climatic data (mean, maximum and minimum daily air temperatures (T), relative humidity (RH) and total rainfall) were recorded, in a meteorological station located 800 m from the vineyard.

In 2013, the maximum temperature was detected at the beginning of August (around 40 C). The total rainfall was 433 mm and mainly occurred in spring, at the end of August (55 mm) and during the second half of September (52 mm) (Figure 1).

Overall, the 2014 vegetative season was marked by average temperatures well below seasonal normal (Figure 1). The highest maximum temperatures (32 °C) were recorded at the end of spring (20 DAF and 21 DAF) and on July 20th (59 DAF). From the second half of April to harvest the total rainfall was abundant (489 mm) and quite frequent both in spring and summer (Figure 1).

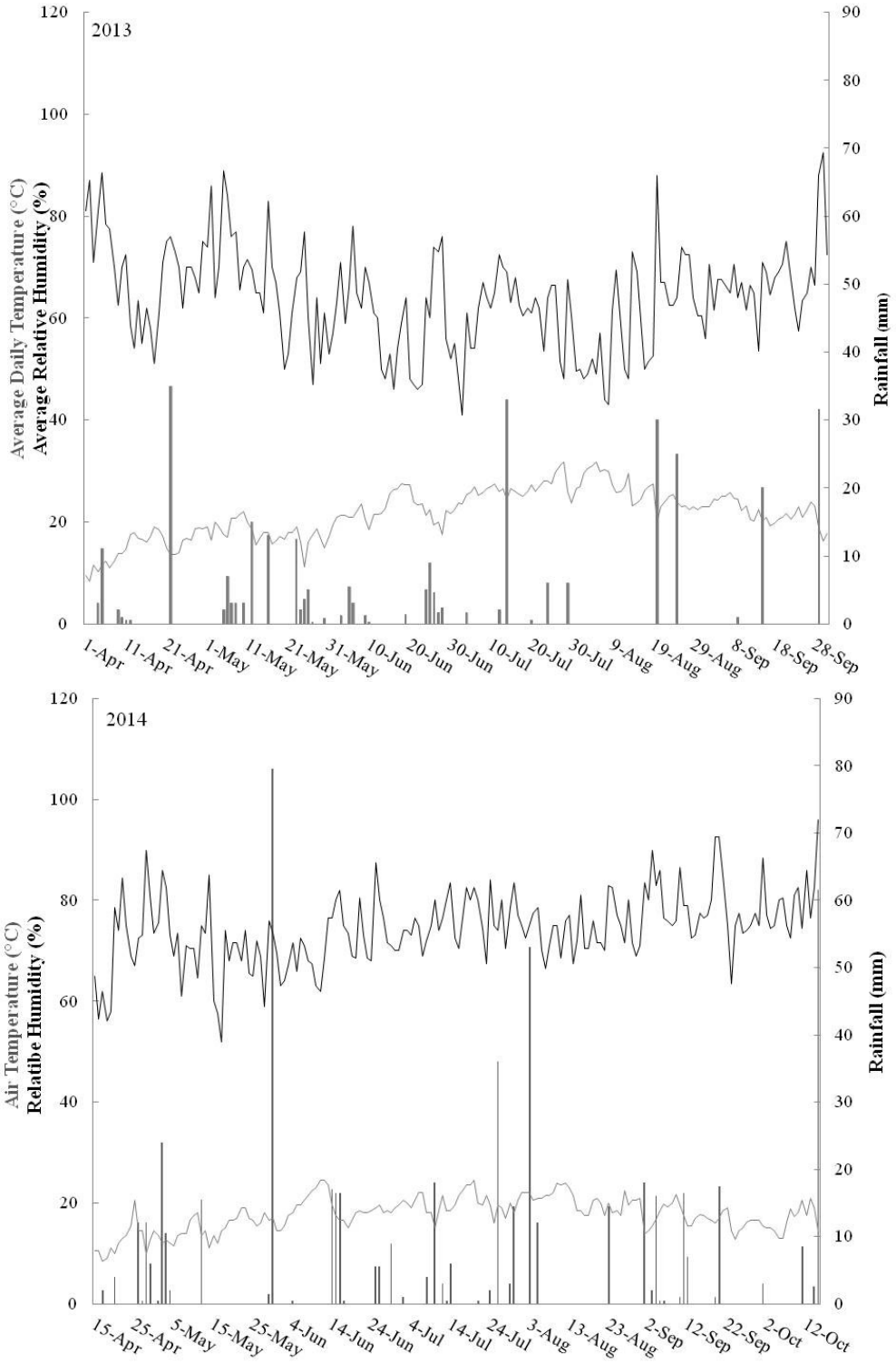

Figure 1. Seasonal trends of average daily air temperature (grey line) and average relative humidity (black line) recorded in 2013 and 2014, 800 m from the trial site. Vertical bars indicate daily rainfall.

Leaf nutritional status and SPAD Index

In 2013, plants submitted to trimming presented similar leaf macro and micronutrients concentration compared to CK vines (Table 1). The concentration of Ca and Mg resulted higher in ET plants compared to vines trimmed in post-veraison (Table 1).

In 2014, trimmed vines showed similar leaf N and Fe concentration to the control; however, higher N and Fe values were detected in ET than in LT leaves (Table 1). Trimming enhanced the concentration of P in the leaves without influencing leaf levels of K, Ca and Mg. Early trimmed vines presented higher leaf B value compared to CK and LT plants (Table 1). Trimming did not modify the concentration of Mn, Zn, Cu and Na.

In both years the trimming treatments did not alter the leaf SPAD values at veraison (Table 1).

Stem water potential

The lowest values of stem water potential were detected in the first year due to the climatic conditions (Figure 1).Trimming did not modify the values of the stem water potential (-MPa) recorded at 75 DAF in 2013 (CK: 1.3; ET; 1.6; LT; 1.2); at 109 DAF (CK: 0.8; ET; 0.9; LT; 0.8) and at 118 DAF in 2014 (CK: 0.8; ET; 0.8; LT; 0.9).

Leaf gas exchange

Treatment did not influence leaf photosynthetic activity or stomatal conductance, during ripening (Table 2)**.**

Table 1. Leaf mineral elements: Nitrogen, Phosphorous, Calcium, Magnesium (macronutrients); Iron, Manganese, Zinc, Copper, Boron and Sodium (micronutrients) and SPAD index, detected at veraison, in 2013 and 2014, in control, early trimmed and late trimmed cv. Sangiovese vines.

Treatment	N (%)		P(%)		K (%)		Ca (%)		Mg (%)		Fe (ppm)		Mn (ppm)		Zn (ppm)		Cu (ppm)		B (ppm)		Na (ppm)		SPAD Units	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
CK	1.76	1.91ab	0.17	0.17b	1.16	1.26	3.28ab	2.91	0.39ab	0.30	56.3	58.0ab	61.3	48.0	14.0	19.3	111.0	131.0	49.0	38.7b	77.3	73.7	32.4	33.9
ET	1.80	2.16a	0.16	0.20a	1.09	1.22	3.36a	3.06	0.42a	0.31	57.3	64.0a	55.7	45.0	13.7	18.0	99.3	164.0	48.3	45.7a	66.0	59.7	30.6	33.6
LT	1.76	1.81b	0.19	0.19a	1.30	1.30	3.17b	3.01	0.33b	0.34	79.0	53.0b	57.3	49.0	14.0	20.7	119.7	158.3	45.0	39.3b	72.0	68.0	30.5	31.1
Significance	*n.s.*	*	*n.s.*	*	*n.s.*	*n.s.*	*	*n.s.*	*	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*Significant at $P \leq 0.05$; n.s., not significant ($P=0.05$). Means followed by different letter in each column are significantly different according to the Student- Newman-Keuls test. CK, control vines; ET, early trimmed vines; LT, late trimmed vines.

Table 2. Net photosynthesis, stomatal conductance measured on control, early trimmed and late trimmed cv. Sangiovese vines.

2013	**75 DAF**				**113 DAF**				**127 DAF**			
	Net photosynthesis		**Stomatal Conductance**		**Net photosynthesis**		**Stomatal Conductance**		**Net photosynthesis**		**Stomatal Conductance**	
	µmol CO_2 m^{-2} s^{-1}		**mol H_2O m^{-2} s^{-1}**		**µmol CO_2 m^{-2} s^{-1}**		**mol H_2O m^{-2} s^{-1}**		**µmol CO_2 m^{-2} s^{-1}**		**mol H_2O m^{-2} s^{-1}**	
Treatments	**9:00-10:30 AM**	**3:00-4:30 PM**	**9:00-10:30 AM**	**3:00-4:30 PM**	**9:00-10:30 AM**	**3:00-4:30 PM**	**9:00-10:30 AM**	**3:00-4:30 PM**	**9:00-10:30 AM**	**3:00-4:30 PM**	**9:00-10:30 AM**	**3:00-4:30 PM**
CK	6.0	8.2	0.097	0.119	8.1	8.4	0.198	0.221	8.8	ND†	0.195	ND
ET	6.0	5.2	0.083	0.104	6.7	7.5	0.283	0.138	7.4	ND	0.122	ND
LT	4.0	7.1	0.067	0.084	8.1	7.0	0.199	0.148	10.3	ND	0.246	ND
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	ND	*n.s.*	ND
2014	**70 DAF**				**109 DAF**				**118 DAF**			
CK	5.6	6.8	0.096	0.129	11.2	8.3	0.405	0.315	7.6	7.8	0.286	0.299
ET	5.8	6.1	0.099	0.114	8.9	10.9	0.357	0.371	7.3	8.6	0.267	0.297
LT	8.3	6.6	0.111	0.087	9.9	9.0	0.351	0.354	7.3	7.3	0.263	0.300
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student-Newman-Keuls test.

CK, control vines; ET, early trimmed vines; LT, late trimmed vines; †ND, not detected.

Vegetative and productive parameters

In both years, vines trimmed at pea size presented, at the end of the vegetative growth (end of July), a similar total leaf area compared to control and post-veraison trimmed plants. However, early trimmed vines were characterized by a different composition of TLA, presenting, in both years, significantly lower main shoots leaf area and higher laterals leaf area compared to the CK and LT treatments. In 2013 and 2014, early trimmed vines presented longer laterals, with higher number of nodes and higher average laterals leaf area compared to control and LT vines (Table 3). The imposition of LT treatment removed 34% and 35% of total leaf area in 2013 and 2014, respectively and was not followed by formation of new laterals (Table 3). In 2013, after the application of LT treatment, the highest value of SLA was detected in CK plants, LLA resulted higher in ET vines with respect to CK and LT plants, while TLA was higher in control and ET vines. In 2014, ET vines presented lower SLA compared to control plants, but higher than vines subjected to LT (Table 3). The highest values of LLA and TLA were measured in ET plants; LT vines did not change LLA, while decreasing TLA compared to CK plants (Table 3).

Early trimming increased the number of nodes, lateral length and single leaf area in both years (Table 3). Late trimming decreased the number of laterals in both years, their length and single leaf area in 2013 (Table 3). In both years, LT laterals displayed a lower growth than ET laterals (Table 3).

Table 3. Leaf area of main shoots, laterals and total leaf area per plant, number of laterals per shoot, lateral length, number of nodes per lateral, lateral single leaf area, in control, early trimmed and late trimmed cv. Sangiovese vines, after the imposition of LT treatment (86 DAF, 2013; 92 DAF, 2014).

Treatment	SLA (m^2)		LLA (m^2)		TLA (m^2)		Lateral/shoot (N°)		Lateral length (cm)		Lateral nodes (N°)		Lateral leaf area (cm^2)	
	2013	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**
CK	4.4a	4.2a	2.1b	1.4b	6.5a	5.6b	16.0a	13.7a	4.81c	3.53b	1.95b	1.60b	108.21c	86.73b
ET	3.0b	3.5b	2.9a	3.0a	6.0a	6.4a	11.5b	11.3ab	9.24a	8.95a	3.05a	3.70a	197.96a	218.27a
LT	2.8b	2.9c	1.5b	1.1b	4.3b	4.1c	8.2c	7.7b	7.13b	5.78ab	2.29b	1.91b	152.68b	124.96b
Significance	***	**	**	**	***	***	***	*	***	*	***	**	***	**

*Significant at $P \leq 0.05$; ** significant at $P \leq 0.01$; *** significant at $P \leq 0.001$. Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test. CK, control vines; ET, early trimmed vines; LT, late trimmed vines. SLA, main shoots leaf area; LLA, laterals leaf area; TLA, total leaf area per plant (SLA + LLA**).**

In 2013 and 2014, post-veraison trimming decreased bunch weight, plant productivity and pruning wood weight (Table 4).

Trimming did not alter LA/yield ratio and Ravaz index (Table 4). Trimmed plants presented similar LA/yield ratio compared to CK vines; however, in 2014, ET plants displayed a higher LA/yield as compared to LT vines (Table 4).

Table 4. Bunch number per plant, plant productivity, bunch weight, number of berries per cluster, bunch compactness and discoloration, pruning wood weight, Ravaz Index and LA/yield ratio, recorded in 2013 and 2014, in control, early trimmed and late trimmed cv. Sangiovese vines.

	2013				2014			
Parameters	**CK**	**ET**	**LT**	***Significance***	**CK**	**ET**	**LT**	***Significance***
Bunch (N°/plant)	13	13	13	*n.s.*	15	15	15	*n.s.*
Productivity (kg/plant)	4.37a	4.63a	3.61b	*	6.57a	6.48a	4.94b	*
Bunch weight (kg)	0.329a	0.344a	0.237b	*	0.434a	0.423a	0.327b	***
Berries (N°/bunch)	144	167	130	*n.s.*	132	113	111	*n.s.*
Bunch compactness (OIV rating)	7.4a	7.9a	6.7b	*n.s.*	8.3a	8.4a	6.9b	**
Bunch discoloration (%)	25.5b	36.9a	25.4b	*	89.2a	91.0a	73.1b	**
Pruning wood weigh (kg/plant)	0.675a	0.661a	0.485b	*	0.662a	0.653a	0.464b	*
Ravaz Index	6.7	7.1	7.6	*n.s.*	11.1	10.2	10.8	*n.s.*
LA/yield	1.68	1.39	1.36	*n.s.*	0.87ab	1.03a	0.82b	***

*Significant at $P \leq 0.05$; ** significant at $P \leq 0.01$; *** significant at $P \leq 0.001$; n.s., not significant ($P=0.05$). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test (bunch and berries number, plant productivity, pruning wood weight) and Kruskal-Wallis test, followed by Dunn's comparison test (bunch weight, compactness, discoloration, Ravaz Index, Leaf area/yield ratio). CK, control vines; ET, early trimmed vines; LT, late trimmed vines.

Trimming did not modify the percentage of bud burst, expressed as shoots from count nodes/count nodes, and their fruitfulness (Table 5).

Table 5. Budburst percentage expressed as: shoots from count nodes. Fruitfulness expressed as inflorescences/shoots from count nodes, in control, early trimmed and late trimmed cv. Sangiovese vines.

Treatments	Budburst SCN/CN (%)		Fruitfulness INF/SCN	
	2014	2015	2014	2015
CK	114.56	114.17	1.52	1.49
ET	108.48	108.52	1.48	1.32
LT	109.44	109.57	1.37	1.38
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*

n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Kruskal-Wallis test, followed by Dunn's comparison test. CK, control vines; ET, early trimmed vines; LT, late trimmed vines. CN; count nodes, SCN, shoots from count nodes; INF, inflorescences. Data were collected after one (2014) or two years (2015) of treatment imposition.

Bunch qualitative parameters

Trimming did not influence number of berries per bunch (Table 4), cluster and rachis length and width (data not shown). Late trimming decreased bunch compactness (Table 3). In 2014, the severity of *Botrytis* cluster rot (57% in control plants) was markedly reduced by late trimming (16%) and to lesser extent by early trimming (30%). Late trimmed vines presented lower bunch discoloration compared to early trimmed ones (Table 4).

Berry technological parameters

In 2013, all treatments presented higher values of TSS, pH and lower values of titratable acidity at harvest compared to 2014, due to the meteorological conditions that did not allow the grapes to fully ripen (Table 6). In both years, late trimming decreased berry weight at harvest (Table 6). Trimming treatments did not change berry total soluble solids, pH and titratable acidity concentration during ripening in either of the two years (Table 6).

Table 6. Berry weight (g), technological parameters (total soluble solids, pH, titratable acidity), recorded during ripening in 2013 and 2014, in control, early trimmed and late trimmed cv. Sangiovese vines.

2013	**75 DAF**	**101 DAF**	**113 DAF**	**125 DAF**
		Berry weight (g)		
CK	1.7	2.4	2.5a	2.5a
ET	1.6	2.2	2.3ab	2.4ab
LT	1.5	2.1	2.1b	2.3b
Significance	*n.s.*	*n.s.*	*	*
		TSS (°Brix)		
CK	12.6	19.1	21.3	23.6
ET	12.1	19.5	22.7	23.9
LT	13.2	20.7	22.3	24.3
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
		pH		
CK	2.76	3.08	3.21	3.25
ET	2.79	3.06	3.19	3.23
LT	2.81	3.12	3.22	3.27
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
		TA (g/L of tartaric acid)		
CK	22.5	9.6	7.7	7.0
ET	21.7	9.2	7.6	7.1
LT	21.2	9.1	7.5	7.0
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
2014	**71 DAF**	**106 DAF**	**120 DAF**	**125 DAF**
		Berry weight (g)		
CK	1.95a	2.6a	2.9	2.90a
ET	1.81b	2.4b	2.7	2.69a
LT	1.81b	2.5b	2.5	2.51b
Significance	***	**	*n.s.*	***
		TSS (°Brix)		
CK	10.9	17.0	18.6	18.8
ET	10.0	17.3	19.3	19.2
LT	10.2	18.0	19.8	19.9
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
		pH		
CK	2.61	3.02	3.15	3.17
ET	2.61	3.03	3.20	3.15
LT	2.60	3.02	3.17	3.19
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
		TA (g/L of tartaric acid)		
CK	23.7	10.3	8.7	8.3
ET	24.9	10.0	7.9	8.2
LT	22.9	9.7	8.1	8.1
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*Significant at P ≤ *0.05*; ** significant at P ≤ *0.01*; *** significant at P ≤ *0.001*; n.s., not significant (*P=0.05*). Means followed by different letter in each column are significantly different according to the Student- Newman-Keuls test. CK, control vines; ET, early trimmed vines; LT, light trimmed vines.

Organic acids

In 2013 and 2014, all treatments presented similar tartaric acid concentrations (mg/g of FW) at harvest. Noteworthy, a transient decrease in the concentrations of tartaric acid took place at the beginning of September in both years, regardless of the treatments (Table 7). Similar concentrations of tartaric acid were detected in 2014 for CK plants, compared to the previous season, however, in trimmed plants the highest values were reached in 2013 (Table 7). In both years, late trimming reduced the concentration of malic acid in the berry; early trimming produced a similar effect only in 2013.

Table 7. Tartaric and malic acid concentrations, recorded during ripening in 2013 and 2014, in control, early trimmed and late trimmed cv. Sangiovese vines.

	Organic acids (mg/g of FW)							
2013	**75 DAF**		**101 DAF**		**113 DAF**		**127 DAF**	
Treatment	**Tartaric**	**Malic**	**Tartaric**	**Malic**	**Tartaric**	**Malic**	**Tartaric**	**Malic**
CK	13.15	6.25	7.06	2.52	7.51	1.40	7.87	1.89a
ET	11.27	6.10	6.21	2.14	8.18	1.11	8.19	1.36b
LT	11.52	5.69	5.44	2.00	8.94	1.12	8.61	1.37b
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*
2014	**71 DAF**		**106 DAF**		**120 DAF**		**125 DAF**	
CK	10.49	8.46	7.02	3.51a	7.76	2.88	7.98	2.94a
ET	11.40	9.34	5.91	3.44a	8.42	2.65	7.41	2.78a
LT	12.00	9.11	5.42	2.96b	7.20	2.40	8.25	2.29b
Significance	*n.s.*	*n.s.*	*n.s.*	**	*n.s.*	*n.s.*	*n.s.*	*

*Significant at $P \leq 0.05$; n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test. CK, control vines; ET, early trimmed vines; LT, late trimmed vines.

Berry skin total anthocyanins and phenolics

In 2013, trimmed plants presented at harvest higher berry skin anthocyanins concentration compared to untreated control (Figure 2). In 2014, only vines submitted to late trimming displayed enhanced anthocyanin levels at harvest (Figure 2). In case of late trimming, in both years, such effect was also recorded two weeks after treatment imposition (Figure 2).

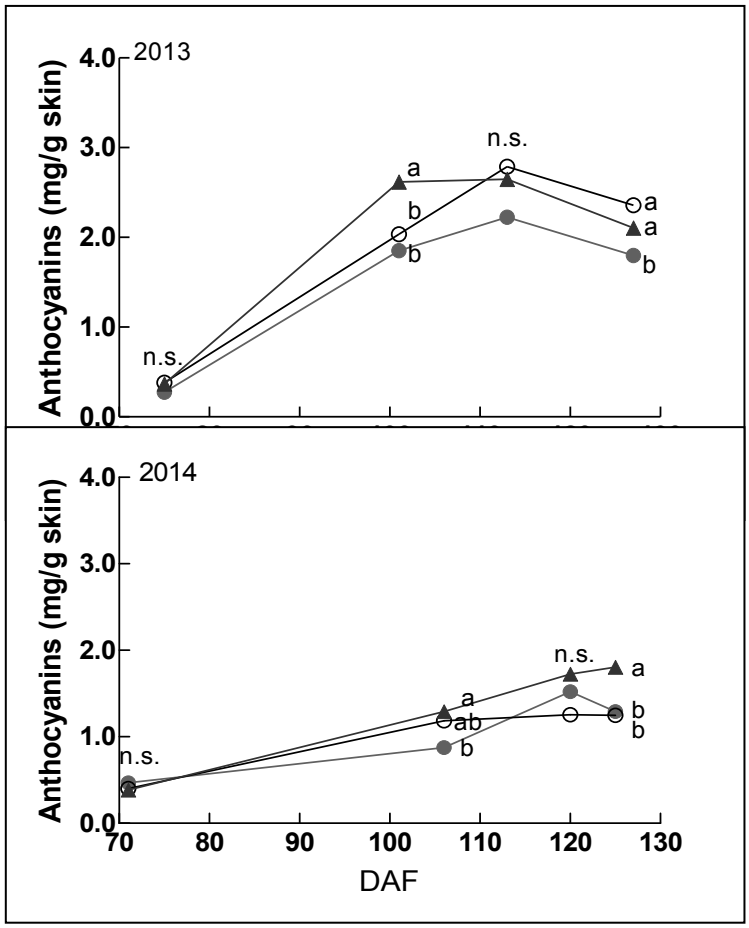

Figure 2. Seasonal trends of anthocyanins concentration, recorded in 2013 and 2014 on cv. Sangiovese control (CK,●) vines and plants submitted to early (ET,○) or late (LT,▲) trimming. Veraison occurred at 75 Days After Flowering (DAF) in 2013 and 71 DAF in 2014. Data are means of n=3 values. Different letters indicate significant differences according to the Student- Newman-Keuls test (*P=0.05*).

In 2013, trimming enhanced the level of berry skins total phenolics at harvest, compared to untreated control (Figure 3). In 2014, only vines trimmed in post-veraison presented higher values than control plants (Figure 3). In 2013, at 113 DAF, significantly higher concentrations were detected in skins of ET plants respect to controls, while LT presented similar concentrations compared to the other treatments. In 2014, at 120 DAF, LT plants, showed significantly higher concentrations compared to CK, while ET vines showed similar values to both CK and LT plants (Figure 3).

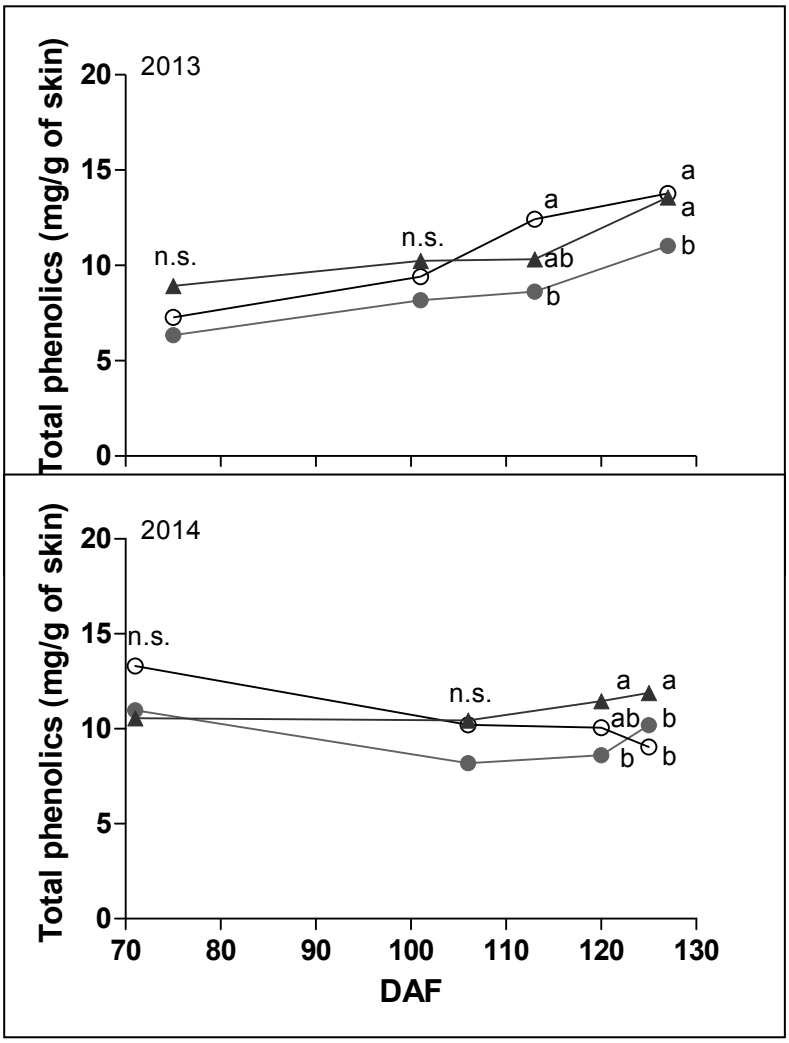

Figure 3. Seasonal trends of total phenolics concentration, recorded in 2013 and 2014 on cv. Sangiovese control (CK,●) vines and plants submitted to early (ET, ○) or late (LT,▲) trimming. Veraison occurred at 75 Days After Flowering (DAF) in 2013 and 71 DAF in 2014. Data are means of n=3 values. Different letters indicate significant differences according to the Student- Newman-Keuls test (*P=0.05*).

Wine chemical analyses

On vintage 2013, the experimental Sangiovese wines were characterized by high alcohol strength (average range: 13.6–14.1%), dry matter (25.0–27.0 g/L), total acidity (6.8–7.0 mg/L), volatile acidity (0.35–0.37 g/L) and pH (3.45–3.54) thus indicating a satisfactory degree of grape ripeness at harvest. Moreover, the volatile acidity and tonality reached

standard range for Sangiovese wines. With regard to the effects of the treatments (early and late trimming), the statistical analysis did not highlight differences among wines.

Wines from harvest 2014, compared to 2013, showed a decrease in all of the chemical parameters considered with value, such as alcohol strength (average range: 9.9–11.1%), dry matter (20.9–23.0 g/L), and pH (3.27–3.38) and the exception of total acidity (6.1–7.2 mg/L) and volatile acidity (0.25–0.27 g/L) quite below the standard average for Sangiovese wine. Statistics confirmed the outcomes of 2013 vintage when early and late trimming did not affect the chemical characteristics of the wines.

Tannins and color components in wine

Due to the importance of polyphenolic compounds that affect the quality of red wines in terms of astringency, bitterness and color, further analyses were carried out on tannins and color components. Total red color (TCO), co-pigmentation (COP), large (LPP) and small polymeric pigments (SPP), tannins (TNN), non-tannin total iron-reactive phenolics (IRP) and anthocyanins (ANT) were analyzed in wines produced in the two vintages at three months (wine 2013: CK/13, ET/13, LT/13; wines 2014: CK/14, ET/14, LT/14) and 15 months (wines 2013: CK/13_15, ET/13_15, LT/13_15; wines 2014: CK/14_15, ET/14_15, LT/14_15) from the end of fermentation. In wines of 2013, these compounds were also analyzed in 2016 after 27 months of storage (CK/13_16, ET/13_16, LT/13_16) to evaluate its effect on composition. The average values from the vinification replicates provided data for Principal Component Analysis (PCA). Two significant principal components accounted for 92.6% variation (PC1: 66.9%; PC2: 25.7%). As an overview, wines produced in vintages 2013 and 2014 resulted separated in the right and left side of the bidimensional

space, respectively, thus demonstrating considerable differences in terms of color due to the year of the harvest (Figure 4).

Examination of the attribute space showed that the TCO, LPP, SPP, TNN and IRP variables had the highest factor loadings on the positive half of the first dimension whereas COP was the most dominant attribute for the positive half of the second principal component. Within each vintage, samples were mainly grouped according to the duration of the storage as a demonstration of its effect on color and on phenolic components. Moreover, in all groups CK resulted always separated with respect to ET and LT thus highlighting differences in wine color components and phenols content between control and trimmed trials. The co-pigmentation was the main characteristic for CK/13 compared to ET/13 and LT/13. However, in the comparison, LT/13 had higher factor loadings for SPP, IRP, TNN, TCO and ANT than CK/13 and ET/13. The ET/13 had moderate loadings showing intermediate characteristics between CK/13 and LT/13 for all parameters. Analyses of these wines after 15 months showed a deep variation of color components and tannins. Differences between CK/13_15 and the other two wines were still present, with LT/13_15 characterized by the highest loading factors for SPP, IRP, TNN, TCO and ANT compared to CK/13_15 and ET/13_15. The CK/13_15 was no longer characterized by co-pigmentation which decreased in all wines regardless of the viticultural practice adopted. During twelve months storage the TNN increased in all samples whereas IRP were similar to the values determined after three months from the end of fermentation. Analyses performed at twenty-seven months from the end of fermentation demonstrated that LT/13_16 had highest loading factors for SPP, IRP, TNN, TCO and ANT compared to CK/13_16 and ET/13_16 (Figure 5). As a parameter of practical interest in LT/13_16 the TCO achieved 3.7 AU, whereas it was 2.2 and 2.9 in CT/13_16 and ET/13_16,

respectively. Accordingly, among wines produced in 2014 (left side of the two dimensional plot), LTs reached the highest average values for all parameters with a similar loading factor in the first (SPP, IRP, TNN, TCO, ANT) and second (LPP, COP) component. Wines CK/14 and ET/15 had high loading factors for SPP, IRP, TNN, TCO, ANT with ET/15 obtaining intermediate average values between CK/14 and LT/14 (Figure 5). After fifteen months storage, all 2014 wines were characterized by SPP, IRP, TNN, TCO, ANT content with LT/14_16 still having higher average values for all parameters.

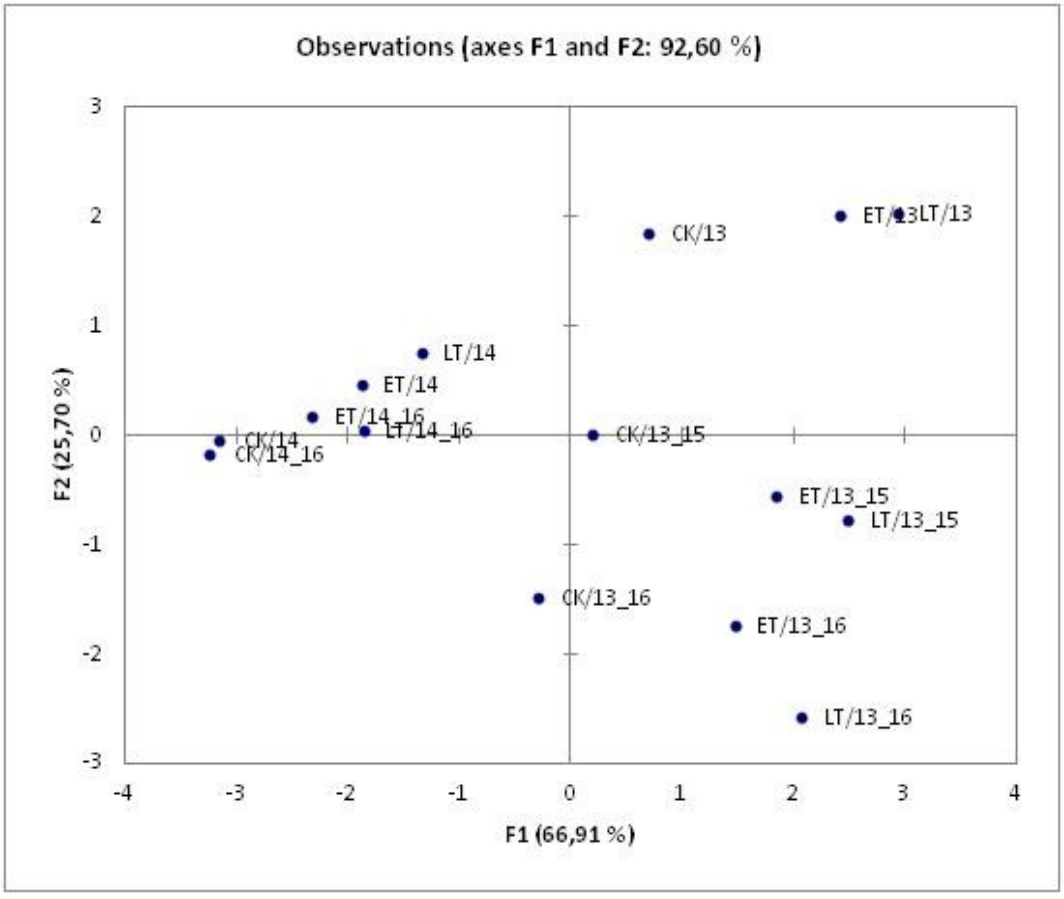

Figure 4. Score plot of average color components, tannins and total phenols data obtained from three vinifications for each trials in vintages 2013 (CK/13, ET/13, LT/13) and 2014 (CK/14, ET/14, LT/14). For 2013 wines, analyses were repeated after 15 (CK/13_15, ET/13_15, LT/13_15) and 27 (CK/13_16, ET/13_16, LT/13_16) months from the end of fermentation. For 2014 wines, analyses

were repeated after 15 months (CK/14_16, ET/14_16, LT/14_16). CK: control vines; ET, early trimmed vines; LT, late trimmed vines.

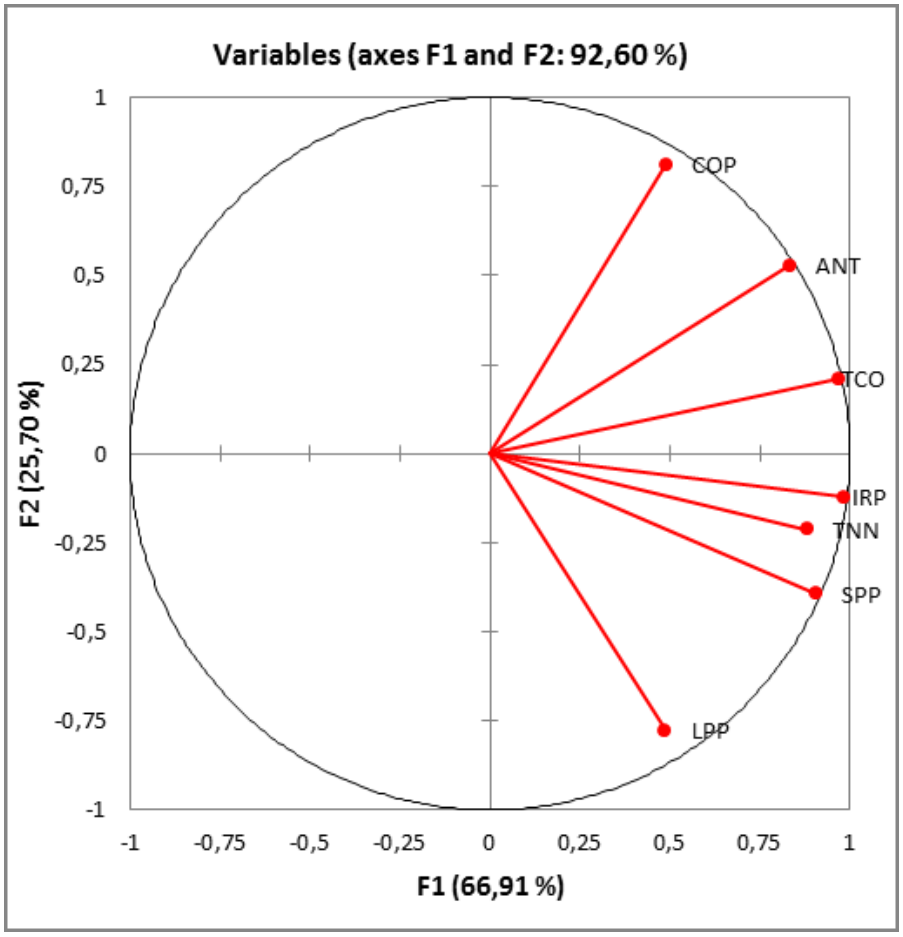

Figure 5. Loading plot of color components, tannins and total phenols determined in cv. Sangiovese wines produced from plots under different canopy management treatments. CK: control vines; ET, early trimmed vines; LT, late trimmed vines.

Discussion

The effects of trimming, performed in two different timings, on vine physiology, grape and wine composition of organically cultivated cv. Sangiovese, have been investigated. Our results highlighted, in two seasons characterized by contrasting climatic conditions (Figure 1), the relevance of trimming in the last phase of berry ripening for improving grape berry and wine color in cv. Sangiovese. To our knowledge, this is the first study comparing post-veraison trimmed plants with untrimmed controls.

In both years, late trimming increased anthocyanin concentration at harvest; this effect was also observed two weeks after treatment imposition (Figure 2). Early trimming produced analogous results at harvest only in the first year of the experiment. At harvest, the effect of trimming practices on berry skin total phenolics was similar to that observed for anthocyanins at harvest (Figure 3).

The lower concentrations of berry skin anthocyanin and total phenolics, berry juice TSS, pH, the higher TA and organic acids values detected in 2014 as compared to 2013, are related to the different climatic conditions during ripening (Figure 1), confirmed also by plant water status (stem water potential).

Our data showed that trimming did not modify the main chemical characteristics of wine (e.g. alcohol strength, dry matter, total and volatile acidity and pH), regardless of the year. However, a sensible increase in color component and tannins was evident in wines obtained in vintages 2013 and 2014 from vines submitted to post-veraison trimming.

The higher rainfall (489 mm) and lower average daily temperature (18° C) in the 2014 growing season, determined a general reduction of wine color and total polyphenols.

The overview of the data collected in wines produced in vintages 2013 and 2014 and monitored up to 27 months from the end of fermentation shows that throughout the storage a decrease in co-pigmentation, total anthocyanins content, tannins and total color, even to a lower extent, occurred. Conversely, small and large polymeric pigments increase in content while total polyphenols are stable. The chemistry behind the color change in red wines during storage is complex (Boulton, 2011). Polymeric pigments - via the condensation reaction of tannin with anthocyanins, with or without acetaldehyde-bridged complexes - are most stable to color change. Therefore, their formation is foreseen in red wine. Although tannins offer a substitute reaction pathway for anthocyanins, the polymerization mechanisms are largely affected by the wine type considered. In particular, the ability to drive the polymeric pigment reaction forward depends on many factors, including anthocyanins, tannins, acetaldehyde, pH, SO_2 and temperature. Our results suggest that in both vintages, late trimming carried out at 15 °Bx showed to be effective on quality of wine by increasing all of the parameters correlated to color.

The effects of post-veraison trimming on wines were amplified by ageing, thus indicating that the enological benefits brought about by this late agronomic intervention can be adopted for high quality wines produced by Sangiovese grapes.

Despite the different agronomic techniques to increase anthocyanin levels in grapes (e.g. leaf removal, application of phytoregulators), this is the first time that an increase in anthocyanin concentration in the berries is attained through the imposition of post-veraison trimming. An increase in the accumulation rate of anthocyanins emerged after late trimming imposition (Figure 2), presumably due to increased anthocyanin synthesis. Subsequently, in the last phase of berry ripening a decrease in anthocyanin concentration was observed in all treatments (Figure 2) favoured by highest temperatures measured at the beginning of

September on exposed bunches, ranging from 35 to 44 °C. In the second year (Figure 2) the accumulation rate in post-veraison treated plants was lower compared to 2013 and continued until harvest, when the berry skin anthocyanin from post-veraison trimmed plants levels were 0.5 mg/g higher than those from controls. This is probably due also to the lowest temperatures detected in 2014 on LT exposed bunches that since the beginning of Septembert did not exceed 34 °C. In fact, the accumulation of anthocyanins occurs in berry skins from veraison to full maturity, when the rate of synthesis levels off (Castellarin et al., 2012).

In both years, post-veraison trimming, applied once during the same vegetative season, did not modify the concentration of TSS, since veraison to harvest (Table 6). In fact, a decrease in total soluble solids generally was achieved after severe post-veraison shoot trimmings (8-10 maintained nodes), performed on vines already subjected to trimming in the same vegetative season (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016).

In previous research the effect of summer pruning practices (e.g. shoot trimming, leaf removal, etc.) was interpreted primarily in terms of source-sink relations (Filippetti et al., 2015) and, in particular, by focusing on the leaf photosynthetic capacity, depending on its age (Poni et al., 2006, Intrieri et al., 2008, Poni et al., 2013; Palliotti et al., 2013). However, some authors (Poni et al., 2013, Tessarin et al., 2014, Bondada et al.,, 2016) highlighted the importance of considering the contributions of other factors, such as hormons, for elucidating the observed phenomena.

Our data showed that the removal of the shoot apex, both in case of post-veraison and early trimming had a general positive effect on cv. Sangiovese berry skin anthocyanins and phenolics, particularly when performed in post-veraison (Figures 2 and 3). This organ, characterized by a the presence of shoot apex meristem (SAM, Vernoux et al., 2010) plays a

pivotal role in auxins synthesis and translocation, being this the active site where auxins synthetized in different plant organs. They enter in the polar transport pathway and, along with the phloem transport (Michniewicz et al., 2007), can reach the berry. Treating bunches with auxins both in pre-veraison (Davies et al., 1997) or at veraison (Jeong et al., 2004) produced negative effects on anthocyanin accumulation and genes related to their biosynthesis. Moreover, Davies et al., (1997) observed that the effect of auxins was more marked on anthocyanins than on sugars. In early trimmed vines, the development of lateral shoots (Table 4), could have mitigated the effect of shoot apex removal, on berry total anthocyanins. In trimmed shoots the possible compensatory role of laterals apex in synthesizing and controlling the transport of auxins, should be further investigated.

The decrease of malic acid detected in both years at harvest in post-veraison trimmed plants and in the first year also on early trimmed vines (Table 7) compared to control, suggests the possible contribution of auxins to berry ripening. Such decrease was also evident when results were expressed on a per berry basis (data not shown), In fact, the effects of auxins on malic acid have been reported in literature (Böttcher et al., 2012, Ziliotto et al., 2012). In particular, Böttcher et al., (2012), observed that treating bunches in pre-veraison reduced the rate of decline in malic acid levels during ripening. In our research, no changes in berry temperature were observed among treatments during ripening (data not shown), highlighting that the decrease in malate concentration observed in trimmed plants could be linked to factors other than heating. On the other hand, trimming did not influence the concentration of tartaric acid (Table 7) that as it is known from literature is characterized by a different metabolism than malic (Ford, 2012).

We cannot also exclude that trimming, causing a wound, may have induced the production of abscisic acid (ABA), (Leòn et al., 2011) and/or ethylene (Sun et al., 2007) and a related

increase in berry skins anthocyanin concentration, Figure 2 (Jensen et al., 1975; Kataoka et al., 1982, Wicks and Kliewer, 1983). Abscisic acid can be translocated from vegetative organs to the berry via the phloem (Shiozaki et al., 1999, Antolin et al., 2003, Castellarin et al., 2011). Different studies demonstrated that ABA treatment promoted an increase in anthocyanins rather than an advancement of sugar accumulation when applied at veraison (Kataoka et al., 1982, Mori et al., 2005, Cantin et al., 2007, Peppi et al., 2007, Peppi and Fidelibus, 2008) or in post-veraison (Kataoka et al., 1982, Peppi et al., 2007). Noteworthy, Wicks and Kliewer (1983) demonstrated that anthocyanins and total phenolic levels can change without any significant variation in soluble skin carbohydrates after treatment with the ethylene-releasing molecule ethephon. The possible role of the different plant hormones on berry ripening, after the imposition of canopy managent practices, should be further investigated in order to fully understand the implications of agronomical practices.

Treatments did not modify the leaf SPAD index and nutritional status (Table 1) that resulted fully satisfactory in all plants (Penazzi et al., 2011); moreover, leaves showed appreciable photosynthetic activity also in the last phase of ripening (Table 2).

Post-veraison trimming, in contrast to early trimming that promoted laterals re-growth, determined late in the season a permanent reduction (-34%, 2013; -35%, 2014) of total leaf area, including a portion of the main shoot with relevant photosinthetic activity. Plants submitted to LT presented lower TLA compared to the other treatments in both years (Table 3) and a compensatory lateral re-growth was not recorded. In particular, trimming in post-veraison allowed to contain the development of lateral shoots compared to early trimming. In fact, LT laterals displayed a lower growth than those of ET ones (Table 3). Moreover, no compensatory increase in photosynthetic activity or stomatal conductance, based on single leaf gas-exchange readings, in the leaves of the main shoot was observed after the

application of post-veraison trimming (Table 2). Early trimming induced a diverse canopy developement and leaf area composition, due to a more pronounced development of laterals, confirmed by the increased number of nodes, lateral length and single leaf area detected in both years (Table 3).

Lateral development in early trimmed vines could in part explain why the effect of this treatment on berry color was not as clear as those of post-veraison trimming.

In 2013, since 86 DAF the LA/yield ratio was similar in all treatments (Table 4). In 2014, plants submitted to early trimming presented similar values of LA/yield ratio respect to CK but higher when compared to LT vines, whereas in this latter did not differ from CK ones (Table 4). Seasonal differences in cluster weight altered LA/yield ratio, in fact in 2014 values were lower than those detected in 2013. It has been reported that above 1 m^2/kg of fruit, the sugar concentration usually tends to reach a plateau and becomes less responsive to source-sink modulation (Kliewer and Dokoozlian, 2005). Moreover, it has been shown that an increase of LA/yield ratio, when values are in the range of 0.8-1.2 m^2/kg, may produce higher concentration of TSS, anthocyanins and total phenols and rise up color intensity (Reynolds et al., 1994, Guidoni et al., 2002, Pastore et al., 2011). However, in our work, trimming treatments, despite the observed changes in canopy morphology, LA composition (Table 3), LA/yield ratio values (Table 3), did not modify the berry TSS, pH and TA at harvest (Table 6). This similarity could be explained by the non limiting values of LA/yield ratio in both years (Table 6). Moreover, LT vines were not limited enough by treatment imposition to affect the berry soluble solids concentration at harvest, as reported in previous studies (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016). Our data clearly demonstrated post-veraison shoot trimming to be highly effective in reducing berry weight (Table 6), bunch weight and plant productivity at harvest (Table 3). The lower value of berry

weight was not related to a decrease in soluble solids concentration (Table 6) suggesting that the effects of post-veraison trimming on berry color may be consequences of the shoot apex removal, rather than the severity of trimming. Our data indicate that late trimming can be a cheap and effective strategy to control plant productivity and the degree of bunch compactness (Table 4). Therefore this practice could be a valuable alternative to cluster thinning, a costly intervention which can also induce compensation phenomena (increased berry weight and bunch compactness) together with an increase in the berry soluble solids concentration (Pastore et al., 2011).

Moreover, in both years, post-veraison trimming decreased bunch compactness (loosening of the bunch) in cv. Sangiovese (Table 4), a cultivar well-known for its vigorous growth and large sized compact clusters of varying degree (moderately compact, semi-compact, and compact) (Nelson-Kluk, 2006), whereas the rachis weight, length and width were similar in all treatments (data not shown). A reduction of berry and bunch weight was observed in cvs. Grenache and Tempranillo after a severe shoot trimming imposed after berry set, presumably due to case laterals regrowth (Martinez de Toda et al., 2013). In contrast, our data showed that early trimming did not influence berry and bunch weight compared to control and may favor the production of compact bunches (Table 4). A reduction of berry anthocyanin concentration could be also associated to increased bunch compactness. However, this is not always true in the case of ET plants, presenting higher total anthocyanin concentration compared to CK plants, in the first year, despite a similar level of bunch compactness.

Our data showed that the loosening of clusters in LT vines was related to ait reduction in berry size (Table 6) and bunch weight (Table 4). As a consequence, a reduced yield per vine was expected as berry and bunch weights are a function of plant productivity (yield per

vine). These data are consistent with the findings of a multi-year experiment, concerning the effect of post-veraison trimming practices on cv. Sangiovese, conducted in a nearby organic vineyard (Rombolà et al., 2011, Bondada et al., 2016). Possible reasons concerning this reduction in berry and cluster weight have been discussed in our previous work (Bondada et al., 2016). For example, the stress caused by post-veraison trimming (Candolfi-Vasconcelos et al., 1994) might have accelerated an early synthesis of callose reducing sugar export into the berries and the fact that post-veraison berry expansion relies on phloem influx (Bondada et al., 2005, Keller et al., 2014), a resistant phloem will reduce berry size by reducing its sink strength.

Due to the lack of effective products against *Botrytis* cluster rot, the loosening of the clusters is a highly useful after feature particularly in organic viticulture as loosened clusters by virtue of increased epicuticular wax load and cuticle thickness become less susceptible to cluster rots (Martin 1990). As the fruit expands the density of the cuticle decreases (Comménil et al., 1999) and a weak cuticle could predispose the fruit to fungal attack. In our experiment, late trimming reduced the severity of *Botrytis* cluster rot when the infection was particularly severe (2014). It has been shown that phenolic compounds may exert a fungicide action (Goetz et al., 1999), therefore, the highest levels of berry skins phenolic compounds in post-veraison trimmed plants (Figure 3) could have played an important role in controlling the severity of *Botrytis* cluster rot. In both years, LT plants displayed lower bunch discoloration than early trimmed ones (Table 4). This reduction together with the lower severity of *Botrytis* cluster rot in late trimmed plants could also be explained by an improved air circulation and light penetration at cluster level, providing more uniform berry ripening.

Conclusions

The key agronomic and oenological relevance of post-veraison trimming for achieving substantial improvements in berry and wine quality in cv. Sangiovese clearly emerged in two vegetative seasons displaying strongly contrasting climatic conditions during ripening. The impact of this practice should be contestualized in the light of vineyard plantation and management, therefore considering vine spacing, training system, winter pruning, canopy architecture, soil management, etc. The improvement in berry color through post-veraison trimming was attained without using external inputs (e.g. phytoregulators) and without modifying the berry TSS and other technological parameters. The reduction in yield per plant in late trimmed compared to pea-size trimmed and control vines showed that this practice should be considered as a valuable alternative to cluster thinning, while the improved cluster morphology represents important results particularly for organic growers, lacking effective products against bunch rot. In both years, post-veraison trimming had a positive effect also on wine quality. Trimming at pea-size was not always as effective as late trimming in improving berry skin color and its impact seemed more dependent to climatic conditions. Early trimming, despite the different leaf area composition, particularly due to a greater development of lateral shoots, did not modify the berry technological parameters. This practice produced more compact and discolored bunches with respect to post-veraison trimming.

Avoiding trimming for providing some shade to the cluster ("accucciatura") or twisting long shoots on the top wire with the aim to preserve the shoot apex ("accapannatura") could decrease anthocyanin concentration and favor the production of compact bunches. Therefore

the application of the latter techniques should be carefully considered, implemented through peculiar shoots positioning (e.g. semi ballerina effect) and possibly associated to late season defoliations.

In future research, the effects of shoot tipping/topping and its possible consequences on the plant hormonal status should be further investigated. Morevorer, also the possible drawbacks due to trimming imposition in warm periods should be considered, together with the possibility of implementing this practice also in cold climates.

The enological benefits induced by late trimming can be particularly appreciated in aged wines and represents a new approach for obtaining higher quality wines.

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CHAPTER 2: Effects of bunch- zone late defoliations on berry composition and wine quality in organically-cultivated cv. Sangiovese (*Vitis vinifera* L.)

Paola Tessarin[1], Arianna Ricci[2], Giuseppina Paola Parpinello[2], Adamo Domenico Rombolà[1*]

[1]Department of Agricultural Sciences, School of Agriculture and Veterinary Medicine, Alma Mater Studiorum, University of Bologna, Viale G. Fanin 44, 40127 Bologna Italy.

[2]Department of Agricultural and Food Sciences, School of Agriculture and Veterinary Medicine, Alma Mater Studiorum, University of Bologna, Piazza Goidanich 60, 47521 Cesena, Italy.

Corresponding author: [*]E-mail adamo.rombola@unibo.it Tel:+390512096461, Fax+39051 2096401.

Abstract

Late defoliation (imposed at the onset of veraison or later) is a practice frequently adopted, enabling to establish a microclimate contrasting the development of fungal diseases. For the first time, the implications of late defoliations and shoot positioning on young and aged organic cv. Sangiovese wines were investigated. In 2013 and 2014, vines submitted to post-veraison (15 Brix), pre-harvest late defoliations and post-veraison shoot positioning were compared with untreated controls to assess the effects on vine physiology, grape and wine quality. In different seasons, late defoliations enhanced berry skin flavonols concentration without modifying anthocyanins levels, berry soluble solids, pH and titratable acidity. Late defoliations and shoot positioning did not alter bunch morphology and limited the severity of *Botrytis* cluster rot. A marked reduction of Leaf area/yield ratio occurred in late defoliated vines. Treatments did not modify leaf photosynthetic activity and stomatal conductance. The percentage of budburst of count nodes and their fruitfulness was not influenced by treatment imposition. The enological benefits of late defoliations and shoot positioning can be fully appreciated in both young and aged cv. wines. These canopy management practices positively influenced wine components that might have a marked effect on the final color intensity, without modifying the basic chemical characteristics of wine. The enological results constitute a significant contribution toward the enhancement of wine quality.

Key words: *canopy management, summer pruning, organic viticulture and wine, anthocyanins, flavonols, cluster rot.*

Introduction

Organic cultivation method is gaining popularity, especially in the viticultural sector (as found in the O.I.V website (www.oiv.int), 2015, Parpinello et al., 2015, Botelho et al., 2016, Tessarin et al., 2016) due to the cultural and social role played by wine-making and the attention paid to the whole production cycle. In organic vineyards, all field strategies including canopy and soil management are aimed to safeguard and enhance plant health and resilience under biotic and abiotic stresses (Botelho et al., 2016). Because of the need to adhere to the principles of organic viticulture, organic grape growers seek out a wide repertoire of summer pruning practices (e.g. shoot topping/trimming, leaf removal, shoot positioning) in order to preserve grape quality until harvest and provide healthy grapes to the winery. Currently there is little information on the effects of canopy management practices in organic vineyards (Rombolà et al., 2011, Bondada et al., 2016, Tessarin et al., 2016).

Color is one of the most important organoleptic parameters of red wine, strictly influencing the quality evaluation of this product. The color of young red wines is mainly due to berry skin anthocyanins which are unstable compounds so that much of the initial color is lost during fermentation and maturation. Co-pigmentation consists in molecular associations between anthocyanins pigments and other organic molecules that are present in the solution often reported as cofactors and can account for between 30% and 50% of the color in young red wines (Boulton, 2001). Noteworthy, anthocyanin-co-pigment complexes intermediates maintain the anthocyanins in the wine and also contribute to the formation of more stable associations during the slow process that involves polymeric pigments formations in the older wine (Boulton, 2001). For the above mentioned reasons the concentration of anthocyanins and co-pigments at harvest is a crucial factor for determining the extent of

color in mature wine; therefore all viticultural practices that may enhance the anthocyanin and/or flavonol content in grapes may influence wine quality.

Late defoliation (imposed at the onset of veraison or later) is a frequently adopted practice, that establishing conditions that curtail the development of fungal diseases (e.g. *Botrytis* cluster rot), thus allowing healthy bunches to be maintained on the vine up to the beginning of autumn for the attainment of adequate levels of phenolic and aromatic compounds. The effects of basal leaf removal at veraison (Matus et al., 2008, Pastore et al., 2013) or end of veraison (Tessarin et al., 2014) were discussed in previous papers concerning different varieties such as cvs. Sangiovese, Cabernet Sauvignon and Uva Longanesi. For this latter variety the enolological implications on young wines were also assessed. Moreover, the effects of different intensities and modalities of basal late defoliation (imposed at complete veraison, or complete veraison and pre-harvest) were investigated on cv. Nero di Troia grapes and wines at racking (Baiano et al., 2015). Other authors focused on the implication of post-veraison defoliation of leaves inserted above the bunch zone on cv. Sangiovese potted vine (Poni et al., 2013) or on field conditions, assessing also the effects on wine quality (Palliotti et al., 2013).

Nowadays, there is still scant information on the implications of post-veraison and pre-harvest basal leaf removal on vine performance, grapes and wine quality (Baiano et al., 2015), even though these practices are commonly adopted, not only in organic vineyards. In a context of climate change, where high temperatures are frequent in the first phases of berry ripening, the protection of clusters from direct solar radiation could help to preserve berry color.

Therefore we decided to investigate the effects of post-veraison, pre-harvest defoliation and post-veraison shoot positioning on organically cultivated cv. Sangiovese, on vine

physiology, grape and wine composition, with particular regard to the impact on berry and wine color components.

Materials and Methods

Plant material and experimental layout

The experiment was conducted in 2013-2014, in a mature vineyard planted in 2003, with cv. Sangiovese grapes (clone FEDIT 30 ESAVE), *Vitis vinifera* L., grafted onto Kober 5BB, trained to cordon de Royat training system (VSP). The vineyard was located in Tebano (Faenza, RA), Italy (44°17′7″ N, 11°52′59′E, 117 m a.s.l.), on a medium slope, with southeast/northwest and downhill-oriented rows. Vines were spaced 2.8 m within the row and 3.0 m between rows (3,571 plants/ha). Starting in 2007, the vineyard was managed as organic, in accordance with Reg. EC 834/2007 (EC, 2007).

Beginning in 2007, no irrigation water was applied, and the vineyard was not fertilized. The loamy clay alkaline soils of the vineyard presented medium organic matter (2.2 %) and nitrogen (1.5 ‰) concentration, high levels of carbonates (total carbonates 14.7 %; active lime: 6.7 %), medium-high concentration of assimilable phosphorus (P: 10 μg/g) and potassium ions (K: 188 μg/g), as well as assimilable iron (23 μg/g) and manganese (11.07 μg/g). Available copper in the soil showed high values, around 19 μg/g. Annually, at the end of each vegetative season, herbaceous species were sowed in alternate planting rows, such as fava bean (*Vicia faba*), barley (*Hordeum vulgare*) and subterranean clover (*Trifolium subterraneum*). Soil was managed by mowing the vegetation during late spring, which maintained biomass on the soil surface. In October 2014, subterranean clover was sowed also along each row.

The vineyard was treated against diseases and pests, using products allowed by the EC Regulation (EC, 2002). Treatments consisted mainly of copper (an average of 6 kg/ha/year) and sulfur (an average of 70 kg/ha/year), enabling control of fungal pathogens (*Plasmopara viticola*, *Uncinula necator* and *Botrytis cinerea*). At the end of February vines were spur-pruned to two count nodes/buds equating to 12-14 buds per vine. The noncount shoots (shoots arising from base buds of the spur) were removed at the beginning of the season by leaving 12 shoots with uniformed distribution per meter of cordon. The cluster number was adjusted by cluster thinning at veraison, in order to maintain a maximum number of 16 bunches per plant.

The experimental design included 4 treatments: a control (CK), non-treated vines; two timing of defoliation: post-veraison defoliation (DEF I), imposed in post-veraison, when the berries reached 15 Brix (86 and 92 days after flowering (DAF) in 2013 and 2014, respectively), pre-harvest defoliation (DEF II, imposed at 101 and 106 DAF in 2013 and 2014, respectively) and shoot positioning through "semi-ballerina" effect (SB, performed at 86 and 92 days after flowering (DAF) in 2013 and 2014, respectively). Each treatment was replicated three times in a completely randomized experimental design, with 30 monitored vines per treatment, for a total of 120 plants. The control treatment (CK) presented long shoots of 24 nodes, falling from both side of the canopy, without obstructing the passage of agricultural machineries. Defoliation treatments were performed on plants with long shoots (24 nodes) by manually removing all main shoots leaves and laterals up to the eightth node. The removed leaves and laterals were immediately kept away from the experimental plots, in order to avoid release of nutrients in the soil and subsequent uptake from the vines. The "semi-ballerina" effect (Dry, 2011) consisted in positioning long shoots (24 nodes) to provide some shading to the bunches in the hottest hours of the afternoon.

Leaf macro and micronutrients and SPAD index

At veraison (75 DAF, 2013; 71 DAF, 2014) 20 mature, exposed and completely expanded leaves per experimental plot (60 leaf per treatments), inserted at the fourth node above the first bunch were collected, in order to monitor the leaf nutritional status. Leaves were sampled from shoots originating from true buds, bearing at least one bunch. The leaf chlorophyll index was detected with a Minolta SPAD-502 chlorophyll meter (Minolta, Osaka, Japan) by performing five measurements per leaf blade.

Leaf blades, deprived of petioles, were washed in a detergent solution (HCl 0.1 N + Tween 20 0.1 %) to remove nutrients that may have been present on the leaf, rinsed with distilled water, dried at 65 °C until constant weight, then weighed and finally milled.

Leaf blades were dried at 70 °C, and ground (sieve < 0.5 mm). Total Nitrogen was determined by the Kjeldhal method and phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and boron (B) were analyzed as described in García-Escudero et al. (2013).

Leaf gas exchange

Measurement of leaf gas exchange was determined on two plants per experimental plot (6 plants per treatment) using an infrared gas analyzer (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-Cor, Inc., Lincoln, NE, USA). Measurements were performed in the morning between 0900 and 1030 and again in the afternoon between 1500 PM and 1630 PM, on a leaf inserted in the middle third of a shoot (12th node), originating from true buds. Leaves were illuminated by the LI-COR 6400 LED light source providing a photosynthetic photon flux density ca.1200 μmol/(m^2·s). The level of CO_2 was fixed at 380 ppm within the leaf chamber. Net photosynthesis was measured when foliar CO_2 uptake was

steady. The measurements were taken at 3 dates during ripening: at 75, 113 and 127 DAF, in 2013 and 70, 109 and 118 DAF, in 2014.

Stem water potential

Stem water potential (Mpa) was measured at midday during berry ripening (75 DAF, 2013; 109 DAF and 118 DAF, 2014) on two plants per experimental plot (sixplants per treatment), by using the Scholander pressure chamber method. From each vine, one mature, completely expanded, exposed, healthy non transpiring leaf, inserted at the 12^{th} node, was selected. About 60 minutes before midday measurement leaves for determination of ψ stem were enclosed in plastic bags covered with aluminum foils.

Vegetative-productive parameters

The vines phenological phases were monitored. After one (April 2014) and two years (April 2015) of treatment imposition the percentage of bud burst (number of shoots from count nodes (SCN)/ count nodes (CN) and the fruitfulness of count nodes (number of inflorescences (INF)/ SCN were detected on 30 plants per treatment, when inflorescences were clearly visible (BBCH 53).

The contributions of primary and lateral leaves were measured separately per position through a LI-3000A leaf area meter (Li-Cor Biosciences, Lincoln, NE, USA) on nine representative fruiting shoots per treatment, at the end of the vegetative growth, sampled from three additional vines. After the shoot sampling these vines were not used for any additional sampling or measurements. The total leaf area per vine (TLA), the total main shoots leaf area (SLA) and the total laterals leaf area (LLA) per plant were estimated by multiplying the average leaf area per shoot by the number of shoots per vine. The number of

laterals per shoot, the length and the number of nodes and the average leaf area of each lateral were also determined. At harvest (127 DAF, 2013; 125 DAF, 2014), productive parameters, such as number of clusters per plant, productivity per vine and bunch weight (Digital Dynamometer, Wunder SA-Bi S.r.l, Milan, Italy) and leaf area (LA/yield) ratio were determined on 10 plants per experimental plot (30 vines per treatment).

After leaves abscission, the pruning wood weight (kg) was detected and the Ravaz Index (yield/ pruning wood ratio) calculated on 10 plants per experimental plot (30 vines per treatment).

Berry growth and technological parameters

The following parameters were analysed: berry weight, expressed as g per berry (technical balance Gibertini Elettronica S.r.l., Milan, Italy); total soluble solids (TSS; Brix; Electronic Refractometer Maselli Misure S.P.A., Parma, Italy); titratable acidity (TA; expressed as g/L of tartaric acid) and pH (Crison Compact Titrator, Crison Instrument SA, Barcelona, Spain) were periodically determined by collecting 50 berries per replicate at harvest.

Berry skin anthocyanin and flavonol analysis

Additional samples (20 berries) were collected at harvest for determining anthocyanins and flavonols qualitative and quantitative profile. The skin extract from each sample was analyzed according to Mattivi et al. 2006, using an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a PDA detector and a reversed-phase column RP18 250 × 4.6 mm (5-µm particle size) (Phenomenex, Castel Maggiore, BO, Italy). Anthocyanins were determined by measuring absorbance at 520 nm. A calibration curve was established with malvidin-3-

glucoside standard (Lab Service Analytica Srl, Anzola Emilia, BO, Italy) and the anthocyanins were expressed as mg/g skin.

Flavonols were determined by measuring absorbance at 360 nm; a calibration curve was established with quercetin-3-O-glucopyranoside standard (Lab Service Analytica Srl, Anzola Emilia, BO, Italy) and flavonols were expressed as mg/g skin.

Qualitative parameters of bunches

At harvest (127 DAF, 2013; 125 DAF, 2014) the qualitative parameters of bunches were measured. For each bunch, the index of cluster compactness (according to the 1983 OIV classification) and the surface area affected by discoloration (%) were detected. The incidence (number affected cluster per vine) and severity (number of affected berries per cluster) of bunch rot were monitored on 10 plants per experimental plot (30 plants per treatment).

One bunch per plant (30 per treatments) was sampled for determining bunch weight, length and width, the number of berries per cluster, the rachis weight, length and width.

Bunch temperature

An assay of bunch temperature values was performed through infrared thermometer Raytek Raynger^{TM} ST (Santa Cruz, CA, USA). The temperature of the basal cluster, inserted on the same shoot, was detected on the sunlight exposed surface and on the shaded portion of 6 bunches per treatment. The measurements of leaf and bunch temperatures were repeated 5 times per days (at 0800, 0900, 1000, 1100, 1400, 1500) and taken at 5 (2013) and 4 dates (2014) during ripening.

Wine chemical and sensory analysis

In both vintages, grapes collected at optimum technological maturity were processed according to organic winemaking protocol proposed by the Italian Association for Organic Farming (AIAB, Italy) in accordance to the dispositions of Reg. CE N. 203/2012 and Reg CE n. 834/2007. Twelve vinifications (CK, DEF I, DEF II and SB repeated for three different vine blocks) of 20 kg of grapes were carried out. Briefly, after destemming and crushing skin and must were placed in stainless steel tanks and treated with sulfur dioxide (as potassium metabisulphite: 10 g/hl, AEB, Italy), complex nutrients (30 g/hl, Nutristart, Lafford, France) and inoculated with OGM-free yeasts (20 g/hl *Saccharomyces cerevisiae*, F15, Laffort, France). Sugar consumption was monitored over time by means of a Babo densimeter throughout fermentation. The tank content was homogenized every day to dissolve the cap into the wine. At zero degree Babo, both free run wine and wine extracted from marc with a piston press (2 bar) were collected and pooled. After the final racking, carried out fifteen days from the end of alcoholic fermentation, the wines were cold-stabilized, then bottled and stored at 10 °C prior to chemical and sensory analyses. Wines were analyzed for alcohol strength (AS, %), dry matter (DM, g/L), pH (U), total acidity (TA, g/L), volatile acidity (VA, g/L)), optical density (OD, AU) at 420, 520 and 620 nm, total color intensity (CI, AU), hue (HUE, AU), total polyphenols (TP, mg/L) at 280 nm according to European official methods (EC, 1990). Moreover, total (SO_2T, mg/L) and free (SO_2F, mg/L) sulphur dioxide (Ripper and Schmitt, 1896), reducing substances (RS, g/L) (Lane and Eynon, 1923). Wines were also analyzed for the following color and phenolics related parameters: total anthocyanins (ANT, AU), total red color (TC, AU), (COP, AU) (Boulton, 2001), large polymeric pigments (LPP, AU), small polymeric pigments (SPP, AU), tannins (TN, mg/L) and non-tannin total iron-reactive phenolics (IRP, mg/L) (Harbertson et al.

2003) carried out by spectrophotometric assay (UV–Vis 1240 mini, Shimadzu, Milano, Italy). All the listed analyses were carried out at the end of alcoholic fermentation. In order to monitor the change of wine composition over time, the analyses of color and phenolic components were repeated 16 months from the end of the fermentation, for vinifications performed on 2014 whereas for those carried out on 2013 analyses were repeated 16 and 28 months from the end of fermentation. Data are presented as mean values obtained from two replicated analyses of each vinification.

Statistical analysis on berries and vegetative-productive parameters data

Analysis of variance and comparison of means of parametric data were performed using SAS 6.04 software (SAS INSTITUTE, CARY, NC, USA) and Student-Newman-Keuls test ($P=0.05$). Non parametric data were subjected to Kruskall Wallis test, followed by Dunn's comparison test ($P=0.05$).

Wines analysis of variance for mean separation and Tukey as post hoc test were carried out with XLSTAT version 2011.1.05 (ADDINSOFT, Anglesey, UK). All statistics were performed with significance at $P = 0.05$.

Results

Climatic conditions

In the two years of the research, climatic data (mean, maximum and minimum daily air temperatures (T), relative humidity (RH) and total rainfall) were recorded, in a meteorological station located 800 m from the vineyard.

In the 2013, the maximum temperatures were detected at the beginning of August (around 40 °C). The total rainfall was 433 mm and mainly occurred in spring, at the end of August (55 mm) and during the second half of September (52 mm).

Overall, the 2014 vegetative season was marked by average temperatures well below seasonal norms. The highest maximum temperatures (32 °C) were recorded at the end of spring (20 DAF and 21 DAF) and on July 20th (59 DAF). From the second half of April to harvest the total rainfall was abundant (489 mm) and quite frequent both in spring and summer.

Leaf nutritional status and SPAD Index

In both years, treatments did not modify leaf nutritional status. Average values for leaf macro nutrients were: N (1.8%), P (0.2%), K (1.2%), Ca (3.1%), Mg (0.3%) and for micro nutrients were: Fe (61.6 ppm), Mn (56.0 ppm), Zn (16.2 ppm), Cu (125.4 ppm), B (43.5 ppm), Na (76.8 ppm). The SPAD index, around 32.8, was similar in all treatments in 2013 and 2014.

Stem water potential

The lowest values of stem water potential were detected in the first year due to the climatic conditions.

Treatments did not modify the values of the stem water potential (-MPa) recorded at 75 DAF in 2013 (CK: 1.3; DEF I: 1.3; DEF II: 1.4; SB: 1.3); at 109 DAF (CK: 0.8; DEF I: 0.7; DEF II: 0.8; SB: 0.8) and at 118 DAF in 2014 (CK: 0.8; DEF I: 0.7; DEF II: 0.9; SB: 0.9).

Leaf gas exchange

Treatments did not influence leaf photosynthetic activity during ripening (Table 1). Also stomatal conductance was not affected by treatment impositions with the only exception of 188 DAF, when plants submitted to post-veraison defoliation 15 presented significantly lower stomatal conductance respect to control, DEF II and SB vines (Table 1).

Table 1. Net photosynthesis and stomatal conductance measured in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

	75 DAF				113 DAF				127 DAF			
	Net photosynthesis		Stomatal Conductance		Net photosynthesis		Stomatal Conductance		Net photosynthesis		Stomatal Conductance	
2013	**µmol CO_2 m^{-2} s^{-1}**		**mol H_2O m^{-2} s^{-1}**		**µmol CO_2 m^{-2} s^{-1}**		**mol H_2O m^{-2} s^{-1}**		**µmol CO_2 m^{-2} s^{-1}**		**mol H_2O m^{-2} s^{-1}**	
Treatments	**0900-1030**	**1500-1630**	**0900-1030**	**1500-1630**	**0900-1030**	**1500-1630**	**0900-1030**	**1500-1630**	**0900-1030**	**1500-1630**	**0900-1030**	**1500-1630**
CK	6.0	8.2	0.097	0.120	8.1	8.4	0.198	0.221	8.8	ND.†	0.195	ND
DEF I	3.2	7.6	0.055	0.132	8.5	7.3	0.215	0.131	7.0	ND.	0.251	ND
DEF II	10.7	11.3	0.164	0.176	11.6	9.2	0.229	0.158	7.5	ND.	0.264	ND.
SB	9.2	8.6	0.166	0.164	6.6	7.3	0.223	0.136	7.8	ND	0.211	ND.
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*ND*	*n.s.*	*ND*
2014	70 DAF				109 DAF				118 DAF			
CK	5.6	6.8	0.096	0.129	11.2	8.3	0.405	0.315	7.6	7.8	0.286	0.299 ab
DEF I	4.8	5.4	0.097	0.085	9.7	8.5	0.315	0.306	8.3	8.6	0.260	0.326 a
DEF II	5.7	6.3	0.114	0.105	7.9	6.9	0.406	0.339	6.5	8.5	0.291	0.254 b
SB	6.7	6.6	0.111	0.093	11.8	8.2	0.369	0.299	8.0	9.2	0.267	0.293 ab
Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*

*Significant at $P \leq 0.05$; n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student Newman-Keuls test.

CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect.; †ND, not detected.

Vegetative and productive parameters

In both years TLA and SLA at harvest were higher in controls and plants submitted to "semi-ballerina" effect, whereas LLA resulted significantly higher only in the first year compared with those of defoliated plants (Table 2).

In 2013, CK and SB vines showed a higher number of laterals per shoot, with higher length and lateral leaf area compared with plants submitted to defoliation; however, this difference was not observed in the second year (Table 2). The imposition of defoliation treatments removed 30-35% of TLA.

In both years, treatments did not modify plant productivity, pruning wood weight and Ravaz Index (Table 3). Treatments did not alter cluster weight compared with that of control vines; however in the first year plants defoliated in post-veraison presented higher values compared with DEF II and SB bunches (Table 3). The highest values of LA/yield ratio were observed in the first year; the imposition of late defoliations reduced LA/yield ratio compared with CK and SB plants in both years (Table 3).

Treatments did not modify the percentage of bud burst, expressed as shoots from count nodes/count nodes, and their fruitfulness (Table 4).

Table 2. Leaf area of main shoots, laterals and total leaf area per plant, number of laterals per shoot, lateral length, number of nodes per lateral, lateral single leaf area at harvest, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

Year	Treatments	SLA (m^2)	LLA (m^2)	TLA (m^2)	Lateral/shoot (N°)	Lateral length (cm)	Lateral nodes (N°)	Lateral leaf area (cm^2)
2013 (127 DAF)	CK	4.41 a	2.08 a	6.49 a	16.0 a	4.81 a	1.96	108.21 a
	DEF I	3.01 b	1.30 b	4.31 b	12.7 b	3.50 b	1.78	85.23 b
	DEF II	3.03 b	1.27 b	4.30 b	12.5 b	3.64 b	1.79	86.33 b
	SB	4.44 a	2.12 a	6.56 a	16.1 a	4.80 a	1.85	105.77 a
	Significance	***	**	***	**	*	*n.s.*	*
2014 (125 DAF)	CK	4.25 a	1.39	5.64	13.7	3.53	1.60	86.73
	DEF I	2.97 b	0.69	3.66	11.6	2.30	1.30	58.83
	DEF II	3.07 b	1.12	4.19	12.0	3.60	1.69	78.88
	SB	4.23 a	1.26	5.49	13.6	2.93	1.47	69.50
	Significance	**	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*Significant at $P \leq 0.05$; ** significant at $P \leq 0.05$; *** significant at $P \leq 0.001$; n.s., not significant ($P=0.05$). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test. CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect.

SLA, main shoots leaf area; LLA, laterals leaf area; TLA, total leaf area per plant (SLA + SLA). DAF, Days After Veraison.

Table 3. Bunch number per plant, plant productivity, bunch weight, number of berries per cluster, bunch compactness and discoloration, recorded at harvest, pruning wood weight, Ravaz Index, LA/yield ratio in 2013 and 2014, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

Parameters	2013					2014				
	CK	**DEF I**	**DEF II**	**SB**	***Significance***	**CK**	**DEF I**	**DEF II**	**SB**	***Significance***
Bunch (N° plant^{-1})	13	13	13	13	*n.s.*	15	15	15	15	*n.s.*
Productivity (kg plant^{-1})	4.4	4.4	4.0	3.8	*n.s.*	6.6	6.7	6.2	6.5	*n.s.*
Bunch weight (kg)	0.329 ab	0.349 a	0.312 b	0.300 b	*	0.434	0.445	0.426	0.422	*n.s.*
Berries (N° bunch $^{-1}$)	144	169	149	147	*n.s.*	132	149	134	134	*n.s.*
Bunch compactness (OIV rating)	7.4	8.4	7.8	7.4	*n.s.*	8.3	8.4	8.4	8.3	*n.s.*
Bunch discoloration (%)	25.5 b	35.2 a	19.6 b	25.8 b	*	89.2 ab	90.8 a	77.5 b	83.5 ab	*
Pruning wood weigh (kg plant-1)	0.675	0.692	0.628	0.653	*n.s.*	0.622	0.691	0.600	0.594	*n.s.*
Ravaz Index	6.67	6.59	6.93	6.09	*n.s.*	11.1	10.2	10.6	11.1	*n.s.*
LA/yield	1.68 a	1.05 b	1.20 b	1.75 a	***	0.87 a	0.55 b	0.59 b	0.88 a	***

*Significant at P $\leq$ *0.05*; *** significant at P$\leq$*0.001*; n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test (bunch and berries number, plant productivity, pruning wood weight) and Kruskal-Wallis test, followed by Dunn's comparison test (bunch weight, compactness, discoloration, Ravaz Index, LA/yield ratio). CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect.

Table 4. Budburst percentage expressed as shoots from count nodes/count nodes. Fruitfulness expressed as inflorescences/shoots from count nodes, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

		Budburst (%)	**Fruitfulness**
Year	**Treatment**	SCN/CN	INF/SCN
2014	CK	114.56	1.52
	DEF I	112.19	1.56
	DEF II	113.31	1.60
	SB	112.01	1.54
	Significance	*n.s.*	*n.s.*
2015	CK	114.17	1.49
	DEF I	107.38	1.47
	DEF II	113.48	1.53
	SB	112.63	1.39
	Significance	*n.s.*	*n.s.*

n.s., not significant (*P=0.05*) according to the Kruskal-Wallis test, followed by Dunn's comparison test. CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect. TCS, total count shoots per plant; CN; count nodes, SCN, shoots from count nodes; INF, inflorescences. Data were collected after one (2014) or two years (2015) of treatment impositi

Bunch qualitative parameters

Treatments did not influence number of berries per bunch (Table 3), cluster and rachis length and width (data not shown). In both years bunches from plants submitted to different canopy management treatments presented similar compactness. In 2014, the severity of *Botrytis* cluster rot (57% in control plants) was markedly reduced by DEF I (37%) and to lesser extent by SB (39%) and DEF II (42%). In the first year, post-veraison defoliation increased bunch discoloration at harvest compared with control and the other treatments (Table 3). In 2014, treatments did not produce any effect on bunch discoloration respect to CK, however SB bunches presented lower values compared with DEF I ones (Table 3).

Berry growth and technological parameters

In 2013, all treatments presented higher values of TSS, pH and lower values of titratable acidity at harvest compared with 2014, due to the meteorological conditions that did not allow the grapes fully ripening (Table 5). Late defoliations and shoot positioning did not change berry total soluble solids, pH and titratable acidity concentration during ripening in none of the two years (Table 5).

Table 5. Berry weight (g), technological parameters (total soluble solids, pH, titratable acidity) recorded at harvest in 2013 and 2014, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

Year	Treatments	Berry weight (g)	TSS (Brix)	pH	TA (g/L tartaric acid)
2013	CK	2.5	23.6	3.25	7.0
	DEF I	2.5	23.9	3.26	6.8
	DEF II	2.5	23.6	3.25	6.8
	SB	2.5	23.9	3.23	7.2
	Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*
2014	CK	2.9	18.8	3.17	8.6
	DEF I	2.9	19.1	3.19	8.3
	DEF II	2.9	19.6	3.20	8.4
	SB	2.7	18.8	3.14	8.5
	Significance	*n.s.*	*n.s.*	*n.s.*	*n.s.*

n.s., not significant (*P=0.05*) according to the Student- Newman-Keuls test. CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect. TSS: total soluble solids; TA: titratable acidity.

Berry skin anthocyanins and flavonols

Treatments did not modify total glucosidated anthocyanins concentration at harvest. A higher percentage of cyanidin-3-glucoside and di-substituted anthocyanins forms, together with a higher 3′4′-OH/3′4′5′-OH ratio, was observed in both years in DEF I and in the second years also in DEF II berry skins compared with CK ones (Table 6). On the other hand, malvidin-3-glucoside and tri-substituted anthocyanins forms were characterized by lower percentage in 2013 and 2014 in DEF I and in the second year also in DEF II berry skins compared to that of untreated plants (Table 6). Shoot positioning did not influence the percentage of berry skin glucosidated anthocyanins compared to CK in none of the two years; however in 2014 higher percentages of petunidin-3-glucoside were observed in SB berry skins respect to DEF II ones (Table 6).

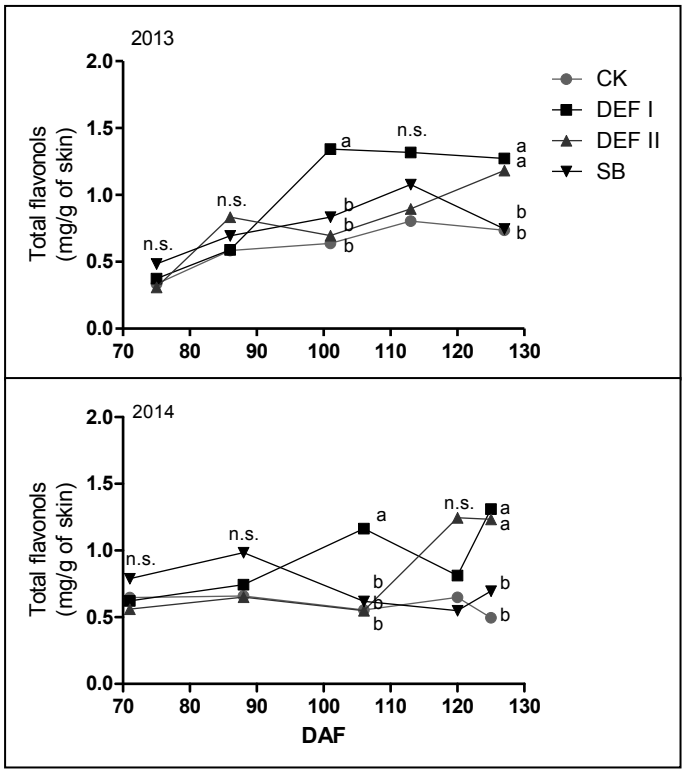
In both years, late defoliations increased the total concentration of berry skins flavonols glycosides at harvest, compared to CK and SB treatments (Table 6, Figure 1). In 2013, no differences in the percentage of glycosidated flavonols were observed among treatments, whereas in the second year lower percentages of myricetin-glucuronide and myricetin-glucoside and higher of kaempferol-glucoside were observed in DEF II berry skin compared with CK; moreover both DEF treatments and shoot positioning increased the percentage of the sum between quercetin-glucoside and 3-O-glucuronide.

Table 6. Total glucosidated anthocyanins concentration, percentage of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, di-substituted forms (3'4'-OH glucosidated anthocyanis), tri-substituted forms (3'4'5'-OH glucosidated anthocyanins), 3'4'-OH/3'4'5'-OH ratio, total glycosilated flavonols, percentage of myricetin-glucuronide, myricetin-glucoside, sum of quercetin-glucoside and quercetin-3-O-glucuronide and kaempferol – glucoside recorded at harvest, in 2013 and 2014, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese).

Parameters	2013					2014				
	CK	**DEF I**	**DEF II**	**SB**	***Significance***	**CK**	**DEF I**	**DEF II**	**SB**	***Significance***
Total-G-anthocyanins (mg/g skin)	3.21	3.38	4.17	3.69	*n.s.*	2.18	2.34	2.29	2.37	*n.s.*
Delphinidin-3-G (%)	13.56	15.64	14.03	13.20	*n.s.*	11.37	13.38	11.02	12.38	*n.s.*
Cyanidin-3-G (%)	20.96 b	29.22 a	24.55 ab	23.62 ab	*	22.19 b	33.48 a	32.1 a	23.21 b	*
Petunidin-3-G (%)	14.30	14.43	14.16	13.86	*n.s.*	11.68 ab	11.75a b	10.99 b	12.67 a	*
Peonidin-3-G (%)	13.70	13.08	14.36	15.39	*n.s.*	14.40	11.74	14.14	13.01	*n.s.*
Malvidin-3-G (%)	37.48 a	27.62 b	32.90 ab	33.93 ab	*	40.36 a	29.65 b	31.75 b	38.73 a	*
3′4′-OH-anthocyanins (%)	34.66 b	42.30 a	38.91 ab	39.01 ab	*	36.59 b	45.22 a	46.24 a	36.23 b	*
3′4′5′-OH-anthocyanins (%)	65.34 a	57.7 b	61.1 ab	60.99 ab	*	63.41 a	54.78 b	53.76 b	63.77 a	*
3′4′-OH/3′4′5′-OH	0.53 b	0.73 a	0.64 ab	0.64 ab	*	0.58 b	0.83 a	0.86 a	0.57 b	*
Total-Flavonols (mg/g skin)	0.736 b	1.273 a	1.182 a	0.745 b	**	0.496 b	1.311 a	1.234 a	0.696 b	***
Myricetin-glucuronide	1.30	1.33	1.57	1.89	*n.s.*	2.29 a	1.32 ab	1.03 b	2.23 a	*
Myricetin-glucoside (%)	9.11	7.38	8.06	9.12	*n.s.*	9.60 a	5.61 ab	4.58 b	7.54 ab	*
Sum of Quercetin glucoside and Quercetin glucuronide (%)	84.62	85.27	84.74	84.3	*n.s.*	83.96 b	85.27 a	85.67 a	85.03 a	*
Kaempferol-glucoside (%)	4.97	6.02	5.63	4.69	*n.s.*	4.15 b	7.80 ab	8.72 a	5.20 ab	*

*Significant at P ≤ *0.05*; *** significant at *P≤0.001*; n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test (bunch and berries number, plant productivity) and Kruskal-Wallis test, followed by Dunn's comparison test (bunch weight, compactness, discoloration). CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect.

Figure 1. Seasonal trends of berry skin glycosylated flavonols, recorded in 2013 and 2014 on cv. Sangiovese control vines and plants submitted to post-veraison, pre-harvest defoliations and shoot positioning through "semi-ballerina" effect . DAF, Days After Veraison. Veraison occurred at 75 Days After Flowering (DAF) in 2013 and 71 DAF in 2014. Data are means of n=3 values. Different letters indicate significant differences according to the Student- Newman-Keuls test (*P=0.05*).



Bunch temperature

In both season, exposed bunches from vines submitted to late defoliations generally showed higher temperatures, after treatments imposition, compared with those of controls, in the hottest hours of the day (Table 7). Vines submitted to shoot positioning through "semi-ballerina" effect presented similar bunch temperatures compared with CK plants, with the exception of some measurements performed at 85 and 101 DAF in 2013, when recorded values were higher and lower, respectively to CK vines (Table 7).

Table 7. Temperature of exposed bunches recorded during ripening in 2013 and 2014, in controls, vines submitted to post-veraison or pre-harvest defoliations or shoot positioning through "semi-ballerina" effect (cv. Sangiovese) vines.

2013	85 DAF		101 DAF		113 DAF	
Treatment	1400-1500	1500-1600	1400-1500	1500-1600	1400-1500	1500-1600
CK	35.7 b	37.0 b	32.3 b	41.3 b	31.0 b	27.7 ab
DEF I	38.0 a	38.3 a	38.7 a	41.0 b	34.3 a	24.0 b
DEF II	36.3 b	33.3 c	36.4 a	44.0 a	33.7 a	27.7 ab
SB	37.3 ab	38.7 a	35.3 ab	35.5 c	30.7 b	29.0 a
Significance	*	**	*	**	*n.s*	*n.s*
2014	96 DAF		109 DAF		118 DAF	
CK	25.2 b	26.1 b	30.8 b	31.4 b	28.2 b	25.5 b
DEF I	26.5 a	28.6 a	41.9 a	43.7 a	34.5 a	29.4 a
DEF II	25.3 b	26.6 b	42.5 a	42.3 a	34.2 a	29.4 a
SB	24.9 b	26.3 b	31.5 b	31.3 b	27.4 b	26.4 b
Significance	***	**	***	***	***	**

*Significant at P ≤ *0.05*;** significant at *P≤0.05*;*** significant at *P≤0.001*; n.s., not significant (*P=0.05*). Means followed by different letter in each row are significantly different according to the Student- Newman-Keuls test. CK, control vines; DEF I, vines defoliated in post-veraison; DEF II, vines defoliated in pre-harvest, SB, vines submitted to "semi-ballerina" effect.

Wine chemical analyses

Wines obtained from controls (CK), different timing of late defoliation (DEFI and DEFII) and shoot positioning, through "semi-ballerina" effect (SB) were analyzed for chemical composition at bottling (Table 8). A one-way ANOVA performed on all treatments did not highlight significant differences among parameters, regardless the year of the vintage. Noteworthy, wines produced in 2014 were characterized by low levels for all of the chemical parameters with range value below the seasonal norm: alcohol (range: 9.9–10.5%), pH (3.23–3.30), dry matter (20.1–22.5), color intensity (1.791–2.427 AU) and total polyphenols (617–739 mg/L), whereas the hue reached high value (0.763–0.888). These results were the consequence of the 2014 season, which was characterized by low average temperatures and high rainfall with marked effect on grape ripening and polyphenols accumulation. In wines of 2013 alcohol strength was in the range between 13.5–14.4% and total polyphenols between 1113–1208 mg/L, titratable acidity (6.5–6.8 g/L) and pH (3.45–4.49). Although significant differences were not found, the highestlevels of alcohol (14.4%) and dry matter (27.6 g/L) in the wines were recorded in SB. It is interesting to stress that although the difference was not significant at α: 5% (p=0.118) wines obtained from vines submitted to pre-harvest defoliation (DEF II) were characterized by higher color intensity (CK: 6.038; DEF I: 7.384; DEF II: 6.474; SB: 7.179) whereas the hue was similar among treatments. The concentration of reducing substances (1.3–1.4 g/L) and free or total sulfur dioxide was similar in all samples. In order to obtain more insight about the evolution of color component over time, the variation of anthocyanins (ANT), tannins (TN) and their impact on the formation of small (SPP), large (LPP), as well as the content of total iron- reactive phenols (IRP) and chromatic

characteristics such as total color (TC) and co-pigmentation (CP) were monitored at bottling (four months after the end of fermentation) and after one year storage in both vintages 2013 and 2014; moreover for 2013's wines the analyses were replicated after two years storage (Table 9). In 2014-wines significant changes in color components were not found either among treatments or based on period of storage. However, at bottling and after 16 months storage DEF I, DEF II and SB wines reached higher value in total color (CK: 0.77; DEF I: 0.91; DEF II: 1.2; SB: 0.99 AU) and iron-reactive phenol (CK: 676; DEF I: 735; DEF II: 843; SB: 788 mg/L), compared with CK. In 2013, the main effect was observed in wines obtained from grapes of plants submitted to post-veraison defoliation and shoot positioning through "semi-ballerina" effect, which were found to be effective for all color compounds, by enhancing them. In particular, the adoption of these two canopy management practices determined higher total color value, compared with CK, and the difference was still shown after two years storage (Table 8). Although with different timing, the same trend was observed for all the other color components such as anthocyanins, large and small polymeric pigments. As predictable, large and small polymeric pigments increased over time during 28 months storage in all treatments and their increase was higher in DEF I (LPP: 3.92 AU; SPP: 0.74 AU) and SB (LPP: 3.79 AU; SPP: 0.86 AU). The total monomeric anthocyanins decreased significantly only after 28 months from the end of fermentation in all treatments and the highest values were observed in DEF II (0.84 AU) and SB (0.9 AU). Co-pigmentation decreased significantly after 16 months storage regardless the management (CK: 0.72 to 0.13 AU; DEF I: 0.73 to 0.11 AU; DEF II: 0.86 to 0.25 AU, SB: 0.84 to 0.12 AU), then a further reduction in COP was monitored in wines stored after 26 months but this difference was not significant anymore (Table 8). The decrease of the co-pigmentation is an expected event during wine storage,

especially during the first year of wine aging as cofactors are oxidized or hydrolyzed (Boulton, 2001). Concentration of tannins and iron-reactive phenols decreased slightly after 26 months storage and reached highest value in DEF I and SB. In conclusion, as a function of the DEF I, DEF II and SB the TC, COP, ANT, LPP, SPP were sensibly higher than in CK thus confirming the results obtained for color intensity on basic analyses.

Table 8. Chemical composition of wines produced in vintages 2013 and 2014 from grape obtained from vines submitted to post-veraison (DEFI), pre-harvest defoliations (DEFI), shoot positioning through "semi-ballerina" effect (SB) and control (CK) in cv. Sangiovese. Data are presented as average value of three vinifications.

	2013					**2014**				
Parameters	**CK**	**DEF I**	**DEF II**	**SB**	***Significance***	**CK**	**DEF I**	**DEF II**	**SB**	***Significance***
ALC (%)	13.6	13.7	13.5	14.4	*n.s.*	9.9	10.3	10.5	9.6	*n.s.*
TA (g/L)	6.8	6.5	6.6	6.8	*n.s.*	6.1	6.2	6.1	6.7	*n.s.*
VA (g/L)	0.35	0.35	0.34	0.33	*n.s.*	0.27	0.23	0.25	0.27	*n.s.*
pH	3.45	3.47	3.49	3.47	*n.s.*	3.27	3.28	3.30	3.23	*n.s.*
DM (g/L)	25.0	25.3	24.9	27.6	*n.s.*	20.1	21.9	22.5	21.8	*n.s.*
RS (g/L)	1.3	1.4	1.3	1.4	*n.s.*	<1	<1	<1	<1	*n.s.*
SO_2T (mg/L)	58	57	56	60	*n.s.*	37	31	30	40	*n.s.*
SO_2F (mg/L)	25	25	25	26	*n.s.*	11	10	12	12	*n.s.*
CI (AU)	6.038	7.384	6.474	7.179	*n.s.*	1.791	2.103	2.427	2.010	*n.s.*
HUE (AU)	0.647	0.648	0.666	0.625	*n.s.*	0.848	0.888	0.763	0.868	*n.s.*
TP (mg/L)	1113	1206	1122	1208	*n.s*	617	665	739	677	*n.s*

*Significant at $P \leq 0.05$; *** significant at $P \leq 0.001$; n.s., not significant ($P=0.05$). Means followed by different letter in each row are significantly different according to the ANOVA test followed by the *post-hoc* Tukey's test. Legend: ALC: alcohol strength; TA: Titratable acidity; VA: Volatile acidity; DM: Total dry matter; RS: Reducing sugars; SO_2T: Total sulphure dioxide; SO_2F: Free sulphur dioxide; CI: Color intensity; HUE: Color hue;

TP: Total polyphenols; MA: Malic acid; LA: Lactic acid; nd: not detectable. Data are the mean value of three indipendent vinifications of 20 Kg (replicates).

Table 9. Statistical analysis (One-way ANOVA) of the phenolic and color components of Sangiovese red wines submitted to post-veraison (DEFI), pre-harvest defoliations (DEFI), shoot positioning through "semi-ballerina" effect (SB) and control (CK) in cv. Sangiovese. Data are presented as mean value of three vinifications.

ANOVA factor	Year	TC (AU)	COP (AU)	ANT (AU)	LPP (AU)	SPP (AU)	TN (mg/L)	IRP (mg/L)
CK	2013(4)	3.3ab	0.72ab	1.6ab	0.50c	0.52b	284	1285
	2013 (16)	2.3b	0.13c	1.0bcd	0.77c	0.50b	393	1307
	2013(28)	2.2b	0.05c	0.7d	2.69ab	0.67ab	267	1244
DEF I	2013(4)	3.9a	0.73ab	1.8a	0.72c	0.62ab	383	1487
	2013(16)	2.9ab	0.11c	1.3abcd	0.99c	0.70ab	503	1506
	2013(28)	2.7ab	0.00c	0.84cd	3.92a	0.74ab	429	1357
DEF II	2013(4)	3.4ab	0.86a	1.5abc	0.50c	0.52b	289	1305
	2013(16)	2.8ab	0.25bc	0.8cd	1.37bc	0.68ab	452	1322
	2013(28)	2.3b	0.00c	0.6d	3.37a	0.66ab	294	1147
SB	2013(4)	3.9a	0.84a	1.8a	0.61c	0.64ab	393	1511
	2013(16)	3.0ab	0.12c	1.3abcd	1.15c	0.75ab	535	1579
	2013(28)	2.9ab	0.02c	0.9bcd	3.79a	0.86a	433	1484
	p-value	***	***	***	***	**	**n.s.**	**n.s.**
CK	2014(4)	0.77	0.00	0.48	0.19	0.19	178	676
	2014(16)	0.76	0.04	0.25	0.33	0.25	164	642
DEF I	2014(4)	0.91	0.05	0.52	0.21	0.22	176	735
	2014(16)	0.83	0.04	0.32	0.33	0.27	155	686
DEF II	2014(4)	1.2	0.07	0.76	0.22	0.25	220	843
	2014(16)	1.1	0.07	0.45	0.37	0.31	186	768
SB	2014(4)	0.99	0.11	0.56	0.19	0.21	223	788
	2014(16)	0.93	0.09	0.37	0.31	0.28	187	730
	p-value	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**

Legend: between parenthesis months form the end of fermentation; TC: total color; COP: co-pigmentation; ANT: anthocyanins; LPP: large polymeric pigment; SPP: small polymeric pigment; TN: tannins; IRP: iron reactive phenolics. The letters represent the results of Tukey's comparison post hoc test: different letters on the column indicate means significantly different ($\alpha = 0.05$) among different wines.

Discussion

Our on-field research has been focused on gaining further knowledge on the effects of late defoliations through a wine-oriented strategy for improving its organoleptic quality, with particular attention to color parameters.

Little information is available on the effects of late defoliations on wine parameters. To our knowledge this is the first investigation on the multiple effects of this practice on berry, young and aged wine (Table 8).

As regard wines, the adoption of post-veraison or pre-harvest late defoliations and shoot positioning through "semi-ballerina" effect did not significantly affect the basic chemical characteristics of wines, such as alcohol strength, titratable acidity, volatile acidity, dry matter and pH in both vintages 2013 and 2014. These results are not consistent with Baiano et al. (2015) who found that late defoliations might have a significant effect on chemical composition of wines produced from cv. Nero di Troia. As regard color component (TC, COP. ANT, LPP, SPP), tannins (TN) and iron-reactive phenols (IRP), although significant differences were not found, late defoliations and shoot positioning enabled to obtain, in vintage 2013, wines with enhanced color characteristics, compared with control (Table 8). In particular, DEF I and SB wines were characterized by higher total color, monomeric anthocyanins, large and small polymeric pigments, tannins and iron-reactive phenols at bottling. In these wines differences were still present after two years storage. These results are consistent with Baiano et al., (2015). The same trend was observed in wines produced in 2014 at bottling and after one year of storage although the effect of these canopy management practices was more difficult to evaluate due to the characteristics of grape that produced wines with low alcohol and polyphenols content. All together these components might have a sensible effect on the final color intensity of the wine with a sensory impact on

wine evaluation. Moreover these characteristics are of well known importance in the production of wines that undertake long aging. The loss of tannins and anthocyanins during storage may be explained by anthocyanins degradation or incorporation of these compounds into oligomeric and polymeric pigments with a general preferential formation of pigmented tannin-anthocyanin polymers (LPP) over anthocyanin-acetaldehyde cross-linked oligomers and pyranoanthocyanins (SPP) during aging (Harbertson et al., 2013). This hypothesis was strengthened by the results obtained on polymeric pigments. In fact, after one year of storage the total polymeric pigments, fractioned in LPP and SPP, increased significantly. Noteworthy, in this work the positive effects of late defoliations treatments on the color of wine are not due to an enhanced berry skin anthocyanins concentration. In fact, in both seasons, similar levels of anthocyanins were detected at harvest in all treatments (Table 6).

The impact of both post-veraison and pre-harvest defoliations on total flavonols accumulation during ripening (Figure 1) and flavonols concentration at harvest (Table 6) emerged in the two contrasting seasons (Figure 1). As evident from Figure 1, the increase in flavonols concentration started after the imposition of defoliation treatments both for DEF I (applied at 86 DAF, 2013; 92 DAF, 2014) and DEF II (101 DAF, 2013; 106 DAF, 2014).

Total flavonols were higher in DEF I and DEF II berry skins compared with CK and SB at harvest. In particular, late defoliation treatments induced the biosynthesis of flavonols characterized by an increase of total flavonols concentration for DEF I up to 0.54 and 0.81 mg/g of skin and for DEF II of 0.45 and 0.74 mg/g of skin in 2013 and 2014 respectively, compared with CK (Table 6). Moreover total flavonols levels at harvest, in berry skins of plants submitted to late defoliation treatments, resulted higher in 2014 compared with the previous year, whereas in CK and SB berry skins higher values were observed in 2013. This suggests that late defoliations may enhance total flavonols levels also in anomalous

vegetative seasons. Moreover, the berry skins total flavonols concentrations recorded in DEF I and DEF II vines were higher compared with those detected in the same cultivar after the imposition of pre-bloom of veraison leaf removal treatment, in a vineyard following Integrated Pest Management (Pastore et al., 2013). The application of glyphosate, the most widely used herbicide in the world, which is forbidden in organic vineyards, could in part explain the observed differences. In fact, the herbicidal effect of glyphosate can be ascribed to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), an enzyme of the shikimate pathway, blocking the biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan. The blockage of the shikimate pathway results in substantial accumulation of shikimate in plant tissues provoking a decrease of lignin and flavonoid concentrations (Franz, et al. 1997) this latter has been recently observed also in grape berry (Donnini et al., 2016). The percentage of individual flavonols at harvest resulted similar in DEF I and DEF II berries (Table 6). When changes in the abundance of individual flavonols were observed (2014), a higher percentage of the sum of quercetin-glucoside and glucuronide was recorded in berry skins from plants submitted to late defoliation treatments; moreover DEF II plants showed also higher percentage of kaempferol-glucoside and lower of myricetin-glucoronide and myricetin-glucoside compared with CK plants (Table 6). An increase in cv. Sangiovese berry skins flavonols concentration was also observed after leaf and laterals removal imposed in pre-bloom or at veraison (Pastore et al., 2013) and similarly to what happened in our case, higher quercetin and kaempferol percentages were detected compared with control plants. The effects of increased bunch exposure on berry skins flavonols seem cultivar dependent. It has been shown that concentration of quercetin-glucoside in Pinot Noir (Price et al., 1995) and Shiraz (Haselgrove et al., 2000) berries was strongly correlated with the degree of fruit exposure (Price et al., 1995) and that bunch shading significantly reduced

flavonols levels in Shiraz (Downey et al., 2004, Ristic et al., 2007) and Cabernet Sauvignon berry skins (Matus et al. 2009). Conversely, flavonols were the most drastically reduced flavonoids following leaf removal treatments in cv. Cabernet Sauvignon (Matus et al. 2009). Furthermore, late season basal defoliations on cv. Nero di Troia have been shown to reduce the flavonols myricetin-3-glucoside, quercetin-3-glucoside, quercetin-3-glucuronide and laricitrin-3-rhamnose-7-trihydroxycinnamic acid while increasing laricitrin-3-glucoside at harvest (Baiano et al. 2015).

The bunches of plants submitted to post-veraison and pre-harvest defoliations showed higher temperatures after treatment imposition compared with those of CK and SB vines, during the hottest hours of the day (Table 7). However, previous research showed that temperature may have little or no impact on berry flavonols biosynthesis (Price et al., 1995, Haselgrove et al., 2000, Spayd et al., 2002, Mori et al., 2005). Therefore the effects of late DEFs imposition on total flavonols concentration at harvest may be explained, in part, by the increased light condition in the bunch zone, rather than increased bunch temperatures (Table 7).

Flavonols may play a crucial role as co-pigments in young red wine, stabilizing anthocyanin and creating stable association to form polymeric pigments whose importance for the color of older red wines is known. For instance, iconic wines, such as. Brunello di Montalcino, are characterized by prolonged aging period (in oak barrels and in bottles), aimed to fully express color and the other organoleptic properties. Previous research mainly focused on agronomic approaches such as early defoliation for enhancing grape berry quality. In fact this practice has been shown to produce a reduction in bunch weight, promote the loosening of clusters and improve berry quality, through increased soluble solids and anthocyanin concentration (Poni et al., 2006, Intrieri et al., 2008, Pastore et al., 2013). However, other

research suggests that defoliation just before anthesis should be carefully applied because it can decrease must TA, increase must pH (Risco et al., 2014) enhancing malic acid degradation (Gatti et al., 2015) with negative consequences on wine quality. Futhermore, it is still unclear the possible effect of early defoliation on grapes ripening disorders, such as sunburn, (Pastore et al., 2013, Gatti et al., 2015) and what the long-term effects of exposure to light radiation on epicuticular waxes are. In a context of climate change, where several agronomic approaches are oriented to produce wine with moderated alcohol content (Rombolà et al., 2011, Palliotti et al., 2013, Bondada et al., 2016) the increase in soluble solids concentration observed after the imposition of early defoliation practice should be carefully considered, together with the possible implications on grape seeds. In our study neither DEFs nor SB treatments modified berry weight and technological parameters (TSS, pH and TA) at harvest compared with control (Table 5). The lower concentrations of total glucosidated anthocyanins, berry juice TSS, pH and the higher TA values detected in 2014 as compared with 2013, were related to the different climatic conditions during ripening, confirmed also by plant water status (stem water potential). The effect of late leaf removal on berry TSS seems to be dependent on the variety, on the portion of the canopy that is influenced by defoliation and by the severity of treatment. It has been shown that the removal of the bunch zone leaves and laterals at veraison (8 Brix) in cv. Sangiovese (Pastore et al., 2013) and in cv. Cabernet Sauvignon vines (Matus et al., 2009) did not modify berry TSS at harvest. However, a light basal defoliation performed at the beginning of veraison (10 Brix) on cv. Uva Longanesi (Tessarin et al., 2014) markedly contributed to the reduction of berry total soluble solids and consequently of wine alcohol content (-0.5% TAV) and caused a decrease in the berry skin anthocyanins concentration, not revealed by wines analysis. These results suggested that besides the effects on plant photosynthetic capacity

and the alteration of light and thermal conditions at bunch level, late defoliations could also modify berry metabolism through additional multiple mechanisms, such as the synthesis of hormones (Tessarin et al., 2014). Differently, cv. Nero di Troia grapevines submitted to diverse intensities and modalities of late defoliations (imposed at complete veraison, or complete veraison and pre-harvest) generally showed higher TSS and lower titratable acidity compared to grapes from non-defoliated vines (Baiano et al., 2015). The removal of leaves inserted above the bunch zone in post-veraison (12 Brix), on cv. Sangiovese potted vines was more effective than defoliation imposed in berry growth lag-phase in temporarily decreasing soluble solids concentration compared with the control (Poni et al., 2013). In addition, mechanical defoliation applied in post-veraison (16–17 Brix), by removing at least 30–35% of vine leaf area apical to the bunch zone has been shown to be an easy and economically viable technique for delaying sugar accumulation in the berries and for limiting the alcohol content of young wines (Palliotti et al., 2013).

Seasonal differences in cluster weight altered LA/yield ratio. In fact in 2014, values were lower than those detected in 2013 (Table 3). In both years, late leaf removals markedly reduced LA/ yield ratio compared with CK and vines submitted to shoot positioning through "semi-ballerina" effect (Table 3). In 2013, all treatments presented non-limiting values of LA/yield ratio (Kliewer and Dokoozlian, 2005). However, in the second year, plants submitted to late defoliations treatments displayed lower Leaf area/yield ratio values (Table 4) relative to the0.8-1.2 m^2/kg range suggested as an appropriate target (Kliewer and Dokoozlian, 2005). In previous research, a reduction in TSS at harvest was achieved only when the LA/yield ratio was lower than 0.8–1.2 m^2/kg (Kliewer and Dokoozlian 2005, Poni et al., 2013). In our work, the decrease of the abundant TLA (-30-35%) occurred in post-veraison for DEF I plants and even later in DEF II vines and did not result into an enhanced

photosynthetic activity of the remaining leaves compared with CK (Table 1). Noteworthy, plants were characterized byseveral, healthy, efficient remaining leaves (Table 1) displaying satisfactory photosynthetic activity until harvest (Table 1). By contrast, in other studies grapevine leaves showed notable Pn compensation when a significant portion of LA was removed (Poni et al. 2013). A lower photosynthetic activity of basal leaves since post-veraison, with respect to that of intermediate and apical leaves, could, in part, explain the limited impact on the source-sink balance observed in DEFs vines, despite the removal of 30-35% of the TLA (Kriedman et al., 1970). In both years, treatments did not modify the leaf SPAD index and nutritional status that resulted fully satisfactory in all plants. Moreover no changes in the percentage of budburst of count nodes and their fruitfulness were observed after the first and the second year of treatment imposition (Table 5), suggesting that the reiteration of late defoliations did not affect bud fertility. However, the imposition of early defoliation should be carefully evaluated and probably be limited to specific seasons, due to a possible cumulative negative effect on vine bud fertility (Risco et al., 2014).

Concerning the relative abundance of the single anthocyanin glucosidated forms, some changes were observed, in particular, the 3′4′-OH/3′4′5′-OH anthocyanin ratio at harvest was always higher in DEF I berries and in the second year also in DEF II compared with CK and SB ones. This could be explained by a metabolic shift, characterized by a higher accumulation of cyanidin and lower of malvidin-3-glucoside in plants submitted to late leaf and laterals removal in the bunch zone (Table 6). Similarly, Pastore et al., 2013 detected in cv. Sangiovese vines, submitted to veraison-leaf removal in the bunch zone, higher percentage of cyanidin-3-glucoside and lower of malvidin-3-glucoside, despite no changes in the concentration of total anthocyanins compared with CK at harvest. The modifications of anthocyanins percentage may be, in part, explained by the increased light interception by

bunches of canopies submitted to late defoliations treatments that seems to favor an increase in the tri-substituted anthocyanins forms and a decrease in the di-substituted ones, particularly of cyanidin-3-glucoside. In our work the higher coverture of bunches in CK vines and plants submitted to shoot positioning through "semi-ballerina" effect did not affect total anthocyanin concentration at harvest (Table 6). Similarly, shading bunch conditions are commonly observed also in other widespread training system such as GDC, Casarsa. Downey et al., (2004), demonstrated that cv. Shiraz bunches inserted in light excluding boxes could color normally, showing a greater proportion of the di-oxygenated anthocyanins. Moreover, also total cluster shading of cv. Pinot Noir bunches resulted in minimal differences in anthocyanin concentration and composition (Cortell and Kennedy, 2006). Noteworthy, the influence of bunch sunlight exclusion on berry anthocyanins may be also dependent on the period of treatment impositions (Li et al., 2013). In particular, it has been reported that clusters totally shaded from one week pre-veraison to one week post-veraison accumulated less anthocyanins than control (clusters exposed to sunlight during the entire developmental period) at one week post-veraison; however, their re-exposure to sunlight resulted, at maturity, in recovery of anthocyanins at similar levels to the control (Li et al., 2013). The effects of light on fruit composition, in particular on anthocyanin concentration are also closely dependent to the elevation of berry temperature as a consequence of increased sunlight exposure, because high berry temperature can inhibit color development (Bergqvist et al., 2001, Mori et al., 2005). Although the highest temperatures recorded on bunches of DEFs vines, after treatments impositions (Table 7), the total glucosidated anthocyanins were similar in all treatments, while changes in anthocyanins composition were observed. This seems to suggest that bunch exposition to higher temperatures may change the proportion of total glicosidated anthocyanins (Table 6).

Late defoliations and shoot-positioning did not influence the morphology of clusters, the number of berries per bunch (Table 3), cluster and rachis length and width (data not shown). Despite in both years bunches from plants submitted to different canopy management treatments presented similar compactness, in 2014 the severity of *Botrytis* cluster rot was markedly reduced by DEF I and to lesser extent by shoot positioning and defoliation in pre-harvest. Therefore, our data suggest that in a season characterized by higher rainfall during the ripening period, it is important to avoid over-shading and perform preferentially in post-veraison the removal of leaves and laterals in the bunch zone. The possibility to reduce *Botrytis* in cv. Sangiovese bunches classified as moderately compact, semi compact, and compact (Nelson-Kluk, 2006), thus more subjected to cluster rot infection, particularly in rainy seasons, such as 2014, is of paramount importance for organic growers, lacking effective products against this pathogen. A recent study focused on the application of chitosan, known for its effectiveness against the bunch rot, as an alternative for triggering plant physiological responses, enhancing defenses mechanisms on organic cv. Cabernet Sauvignon and Sangiovese grapes; the observed increase in phenolic acids and nitrogenous compounds, especially ɤ-aminobytyric acid (GABA), in the pulp of cv. Cabernet Sauvignon grapes suggests changes in stress response (Tessarin et al., 2016).

In the first year, post-veraison defoliation increased the severity of bunch discoloration at harvest compared with control and the other treatments (Table 3). This result that can be explained by the prolonged bunch exposure to highest temperature, characterizing 2013 ripening period. In the context of climate change, where very high temperatures occur frequently during ripening, the possibility to anticipate defoliation until post-veraison should be carefully considered, in order to avoid losses of anthocyanins (Mori et al., 2005) and for limiting the incidence of berry ripening disorders.

Noteworthy, important results have been recently achieved in the field of mechanization, thanks to the development of peculiar sensors allowing leaf removal also in pre-harvest, without producing damage to the bunches in the latter phase of ripening. Furthermore, late defoliation also enables a considerable saving of time during harvest. Also for these reasons the implications of late defoliations on vine physiology and berry quality deserve further investigation and possibly need to be extended to other varieties in order to deepen knowledge on this agronomic approach that has been scarcely explored. This agronomic technique could be extended also to other varieties, such as Nebbiolo and Gaglioppo that similarly to cv. Sangiovese are characterized by poor color.

Conclusions

The enological benefits induced by post-veraison, pre-harvest leaf removal and shoot positioning through "semi-ballerina" effect can be fully appreciated in both young and aged cv. Sangiovese wines. In particular, these canopy management practices positively influenced wine components that might have a marked effect on the final color intensity, without modifying the basic chemical characteristics of wine.

The positive implications of late defoliations on wine color are not related to an enhanced berry skin anthocyanins concentration.

A clear effect of late defoliations on total flavonols concentration emerged in the two contrasting seasons and seems to be ascribable to enhanced light condition in the bunch zone, rather than increased bunch temperatures.

Although vines submitted to late defoliations showed a marked reduction of Leaf area/yield ratio compared with controls and vines submitted to shoot positioning through "semi-ballerina effect", the berry technological parameters (total soluble solids, pH and titratable

acidity), the percentage of budburst of count nodes and their fruitfulness, observed after the first and the second year of treatment imposition, were not modified.

Data suggest that in season with high rainfall during the ripening period, it is important not to exaggerate with coverture and the bunch zone removal of leaves and laterals should be preferably performed in post-veraison, rather than in pre-harvest.

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CHAPTER 3: ABA-mediated transcriptional reprogramming of secondary metabolism in cultured cells from Sangiovese mature grapes

Part of this work is included in a Manuscript in preparation:

Tessarin P., Lugli, A., Conde, A., Martins, V., Bregoli, A.M., Rombolà. A.D.

Introduction

Sangiovese is one of the most widespread grape varieties in Italy, covering around 70,300 ha, which constituted 4% of the national grape growing area (Unione Italiana Vini). Sangiovese displays a relevant agronomic and economic importance since this cultivar is used for producing hundreds of different wines, such as Brunello di Montalcino, Chianti Classico, Morellino di Scansano, Rosso Piceno Superiore, Romagna Sangiovese (Bergamini et al., 2012). Sangiovese is characterized by a great number of clones due to its high reactivity and variability of response to cultural and environmental conditions (Rustioni et al., 2013). This cultivar displays a delicate anthocyanin profile (Mattivi et al., 2006, Castellarin et al., 2012). Furthermore, particularly in warm season, this variety is subjected to a marked uncoupling between technological and phenolic maturity (Rombolà et al., 2011, Palliotti et al., 2013, Poni et al., 2013, Rombolà et al., 2015, Bondada et al., 2016, Tessarin et al., 2016), a phenomenon that can seriously compromise the overall quality of the final product due to its negative implications on wine chemical and sensory properties (Palliotti et al., 2014). In the context of climate change, several agronomic approaches, such as canopy management practices (Rombolà et al., 2011, Palliotti et al., 2013, Poni et al., 2013, Rombolà et al. 2015, Bondada et al., 2016, Tessarin et al. 2016), have been implemented to solve this issue and improve berry and wine quality. In some studies the implications of summer pruning practices (e.g. shoot trimming, leaf removal, etc.) were interpreted primarily in terms of source-sink relations and by mainly focusing on the leaf photosynthetic capacity, depending on its age (Poni et al., 2006, Intrieri et al., 2008, Poni et al., 2013, Palliotti et al., 2013). However, some authors (Poni et al., 2013, Tessarin et al., 2014, Bondada et al., 2016) suggested the additional contribution of other factors, such as hormones, for elucidating the observed phenomena. In fact, canopy management practices,

such as shoot trimming, characterized by the removal of the shoot apex may also impact the vine hormonal status causing changes in auxins distribution and/or ethylene (Léon et al., 2011) and the production of abscisic acid (Sun et al., 2007) in response to cut wound. The effects of endogenous and exogenous hormones on berry ripening have been analyzed in several studies (Kuhn et al., 2013). In particular, the enhancement of ripening by ABA in grapevine has been observed at the compositional (Coombe and Hale, 1973, Pirie and Mullins, 1976, Wheeler et al., 2009) and, in some cases, at the molecular level (Ban et al., 2003, Jeong et al., 2004, Giribaldi et al., 2010).

Moreover, different studies showed that a treatment with this hormone at veraison (Kataoka et al., 1982, Mori et al., 2005, Cantin et al., 2007, Peppi et al., 2007, Peppi and Fidelibus, 2008) or in post-veraison (Kataoka et al., 1982, Peppi et al., 2007) promoted an increase in anthocyanins.

Sangiovese grapes are quite refractory/recalcitrant regarding anthocyanins production, so it is important to understand secondary metabolism at the cellular level, and for that the attainment of cultured cells from the grapes as biological model could be of great interest.

The *in vitro* cultures represent a valid technique for investigating, in controlled conditions, the relations between the mechanisms regulating the secondary metabolism in plant cells and the changes induced by environmental, pedoclimatic and agronomic factors. Therefore we were stimulated to establish an experiment aimed to obtain, for the first time, cell cultures from grape berries sampled in an organic cv. Sangiovese vineyard, where, in a parallel research, the impact of different canopy management practices on cv. Sangiovese vines has been assessed (Rombolà et al., 2011, Rombolà et al., 2015, Bondada et al., 2016, Tessarin et al., 2016). Moreover, we want to explore the influence of auxins and abscisic acid

application on the genes involved in the secondary metabolism of phenolic compounds of *in vitro* cv. Sangiovese cells cultures.

Material and Methods

Field material

On September 17th, 2013, 113 days after flowering, 20 not completely mature grape berries were collected in an organic vineyard of cv. Sangiovese (clone FEDIT 30 ESAVE) grafted onto KOBER 5BB, trained to Cordon du Royat. The vineyard, hosting a long-term experiment coordinated by the University of Bologna, is located in Tebano (Faenza, RA), Emila Romagna Region, Italy (44°17'7''N, 11°52'59'E, 117 m a.s.l.), on a medium slope, with southeast/northwest and downhill-oriented rows. Starting in 2007, the vineyard was managed as organic in accordance with Reg. EC 834/2007 (EC, 2007). Beginning in 2007, no irrigation water was applied and the vineyard was not fertilized. Berries that were free of physical injuries and uniform in size were sampled and transported in refrigerated boxes to the laboratory and used within 12 h.

Establishment of calli cultures from grape berry mesocarp and exocarp explants

Berries were washed with running water for 10 minutes, their surface was sterilized with sodium chloride (0.5% chlorine) for 20 minutes, in a flow chamber, than througly washed with sterilized water. Berries were sliced in half and immediately transferred on a solid medium (0.8% agar) containing: Gamborg B5 medium (Duchefa Biochemie) supplemented with 2% sucrose, 1.0 µg mL^{-1} BAP, 0.5 µg mL^{-1} NAA, at pH 5.8. The Petri dishes were incubated at 25 °C, photoperiod 16 hours, light intensity 50 µmol^{-2}s^{-1}. The state of the berries was monitored to confirm there was no contamination. After 3 weeks, berries were sliced in pieces of c.a. 1 cm^2, characterized by both exocarp and mesocarp and then

transferred into new Petri dishes. Several subcultures were performed on the same solid medium.

Establishment of cell suspensions

The *callus* obtained from cv. Sangiovese berries was subcultured in a liquid medium containing Gamborg B5 medium (Duchefa Biochemie), 4% sucrose, 250 mg/L casein, 0.1 mg/L (NAA), 0.3 mg/L kinetin at pH 5.8 to obtain cell suspensions. Cultures were maintained in 250-mL flasks on an orbital shaker (100 rpm), at 25 ºC, under a photoperiod of 16 h, light intensity 50 $\mu mol^{-2}s^{-1}$. During maintenance, cells were sub-cultured weekly by transferring 10-mL aliquots into 40 mL of fresh medium.

Aliquots of 10-mL were transferred into 40 mL of two new fresh liquid media containing Gamborg B5, 4% sucrose, 250 mg/L casein, 0.3 mg/L kinetin at pH 5.8 and different concentrations of auxin (NAA): 0.1 mg/L or 0.05 mg/L, respectively. Each treatment was replicated twice.

Elicitation with Abscisic Acid

Five-day old grown cells at the beginning of the exponential growth phase were subjected to elicitation with 150 µM abscisic acid (ABA) (Vuong et al., 2014). Both cultural media containing 1 mg/L or 0.05 mg/L NAA, respectively were elicitated with abscisic acid. Four treatments were compared: control (liquid media 1, L1): Gamborg B5 medium, supplemented with 4% sucrose, 250 mg/L casein, 0.3 mg/L kinetin, 0.1 mg/L NAA at pH 5.8; Liquid media 2, L2: Gamborg B5 medium, supplemented with 4% sucrose, 250 mg/L casein, 0.3 mg/L kinetin, 0.1 mg/L NAA, 150 uM ABA, at pH 5.8; Liquid media 3, L3: Gamborg B5 medium, supplemented with 4% sucrose, 250 mg/L casein, 0.3 mg/L kinetin, 0.05 mg/L NAA, at pH 5.8; Liquid media 4, L4: Gamborg B5 medium, supplemented with 4% sucrose, 250 mg/L casein, 0.3 mg/L kinetin, 0.05 mg/L NAA, 150 uM ABA, at pH 5.8.

The ABA solution was sterilized by filtration with 0.2 µm Millipore filters (Minisart®, Sartorius, Germany). Cell aliquots were collected for RNA extraction 8 days after elicitation with ABA (Vuong et al., 2014).

RNA extraction

RNA extraction was performed with RNeasy Plant Mini Kit (Qiagen) using a different extraction buffer. The samples was mixed with 1 mL of extraction buffer (2% [w/v] CTAB, 2% [w/v] PVPP, 300 mM Tris HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 2% β-mercaptoetanol) and incubated at 60 ºC for 20 min in agitation. Treatment with DNase I (Qiagen) was applied. RNA integrity was confirmed with agarose gels stained with SYBR Safe (InvitrogenTM, Life Technologies) and Nanodrop (Thermo Fisher Scientific Inc.) was used for RNA quantification.

Real-time qPCR

Real-time qPCR were used for gene expression analysis of the genes *VvPAL*, *VvCHS*, *VvUFGT* and *VvABCC1*. cDNA was synthesized from 1 µg of total RNA using Omniscript Reverse Transcription Kit (Qiagen). Real-time PCR analysis was performed with QuantiTect SYBR Green PCR Kit (Qiagen) using 1 µL cDNA (diluted 1:10 in ultra-pure distilled water) in a final reaction volume of 10 µL per well. Polymerase was activated with an initial step of 15 min at 95 °C to enzyme activation, the double strand denaturation occurred at 94ºC for 15s, the annealing temperature was 55 ºC during 30s and the extension temperature was 72 ºC during 30s. Amplification was performed using 40 cycles. For reference genes *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was selected, as these genes were proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al., 2006). Gene specific primer pairs used for each target or reference gene are listed on Table 1. Melting curve analysis was performed for specific gene

amplification confirmation. The expression values were normalized by the average of the expression of the reference genes as described by Pfaffl (2001). Primers for *VvGAPDH*, *VvUFGT1* and *VvABCC1* were made using QuantPrime qPCR primer design tool (Arvidsson et al., 2008).

Table 1. Gene specific primer pairs used for determination of gene expression by real-time PCR.

Gene	Assession Number	Primers	Reference
VvGAPDH	XM_002263109	F: 5'- CTTTCCGTGTTCCTACTGTTG-3′ R: 5'- CCTCTGACTCCTCCTTGAT-3′	Custom made using QuantPrime software
VvPAL1	GSVIVG01025703001	F: 5′-CCGAACCGAATCAAGGACTG-3′ R: 5′-GTTCCAGCCACTGAGACAAT-3′	Boubakri et al. 2013
VvCHS1	GSVIVT00037967001 (8x)	F: 5′-CGAGCTCACCACCGAGCACCTTACCT-3′ R: 5′-CCGCTCGAGTGTTGGCTACCTGCTTCACT-3′	Boubakri et al. 2013
VvUFGT1	GSVIVT01024419001	F: 5′-TGCAGGGCCTAACTCACTCT-3′ R: 5′-GCAGTCGCCTTAGGTAGCAC-3′	Custom made using QuantPrime software
VvABCC1	GSVIVT01028722001	F: 5′-CTCCACTGGTCCTCTGCTTC-3′ R: 5′-AGCCTGCTTCGAAAGTACCA-3′	Custom made using QuantPrime software

Statistical Analysis

Gene expression data for each treatment was compared by one-way ANOVA analysis, and Tukey's post-test (*P=0.05*) for comparison of means of parametric data, using Prism® 5 (GraphPad Software, Inc.).

Results

To study the effects of hormones on the grape berry cv. Sangiovese and their effect on phenolic metabolism at the molecular level, a *calli* culture from grape berry's mesocarp and exocarp was established (Figure 1).

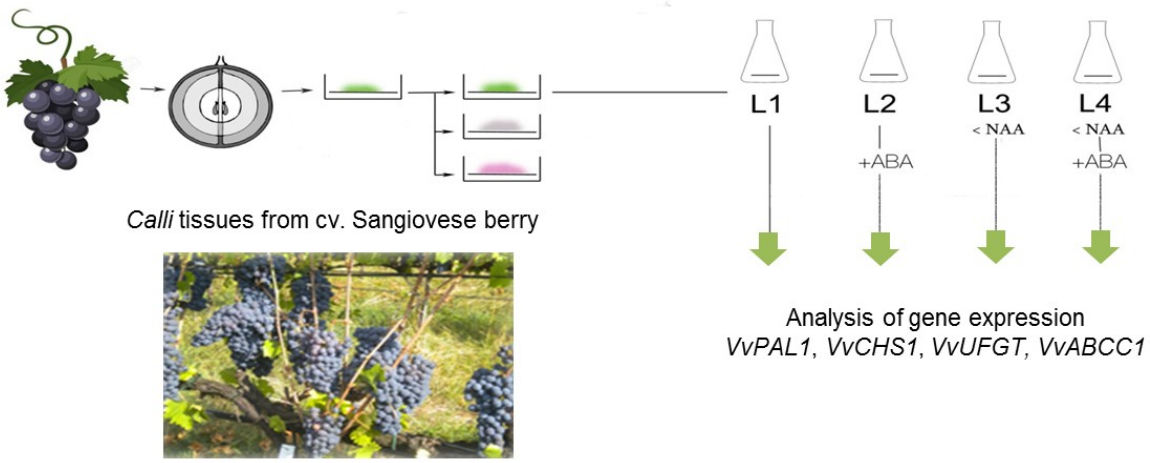

Figure 1. Experimental summary of the obtention of different lines of *calli* tissue from cv. Sangiovese berry exocarp and mesocarp, and analysis of the expression of *VvPAL1*, *VvCHS1*, *VvUFGT* and *VvABCC1* after ABA-elicitation of cell suspensions grown in different concentrations of auxins.

Calli induction was observed c.a.100 days after samples collection (Figure 2). A repeated selection and subcultivation of different-colored *calli* clusters enabled the obtention, after 7 month from the first transplant, of 3 different *calli* lines characterized by white, pink and green color (Figure 2).

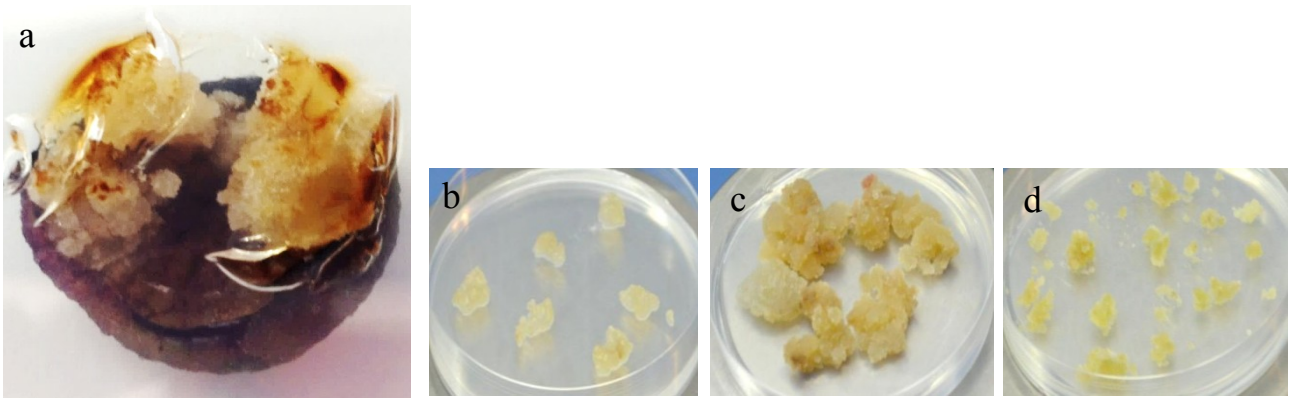

Figure 2. *Callus* induction from cv. Sangiovese grape berry tissues (a). Established *calli* lines were characterized by white (b), pink (c) and green (d) color.

The expression of *VvPAL1*, *VvCHS1*, *VvUFGT* and *VvABCC1* genes was studied by Real-Time PCR on the green *calli* line which showed better growth.

As shown in Figure 3 the transcript levels of a phenylalanine ammonia lyase gene (*VvPAL1*), that encodes an enzyme catalyzing the first step in the phenylpropanoid pathway in which trans-cinnamic acid is produced, increased abruptly in cells elicited with 150 µM ABA (up to ~150- fold) and this stimulation in gene expression was even more evident when ABA was added to cultured cells growing in a medium with low-NAA (0.05 mg/L) (Figure 3). Moreover, the steady-state transcript levels of *VvPAL1* were similar between cells cultivated in a medium with low-NAA (in the absence of ABA) and control conditions (Figure 3).

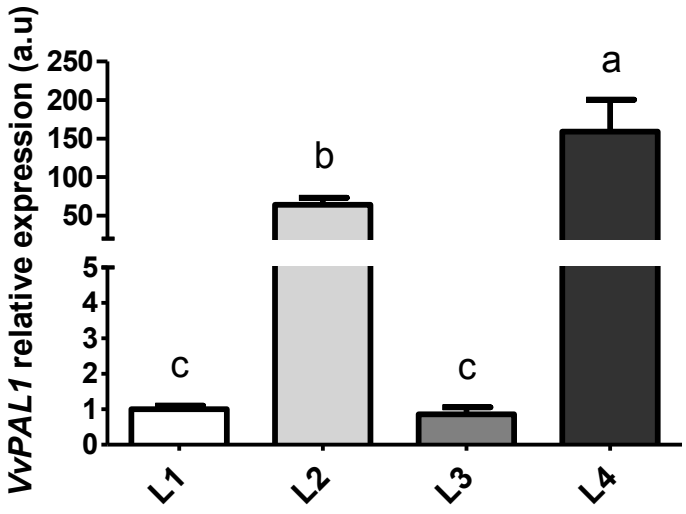

Figure 3. Hormonal regulation of the secondary metabolism in cell cultures from cv. Sangiovese grapes. Effect of the elicitation with 150 µM ABA on the expression of *VvPAL1*. Different letters above each column indicate significative differences according to one-way ANOVA analysis (*P = 0.05*). Results show the mean ± SEM of n=3 measures. L1: control (Gamborg B5 medium + 4% sucrose); L2: 0.1 mg/L NAA+150 µM ABA; L3: 0.05 mg/L NAA, L4: 0.05 mg/L NAA+150 µM ABA).

Transcriptional changes of *VvCHS1* which codes for chalcone synthase - that initiates the flavonoid pathway - were also studied (Figure 4). The enzyme catalyzes the conversion of 4-coumaroyl-CoA and malonyl-CoA to naringeninchalcone. As can be seen, the transcript levels of *VvCHS1* increased drastically (up to ~18000-fold) in cells elicited with 150 µM ABA, but the stimulation mediated by ABA was much less strong when the culture medium contained low-NAA (Figure 4). When the effect of low-NAA was studied in the absence of ABA elicitation, the steady-state transcript levels of *VvCHS1* were lower than in control conditions, which could account for the observed reduction in the number of *VvCHS1* transcripts from the condition ABA/normal NAA to the condition ABA/low-NAA (Figure 4).

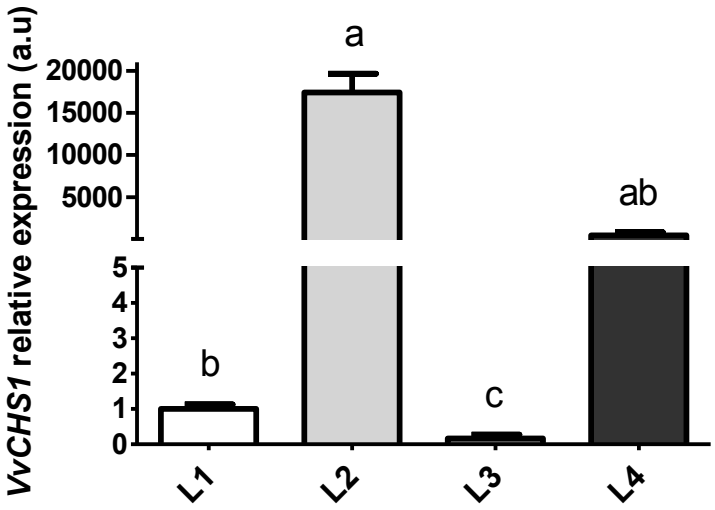

Figure 4. Hormonal regulation of the secondary metabolism in cell cultures from cv. Sangiovese grapes. Effect of the elicitation with 150 µM ABA on the expression of *VvCHS1*. Different letters above each column indicate significative differences according to one-way ANOVA analysis (*P = 0.05*). Results show the mean ± SEM of n=3 measures. L1: control (Gamborg B5 medium + 4% sucrose); L2: 0.1 mg/L NAA+150 µM ABA; L3: 0.05 mg/L NAA, L4: 0.05 mg/L NAA+150 µM ABA).

The enzyme UDP-glucose:flavonol 3-*O*-glucosyl transferase (UFGT) catalyzes the final step of anthocyanin biosynthesis. The transcript levels of *VvUFGT* increased drastically (up to ~20000-fold) in cv. Sangiovese cells elicited with 150 µM ABA. This increase was more marked in presence of higher concentration of NAA (Figure 5). Moreover, PCR analysis did not detect transcritpt levels of *VvABCC1*.

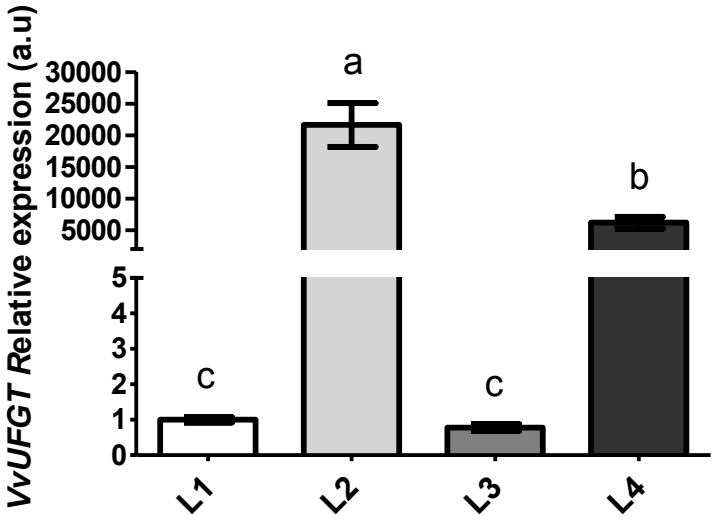

Figure 5. Hormonal regulation of the secondary metabolism in cell cultures from cv. Sangiovese grapes. Effect of the elicitation with 150 µM ABA on the expression of *VvUFGT*. Different letters above each column indicate significative differences according to the one-way ANOVA analysis (*P = 0.05*). Results show the mean ± SEM of n=3 measures. L1: control (Gamborg B5 medium + 4% sucrose); L2: 0.1 mg/L NAA+150 µM ABA; L3: 0.05 mg/L NAA, L4: 0.05 mg/L NAA+150 µM ABA).

Discussion

Sangiovese grapes are quite refractory/recalcitrant regarding anthocyanins production, so it is important to understand secondary metabolism at the cellular level. The obtention of cultured cells from the grapes could be a biological model of great interest. In our work we were able to produce for the first time cultured cells from cv. Sangiovese mature grapes. These cells were amenable to study secondary metabolism as key genes (*VvPAL1, VvCHS1* and *VvUFGT*) responded to ABA elicitation.
Data on gene expression show that phytoregulators influenced the secondary metabolism in cell suspensions (Figures 3, 4 and 5).

The transcript levels of a phenylalanine ammonia lyase gene (*VvPAL1*), encoding an enzyme catalyzing the first step in the phenylpropanoid pathway in which trans-cinnamic acid is produced, increased abruptly in cells elicited with 150 µM ABA (Figure 3). Previous studies showed that abscisic acid promotes the expression of several genes related to the initial reactions of the metabolic pathways of biosynthesis of flavonols, in cell culture of *Vitis vinifera* cv. Cabernet Sauvignon, including *VvC4H, VvCHI, VvCHI2* and *VvPAL* (Gagné et al., 2011).

The effect of the lower auxins concentration on *VvPAL1* gene expression resulted significantly evident only in cells elicitated with ABA (Figure 3). Data concerning *VvPAL1* gene expression suggest a synergic action of ABA and auxins, similarly to what happen in the berry when the accumulation of sugars begins.

In this phase, the level of auxins tend to decrease and abscisic acid starts to exert its role in promoting berry ripening (Böttcher et al., 2012). The metabolism of sugars is influenced by the reduction of auxins levels, since this decrease determines an increase in the activity of the invertase which hydrolyzes sucrose in fructose and glucose (Davies et al., 2009). The accumulation of sugars seems to be related to the sink activity induced in the berries by ABA stimulating the accumulation of anthocyanins in the berry skin through the activation of the enzyme PAL and consequently of the flavonoids pathway (Kataoka et al., 1984). In berry skins the activity of this enzyme reaches his maximum level immediately after veraison (Hiratsuka et al., 2001) concomitantly to the increase of ABA concentration (Davies et al., 2009).

Dunlap and Robacker (1991), studying senescence in ovaries of *Cucumis melo* inflorescences, observed that ABA supply induces a decrease in the levels of free auxin and increases the esterified (inactive) forms. Moreover, it has been reported that flavonoids,

whose synthesis is stimulated by abscisic acid, negatively regulate the transport of auxins (Brown et al., 2001). The transcript levels of *VvCHS1,* codifying for the enzyme chalcone synthase increased markedly in cells elicited with 150 µM ABA (Figure 4). Since the enzyme chalcone syntase competes with stilbene synthase for the same substrate (4-coumaroyl-CoA), this suggest that lower level of NAA could enhance the production of stilbenes (trans-resveratrol and derivatives) through the activation of stilbene synthase. Once activated, the synthesis of stilbenes generally prevails on that of flavonoids (Melchior and Kindl, 1990). For these reasons future research should be addressed to investigate the expression of the genes *VvSTS* and the activity of the enzyme STS in relation to different concentrations of auxins.

Like *VvCHS1*, the *VvUFGT* gene that codes the glicosylation of cianidin, pelargonidin and delphinidin was highly expressed in cells elicitated with ABA. These genes are more responsive to hormonal stimuli, suggesting that the initial reactions of the phenylpropanoid pathway are less influenced by external factors (Figures 4 and 5). PAL is a crucial enzyme in the metabolism of phenylpropanoids (Wen et al., 2005). The activity of this enzyme is more difficult to influence and is noticeably induced only as a consequence of strong abiotic stresses (Dixon and Palva, 1995). For this reason PAL is often used as an indicator of the reaction mechanisms activated by plant (Sgarbi et al., 2003).

PCR analysis did not detect transcript levels of *VvABCC1* that codes for a tonoplast S-conjugated anthocyanins transporter suggesting the enzyme is not operating in the experimental conditions, probably because no sufficient amounts of anthocyanins were produced in response to the observed strong shifts in *VvPAL1* and *VvCHS1* expression, which goes in agreement with the lack of change in the color phenotype of the cultures.

In our study on cv. Sangiovese we observed that the elicitation with 150 µM ABA compromised the viability of cultured cells, therefore in future studies we should test the effect of lower concentrations of ABA, which could favor the continued growth of the cell culture and, eventually, the effective production of anthocyanins.

Canopy management practices, such as shoot trimming, characterized by the removal of the shoot apex, not only influence the canopy architecture, but may also impact vine hormonal status causing changes in auxins distribution and the production of ethylene (Léon et al., 2011) and/or abscisic acid (Sun et al., 2007) in response to cut wound. Recently, it has been reported that, in two subsequent years with contrasting climatic conditions, post-veraison trimming increased berry skin anthocyanin and phenolic concentration without modifying berry soluble solids (Tessarin et al., 2016). The possible role of the different plant hormones on berry composition, after the imposition of specific canopy management practices, should be further investigated.

In future studies a careful transcriptomic analysis of the secondary metabolism will be performed, in addition to a detailed metabolomic analysis to quantify key metabolites.

Conclusions

In the present work, we report for the first time the establishment of cell cultures from cv. Sangiovese berry tissues and explore the influence of auxins and ABA on the expression of genes involved in the secondary metabolism of phenolic compounds in this cultivar. Results showed that the elicitation of cell suspensions with ABA caused an increase in the expression of *VvPAL1*, *VvCHS1*, *VvUFGT* genes, while transcripts of *VvABCC1* were not detected. This approach indicates that *in vitro* cultures cv. Sangiovese can be used as a model for investigating, in controlled conditions, the relations between the mechanisms

regulating the secondary metabolism in grape cells and the changes possibly induced by agronomic factors in this economically-relevant cultivar.

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Discussion

The PhD Thesis provided clear scientific evidences on the implications of different post-veraison canopy management practices (trimming, defoliation, shoot positioning) on vine physiology, grape and wine quality of organically-cultivated cv. Sangiovese.

The Research work, conducted in the context of climate change, demonstrated that late trimming (post-veraison) and both post-veraison and pre-harvest defoliations are valuable agronomical techniques for improving the chemical properties and sanitary status of organic cv. Sangiovese grapes. In particular, post-veraison trimming and late defoliations resulted effective strategies for enhancing, in contrasting seasons, the concentration of berry skins phenolic compounds, without altering soluble solids levels in the berry (Chapter 1, Table 6, Figures 2 and 3, Chapter 2, Table 5, Figure 1). These results led to significant improvements for the color of young and aged wines (Chapter 1, Figures 6 and 7, Chapter 2, Table 9).

For the first time, post-veraison trimmed plants (Figure 4) were compared with untrimmed controls (Figure5), assessing the effects on vine physiology, grape and wine quality.

Figure 4. Plants of cv. Sangiovese submitted to post-veraison (late) trimming.

Figure 5. Plants of cv. Sangiovese characterized by long shoots (controls).

Results highlighted, in two seasons characterized by contrasting climatic conditions, the relevance of trimming in the last phase of berry ripening for improving grape berry and wine color in cv. Sangiovese. Despite the different agronomic techniques to increase anthocyanin levels in grapes (e.g. leaf removal, application of phytoregulators), this is the first time that an enhancement in berry skins anthocyanins and total phenolics concentration is attained through the imposition of post-veraison trimming (Chapter 1, Figures 2 and 3). Moreover, late trimming, differently from other studies (Rombolà et al., 2011, Filippetti et al., 2015, Bondada et al., 2016), was imposed only once in the vegetative season.

In previous papers, the effect of summer pruning practices (e.g. shoot trimming, leaf removal, etc.) was interpreted primarily in terms of source-sink relations (Filippetti et al., 2015) and, in particular, by focusing on the leaf photosynthetic capacity, depending on its age (Poni et al., 2006, Intrieri et al., 2008, Poni et al., 2013, Palliotti et al., 2013). Recently, some authors (Poni et al., 2013, Tessarin et al., 2014, Bondada et al., 2016) highlighted the

importance of considering the contributions of other physiological factors, such as hormones, for elucidating the observed phenomena.

Interestingly, data showed that the removal of the shoot apex, both in case of post-veraison (Figure 4) and early trimming (Figure 6), had a general positive effect on cv. Sangiovese berry skin anthocyanins and phenolics, particularly when performed in post-veraison.

Figure 6. Plants of cv. Sangiovese submitted to trimming at pea-size (early trimming).

This organ, characterized by a the presence of shoot apex meristem (SAM, Vernoux et al., 2010) plays a pivotal role in auxins synthesis and translocation, being this the active site where auxins synthetized in different plant organs. Treating bunches with auxins both in pre-veraison (Davies et al., 1997) or at veraison (Jeong et al., 2004) produced negative effects on anthocyanin accumulation and genes related to their biosynthesis. Moreover, Davies et al., (1997) observed that the effect of auxins was more marked on anthocyanins than on sugars. In vines trimmed at pea-size, the development of lateral shoots (Chapter 1, Table 2) could have mitigated the effect of shoot apex removal, on berry total anthocyanins (Chapter 1, Figure 2). Also the decrease of malic acid concentration in both years detected at harvest in

post-veraison trimmed plants and, in the first year, also on early trimmed vines compared to control, suggests the possible contribution of auxins to berry ripening (Chapter 1, Table 7). In fact, the effects of auxins on malic acid has been reported in literature (Böttcher et al., 2012, Ziliotto et al., 2012). In particular, Böttcher et al., (2012), observed that treating bunches in pre-veraison reduced the rate of decline in malic acid levels during ripening. It was also possible that trimming, causing a wound, may have induced the production of abscisic acid (ABA), (Leòn et al., 2011) and/or ethylene (Sun et al., 2007) and a related increase in berry skins anthocyanin concentration (Jensen et al., 1975, Kataoka et al., 1982, Wicks and Kliewer, 1983). Noteworthy, Wicks and Kliewer (1983) demonstrated that anthocyanins and total phenolic levels can change without any significant variation in soluble skin carbohydrates after treatment with the ethylene-releasing molecule ethephon. The parallel *in vitro* experiment conducted on organic cv. Sangiovese cell suspensions obtained from berries sampled in the same experimental vineyard showed that transcript levels of *VvPAL1* increased abruptly in cells elicited with 150 µM ABA (Chapter 3, Figure 4). Interestingly, the effect of the lower concentration of auxins on *VvPAL1* relative expression resulted significantly evident only in cells elicitated with ABA (Chapter 3, Figure 4). In particular, the genes *VvCHS1* and *VvUFGT* were more responsive to hormonal stimuli (Chapter 3, Figures 5 and 6), suggesting that the initial reactions of the phenylpropanoid pathway are less influenced by external factors. Avoiding trimming for providing some shade to the cluster ("accucciatura") or twisting long shoots on the top wire with the aim to preserve the shoot apex ("accapannatura") could decrease anthocyanins and total phenolics concentration. Therefore, the application of these techniques should be carefully evaluated, possibly associated to late season defoliations and specific soil management practices (e.g. intercropping and late mowing of herbaceous species) (Rombolà et al., 2014, Sevindik et al.,

2014, Rombolà, 2015, Rombolà et al., 2015, Botelho et al., 2016). Late defoliation (Figure 7) represented a valuable technique for enhancing falvonols levels in berries of plants characterized by long shoots and for improving young and aged wine quality (Chapter 2, Table 6, Table 9).

Figure 7. Plants of cv. Sangiovese submitted to late defoliation.

Interestingly, the positive effects of post-veraison and pre-harvest leaf removal on wine colorwere not related to an enhanced berry skin anthocyanins concentration, as in case of post-veraison trimming, but to a marked increase in flavonols concentrations after treatments imposition (Chapter 2, Table 6, Figure 1). In fact, flavonols may play a crucial role as co-pigments in young red wine, stabilizing anthocyanin and creating stable association to form polymeric pigments whose importance for the color of older red wines is known (Boulton, 2011). The possibility to strengthen the color properties of wine, independently from high total anthocyanin concentration is of paramount importance for all those cultivar that like Sangiovese present delicate anthocyanin profile. The effects of late defoliations imposition on total flavonols concentration seem to be ascribable to increased light condition in the

bunch zone (photosynthetically active radiation ranging from 1100 to 2000 μmol/(m^2 ·s), rather than increased bunch temperatures (Chapter 2, Table 7). Interestingly, light may have a strong influence also on multiple aspects of the auxin system, such as controlling auxin levels, transport and responsiveness (Halliday et al., 2009). For this reason, in future Research, the obtained cell suspensions could be used to further investigate, in controlled conditions, also the effects of different light conditions and hormones concentrations on the secondary metabolism of cv. Sangiovese berry cells.

Previous papers mainly focused to agronomic techniques such as early defoliation for enhancing grape berry quality. In fact this practice has been shown to produce a reduction in bunch weight, promote the loosening of clusters and improve berry quality, through increased soluble solids and anthocyanin concentration (Poni et al., 2006, Intrieri et al., 2008, Diago et al., 2012, Pastore et al., 2013). However, in a context of climate change, where several agronomic approaches are oriented to delay sugar accumulation and produce wine with moderated alcohol content (Rombolà et al., 2011, Palliotti et al., 2013, Palliotti, 2014, Bondada et al., 2016), the increase in soluble solids concentration due to the imposition of early defoliation should be carefully considered, together with the possible implications of this practice on grape seeds, must titratable acidity and pH (Diago et al., 2012, Risco et al., 2014, Gatti et al., 2015). For this reason, the possibility of disposing of summer pruning practices (e.g. late trimming and defoliations) that did not alter berry technological parameters, while enhancing berry skin phenolics concentration may represent a trump card for growers facing climate change. Concerning cluster morphology also late trimming enabled to contain plant productivity and loosened clusters (Chapter 1, Table 4), therefore it could be considered as a simple, cheap, valuable alternative to cluster thinning. These results are consistent with the findings of a four-year experiment, concerning the

effects of post-veraison trimming practices on cv. Sangiovese, conducted in a nearby organic vineyard (Rombolà et al., 2011, Bondada et al., 2016). The loosening of clusters in late trimmed plants also enabled to contain *Botrytis* cluster rot. Differently from late trimming, late defoliations and shoot positioning thorough "semi-ballerina" effect (Figure 8) did not influence the morphology of clusters (Chapter 2, Table 3).

Figure 8. Plants of cv. Sangiovese submitted to shoot positioning thorough semi-ballerina effect.

Nevertheless, the severity of *Botrytis* cluster rot was markedly reduced by post-veraison defoliation and to lesser extent by shoot positioning and defoliation in pre-harvest. Results suggest that in season characterized by higher rainfall during the ripening period, it is important to avoid over-shading and the leaves and laterals should be preferably removedin post-veraison. The possibility to reduce *Botrytis* cluster rot (Figure 9), particularly in rainy growing seasons (e.g. 2014), through specific canopy management practices, is crucial for organic growers, lacking effective products against this pathogen.

Figure 9. Bunch of cv. Sangiovese affected by *Botrytis* cluster rot.

This especially in the case of Sangiovese (Figure 10) (Nelson-Kluk, 2006) or other varieties that tends to present particularly compact clusters.

Figure 10. Compact cluster of cv. Sangiovese.

Treatments did not modify the leaf SPAD index and nutritional status that resulted fully satisfactory (Penazzi et al., 2011). Despite the removal of (30-35%) of the total leaf area, leaves showed appreciable photosynthetic activity also in the last phase of berry ripening (Chapter 1, Tables 1 and 2, Chapter 2, Table 1). Moreover, no compensatory increase in photosynthetic activity or stomatal conductance, based on single leaf gas-exchange readings, in the leaves of the main shoot was observed after the application of late trimming or late defoliations (Chapter 1, Table 2, Chapter 2, Table 1). Noteworthy, nor late trimming, neither late defoliations did not change the percentage of budburst of count nodes and their fruitfulness after the first and the second year of treatment imposition, suggesting that the reiteration of these practices did not affect bud fertility (Chapter 1, Table 5, Chapter 2, Table 4). Differently, the possibility to impose early defoliation should be carefully and probably be limited to specific seasons, due to a possible cumulative negative effect on vine bud fertility (Risco et al., 2014).

The peculiar soil management adopted in the experimental vineyard (Figure 11), allowed to contain shoots growth and to delay trimming until post veraison and late defoliation even later. The possibility to reduce the number of mechanical intervention in the field should be carefully considered due to its positive implications on the growers' incomes, soil structure and environment.

Figure 11. Soil management in the organic experimental vineyard.

Conclusions

The key agronomic and enological relevance of post-veraison trimming, post-veraison and pre-harvest defoliations for achieving substantial improvements in berry and wine quality in organically-cultivated cv. Sangiovese emerged in two contrasting vegetative seasons. The impact of these practices should be contextualized in the light of vineyard plantation and management, therefore considering vine spacing, training system, winter pruning, canopy architecture, soil management, etc.

The improvement in berry skins phenolic concentration was attained without using external inputs (e.g. phytoregulators) and without modifying the berry total soluble solids and other technological parameters.

In both years, the enological benefits induced by late trimming and late defoliations has been dempnstrated in both young and aged wines, therefore these practices represents an innovative approach for obtaining higher quality wines.

Trimming at pea-size was not always as effective as late trimming in improving berry skin color and its impact seemed more dependent to climatic conditions. In future Research, the possibility to combine late trimming and late defoliations practices for improving total anthocyanins and flavonols concentrations and the grapes sanitary status should also be assessed.

Maintaining long shoots for providing some shade to the cluster could decrease anthocyanin concentration and favor the production of compact bunches. Therefore, the application of this technique should be carefully evaluated, and possibly associated to late season defoliations and peculiar soil management.

Experimental data strongly designated late trimming, a practice proved to contain yield and bunch compactness, as a valuable alternative to cluster thinning.

Late trimming and defoliations reduced the severity of *Botrytis* cluster rot. However, in order to preserve the grapes sanitary status, in seasons with high rainfall during the ripening period, the bunch-zone removal of leaves and laterals should be preferably performed in post-veraison.

The innovative results obtained within the PhD Thesis constitute a breakthrough in Vineyard Management, a fascinating bridge between vine physiology, climatic change and berry composition, disclosing novel scientific paths from vineyards to wine.

Data displays a novel potentiality of canopy management practices, whose beneficial effects represent a tangible opportunity, not only for organic growers, but also for those following conventional or Integrated Pest Management.

For the first time, cell cultures from cv. Sangiovese berry tissues were obtained and enabled to investigate, in controlled conditions, the relations between mechanisms regulating secondary metabolism in grapevine cells and changes induced by environmental and agronomic factors.

The Doctoral Dissertation strongly highlights the need to consider, for a proper interpretation of the multiple modifications induced by canopy management strategies, physiological mechanisms other than the canonic source-sink relationships, in particular their impact on the vine hormonal status.

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