THE IMPACT OF MECHANICAL LEAF REMOVAL ON GRAPEVINE PHYSIOLOGY, RIPENING-RELATED PHYTOHORMONE BIOLOGY, AND FRUIT QUALITY IN (VITIS VINIFERA L.) MERLOT

By

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ABSTRACT

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Removal of basal leaves early in the vegetative and reproductive development of grapevines is a tool used to decrease fruit set, lower cluster rot severity, and improve fruit quality. However, the considerable time required for implementation limits its use by grape growers. Efficient mechanization can potentially mitigate these issues, studies are lacking in a cool climate setting where short seasons and humid summers limit grape production. Therefore, the goal of these studies were to compare mechanical leaf removal with the manual removal of six leaves at the pre-bloom and after-bloom phenological stages over two seasons in Pinot Grigio, a tight-clustered cultivar susceptible to bunch rot, and Merlot, which reaches suboptimal fruit quality in some seasons.

For Pinot Grigio (Chapter 3), the loss of fruit to gray mold was lowered by all leaf removal treatments in the drier 2017 season, but only manual treatments mitigated loss from sour rot in that year. This indicates that a clear fruit zone and reduced cluster compactness are both needed to lower the effect of cluster rot disease. Only pre-bloom treatments enhanced fruit quality, likely driven by a similar reduction in cluster compactness. The results also suggested that pre-bloom manual removal can be an effective means to reduce fruit loss to sour rot severity.

For Merlot (Chapter 4), berry total soluble solids were highest with pre-bloom mechanical treatment. Furthermore, metabolomics analysis revealed that this treatment favored the accumulation of significantly more disubstituted anthocyanins and flavonols

and OH-substituted anthocyanins compared with manual application. Given that vine balance was similar between treatments, increased ripening with PB-ME is likely due to enhanced microclimate conditions and higher carbon partitioning through a younger canopy containing basal leaf fragments proximal to fruit.

Despite these results, it was not clear which factor was controlling the increase in fruit quality in response to pre-bloom mechanical leaf removal. In Chapter 5, an experiment was established where 60% of leaf area was removed from shoots in three ways: 1) manual removal of 5 leaves (PB-MA), 2) mechanical removal (PB-ME), and 3) simulated mechanical removal (PB-SIM), which was implemented to understand whether PB-ME improves fruit quality via enhanced microclimate conditions, or stress. Major phenylpropanoid classes were enhanced by PB-ME, however neither ABA nor ethylene were similarly altered, suggesting their lack of involvement in promoting phenylpropanoid biosynthesis in response to ELR. Instead, the leaf area at nodes above the fruit-zone was lower in PB-ME compared to C, which increased post-veraison fruit temperature (+2.8°C). These parameters correlated with anthocyanins at harvest. In conclusion, skin phenylpropanoid concentrations are influenced by canopy density above the fruit-zone.

Finally, in Chapter 6, the influence of vine balance and light exposure on fruit quality parameters were compared in two locations (Michigan, Italy). Primary metabolism was not significantly altered with the excepting of titratable acidity being decreased by LR8 having the greatest light exposure during ripening. Flavonol biosynthesis was significantly altered by light exposure in both locations, but not by vine balance. The results indicate that fruit exposure to light, rather than source-to-sink balance has a greater influence on flavonoid biosynthesis in grape berries.

Dedicated to my wife, Kristen

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CHAPTER ONE

Introduction

Grape is among the most widely planted and valuable fruit crops in the world, and its importance is seen in the variety of products made from its fruit, including wine, juice, table grapes and raisins (Keller 2015). Among these uses, wine grapes (*Vitis vinifera*) resulted in a global wine production of 259 mhl in 2016 (OIV, 2017). Both production and quality of fruit are highly related to the ripening process, and multiple physiological and environmental factors affect the ability of grapevine to adequately ripen fruit.

The metabolites comprising fruit quality in grape

Fruit quality perception is generally subjective to an individual's genetic makeup; however, dozens of quantifiable chemicals exist which have been determined to represent grape quality. These compounds accumulate and interact leading up to harvest in a cultivar-dependent manor, and are highly regulated by environmental conditions in the vineyard (Zoratti et al. 2014). They comprise the visual, gustatory, olfactory, and somatosensory aspects of quality perception - and while their concentration is critical - the threshold at which they can be seen, felt and sensed is most important. Because of this, modern analytical methods used for detection are often paired or related with sensory methods that allow for this connection to be made (Gambetta et al. 2016, Guth 1997, Lee and Noble 2006). Given that human perception of the compounds important to fruit quality varies from person to person, studying them with their threshold of detection in mind is a close to impossible task. However, the concentration and basic interactions between the constituents comprising quality in grape can be manipulated and studied; and their impacts on human perception elucidated afterwards.

Primary metabolites

The compounds which constitute fruit quality in grape are categorized into primary and secondary metabolites. The former includes amino acids, nucleic acids, proteins, fatty acids,

cellulose, and carbohydrates which drive the processes key to life in plants, such as photosynthesis and respiration. The primary metabolites of importance in grape are sugars and acids. Sugars begin to accumulate after growth Phase II (characterized by the onset of veraison) and increase steadily until harvest where they are stored in the vacuoles of mesocarp cells. The most prominent sugars are hexoses (glucose and fructose), followed by trace amounts of sucrose (Lecourieux et al. 2014). Their concentration is cultivar dependent, and is also impacted by the ripening stage and vine health status (Crippen Jr. and Morrison 1986). Among the metabolites mentioned here, sugars have the largest influence on fruit quality. Their concentration (total soluble solids) within the berry is used a measurement for maturity and can be used as a prediction for the final alcohol content in wines (Degu et al. 2016). In addition, they directly influence the biosynthesis of many secondary metabolites (Castellarin et al. 2011a, Larronde et al. 1998, Lecourieux et al. 2014). Taken together, sugar content and its relationship with other metabolites in grapes dictate the approach taken by the winemaker during fermentation. Therefore, adequate accumulation of hexoses in berries is critical for grape development, maturity and defining fruit quality at harvest.

The organic acids of relative abundance in grape include tartaric and malic, each of which greatly contribute to pH, and the organoleptic properties important to fruit quality. In contrast to sugar, these organic acids are present throughout growth Phase I (initial cell expansion) (Hradzina et al. 1984), and decrease sharply at EL32, just before sugar begins to accumulate at veraison. This tradeoff, and subsequent ratio of total sugars to total acids (Brix°:TA) at harvest is one of the most important components to balance and quality in grapes and wine (Noordeloos and Nagel 1972), and is often related to growing season length. Tartaric acid is unique to grape, and is the prominent acid found throughout maturation (McGovern et al. 2004).

Secondary metabolites

Secondary metabolites stem from primary metabolites and are described as compounds not necessary to life in plants. In grape, they include: terpenes and steroids (which derive from carbohydrate and fatty acid metabolism) and phenolic compounds and alkaloids (coming from sugar and amino acid metabolism) (Ali et al. 2010). Despite their non-essential status, they play important roles in signaling and attraction, and self-defense against abiotic and/ or biotic stresses (Lemut et al. 2013). Specifically, the internal interactions between stress phytohormones, and external reactions with insects, pathogens, radiation, and temperature involve these compounds (Kliebenstein et al. 2005). plants and pathogens. In grape, over 500 phytochemicals have been identified that contribute towards one of these processes. Their concentration is typically highest as a result of a plant undergoing severe or various forms of stress (Zamboni et al. 2006).

Non-volatile compounds

The phytochemical class of non-volatile secondary metabolites having the greatest impact on grape characteristics are the phenolic compounds. They affect the appearance, aroma, taste, and mouthfeel of fruit, and the subsequent wine. Phenolics are found in the skins and seeds of the fruit in addition to the stems (Ali et al. 2010), and concentrate highest at 50 days post-bloom; decreasing thereafter until harvest (Ristic and Iland 2005). The three most prominent classes of phenolics include flavonoids, phenolic acids and stilbenoids (Ali et al. 2010).

Flavonoids

Flavonoids represent the largest group of secondary metabolites found in grape, and are synthesized from the shikimic and polyketide pathways (Ali et al. 2010). These compounds are located in the skins and seeds and vary in concentration between cultivars. In red wine grapes, approximately 80-90% of the total phenolic content stem from three classes of flavonoid

compounds – anthocyanins, flavonols and flavan-3-ols (Jeong et al. 2008); formed as a result of the flavonoid biosynthetic pathway (Stafford and Ibrahim 1992).

Of the flavonoids found in grape, anthocyanin compounds are among those most studied, due to their role in grape and wine color (Mazza and Francis 1995) and the proposed health benefits of red wine (Castañeda-Ovando et al. 2009). In addition, anthocyanins comprise the second largest class of phenolics in red grapes (Harbertson et al. 2003). Anthocyanins start to accumulate in grape skins after the onset of the veraison stage, when sugar is initially transported to berries. This transportation of sugar regulates specific genes involved in the anthocyanin biosynthesis pathway (Lecourieux et al. 2014), with sucrose being the most related to anthocyanin accumulation, followed by glucose, and fructose (Larronde et al. 1998). In their natural, glycosylated form, these compounds are referred to as anthocyanins, however, when glucose is not acetylated by hydroxycinnamic acids to a 3-monoglucoside, they are referred to as anthocyanidins (aglycone). Depending on their degree of acylation, up to 20 chemically unique anthocyanins compounds have been profiled in a red grape cultivar (Yıldırım and Altındışli 2015), however they typically derive from the five primary anthocyanins found in *vinifera* cultivars: malvidin, cyanidin, peonidin, petunidin and delphinidin. The concentration, hue intensity and stability of each differ greatly (He et al. 2010b), and are highly regulated by the degree of hydroxylation, methylation, acylation (Bobeica et al. 2015, Mazza and Francis 1995), and the genotype (Guidoni et al. 2008). An example of this complexity is seen between Pinot noir and Merlot; two red wine cultivars containing identical anthocyanin profiles, but each expressing vastly different hues due to differences in relation to anthocyanin concentration and hydroxylation. Due to the genetic of anthocyanin concentration and profile, these compounds have been used as a chemotaxonomic classification system for red wine grapes (Mattivi et al. 2006).

Flavanols are another class of flavonoids synthesized in skins that are important to fruit quality. They play an important role as protection from UV radiation, due to their ability to absorb both UV-A (325-400 nm) and UV-B (280-325 nm) wavelengths (Downey et al. 2003). They also contribute to wine color through the co-pigmentation process with anthocyanins (Boulton 2001). Some reports have also established the involvement of flavonols in plant-pathogen interactions (Mattivi et al. 2006). The most common flavonols found in grape are quercetin-3-O-glucoside and quercetin-3-O-glucuronide (Downey et al. 2003), as well as the 3-O-glycoside of myricetin and kaempferol (Mattivi et al. 2006).

Flavan-3-ols, also known as procyanidins, or condensed tannins, are the largest class of phenolics in red grapes, and are found in high concentrations in the seeds (Yilmaz and Toledo 2004), and lower in skins. Flavan-3-ols include: catechin, epicatechin, epigallocatechin and epicatechin gallate (Harbertson et al. 2003) depending on the degree of polymerization, and are bitter compounds in isolation. However, as wine ages the bitter component is lost as they oligomerize and polymerize to form proanthocyanidins, or tannins. Tannins are present in skins before the onset of ripening (Harbertson et al. 2002), and serve as a defense mechanism against pathogens and predators (Bogs et al. 2007). The capacity of red wine to age is greatly enhanced by the presence of tannins in fruit (Corder et al. 2006), which is related to their ability to bind oxygen and act as antioxidants. They also contribute significantly to the tactile sensation of dryness and roughness, or astringency in red wines, which can be attributed to the ability of these compounds to coagulate the proteins found in saliva that coat the tongue (Polášková et al. 2008). Relating tannin concentration in grape skins and seeds with sensory perception has traditionally been very difficult (Boulet et al. 2017), however, recent advances in analytical procedures indicate that the amount of tannin in the precipitate formed from the interaction between tannin and proteins

can be closely tied with the severity of perceived astringency in the final wine (Harbertson et al. 2002, Rinaldi et al. 2012).

In addition to their individual roles, many metabolites form important interactions, further impacting fruit quality. As a byproduct of fermentation, ethanol extracts anthocyanins from skins, and condensed tannins from skins and seeds to form high-molecular weight polymeric pigments. Studies have revealed an interaction between tannin and anthocyanins (Boulton 2001), which changes as wine ages. Young wines are primarily comprised of monomeric anthocyanins, while aged wines mainly consist of polymeric pigments (Waterhouse and Zhu 2019). Unlike monomeric anthocyanins, these pigments are very stable under a wide range of pH (Harbertson et al. 2002), and likely make up the majority of the hue of red wines.

Phenolic acids

Simple phenolics (phenolic acids) include derivatives of hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA), which stem from the phenylalanine pathway (Hradzina et al. 1984). Outside the flavonoid group of secondary metabolites, HCA's are the largest class of phenolic compounds in fruits (Zoratti et al. 2014). They accumulate before veraison in the skins and pulp, and comprise the largest group of phenols in juice (Teixeira et al. 2013). An important HBA in grape is gallic acid, found in concentrations between 0.3 – 4.8 mg/l.

Volatile compounds

Aroma perception comprises the majority of flavor (Polášková et al. 2008). Many aromatic compounds stemming from grapes have been detected and help to make each cultivar distinct. The majority of secondary compounds in grapes are glycosylated, meaning that they are bound and unable to volatilize. During the winemaking process, thermovinification and enzyme addition practices aid in breaking this bond (Delcroix et al. 1994), and create a complex aroma and flavor

profile in the wine that far exceeds that of the fruit. Therefore, the presence of aroma precursors in the fruit help to dictate fruit quality.

The family of compounds called terpenes are present in large concentrations in grape, and contribute to the 'fruity' aromatic characteristics of many white grapes. While monoterpenes sesquiterpenes and diterpenes exist in grape, monoterpenes comprise the majority, including linalool, geraniol, nerol and citronellol (Schüttler et al. 2015). Although they accumulate similarly to non-volatile metabolites in relation to the berry's maturity, they are different in that they are found in the skin and pulp of grapes. They are present in both the free and glyosidic forms (Godshaw et al. 2019), also referred to as Free Volatile Terpenes (FVT) and Potential Volatile Terpenes (PVT). Stemming from the carotenoid biosynthesis pathway, norisoprenoids also contribute to the aroma profile of many cultivars. ß-demascenone is the most recognized of these compounds in grape and contributes a cooked-apple or raspberry flavor to select red and white wines alike. Thiols are a class of aroma compounds which are classified as an organic compound containing a sulfyhydryl (-SH) group. They represent many citrus, fruity and black currant characteristics of aromatic wines, such as Sauvignon blanc (Capone et al. 2018).

The presence of some compounds which negatively impact flavor and aroma can be as important to determining grape and wine quality as the desirable compounds previously mentioned. Methoxypyrazines are a class of compounds that impart a 'green' (bell pepper, green bean) flavor and aroma to select Bordeaux cultivars, including Merlot (Sivilotti et al. 2016). In contrast to other volatile compounds, they are present early during maturation, and decrease in concentration as fruit ripen (Polášková et al. 2008). Their existence in grapes is not always considered to be undesirable, and are characteristic of some wines, such as in New Zealand

‘Marlborough Sauvignon Blanc.’ However, in high quantities, the presence of methoxypyrazines in grapes are not desired.

While concentration is important, it is the balance of the aforementioned volatile and nonvolatile compounds comprising a grape’s taste and aroma profiles that determine quality of flavor (Polášková et al. 2008). The concentration and sensory impact of these compounds change as grape must is fermented into wine, but despite this, compounds and precursors located in the fruit can be used as an indicator of final wine quality. Therefore, a goal of viticulture is to increase and maintain the availability of these components to ensure optimal fruit and wine quality can be attained.

CHAPTER TWO

LITERATURE REVIEW

Environmental limitations of Michigan's viticulture areas

Ideal growing conditions for *Vitis vinifera* consist of temperate climate regions with warm, dry summers seeing moderate precipitation, and mild winters (Ali et al. 2010). While these conditions are not unattainable in select viticulture areas, often, one or more of these parameters are far from desirable for a given wine grape growing area. Such is the case in Michigan, a cool climate viticulture region.

Michigan has five American Viticulture Areas (AVAs), encompassing a total of 2850 acres of wine grapes located in the Northwest and Southwest areas of the state (Vanderweide et al. 2017). Despite possessing the affinity for growing wine grapes, viticulture is certainly tested here – long, warm summers are replaced by short and variable ones, rain is often heavy during the final months of maturation, and winters are severe.

Short and variable growing seasons

In Michigan, environmental conditions that influence ripening are highly variable from year to year. According to the Winkler GGD scale (Winkler and Amerine, 1974), Michigan's two primary growing regions, Northwest and Southwest, fall into regions 1a-1b (1,500F°-2,500F°/850C°-1839C°) and regions 1a-III (1,800F°-3,300F°/982°-1816C°), respectively. This variability indicates that the capacity of vines to reach full maturity on a consistent basis is restricted in most seasons due to a lack of adequate temperature accumulation. Because of this, the potential for slowed and lacking fruit technological maturity during maturation exists, impacting fruit quality at harvest. A favorable shift in climactic conditions has occurred over the past decades that has led to an increase in vinifera acreage (Schultze et al. 2014), especially in economically important cultivars such as Merlot, Cabernet Franc and Cabernet Sauvignon (Schultze et al. 2016). Despite continuously increasing season length due to climate change, the number of potential frost

events (dictating vine viability), and seasonal variability are not expected to change in the future (Schultze et al. 2014).

Precipitation near harvest

Michigan typically receives above average rainfall throughout the course of the growing season. However, approximately 30-40% coincides with grape ripening, from veraison (EL35) in August through full maturity (EL38) in October (Schultze et al. 2016). This impedes the ripening process, and further impacts fruit quality. (Zhuang et al. 2014) reported a two-week delay in harvest because of heavy rainfall during the 2011 season compared with 2012 in Michigan's largest growing region.

Many of the cold-resistant vinifera cultivars important to Michigan's wine industry are characterized by their tight clustered morphology, allowing little airflow between clusters after veraison. As a result, they are highly sensitive to *Botrytis cinerea*, also referred to as 'bunch rot' (Mosetti et al. 2016). *Botrytis cinerea* in cool and rainy climates leads to the proliferation of sour rot, which converts glucose and fructose into acetic acid and other metabolites including: glycerol, ethyl acetate, ethanol, acetaldehyde, and galacturonic and gluconic acids (Zoecklein, Williams, and Duncan 2000), rendering fruit unfit for processing and fermentation. Because of this, incidence of rot in clusters is more likely to be a determinant for harvest date than TSS or other fruit quality parameters (Mosetti et al. 2016), further complicating the winemaking process due to lack of metabolite accumulation in fruit.

Taking all this into consideration, Michigan's climactic conditions do not favor production of cultivars requiring warm, seasonal temperatures, such as Merlot, or tight-clustered varieties, like Pinot Grigio, which are severely impacted by sour rot.

Pre-bloom leaf removal to mitigate these challenges

Michigan, and the greater Great Lakes grape growing region is faced with many challenges that impact fruit and wine quality. Two main viticultural practices have been studied in depth regarding their ability to improve fruit quality under a variety of extreme environmental conditions: cluster thinning and leaf removal.

According to *Vitis vinifera* characteristics, cluster thinning involves the removal of a cluster from select shoots, typically the apical cluster, in an effort to increase the sink strength of the basal cluster and improve metabolite accumulation (Preszler et al. 2013). This practice is normally performed at veraison, or before the induction of sugar accumulation into fruit. Early cluster thinning (prior to bloom) was reported to be an effective strategy to control the potential over-crop of Sangiovese and enhance grape phenolic compounds (Gatti et al. 2012). However, (Frioni et al. 2017a) indicated that the effects of cluster thinning are highly modulated by seasonal temperatures; having little to no impact in warm seasons and a clear response in cool seasons when paired with leaf removal. Reports comparing cluster thinning and leaf removal have shown leaf removal to be more effective at improving fruit quality than cluster thinning in high yield cultivars Teran (Gatti et al. 2012) and Sangiovese (Bubola et al. 2017). In addition, cluster thinning in low yield cultivars decreases yield below an economically viable status in low yield cultivars. Cluster thinning was reported to reduce Riesling growers net returns (Preszler et al. 2013). Therefore, an approach to improving fruit quality in both high and low yield cultivars cannot solely consist of cluster thinning and should take into account the response of all existing clusters.

Leaf removal is a viticultural technique that involves the removal of some leaves along shoots, and the position and number of leaves removed, as well as the timing of removal are dependent on the specific goals of a growing region. In warm climates, leaf removal can be utilized

to delay ripening and increase polyphenol concentration (Poni et al. 2013; Javier Tardaguila et al. 2010). In cool climates, which includes Michigan, the goal of leaf removal is to enhance fruit technological maturity through increased microclimate conditions and decreased yield loss due to sour rot by reduced cluster compactness and improved spray efficiency (Acimovic et al. 2016, Guidoni et al. 2008, Mosetti et al. 2016, Zhuang et al. 2014).

Timing of leaf removal on physiological processes

Throughout the growing season, grapevine is continuously exposed a complex web of internal and environmental signals which regulate ripening, and subsequently, fruit quality at harvest. Often, this also includes exposure to many biotic and abiotic stresses, and the timing of their occurrence greatly effects maturation. The application of leaf removal at various timings along the growing season alters both internal processes and environmental factors that lead to different responses of the vine in terms of physiology and fruit quality. The three most researched timings of leaf removal are: (1) 'early,' which includes application at 'pre-bloom' (EL17) through 'bloom' (EL23); (2) 'after-bloom' (EL27), and (3) 'veraison' (EL35). Leaf removal at veraison (EL35) is considered the standard practice in most growing regions. The following processes are described as follows, and their impact by leaf removal further explained in terms of ripening and fruit quality.

Sucrose allocation and fruit set

Prior to female meiosis in grapevine, developmental processes are primarily dependent on starch mobilization from woody storage organs, such as trunks, canes and roots. Although developing inflorescences and flowers are small in comparison with other sinks, the flowering process is demanding of the grapevine (Carmo Vasconcelos et al. 2009, Lebon et al. 2008). At this point, inflorescences and flowers are the most relevant developing organs, called sinks. The ability of the vine to set flowers is cultivar dependent, with some cultivars being sensitive to abscission

(Gewürztraminer) and others resistant (Pinot noir) (Lebon et al. 2005). This is related to the enzymatic activity (Lebon et al. 2016) and presence of sugars in the anthers and ovules of flowers at this stage. In Gewürztraminer, female and male meiosis occurred one week after Pinot noir, and was shown to have similar starch concentrations in anthers and ovules, however, much less sugar than Pinot noir (Lebon et al. 2005). Because flowering is a delicate transformation process, environmental stresses can also have a severe impact on fruit set (Carmo Vasconcelos et al. 2009, Ebadi et al. 1995, Srinivasan and Mullins 1981).

The sensitivity of a cultivar to abscission can be further amplified by leaf removal depending on timing and severity. Leaf removal at veraison has no effect on fruit set (Molitor et al. 2011, Tardaguila et al. 2008) due to the fact that this timing occurs approximately 8 weeks after fertilization is finished in most cultivars (Carmo Vasconcelos et al. 2009). Although after-bloom leaf removal also succeeds the completion of fertilization, some research points to a slight contribution from this timing on the reduction of fruit set (Poni et al. 2006; Molitor et al. 2011; Intrieri et al. 2008), including those where the study directly compared after-bloom and veraison treatments (Pastore et al. 2013). Little is known regarding the mechanisms controlling this phenomenon; however, it has been shown that carbohydrate availability to berries is not involved (Poni et al. 2006). Still, others did not see the same effect (Sternad Lemut et al. 2011, Zhuang et al. 2014). Leaf removal at pre-bloom has consistently led to a reduction in fruit set in both red and white cultivars (Acimovic et al. 2016, Molitor et al. 2011, Poni et al. 2009, Sabbatini and Stanley Howell 2010, Tardaguila et al. 2010). Carbon deprivation from leaf removal at this stage induces stress at meiosis, reducing the flow of hexoses and decreasing flower fertility (Lebon et al. 2004).

The severity of leaf removal at either pre-bloom or after-bloom greatly affects fruit set, as well as developmental processes throughout ripening. A threshold experiment involving (*Vitis*

vinifera L.) Pinot noir found that between removing 4, 6, 8 or 10 leaves, only removal of 6 and 8 leaves induced the desired effect on reducing fruit set and improving fruit quality. Removal of 4 leaves had little to no effect, while 10 leaves induced a severe carbon stress on vines, decreasing yield below an economically viable status (Acimovic et al. 2016). This highlights the ability of leaf removal performed at pre-bloom to induce high carbon stress conditions on the vine if performed out of balance in relation vine vigor, impacting fruit quality. Under severe stress relating to a reduced ability to allocate carbon, the grape berry manages the metabolic fate of assimilates in such a way that sugar accumulation is maximally maintained at the expense of secondary metabolites – particularly anthocyanins (Bobeica et al. 2015). In addition, severe defoliation leads to perturbations to the process of carbohydrate replenishment in woody organs at the end of the growing season and can reduce the formation of inflorescences and fruit set in following seasons (Carmo Vasconcelos et al. 2009), impacting yield and fruit quality.

Photosynthesis and hexoses accumulation

After female meiosis, an important switch occurs in the vine (Lebon et al. 2008), and the primary source of energy for developing organs changes from carbon stored in woody organs to photosynthetically fixed carbon. During photosynthesis, chlorophyll containing organelles convert energy from sunlight, water and carbon dioxide into sugars (food) for the plant in the form of sucrose. Leaves that are approximately 30% of their final size are the main contributors to this process, and can export up to 80% of their fixed carbon (Lemoine et al. 2013). It has also been shown that developing inflorescences allocate fixed carbon (Lebon et al. 2004; Carmo Vasconcelos et al. 2009).

Sugar export from leaves and inflorescences through the phloem primarily occurs in the form of sucrose (Conde et al. 2007, Lemoine et al. 2013). Sucrose deriving from photosynthetic

sources is allocated to sinks, such as roots, young leaves, shoots and clusters. Although fruit develops later than other sinks (Wardlaw 1990), competition for assimilates still exists, and sink demand determines prioritization of sucrose allocation (Lemoine et al. 2013). At the time of veraison, sucrose flow is increased, and it has been reported that many genes involved in the ripening process are reported to be signaled and regulated by sucrose (Koch 2004). Sucrose is then either cleaved by invertases in the apoplast into glucose and fructose or degraded by sucrose synthase into uridine-5'-diphosphate (UDP) glucose and fructose for subsequent metabolism, biosynthesis or storage in vacuoles (Koch 1996). The source to sink balance is an important parameter that controls berry sugar, organic acids, and secondary metabolites content with qualitative enological potential (Bobeica et al. 2015, Smeekens et al. 2010). Environmental stresses, such as low photosynthetically active radiation (PAR), low temperature, high temperature, wind, and precipitation can inhibit photosynthesis, as well as sucrose transport in the phloem (Kliewer and Torres 1972; Spayd et al. 2002; Gamalei et al. 1994), and impact the capacity of a vine to reach its fruit quality potential.

Mature leaves located at the base near the cluster zone of the shoot are generally removed during leaf removal in cool climates (Acimovic et al. 2016, Zhuang et al. 2014). These leaves are less photosynthetically active, and their ability to affect the sink-source relationship is related to the timing and severity of leaf removal performed throughout the growing season (Poni et al. 2006). In addition, carbon stress from leaf removal slows sucrose assimilation to sinks at the beginning of the season and temporarily alters the source-sink status of the vine. However, secondary growth in the form of lateral leaves typically initiates around bloom, and greatly affects hexose accumulation throughout ripening. Lateral formation has been shown to have a compensatory effect in relation to the severity and timing of leaf removal (Hunter and Visser

1990). (Pastore et al. 2013) reported a 17% increase in total lateral leaf area at harvest in the pre-bloom treatment compared with the control, and a total leaf area at harvest not significant from the control. Meanwhile, the treatment at veraison saw an 8% decrease in lateral leaf area from the control, and a total leaf area that was 32% lower than the control at harvest. This was highly correlated with TSS at harvest and was significantly higher than the veraison treatment. The important role of lateral leaf development in carbon fixation and allocation to sinks was also indicated by (Hunter 2000), where lateral leaves removed from early defoliated and undefoliated vines led to significant decrease in yield, and total hexose concentration in berries at harvest.

Hormone signaling and fruit quality

Many endogenous hormones are involved in the non-climacteric ripening process in grape, and their biosynthesis relative to environmental conditions influences additional physiological and metabolic processes (Lurie et al. 2009). Their expression occurs at various stages throughout the growing season; however, many interact, either synergistically or antagonistically in response to development processes and to biotic and abiotic stress conditions (Deluc et al. 2007; Fortes et al. 2011) arising from environmental stresses. This is because some hormones are promoters, while others are inhibitors of ripening, which trade off at key phenological stages. The expression of four hormones: Abscisic acid (ABA), Ethylene, Jasmonic acid (JA) have been linked to development and stress conditions in grape relating to fruit quality parameters.

Ripening promoters: abscisic acid (ABA) and ethylene

As grape is a non-climacteric fruit and does not require a burst of ethylene to induce ripening, a series of other hormones including ABA have been cited to play a combined and integrated role in this process (Fortes, Teixeira, and Agudelo-Romero 2015). Among them, ABA is the most important (Mcatee et al. 2013), and its contribution best defined. ABA levels increase

at veraison, and do so in tandem with characteristic developmental processes, such as softening, and important metabolites, including as hexoses, and anthocyanins. Multiple transcriptomics studies have indicated a higher expression of ABA biosynthesis genes at the time of veraison (Deluc et al. 2007; Fortes et al. 2011). This indicates that ABA is central to the ripening processes that originate at veraison (Castellarin et al. 2011; Kuhn et al. 2014). In addition to its role in ripening, ABA regulates some abiotic stress responses, as indicated by an activation later (EL36) in maturation. This stress induced upregulation of ABA has in turn been shown to increase the accumulation of anthocyanins in (*Vitis vinifera* L.) Shiraz (Degu et al. 2014). (Leng et al. 2014) showed that an external application of ABA increased tannins and anthocyanins at veraison, indicating that ABA co-regulates the enzymes involved in the biosynthesis of these metabolites. The response of ABA to defoliation at any timing in grape has not been reported in the literature, however speculations regarding its involvement in this process have been made. (Poni et al. 2013) hypothesized that the decrease in hexoses accumulation in berries after veraison leaf removal reduces ABA, due to ABA being synthesized in the chloroplasts of source tissues. This may, in turn, downregulate expression of some genes that regulate transport to sinks, including invertases and a monosaccharide transporter (Çakir et al. 2003).

Along with ABA, ethylene has been noted as an important promoter of ripening. Despite the reduced role of ethylene in non-climacteric fruit, production of ethylene in grape still occurs in low levels is related to berry diameter (Chervin et al. 2004). Ethylene primarily occurs in green berries, before the induction of veraison, as indicated by an increase in the ethylene biosynthesis enzymes ACC synthase and ACC oxidase (Deluc et al. 2007), and subsequent down-regulation after veraison. However, the identification of positive markers for ripening genes (Agudelo-Romero et al. 2013) and presence of ethylene signaling activity in grape skins late in ripening

(Cramer et al. 2014) indicate that the ethylene-mediated signaling pathway is active during ripening (Fortes et al. 2015). Due to its involvement in ripening, ethylene has been correlated with the presence of some metabolites. In the study, (Cramer et al. 2014) demonstrated a relationship between ERF transcription factors with the accumulation of lipoxygenases and terpene synthases; enzymes important to flavor development in red and white grapes. ACC synthase genes have also been indicated as early responders to wound stress (Reymond 2000), followed by ethylene response genes, and ethylene response transcription factors. This implicates ethylene as a chemical messenger in response to wounding (Donnell et al. 1996). However, distinguishing ethylene's role in developmental termination (flower senescence, ripening, fruit abscission, leaf senescence) (Pech et al., 2012) from its proposed modulation of wounding and pathogen resistance (Cantu et al., 2009; Van der Ent, 2012) is difficult, as the former often facilitates the latter.

Ripening inhibitor and abiotic stress response: jasmonic acid (JA)

Jasmonates include jasmonic aid (JA) and methyl jasmonate (MeJA), however, JA is often found in higher amounts in grape. JA is present in significant concentrations in green berries, and has been reported to decrease in ripe fruits, leading to the conclusion that JA degradation allows for initiation of the ripening process (Kondo and Fukuda 2001). (Fortes et al. 2011) showed that mRNAs associated with jasmonic acid biosynthesis decreased from EL32 to EL35 and EL36. Jasmonic acid is key to plant defense against herbivores and necrotrophic pathogens, and mediates responses to mechanical stress, UV stress, cold stress, and desiccation stress through trichome formation and regulation in Arabidopsis (Wasternack and Strnad 2016). (Zenoni et al. 2017) also showed that Jasmonate concentrations were significantly higher in five wine grape cultivars at veraison in response to the stress associated with pre-bloom leaf removal. Trichome function has also been shown to involve JA, which leads to the biosynthesis of some secondary compounds,

such as anthocyanins (Qi et al. 2011). In green strawberry fruit, external application of methyl jasmonate induced softening, and anthocyanin accumulation, as well as enhanced expression of multiple anthocyanin biosynthesis related phenylpropanoid genes (Concha et al. 2013). In vivo applications of methyl jasmonate in cell cultures lead to an increase in the stilbene trans-resveratrol (Xu, Zhan, and Huang 2015) and terpenoids (Hampel et al. 2005). (Ju et al. 2016) recently showed that application of MeJA improved the lipoxygenase activity, thus catalyzed more the conversion of linoleic acid into volatile aromas and increased synthesis fatty acid-derived volatile aromas.

Timing of leaf removal on environmental and fruit quality parameters

The goal of performing leaf removal in all growing regions is to partially mitigate the effects of existing climactic conditions, and the three primary environmental parameters improved by leaf removal in Michigan are radiation, temperature and precipitation; each playing a critical role in the ripening process. However, the effects of leaf removal on fruit quality at harvest can change dramatically accordingly with the climate (Percival and Fisher 1994; Poni et al. 2006; Lemut et al. 2013; Lee and Skinkis 2013)

Radiation and temperature

In addition to its role in photosynthesis, sunlight is integral in many developmental processes in higher plants, including ripening (Müller-Xing et al. 2014). As sunlight levels increase during the growing season's first months, grape berries acclimate by increasing the expression of genes dictating early and late flavonoid biosynthesis by way of the phenylpropanoid pathway (Zoratti et al. 2014), reflecting the important role of flavonoids in photo-protection (Downey, Harvey, and Robinson 2004). This leads to an elevation in anthocyanin, proanthocyanidins and flavanol concentrations (Azuma et al. 2012, Cortell and Kennedy 2006, Fujita et al. 2007, Jeong et al. 2004, Matus et al. 2009, Pereira et al. 2006). Whereas, other studies have illustrated UV-

light's limited effect on proanthocyanins and cinnamic acids (Koyama et al. 2012). Anthocyanins (Spayd et al. 2002) and flavonols (Azuma et al. 2012) have been found to be significantly affected by radiation, regardless of temperature. However, flavonoid biosynthesis also seems to be influenced by light in a cultivar-specific manner. For instance, among grapevines, sunlight induces both anthocyanin and flavonol accumulation in Cabernet Sauvignon (Matus et al. 2009) while only flavonol production is induced in Shiraz grapes (Downey, Harvey, and Robinson 2004). The degradation of malate during veraison and ripening is also related to light exposure (Poni et al. 2006; Diago et al. 2012). In some instances, the intensity of UV-light exceeds the potential of these polyphenols to protect fruit, leading to stress conditions such a sunburn, and producing reactive oxygen species (ROS) that can damage cellular components and DNA (Müller-Xing et al. 2014). Some cooler growing regions see low radiation throughout the day, which can lead to a decrease in anthocyanin biosynthesis (Bergqvist et al. 2001). Thus, adequate sunlight is critical, dictating fruit quality through many channels.

The capacity of a grapevine to produce quality fruit is highly related to seasonal accumulation of temperatures, or growing degree days (GGDs), and the status of sunlight intensity often effects berry temperature (Spayd et al. 2002). Their relationship can be seen during the day (Bergqvist et al. 2001). (Smart and Sinclair 1976) showed that shaded fruit had a surface temperature 2.4°C above ambient temperatures, while fruit exposed to light were approximately 12°C higher. While this typically favors the ripening process, heat and cold stress experienced during diurnal fluctuations can have an inhibitory effect on the accumulation of metabolites. In 'Tokay' grapes, day temperatures between 15-25°C, and night temperatures between 10-20°C saw optimal anthocyanin accumulation compared to higher temperatures that reached 35°C and 30°C during the day and night, respectively (Kliewer and Torres 1972). Further studies have revealed

that high temperatures lead to the degradation, rather than inhibition of the flavonoid biosynthesis pathway and anthocyanins (Mori et al. 2007), and decrease expression of genes involved in flavonoid biosynthesis (Yamane et al. 2006). In contrast, low temperatures, and cold events also prevented the accumulation of hexoses and anthocyanins in Merlot (Spayd et al. 2002). Inconsistency between growing seasons can impact the final concentration and composition of anthocyanins and additional flavonoids in skins, which subsequently control grape color (Baranac et al. 1997) and other aspects of fruit quality.

One of the primary goals of leaf removal in cool climates is to increase the penetration of radiation and temperatures in the fruit zone. The timing of leaf removal plays an important role in the ability of sunlight and temperature to influence fruit quality, as the metabolites previously mentioned are synthesized at various points throughout maturation. Leaf removal at veraison provides improved radiation and temperatures during the induction of the ripening phase (Phase II), where sugar and anthocyanins already have begun to accumulate in berries. Because of this, mixed results are seen in fruit quality in response to defoliation at this time. In all reviewed cases, no change in Brix between veraison treatments and the control (Molitor et al. 2011, Osrečak et al. 2016, Pastore et al. 2013, Percival and Fisher 1994). (Pastore et al. 2013) found a significant increase in pH in the veraison treatment and decrease in TA, while no change in pH or TA was reported in multiple studies (Percival and Fisher 1994; Molitor et al. 2011; Lee and Skinkis 2013; Osrečak, Karoglan, and Kozina 2016). (Osrečak et al. 2016) found mixed results between year and cultivar regarding an increase in total phenolics in response to the leaf removal treatment at veraison. Hydroxycinnamic acids were only slightly increased in veraison treatments compared to the control (Lemut et al. 2011). Some publications reported a significant increase in anthocyanins concentration at veraison compared with the control (Lemut et al. 2013; Lemut et al. 2011),

however, (Osrečak et al. 2016) saw mixed results and (Pastore et al. 2013) saw a slight, but insignificant decrease. Significant increases in flavonols were reported in veraison treatments against the control (Lemut et al. 2011; Pastore et al. 2013), however, others saw little to no differences (Lemut et al. 2013). Tannin concentrations saw a slight increase from the control, but were not consistent between year and cultivar (Osrečak et al. 2016). The application of leaf removal after veraison leads to decreased biosynthesis of carotenoids, effecting concentrations of norisoprenoids (Ristic et al. 2007) and terpenes (Zoecklein et al. 1998; Reynolds and Wardle 1989). Insignificant difference in sensory attributes of skins and pulp were seen in (Tardaguila et al. 2008).

The implementation of leaf removal at after-bloom increases radiation and temperatures during the first phase of berry ripening (Phase I), otherwise referred to as 'cell expansion.' This is performed just before organic acids begin to accumulate in vacuoles, and biosynthesis of tannins, hydroxycinnamates and precursors of some phenolic compounds has begun (Fortes, Teixeira, and Agudelo-Romero 2015). In some cases, an increase in Brix was seen across all years and cultivars against the control (Tardaguila et al. 2010), however, most were still lower than the pre-bloom treatment (Intrieri et al. 2008). No change was reported in in most cases (Percival and Fisher 1994; Tardaguila et al. 2010; Lee and Skinkis 2013; Komm and Moyer 2015; Mosetti et al. 2016; Sivilotti et al. 2016). In general, no change in pH or TA from the control was seen in most experiments (Percival and Fisher 1994; Intrieri et al. 2008; Tardaguila et al. 2010; Lee and Skinkis 2013; Komm and Moyer 2015; Mosetti et al. 2016; Sivilotti et al. 2016), however, a significant increase was seen in TA in both years of experimentation on Carignan (Tardaguila et al. 2010). All publications reported saw no change in total phenolics compared to the control (Intrieri et al. 2008). At after-bloom, hydroxycinnamic acids were significantly increased in veraison treatments compared to

the control (Lemut et al. 2011). An increase in anthocyanins was seen compared to control (Lemut et al. 2013) and veraison treatments, but were less than pre-bloom treatments (Lemut et al. 2011; Lee and Skinkis 2013), while some reports show similar values to pre-bloom treatments (Intrieri et al. 2008), with a few publications finding no change to anthocyanins against the control (Tardaguila et al. 2010; Sivilotti et al. 2016). At after-bloom, flavonols were significantly higher than control and veraison treatments (Lemut et al. 2011; Lemut et al. 2013). In some papers, mixed results were found regarding tannin increases over the control treatment between year and cultivar (Komm and Moyer 2015), while the majority of studies found no change in tannin from the control (Tardaguila et al. 2010; Lee and Skinkis 2013; Lemut et al. 2013; Sivilotti et al. 2016). Leaf removal performed at the after-bloom phenological stage was reported to enhance tannin sensory attributes of skins and sweetness attributes of pulp compared to veraison and control treatments (Tardaguila et al. 2008) to that of veraison and the control. (Diago et al. 2010) reported very little change in sensory composition in after-bloom defoliated vines compared with the control. Terpenes were no increased compared to the control in (Komm and Moyer 2015). According to (Mosetti et al. 2016), leaf removal at after-bloom did not lead to differences in IBMP or IPMP (methoxypyrazines) concentrations at harvest.

Pre-bloom leaf removal increases radiation and temperatures to the microclimate, before flowers are fertilized, and the start of metabolic processes. This timing provides the most consistent improvement to the metabolite concentration in fruit at harvest. An increase in TSS was seen compared with the control (Poni et al. 2006; Zenoni et al. 2017), and after-bloom treatments (Intrieri et al., 2008; Pastore et al., 2013), while some results were mixed between treatments and years (Acimovic et al. 2016). Still others saw no change between pre-bloom and after-bloom treatments (Intrieri et al. 2008; Lee and Skinkis 2013; Komm and Moyer 2015). Mixed results

were seen for alterations in pH and TA (Acimovic et al. 2016, Intrieri et al. 2008, Zenoni et al. 2017), although many north-south facing canopies reported a decrease in TA in early-leaf removal treatments (Lee and Skinkis 2013; Pastore et al. 2013; Acimovic et al. 2016). Some publications found no change in pH and TA among all treatments. Most results concerning total phenolics at pre-bloom are mixed regarding treatments, year and cultivars tested (Tardaguila et al. 2010; Komm and Moyer 2015; Acimovic et al. 2016), while some saw a consistent increase in total phenolics compared to the control (Poni et al. 2006; Intrieri et al. 2008), with a few seeing no differences against treatments or year (Poni et al. 2006). No differences were seen in any treatment regarding hydroxycinnamic acids by (Lemut et al. 2013). The majority of publications saw an increase in anthocyanins at pre-bloom compared to the control (Poni et al. 2006; Pastore et al. 2013; Zenoni et al. 2017), and after-bloom treatments (Intrieri et al., 2008; Lemut et al., 2013), while some results were mixed between years, treatments, or varieties (Tardaguila et al. 2010), and some reporting no differences in all years and treatments of experimentation (Lee and Skinkis 2013; Acimovic et al. 2016; Sivilotti et al. 2016). A large change was seen in flavonol concentration at pre-bloom compared to control (Pastore et al. 2013), however, differences between pre-bloom and after-bloom treatments were not always significant (Lemut et al. 2013). Inconsistency in improved tannin at pre-bloom compared with after-bloom and control vines (Komm and Moyer 2015), while no change in tannin occurred more frequently (Lee and Skinkis 2013; Lemut et al. 2013; Sivilotti et al. 2016). An increase in terpenes over the control and after-bloom treatments was seen in the Riesling cultivar, but not Sauvignon blanc (Komm and Moyer 2015). When leaf removal is performed early, improved microclimate conditions lead to increased biosynthesis of carotenoids, which promotes accumulation of norisoprenoids, as well as decreases the presence of

methoxypyrazines in fruit (Ryona et al. 2008). (Diago et al. 2010) found that early leaf removal produced mixed results regarding improved aroma perception from year to year.

Precipitation / disease pressure

Reducing yield loss due to *Botrytis cinerea* (sour rot) is a critical goal of leaf removal in cool climates regions that receive precipitation near harvest. Leaf removal, regardless of timing, produces a more open fruit zone, which and increases evaporative potential with the cluster and improves spray efficiency to fruit (Molitor et al. 2011). Therefore, the timing which has the most consistent ability to reduce fruit set and cluster compactness is key. Leaf removal performed at veraison leads to mixed results regarding sour rot incidence, with some researchers reporting improved results (Pastore et al. 2013), while others, inconsistent results between seasons and cultivars (Molitor et al. 2011). Impacts on sour rot are typically improved when performed after-bloom (Gubler et al. 1991, Intrieri et al. 2008) compared to the control, however results are also not reliable. This is likely due to slight fruit set reduction in clusters, of which results also vary at after-bloom. Pre-bloom leaf removal alters fruit set, decreasing cluster compactness and consistently lowering sour rot incidence to a greater degree to that of control (Zoecklein, Williams, and Duncan 2000; Mosetti et al. 2016; Intrieri et al. 2008; Sivilotti et al. 2016) and after-bloom treatments (Komm and Moyer 2015, Molitor et al. 2011, Percival and Fisher 1994).

Mechanization of leaf removal

Economics of leaf removal

Despite its effectiveness, leaf removal performed manually at any point during the growing season is expensive and is reliant on available labor. For this reason, mechanization of leaf removal has become an important option to consider for growers. An article in ‘Practical Winery and Vineyard’ magazine (Vierra, 2005) reported that growers spend up to $130 per acre for manual

leaf removal, while mechanical leaf removal only costs $25 per acre. In addition, mechanical vastly reduces the time it takes to perform this practice, which could lead to more consistent results in the vineyard. This has important implications for growers who are experiencing shortages in labor, however, very little information exists that evaluates the effectiveness of mechanizing leaf removal compared to manual application regarding fruit quality. Two primary types of mechanical leaf removal machines exist that have been reported in the literature. The first is termed a 'leaf-plucker unit,' which utilizes suction force to draw leaves into a grid-partitioned rotating blade, and was studied by (Intrieri et al. 2008, Kemp et al. 2011, Percival and Fisher 1994). This method is limited because drivers must be attentive and drive in accordance with the homogeneity of the canopy, leading to mixed results. The second is a 'pulsed air unit,' that directs compressed air from circular, rotating heads towards the fruit zone, shredding leaves in front of clusters (Tardaguila et al. 2012; Tardaguila et al. 2010). This method is most suitable for vertical shoot position (VSP) and four arm-kniffin trellis systems (Hed and Centinari 2018). Only (Gubler et al. 1991) compared mechanical and manual leaf removal using both types of machines.

Timing of mechanical leaf removal on physiological processes and fruit quality

All applications of leaf removal at veraison (Kemp et al. 2011, Percival and Fisher 1994) were performed using a leaf-plucker unit. Neither saw a significant reduction in fruit set and yield compared with manual or control treatments (Percival and Fisher 1994) reported a small increase in yield at one of two experimental sites. Both papers showed no increase in fruit quality parameters such as TSS, TA (Percival and Fisher 1994) and tannins (Kemp et al. 2011, Percival and Fisher 1994) in relation to the control. (Percival and Fisher 1994) saw no difference in sour rot from the control or manual treatments in one year of the experiment, while significant decrease in sour rot incidence compared to the control was seen in the second year.

After-bloom mechanical leaf removal is reported by (Diago et al. 2010, Gubler et al. 1991, Intrieri et al. 2008, Kemp et al. 2011, Percival and Fisher 1994). Different from application of defoliation at veraison, (Percival and Fisher 1994) saw a slight difference in yield from manual treatments and the control. (Diago et al. 2010) found a large decrease in yield in the mechanical treatment compared with both the control and manual treatment in both years. A significant difference was seen in fruit set and yield reduction between control and mechanical treatments in (Intrieri et al. 2008), however, the manual treatment was slightly more severe. No improvement was reported in basic fruit quality parameters (Percival and Fisher 1994), anthocyanins, total phenolics (Intrieri et al. 2008) or tannins (Kemp et al. 2011) compared to the control, while in (Intrieri et al. 2008), only a similar °Brix increase was found in mechanical and manual treatments from the control. In addition, (Diago et al. 2010) saw no difference in sensory data between manual and mechanical treatments, and both were improved from the control. Monomer flavan-3-ol concentrations were increased in defoliated vines compared with the fruit produced from non-defoliated treatments, however, results were not consistent regarding the timing of the treatment (Kemp et al. 2011). Leaf removal at after-bloom reduced sour rot incidence and severity, however, manual treatments saw a greater effect (Percival and Fisher 1994). Meanwhile, (Gubler et al. 1991) saw comparable results between manual and mechanical application using both types of machines in the first year. Slightly better results were obtained using the leaf-plucking machine the second year; however, this is likely due to application of each machine on different trellis systems.

Previously, three publications have studied mechanical leaf removal at pre-bloom (Intrieri et al. 2008; Tardaguila et al. 2010; Diago, Vilanova, and Tardaguila 2010), with both (Tardaguila et al. 2010; Diago, Vilanova, and Tardaguila 2010) using a pulsed-air machine. In Graciano and Carignan cultivars, fruit set was significantly reduced in mechanical and manual treatments,

however, in Graciano, the mechanical treatment had an additional 10% effect on fruit set reduction compared to the manual, while there was no difference in Carignan. In both varieties, yield followed a similar pattern, although a more severe impact was seen in the mechanical treatment in the second year of the experiment (Tardaguila et al. 2010). Fruit set was reduced compared to the control, but the impact was not as large as the manual treatment. A similar effect on fruit set and yield was seen in (Diago et al. 2010) regarding both years of experimentation. Yield in the mechanical treatment was only partially decreased from the control, and yield in the manual treatment was slightly smaller than the mechanical treatment due to a decrease in berry size (Intrieri et al. 2008). Basic fruit quality parameters were significantly improved from the control, but manual and mechanical treatments were not different from each other. Anthocyanins from the mechanical treatment were significantly higher than the control, however not as improved as the manual treatment. Additionally, the manual treatment saw a large increase in total phenolics, but no difference was seen from the control regarding the mechanical treatment (Intrieri et al. 2008). In Graciano and Carignan cultivars, (Tardaguila et al. 2010) reported no differences between °Brix values in mechanical and manual treatments from the control in the first year of experimentation. However, in the second year, pre-bloom mechanical treatments had significantly higher values than any other treatment. In Graciano, pH and TA values were highest in the pre-bloom mechanical treatment, while in Carignan, pH was highest, and TA was not different from the control and significantly lower than the manual treatment. In both cultivars, anthocyanins and total phenolics were higher in the pre-bloom manual and mechanical treatments than the control or after-bloom treatments, however, were not different from each other. A similar increase in sensory improvement than the after-bloom treatments was reported in the Tempranillo cultivar by (Diago et al. 2010), although no differences were seen between manual and mechanical application. As

these experiments were performed in warm climate growing areas, no incidence of sour rot was reported. Defoliation performed at pre-bloom is the focus of research in both warm and cool climates due to its effect on reducing yield, sour rot and increasing fruit quality. However, a lack of research regarding mechanization of this practice in cool climates and variability of existing results reinforces the need for further studies in this area.

Mechanical leaf removal-induced stress on leaves effects on physiology and fruit quality

Mixed results have been seen regarding papers that report fruit quality data in response to manual versus mechanical leaf removal at pre-bloom. As previously mentioned, some papers indicate no differences between manual and mechanical treatments across many important metabolites (Diago et al. 2010, Intrieri et al. 2008). Meanwhile, in the Graciano cultivar, °Brix, anthocyanins, and total phenolics (Tardaguila et al. 2010) were higher in the mechanical than the manual treatment at pre-bloom in year two of experimentation, although not all were significant. Additionally, preliminary data from the first year of experimentation in **"Objective 1"** described later indicates improved fruit quality in the mechanical pre-bloom treatment over the manual treatment performed at pre-bloom in (*Vitis vinifera* L.) Merlot and Pinot Grigio cultivars. Mechanical leaf removal applied at any timing typically removes less leaf area than manual treatments, leading to a cluster microclimate that is less exposed to radiation and temperatures (Intrieri et al. 2008). This leads to large differences in environmental conditions between manual and mechanical treatments at pre-bloom. Because of this, similar or greater fruit quality in mechanized treatments indicates additional paths to improved fruit quality within the grapevine.

One such pathway could involve an increase in metabolite biosynthesis through the triggering of stress hormones from mechanical stress induction. During the utilization of either

‘pulsed-air’ or ‘leaf-plucker unit’ mechanical leaf removal, the leaves are shredded and ‘wounded’ instead of being removed completely. The response of plants to wounding has not been studied in depth in grapevine, however, wounding occurs in plants in many other forms, whether by insects, or pathogen response (Cheong et al. 2002). ABA, ethylene and JA have been indicated as key pieces in the pathway responding to mechanical wounding in *Arabidopsis thaliana*, potato, and tomato. ABA was shown to be involved in the wounding response in both tomato and potato (Pena-Cortes et al. 1995). In (Cheong et al. 2002), jasmonic acid and ethylene were involved in the mediation between stress response and metabolite pathways in *Arabidopsis thaliana.* Wounding upregulated MYB and F3’5’H genes responsible for positively regulating the phenylpropanoid pathway and flavonoid biosynthesis, respectively, and downregulates additional MYB genes involved in secondary metabolism repression. The response of the vine to mechanical wounding should be further investigated to elucidate the potential for improved fruit quality from these mechanisms.

In summary, pre-bloom leaf removal has the greatest potential for improving fruit quality in cool climate growing regions. Three publications exist in the literature that refer to pre-bloom mechanical leaf removal. Of these, (Diago et al. 2010) and (Tardaguila et al. 2010) utilized a pulsed-air machine on a VSP trellis system, commonly used in Michigan and the Great Lakes growing region for *vinifera* cultivars. Despite previous research in this area, the growing regions represented in publications are Bologna, Italy and La Rioja, Spain, each classified under Region III and IV, respectively, according to the Winkler Growing Index. Because of this, no research has investigated the effects of mechanical pre-bloom leaf removal on sour rot incidence or improved fruit quality in cool climates, where ripening is more strained by environmental conditions. In addition, responses of the vine to mechanical stress could provide an answer to the effectiveness

of mechanical leaf removal treatments with inferior microclimate conditions to that of manual treatments. An in-depth study of the metabolites important to fruit quality will help elucidate the mechanisms by which plants undergo secondary metabolism in response to leaf removal. Therefore, mechanical pre-bloom leaf removal should be further investigated in cool climates due to the positive implications it provides for growers regarding saving money and increased fruit quality.

CHAPTER 3

Early Leaf Removal as a Strategy to Improve Ripening and Lower Cluster Rot in Cool Climate (Vitis vinifera L.) Pinot Grigio

This chapter has been accepted for publication in an open-access format and is accessible in its entirety at:

VanderWeide JV, Frioni T, Ma Z, Stoll M, Poni S, Sabbatini P. 2020. Early Leaf Removal as a Strategy to Improve Ripening and Lower Cluster Rot in Cool Climate (Vitis vinifera L.) Pinot Grigio. American Journal of Enology and Viticulture. Am J Enol Vitic 71:70-79, doi: 10.5344/ajev.2019.19042

ABSTRACT

Removal of basal leaves early in the vegetative and reproductive development of grapevines is a tool used to decrease fruit set, lower cluster rot severity, and improve fruit quality. However, the considerable time required for implementation limits its use by grape growers. Efficient mechanization can potentially mitigate these issues, albeit this practice has not yet been compared to manual application at pre-bloom and after-bloom stages in a cool and humid growing region where cluster rot is the major limitation for yield and fruit quality. The goal of this study was to compare mechanical leaf removal (ME) with the manual (MA) removal of six leaves at the pre-bloom (PB) and after-bloom (AB) phenological stages over two seasons in Pinot Grigio (a tight-clustered cultivar). Fruit set was only decreased in 2017 by MA of six basal leaves at PB (PB-MA); however, PB reduced cluster compactness in each season. The loss of fruit to gray mold was lowered by all leaf removal treatments in the drier 2017 season, but only MA treatments mitigated loss from sour rot in that year. This indicates that a clear fruit zone and reduced cluster compactness are both needed to lower the effect of cluster rot disease. Only PB treatments enhanced fruit quality, likely driven by a similar reduction in cluster compactness. The results suggest that ME at PB may be used to decrease fruit loss to gray mold in dry seasons and enhance fruit Brix. Nevertheless, PB-MA can be an effective means to reduce fruit loss to sour rot in drier seasons and enhance ripening in years with high precipitation during veraison. This information provides a single approach to alleviate two prominent issues facing seasonal management strategies in cool climate viticulture.

CHAPTER 4

Enhancement of Fruit Technological Maturity and Alteration of the Flavonoid Metabolomic Profile in Merlot (Vitis vinifera L.) by Early Mechanical Leaf Removal

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ABSTRACT

Removal of basal leaves near blooms inevitably affects grapevine balance and cluster microclimate conditions, improving fruit quality. Mechanization of this practice allows growers to save time and resources, but to our knowledge, it has not yet been compared with the manual application of this practice in a cool-climate region where seasonal temperatures frequently limit fruit technological maturity and phenolic ripening in red *Vitis vinifera* cultivars. In our research, berry sugar concentration was highest with pre-bloom mechanical treatment (PB-ME). Furthermore, metabolomics analysis revealed that PB-ME favored the accumulation of significantly more disubstituted anthocyanins and flavonols and OH-substituted anthocyanins compared with manual application. Given that vine balance was similar between treatments, increased ripening with PB-ME is likely due to enhanced microclimate conditions and higher carbon partitioning through a younger canopy containing basal leaf fragments proximal to fruit. This information provides an important strategy for consistently ripening red *Vitis vinifera* cultivars in cool climates.

CHAPTER 5

Canopy architecture and fruit microclimate, not ripening-related phytohormones, control phenylpropanoid biosynthesis in response to early leaf removal in Merlot (*Vitis vinifera* L.) grapevines

Submitted.

ABSTRACT

Early leaf removal (ELR) applied in the cluster zone around bloom is a vineyard practice commonly utilized to reduce disease pressure and control yield. In addition, the literature reports that ELR enhances fruit quality, however, little research deciphers the potential factors controlling this response. Here, 60% of leaf area was removed from shoots in three ways: 1) manual removal of 5 leaves (PB-MA), 2) mechanical removal (PB-ME), and 3) simulated mechanical removal (PB-SIM), which was implemented to understand whether PB-ME improves fruit quality via enhanced microclimate conditions, or stress. Yield was reduced in PB-MA and PB-ME, while TSS was not different at harvest; meaning that ELR decreased the efficiency of carbohydrates allocated to fruit. Major phenylpropanoid classes were enhanced by PB-ME, however neither ABA nor ethylene were similarly altered, suggesting their lack of involvement in promoting phenylpropanoid biosynthesis in response to ELR. Instead, the leaf area at nodes above the fruit-zone was lower in PB-ME compared to C, which increased post-veraison fruit temperature (+2.8°C). These parameters correlated with anthocyanins at harvest. In conclusion, skin phenylpropanoid concentrations are influenced by canopy density above the fruit-zone. Additionally, ripening-related phytohormones are not involved in the response of phenylpropanoid biosynthesis in response to ELR.

INTRODUCTION

The transition to veraison and ripening in grape (*Vitis vinifera*) involves a shift in many primary and secondary metabolites that makeup the final composition of the fruit (Dai et al. 2013a). Among the most important primary metabolites are hexoses, such as glucose and fructose. Hexoses accumulation in fruit is the result of sucrose hydrolysis and these metabolites accumulate steadily during ripening until they represent about 20-30% of the final berry weight. Hexoses concentration dictates wine alcohol concentration and are also involved in triggering the biosynthesis of some classes of flavonoids important to fruit quality, such as anthocyanins (Dai et al. 2013b, Zheng et al. 2009). Flavonoids are secondary metabolites deriving from phenylalanine and the phenylpropanoid biosynthetic pathway. Among the flavonoids important to grape quality are anthocyanins, flavonols, and flavan-3-ols. Anthocyanins are located in the berry skin of red cultivars, and are responsible for the pink, red, and purple color of grapes as they undergo veraison and ripening. Their biosynthesis follows that of hexoses accumulation in fruit (Castellarin et al. 2011), and the final concentration at harvest is an important indicator of red wine quality. Flavonols – colorless compounds with structures similar to anthocyanins (Lücker et al. 2010) – accumulate through the entirety of berry growth, but increase significantly during veraison. The biosynthetic pathway controlling flavonols is stimulated in response to high light conditions, acting as a protectant from light stress conditions in many plant tissues (Del-Castillo-Alonso et al. 2016). The rate of vine growth and fruit ripening are largely controlled by endogenous and environmental factors; endogenous factors are mainly related to photosynthesis, plant water relations and hormonal signals. Environmental factors are mainly related to temperature. In particular, growing degree day accumulation drives development [7] while excessive precipitation delays it (Blank et al. 2019). Additionally, radiation and temperature are critical for phenylpropanoid biosynthesis.

Light intensity (Del-Castillo-Alonso et al. 2016, Guilpart et al. 2014), as well as wavelength range (Martínez-Lüscher et al. 2014), are highly influential in dictating production of flavonoids, particularly flavonols. Whereas, fruit temperature has been implicated in affecting the synthesis of select classes of phenylpropanoids, including anthocyanins (Tarara et al. 2008). In addition to the total concentration of anthocyanins, light temperature have been implicated in affecting substitution and acylation pattern (Martínez-Lüscher et al. 2014; Tarara et al. 2008). Photosynthesis provides assimilates for plant growth and development, and as such, the ratio of photosynthesizing leaves to fruit per vine has been suggested to control ripening rate (Kliewer and Dokoozlian 2005; Parker et al. 2014).

Grape is non-climacteric fruit, suggesting that ethylene is not involved in the ripening transition phase, fruit abscission, or post-harvest cellular decomposition. Despite the low endogenous concentrations of ethylene in grape, a number of studies have indicated that exogenous application of ethylene promoters and inhibitors influences grape ripening and quality (Böttcher et al. 2013, Chervin et al. 2004, 2008). In grape, and many other non-climacteric fruits, abscisic acid (ABA) has been shown to initiate and enhance the processes of softening, sugar accumulation, and flavonoid biosynthesis (Pilati et al. 2017; Sun et al. 2019). In addition to phytohormones, the reactive oxygen species hydrogen peroxide (H_2O_2) has been implicated as a signaling molecule in the initiation of veraison (Pilati et al. 2007; Pilati et al. 2014), as well as fruit softening and the chloroplast-to-chromoplast transition (Decros et al. 2019). Recently, Guo et al. revealed that exogenous application of H_2O_2 advanced the rate of ripening in grape by approximately 20 days (Guo et al. 2019), proving the importance of H_2O_2 in this process.

In cool climate viticulture regions such as Michigan, short and cool growing seasons limit proper fruit development of red *vinifera* cultivars in challenging seasons. Early leaf removal has

been adopted in cool climate viticulture regions as a management strategy to improve fruit quality. Performing this practice before bloom reduces fruit set, and therefore yield, leading to a greater leaf to fruit ratio during ripening (Frioni et al. 2018; Acimovic et al. 2016; Poni et al. 2009). Typically, leaves shading the fruit are removed, which allows greater light exposure to the berries. In addition to improving airflow in the fruit-zone which reduces bunch rot, greater radiation has been shown to enhance fruit quality through an increase in hexoses, anthocyanins, and flavonols (Poni et al. 2009; VanderWeide et al. 2018a). In recent years, studies have indicated the potential of mechanizing this practice in cool and warm climates, making it more accessible for growers (VanderWeide et al. 2018b; Hed and Centinari 2018; Tardaguila et al. 2012; Diago, Vilanova, and Tardaguila 2010; Kemp, Harrison, and Creasy 2011; Bubola et al. 2019; Intrieri et al. 2008; 2016; Cook et al. 2015; VanderWeide et al. 2020). Despite the many studies highlighting the impacts of early leaf removal on fruit quality, little information is found in the literature which provides an adequate mechanistic explanation. This, taken together with our previous work in (*Vitis vinifera* L.) Merlot showing the positive effects of early mechanical leaf removal on total soluble solids, anthocyanin, and flavonol concentrations (VanderWeide et al. 2018) suggests that the potential physiological and hormonal causes should be elucidated. Here, the objective of this work was to characterize the influence of environmental, physiological, and hormonal parameters dictating fruit quality in response to early manual and mechanical leaf removal. By simulating the mechanical treatment, we were able to understand whether the enhancement of fruit quality observed previously in PB-ME (VanderWeide et al. 2020, 2018) was due to improved microclimate conditions or stress-related factors.

MATERIALS AND METHODS

Vineyard sites and plant material

In a commercial vineyard in Michigan (MI), USA (41°96’ N; 86°44’ W), 8-year-old grapevines cv. Merlot, clone 14, grafted on 101-14 rootstock were selected for the experiment. Vines were spaced 2.3 m apart, and rows were 3 m wide and planted on Spinks loamy fine soil (U.S. Department of Agriculture, Soil Conservation Service, 1957). A vertically shoot positioned (VSP) Guyot training system was used, and vines were cane-pruned during the 2017-2018 winter to approximately 40 buds per vine. Three weeks post-bloom, shoots with less than two clusters were thinned and tertiary clusters removed to obtain approximately 20 shoots per vine each containing two clusters (one apical cluster, one basal cluster). To prevent chemical damage to flowers and potential alteration to fruit set, sprays were not applied during bloom. Other locally recommended crop protection practices based on scouting experience and weather were followed. Chemicals were rotated to avoid resistance following the Integrated Pest Management program by Michigan State University (Wise et al. 2018). Weather conditions including temperature and precipitation, were recorded during the experiment by an automated weather station in Berrien Springs (MI) from the Michigan Automated Weather Network (MAWN), located 6.0 km from the experimental vineyard. Growing degree days (GDD) were calculated with the Baskerville-Emin method using a base temperature of 10 °C (Baskerville and Emin 1969). No irrigation was used, and standard summer vineyard practices were applied, including mechanical hedging on July 20.

Experimental design

The experiment utilized four rows of vines and followed a randomized complete block design with four treatments and three replicates. Each experimental block spanned all four rows, with each replicate containing 10 vines that were all subjected to treatments. Prior to treatment application,

shoots from vines in all rows were positioned. The four treatments were as follows: undefoliated control (C), pre-bloom manual leaf removal of all leaves from the five most basal nodes (PB-MA), pre-bloom mechanical leaf removal (PM-ME), and pre-bloom simulated leaf removal (PB-SIM). The mechanical treatment was implemented utilizing a pulsed-air technology (Collard®, Bouzy, France) front-mounted, pneumatic remover, addressed at a zone consisting of eight nodes (38 cm). Tractor velocity was 1.6 km/h, and the remover was pulsing air at 0.8 bar from two nozzles, rotating at 1650 rpm. PB-SIM was implemented to test the source of enhanced fruit quality by PB-ME, which we hypothesized to be either due to improved fruit-zone microclimate conditions, or mechanical stress imposed by the machine. The simulated treatment consisted of removing ~75% of the basipetal portion of leaves on the 8-most basal nodes for each fruit bearing shoot using scissors. To do this, a population of 30 Merlot main and lateral leaves ranging in size were initially measured for leaf area, and then slowly reduced in leaf area basipetally using scissors until only 30% of leaf area remained. A picture was taken of these leaves, which was used as a template in the field to reduce leaf area in this treatment. The three treatments removed ~70% of leaf area per shoot and were not significantly different from each other (p=0.699). Within blocks, three 'target' vines were chosen randomly, and three representative shoots were tagged. These shoots and clusters were used to determine the leaf area removed by treatments, shoot length, as well as for cluster morphology and fruit quality measurements at harvest. Phenological stages were defined according to the modified E-L system (Coombe 1995).

Shoot-length and leaf-area measurements

On each 'target' vine, three representative shoots were tagged, and their shoot length measured weekly starting two weeks before bloom until harvest. Each week, 20 shoots were collected from non-experimental guard vines, and returned to campus in a cooler for measurement of shoot length

and leaf area using a leaf area meter (LI-3050AHS, Lambda Instruments Corporation, Nebraska). With this data, correlations between main leaf area (y) and shoot length (x): $y = 17.7*x - 134.4$, $R^2=0.82$. These formulas were then applied to shoot length measurements from each respective week to obtain the leaf area per shoot for control, MA, and SIM treatments. The amount of leaf area removed from MA and SIM treatments was estimated by placing the whole leaves or leaf fragments in a small plastic bag, and returning them to campus in a cooler, where they were subsequently measured using the leaf area meter. Post-treatment application, the areas from each shoot were subtracted from their respective shoot leaf area values obtained from the regression. Leaf area removed in the ME treatment was estimated using twenty non-experimental shoots (also subjected to mechanical treatments) collected on each date of treatment application. A regression was created between shoot length and leaf area on ME shoots, and the resulting values were subtracted from values obtained by the above-mentioned regression between shoot length and leaf area. Following fruit collection at harvest, six shoots of average length from each treatment within blocks were collected in bags and returned to campus in a cooler. For each shoot, detailed measurements of main leaf area and lateral leaf area were taken at each node. Data for these values were then divided into four equal quartiles according to the number of nodes per shoot, with the first quartile representing the bottom 25% of a shoot's leaf area, and the fourth quartile representing the top 25% of a shoot's leaf area.

Fruit set

Fruit set was estimated by using the vitisFlower smartphone application (Aquino et al. 2018) at pre-bloom (EL-17). The application overestimated (8.2%) the true number of inflorescences in images. This was corrected by collecting 20 additional clusters ranging in size, and creating a correlation between the predicted (application images in field) and true number of inflorescences

(counted in the laboratory) ($y=0.782x+81.87$, $r^2=84.1$), similar to the method by Poni et al (Poni et al. 2006). This regression was applied to the values calculated by the application software to obtain the true count. The number of berries per cluster at harvest was divided by the initial floret count (EL-17) to calculate the percentage of fruit set.

Yield components and cluster morphology

Yield was determined at harvest (September 27, 2018). At harvest, tagged clusters were collected, transported to campus, and stored at -20 °C until detailed measurements were made, including: rachis length and weight, berry number and weight. As reported previously by Acimovic (Acimovic et al. 2016), the cluster compactness index (CCI) was calculated by dividing the number of berries per cluster by rachis length.

Basic fruit chemistry analysis

During the season, 30 berries per block were collected weekly from veraison to harvest in Ziploc bags from the basal cluster on tagged shoots, and utilized for analysis of total soluble solids (TSS), pH, and titratable acidity. Samples were returned to campus in a cooler, where processing took place the following day. Fruit was allowed to reach room temperature, whereby berries were crushed, and the free-run juice collected into 100 mL beakers. A digital refractometer (ATA-3810 PAL-1, Pulse, Inc.) was used to measure TSS, and pH with a 370 Thermo Orion pH meter (Thermo Fisher Scientific, Inc.). Total acidity (TA) was analyzed using a Multi-T 2.2 digital titrator (Laboratory Synergy, Inc.) using 10 mL juice diluted with water to 100 mL and titrated with 0.1 M sodium hydroxide to pH 8.2 using an equation to yield the TA (g/L).

Photosynthesis and fruit temperature evaluation

Throughout vine growth and development, photosynthesis and relative chlorophyll were measured using a LI-COR 6400XT (LI-COR Biosciences, Lincoln, NE). Photosynthetic rate was measured using a Li-Cor 6400 XT with CO_2 mixing system and red/blue LED light source (Li-Cor, Lincoln, NE). The Li-Cor 6400 XT was set at a CO_2 concentration of 400 ppm., photosynthetically active radiation of 1000 µmol m^{-2} s^{-1} and 20^{o}C air temperature and ambient relative humidity. Measurements were taken between the hours of 11:00 am and 12:00 pm on fully expanded main leaves at the 8th node from the base of the shoot and replicated four times per plant at each time point.

Sugars analysis

The concentrations of glucose, fructose, and sucrose were quantified in pulp tissue using a Quatro Premier XE LC-MS/MS. For each sample, pulp (without seeds) from 30 whole berries was ground in a mortar and pestle in liquid nitrogen, and the subsequent powder placed in a 15 mL centrifuge tube. Tubes were allowed to reach room temperature and were subsequently centrifuged at 2,000 rpm for 10 m at 4°C to obtain juice. 500 µL of supernatant was placed in a new 15 mL tube, diluted 10:1 with distilled water, and freeze-dried. Material was reconstituted with HPLC-grade water at a ratio of 10-times the total weight of the freeze-dried material. A 2 mL aliquot of this solution was filtered using a 0.45 µm PES syringe filter. 10 µL of this filtrate was diluted in 90 µL of 90% acetonitrile containing 100 µM D-Glucose-$^{13}C_6$ internal standard to precipitate protein from samples. The mixture was vortexed and centrifuged for 5 m at 2000 rpm. This process was repeated using 90% HPLC-grade acetonitrile to create a final 1:1000 dilution from the original juice. The instrumentation consisted of an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA) connected to an Acquity BEH Amide column (2.1 mm

x 100 mm; Waters, Milford, MA) held at 40°C. A gradient of 10 mM NH_4Ac (solvent A) and acetonitrile (solvent B) was used with a mobile phase flow rate of 0.4 mL/m and run time of 10 m. The UPLC was connected to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA), which was equipped with electrospray ionization and operated in negative ion mode. A standard curve containing glucose, fructose, and sucrose was utilized to quantify compounds based on integrated peak areas. Data was processed using Masslynx 4.1 software (Waters, Milford, MA).

Flavonoid analysis

Flavonoid samples were prepared according to Vrhovsek (Vrhovsek et al. 2012) and quantified with the method by Rossetto (Rossetto et al. 2004). Briefly, 1 g of ground skin material was placed in a 15 mL centrifuge tube, followed by 2.5 mL of water/methanol/chloroform (20:40:40). Samples were vortexed for 60 seconds and placed on an orbital shaker for 15 m at room temperature conditions. Next, they were centrifuged at 4°C for 10 m at 1,000 x *g*, and the methanolic fraction moved to an additional 15 mL tube. 1.5 mL of water/methanol (1:2) was added to the remaining mixture to complete a second extraction, and after shaking and centrifugation were repeated, the upper methanolic fraction was combined with the previous supernatant. The volume in this second container holding the methanolic phases was brought up to 5 mL with HPLC grade water with a graduated cylinder. The mixture was vortexed, and 1 mL of the mixture was filtered using a 0.45 µm PTFE syringe filter prior to being transferred to an HPLC vial for analysis. Samples were analyzed by LC-MS using a Waters Acquity UHPLC interfaced to a Waters Xevo G2-XS Q-ToF mass spectrometer according to our previous study (VanderWeide et al. 2018). Phenolic acids, flavonols, flavan-3-ols, proanthocyanins, and anthocyanins were quantified using standards of coumaric acid, rutin, (+)-catechin, procyanidin B2, and keracyanin chloride, respectively.

Quantification of phytohormones and ethylene

Quantification of multiple phytohormones was carried out in the skin and pulp of fruits according to Zeng (et al. 2011). In summary, either 50 mg of freeze-dried skin tissue or 150 µL of juice were extracted in 1 mL of extraction buffer of methanol:water (4:1 v/v), 0.1% formic acid, and 0.1 g/L butylated hydroxytoluene (BHT). Immediately prior to sample extraction, a 100 nM concentration of the labeled internal standard (ABA-d6) was added to the extraction buffer. The mixture was vortexed for 30 s and placed on an orbital shaker at 150 rpm for 16 h at 4°C. Upon removal, samples were vortexed for 10 s and centrifuged at 4°C for 10 m at 12,000 x *g*. Different from the protocol, supernatant was not filtered through a 0.2 µm PTFE filter membrane. 150 µL of the supernatant from each sample was transferred to autosampler vials. A volume of 10 µL was injected on to an Ascentis Express C18 column (2.1650 mm, 2.7 mm; Waters, Milford, MA) held at 50°C. Chromatography was conducted as per section 1.28, with 0.1% aqueous formic acid (solvent A) and methanol (solvent B), and a run time of 6 m. A standard curve containing ABA internal standard was prepared with 1:4 dilutions ranging from 1 µM to 0.25 nM and used to quantify metabolites based on the integrated peak areas. ABA-GE was calculated as relative quantification to ABA-d6. Data was processed using Masslynx 4.1 software (Waters, Milford, MA).

For measurement of ethylene, a cluster was randomly chosen from two tagged vines within each block, and from each, three berries were randomly removed by cutting the pedicle at approximately 3 mm with scissors. Berries were placed in an amber sample vial (20 mm diameter, 40 mL volume), and immediately sealed with a VICI Valco® Mininert™ gas chromatography valve. After three hours of incubation at room temperature conditions, 1 mL of gas was withdrawn from the vial and injected to a gas chromatograph (Carle series 400 AGC; Hach Co., Loveland, CO, USA) fitted

with a stainless steel column (6 m x 2 mm) packed with activated alumina, and ethylene was detected by a flame ionization detector. A certified standard (Matheson Gas Products Inc., Montgomeryville, PA, USA) containing 0.979 μL L^{-1} ethylene, 4.85% CO_2, and 1.95% O_2, balanced with N_2 was used. The rate of ethylene flow was calculated relative to the total berry weight for each vial.

H_2O_2 analysis

25 mg of dry weight skin material was extracted in 0.5 mL phosphate buffer and 2:1 (v/v) chloroform:methanol according to Pilati (Pilati et al. 2007), and hydrogen peroxide was fluorometrically quantified using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit. Reactions were incubated in a clear, 96-well plate reader, measured at 575 nm using a plate reader, and quantified using a standard curve.

Statistical analysis

Data were analyzed by one-way (treatment) ANOVA using IBM SPSS software (IBM, Armonk, NY, USA). Vine growth, indexed as total leaf area measured from two weeks before bloom until harvest, and basic fruit quality seasonal evolution began at fruit set and was completed at harvest. Means were then separated by Tukey's HSD test at α=0.05. Figures were created using Sigma Plot ver. 11.0 (Systat software Inc., San Josè, CA, USA). The heatmap was generated using Heml Heatmap Illustrator Software (Deng et al. 2014), which was $\log_{10}$ transformed, and means of ELR treatments compared to the control using a t-test.

RESULTS AND DISCUSSION

Early leaf removal lowers the efficiency of carbohydrate allocation to fruit

The ELR treatments caused a significant decrease in vine leaf area one week prior to bloom. Leaf area in vines from the SIM treatment regenerated quicker than PB-MA or PB-ME and was not significantly different from C at 21 DAB, while all ELR treatments were similar to the C at 33 DAB (Figure 1A). This suggests that there was no limitation of leaf area in any treatment during ripening. Interestingly, at this same point (33 DAB), photosynthetic rate (Pn) increased in PB-MA (Figure 1B), and was followed by a general trend of PB-MA and PB-ME having a greater Pn until harvest, with one or both being significantly higher than C and PB-SIM at 51, 70, and 97 DAB. This information together suggests that the canopy age and seasonal photosynthetic curve is delayed in PB-MA and PB-ME compared to C, providing a younger and more active canopy for the entirety of the ripening phase. The ability of early leaf removal to improve Pn has been reported previously (Frioni et al. 2018, Palliotti et al. 2011) and the seasonal Pn trend shares similarities with other works. However, the point at which Pn in PB-MA and PB-ME vines exceeded that of the C occurred earlier in our data set than of Poni et al (2006).

The implications of PB-MA and PB-ME having higher Pn are important for fruit quality, as this suggests greater allocation of photosynthates to ripening fruit. Despite this, neither TSS or hexoses concentrations (Figure 2A, Table 1), nor the rate of TSS accumulation (Figure 2B) were different among treatments during ripening or at harvest. The ability of ELR to increase fruit TSS concentration is mixed in the literature. Some authors have reported an increase (Tardaguila et al. 2012), while others saw no change from the control (Acimovic et al. 2016, Hickey et al. 2018). In our previous experiments, pre-bloom mechanical leaf removal enhanced TSS in Merlot and Pinot Grigio across two seasons (VanderWeide et al. 2020, 2018). In these experiments, a difference in

TSS was observed at the outset of veraison, which was not observed here. Previous research suggests TSS concentrations at this period determine TSS at harvest (Sadras and Petrie 2011). In this experiment, the difference may lie in the low yield per vine in PB-ME, which was 60% lower than in previous seasons (Table 2).

When the allocation of carbohydrates to fruit was considered on a whole-vine level, the C treatment was significantly higher than ELR treatments as early as 56 DAB (Figure 1C). This can be attributed to a larger yield (Table 1). This means that the vine was significantly less efficient at producing sugars. This also implies that vine balance and yield played no part in dictating fruit quality components, in line with our recent works on ELR (VanderWeide et al. 2020, 2018).

PB-ME enhanced the phenylpropanoid profile at harvest

Although no modulation to TSS or hexoses occurred, PB-ME, C and PB-SIM treatments had a significantly greater percentage of red berries than PB-MA and PB-ME at the onset of ripening (56 DAB, Figure 2C), and this significance carried over when berry color formation was expressed as a rate (Figure 2D). Despite this, the differences in color development rate between treatments converged during veraison, and no difference was observed following this date. Previous work reported that TSS were initially impeded by leaf removal treatments, but did not change TSS at harvest (Matus et al. 2009). A similar delay in PB-MA and PB-ME was observed here but was non-significant. The shift in berry coloring described in Figure 2 is similar to that of vine leaf area (Figure 1C). This indicates that the initial delay in vine leaf area development in PB-MA and PB-ME impacted the onset of color development. It is likely that this was due to a cumulated deficit of carbohydrate production (Petrie, Trought, and Howell 2000), which is known to influence anthocyanin accumulation in grape skin.

Similar to our previous work, PB-ME significantly increased the anthocyanin and flavonol profile content (VanderWeide et al. 2018). Additionally, greater concentrations of phenolic acids and flavan-3-ols were seen, which led to a significant increase in total phenolics (Figure 3). According to the heatmap phylogenetic tree, total phenolic acids and flavan-3-ols clustered together, similar recent work by (Sun et al. 2019). However, different from this work, flavonols did not cluster together with anthocyanins; they were more similar to phenolic acids and flavan-3-ols instead (Sun et al. 2019). Caftaric acid was the major phenolic acid increased by PB-ME. Bubola et al. reported an increase in this compound in response to manual leaf removal (Bubola et al. 2019). Meanwhile, total flavonols were increased through a significantly higher amount of quercetin-3-glucoside, the most prevalent flavonol in grape, and myricetin glycosides. Previous reports have illustrated how increasing fruit exposure to light consistently improves flavonol biosynthesis, particularly quercetin (Downey, Harvey, and Robinson 2004; Price et al. 1995; Liang et al. 2014). Additionally, quercetin and myricetin are both non-methylated flavonols, which are known to respond to radiation to a greater extent than methylated flavonols (Martínez-Lüscher et al. 2014). Among the two primary flavan-3-ols, (−)-epicatechin increased significantly in response to PB-ME, whereas (+)-catechin was not altered. (−)-Epicatechin was observed to increase in response to high temperature while (+)-catechin did not (Degu et al. 2016), whereas Blancquaert recently showed the opposite effect in one season in leaf removal treatments (Blancquaert et al. 2019). Kemp et al. (2011)revealed that both (+)-catechin and (−)-epicatechin were increased in mechanical leaf removal fruit compared to the non-defoliated control in one year but decreased in the other. Fujita et al. revealed no impact of light on procyanidin concentration at harvest (Fujita et al. 2007), similar to what is reported here. Metabolomic profiling of phenylpropanoids showed a clear effect of PB-ME on both malvidin-based and acylated anthocyanins ($p=0.042$), while other

anthocyanins were not significantly changed from the control, despite mono-substituted (p=0.081), p-coumaroyl-substituted (p=0.069), tri-substituted (p=0.062), and methylated (p=0.053) seeing a large, but non-significant change from the control. As malvidin anthocyanins are the major compound in grape (Lee and Skinkis 2013), this influenced total anthocyanin concentration. These results show a different modulation of the anthocyanin profile form our previous work (VanderWeide et al. 2018), however, led to the same increase in total anthocyanins.

Ripening-related hormones are not involved in increased phenylpropanoid content

Abscisic acid (ABA) is the primary hormone involved in stimulating ripening in non-climacteric fruit, including grape (Gambetta et al. 2010). In addition, both ethylene and H_2O_2 have been implicated as being involved in this process (Sun et al. 2010; Pilati et al. 2014). As such, these three metabolites were quantified to understand their potential role in controlling ripening in response to ELR. Relative to ABA and H_2O_2, the peak in ethylene was observed first during veraison, at 61 DAB for C. Interestingly, the three ELR treatments peaked significantly earlier (4-6 days, data not reported) compared to C. However, no significant differences were reported between treatments at any timepoint during veraison (Figure 4A), suggesting ethylene is not involved in this response. The peak of ethylene occurring earlier in development in ELR treatments was unique among the parameters measured in this study. This information suggests that fruit light exposure may be involved in the timing of the ethylene burst, rather than a mechanism related to primary metabolism or ABA. This is in partial accordance with previous research by Sun et al., who showed that at the onset of veraison, transcript accumulation for ACO, the rate limiting enzyme in ethylene biosynthesis, was significantly decreased in clusters with no light exposure from fruit set to veraison, and highest in clusters completely exposed during the same period (Sun et al. 2019). The peak in ethylene is immediately followed by an ABA peak in the pulp (Figure

4B). This is similar to previous works in grape (Sun et al. 2010), but different from other fruits, such as tomato and melon (Mou et al. 2016; Sun et al. 2013). In the pulp tissue, the peaks of ABA (Figure 4B), its conjugated glucose ester form (ABA-GE), and salicylic acid glycoside (SAG; Supplemental Figure 1) were delayed in PB-ME and significantly higher at 70 DAB compared to other ELR treatments. Both ABA and salicylic acid accumulate in plant tissues experiencing osmotic stress (Miura and Tada 2014), and ripening is recognized as a controlled osmotic stress. The delay in these hormones provides more evidence for the delay in sugar accumulation in the pulp in this treatment. Following this, ABA concentration peaked in skin tissue, where it was significantly lower in PB-ME compared to C and PB-MA (Figure 4C). As PB-ME provided greater exposure of fruit to light than the C, microclimate conditions do not explain this decrease. While transcripts of the ABA rate limiting enzyme NCED have been shown to be modulated by sunlight in berries (Sun et al. 2019), ABA concentration in berry skins were unresponsive to supplemented UV-B light (Berli et al. 2011). Instead, it is likely that ABA translocation from source tissues was limited, or that biosynthesis in the skin was decreased. Finally, at the completion of berry color change, H_2O_2 concentration in the skin peaked (Figure 4D). This is similar to previous reports stating that the peak comes after veraison (Pilati et al. 2007; Guo et al. 2019), however there are contrasting results in the literature (Pilati et al. 2014). Together, this information suggests that neither ethylene, ABA, nor H_2O_2 are involved in promoting fruit quality in response to ELR.

Figure 5 illustrates a strong correlation between fruit TSS, berry color change, and skin ABA concentrations in all treatments. Both hexoses and ABA are understood to be promotors of ripening and specifically, color change in grape (Gambetta et al. 2010; Wang et al. 2017). Despite the significant alteration in yield (Table 1), and initial alterations to berry color change (Figure 2C, D), early leaf removal did not alter the correlation between the parameters central to veraison.

Modified canopy structure and fruit temperature increased phenylpropanoids

Previous works unravelling the involvement of light and temperature in Merlot anthocyanin biosynthesis showed that increased fruit temperature influence the higher proportion of malvidin-based and acylated anthocyanins (Liang et al. 2014, Romboli et al. 2017, Tarara et al. 2008). Two pieces of evidence suggest the same phenomenon occurred in this experiment. First, Figure 6B shows the average leaf area per quartile of shoot length, as described in Figure 6A. In PB-ME, the combination of main and lateral leaf area was significantly lower in the second quartile, due to both the greater range of removal by the machine and the potential stress imposed to lateral buds by the machine's mode of action. All ELR treatments provided an increase of light lateral to clusters, but the lack of leaves above the fruiting zone likely enhanced light penetration from a steeper angle to fruit for a greater portion of the day. Second, Figure 6C shows the fruit temperature during fruit ripening. In PB-ME, fruit temperature was greater than the next highest treatment by an average of 2.8°C from the end of veraison to harvest, providing direct evidence of phenolic biosynthesis modulation by improved microclimate conditions. A significant, negative correlation was drawn between Q2 leaf area with fruit temperature, providing direct evidence to link these parameters (Figure 6A). Interestingly, Q1 (p=0.664), Q3 (p=0.851), and Q4 (p=0.970) were poorly related with fruit temperature, providing further evidence for this relationship. Finally, fruit temperature was significantly correlated with total anthocyanin and total phenolic concentrations in fruit at harvest, similar to previous works (Figure 6B) (Spayd et al. 2002).

While both ethylene promoters and ABA have been shown to improve flavonoid content in fruit (Xu et al. 2019; Chervin et al. 2004), neither hormone was explicitly increased by ELR. In fact, ABA in the skin was significantly lower in PB-ME during mid-veraison (Figure 4C), suggesting no relationship with the increase in phenylpropanoid compounds, and radiation to be

the primary parameter influencing flavonoid concentration in the skin. Recently, Sun et al. established that five flavonoids (mal 3-glu, mal 3-ac-gly, peo 3-ac-gly, myr 3-glu, rutin) served as biomarkers for fruit exposure to light, but that ripening-related phytohormones were not involved in light-regulated phenolic biosynthesis (Sun et al. 2019). Given that three of these compounds were also significantly modulated by PB-ME in our work, and that quantification of ABA and ethylene showed no relation to final phenolic concentrations in fruit, our conclusion that ripening related phytohormones are not involved in the promotion of flavonoid biosynthesis in response to early leaf removal is further supported. Instead, fruit exposure to sunlight (and subsequently fruit temperature) was the main driver of phenolic biosynthesis in Merlot skins.

CONCLUSION

In conclusion, the initial rate of TSS accumulation and berry color change at the start of veraison followed the same pattern as vine leaf area, highest in the control. However, a shift occurred during this period, and all treatments saw similar TSS concentration at the outset of veraison. This shift was likely influenced by the increase in photosynthesis rate per vine during this time, but did not lead to a significantly increase in TSS, glucose, or fructose concentrations at harvest in these treatments. This suggested that early leaf removal produced a younger, more photosynthetically active canopy during the ripening phase. Analysis of shoot leaf area at harvest revealed that while leaf removal treatments had less leaf area compared to the control in the bottom 50% of the shoot, a strong compensation in lateral leaf area led to no differences in total leaf area in the bottom quartile of the shoot. Instead, PB-ME had significantly less total leaf area in the zone of leaves above fruit (~nodes 6-10), leading to higher fruit temperature (+2.8 °C) in this treatment during veraison. As a result, PB-ME produced significantly greater concentrations of phenolic acids, flavonols, flavan-3-ols, and anthocyanins, but not procyanidins in fruit skin. This work revealed

that enhanced fruit-zone microclimate conditions, not ripening-related phytohormones, improved phenylpropanoid biosynthesis in vines subjected to early mechanical leaf removal. This research also suggests the importance of light interception angle to fruit in dictating phenylpropanoid biosynthesis.

Table 1. Total soluble solids (TSS), hexoses, pH, and titratable acidity values in juice at harvest.

Treatment[c]	TSS (°Brix)	Glucose (g/L FW)	Fructose (g/L FW)	Glucose + Fructose (g/L FW)	pH	Titratable Acidity (g/L)
C	17.7 ± 1.24	105 ± 4.81	71.4 ± 2.04	176 ± 6.77	3.78 ± 0.09	5.68 ± 0.35
PB-MA	18.4 ± 1.14	105 ± 2.03	72.5 ± 0.47	178 ± 2.14	3.84 ± 0.02	5.67 ± 0.63
PB-ME	18.8 ± 0.54	108 ± 4.37	76.6 ± 2.70	185 ± 7.05	3.87 ± 0.08	5.18 ± 0.16
PB-SIM	18.5 ± 0.73	110 ± 10.5	75.5 ± 3.30	186 ± 13.8	3.84 ± 0.15	5.44 ± 0.30
p value (trt)	0.617	0.812	0.098	0.552	0.760	0.306

[a]Data were analyzed by one-way ANOVA with treatment as the fixed factor, and when the differences were significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means. [c]C, control; PB-MA, pre-bloom manual leaf removal of 5 leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal.

Table 2. Effects of leaf removal treatments on cluster morphology and yield parameters.

Treatment[c]	Berry weight (g)	Rachis Length (cm)	Cluster Compactness[d]	Yield (kg/ vine)
C	1.20 ± 0.13	15.5 ± 1.7	7.94 ± 2.28 a[b]	4.95 ± 1.56 a
PB-MA	1.06 ± 0.06	15.1 ± 0.87	6.05 ± 0.72 ab	3.85 ± 0.37 ab
PB-ME	1.01 ± 0.07	14.0 ± 0.62	3.08 ± 0.19 b	2.49 ± 0.28 b
PB-SIM	1.03 ± 0.03	13.7 ± 1.0	5.03 ± 0.61 ab	2.78 ± 0.25 b
p value (trt)[a]	0.068	0.255	0.008	0.019

[a]Data were analyzed by one-way ANOVA with treatment as the fixed factor, and when the differences were significant, means were separated with Tukey's HSD test ($p < 0.05$). [b]Different letters identify significantly different means. [c]C, control; PB-MA, pre-bloom manual leaf removal of 5 leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal. [d]Calculated as: (berry number per cluster/ rachis length).

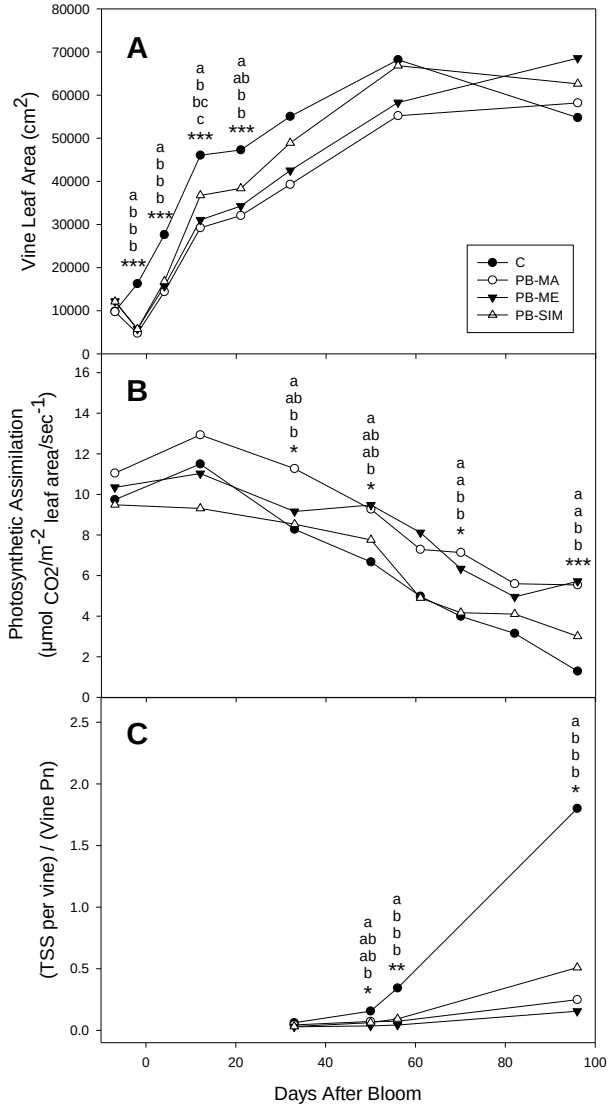

Figure 1. Seasonal evolution of A) vine leaf area, B) photosynthetic rate of main leaves at the 8th node, and C) vine total soluble solids over vine photosynthesis. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. Different letters identify significantly different means at each timepoint. C, control; PB-MA, pre-bloom manual leaf removal of five leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

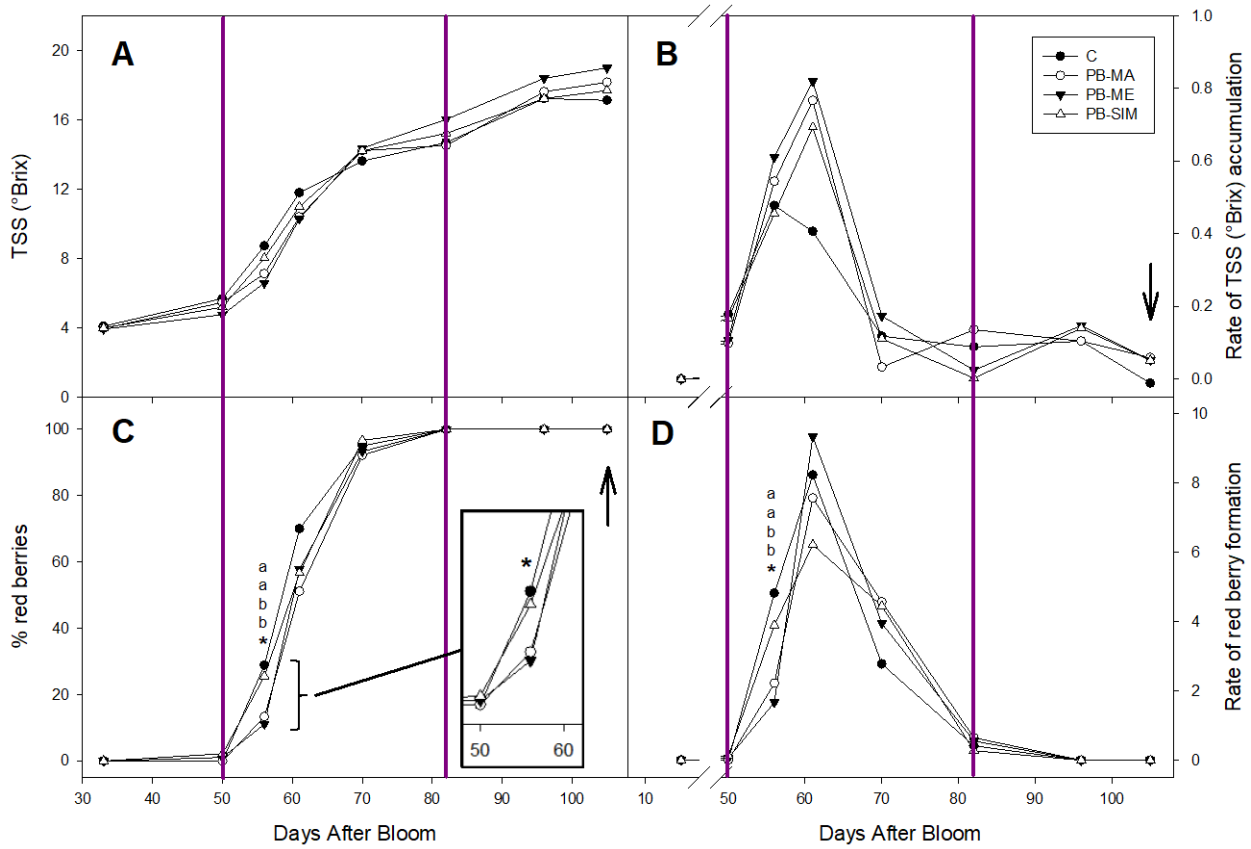

Figure 2. A) Seasonal evolution of total soluble solids (TSS), B) the rate of TSS accumulation, C) percent of red berries (red = appearance of pink, red, or purple color), and D) the rate of red berry formation. Purple lines at 50 and 70 DAB represent the start and end of berry color change, and the arrow marks the date of harvest. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. C, control; PB-MA, pre-bloom manual leaf removal of five leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal. *, $p<0.05$.

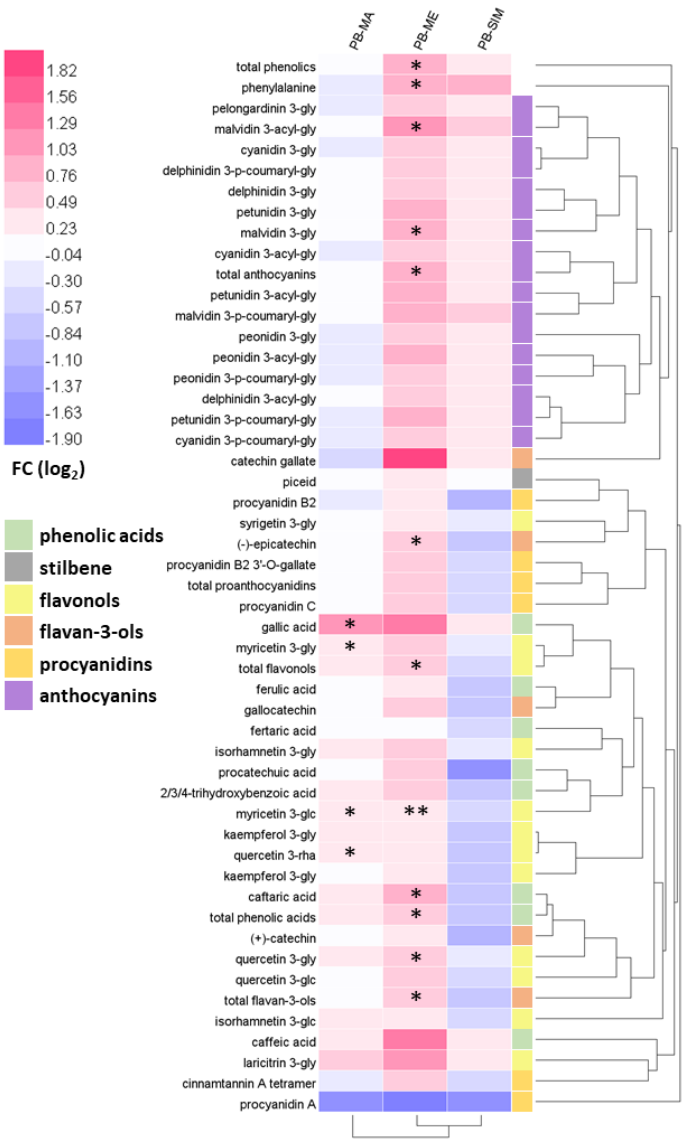

Figure 3. Heatmap illustrating the percent change of phenylpropanoid compounds from the control treatment in harvest-stage fruit from the three leaf removal treatments. Cluster analysis groups the treatments on the x-axis and compounds on the y-axis. Data was log_2 transformed, and each leaf removal treatment compared to the control using a t-test. Red and blue represent an increase and decrease from the control, respectively. *, $p<0.05$. C, control; PB-MA, pre-bloom manual leaf removal of 5 leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal; gly, glycoside; glu, glucoside; glc, glucuronide; rha, rhamnoside; FC, fold change.

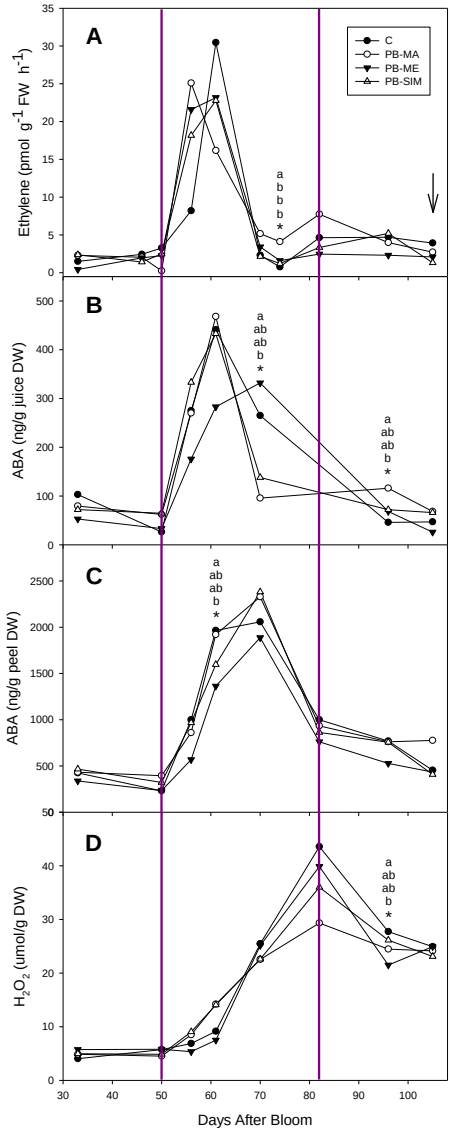

Figure 4. ABA content in A) skin and B) juice, C) ethylene content of whole fruit, and D) H_2O_2 content of berry skins. Purple lines at 50 and 82 DAB represent the start and end of berry color change, and the arrow marks the date of harvest. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. C, control; PB-MA, pre-bloom manual leaf removal of five leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal. *, $p<0.05$.

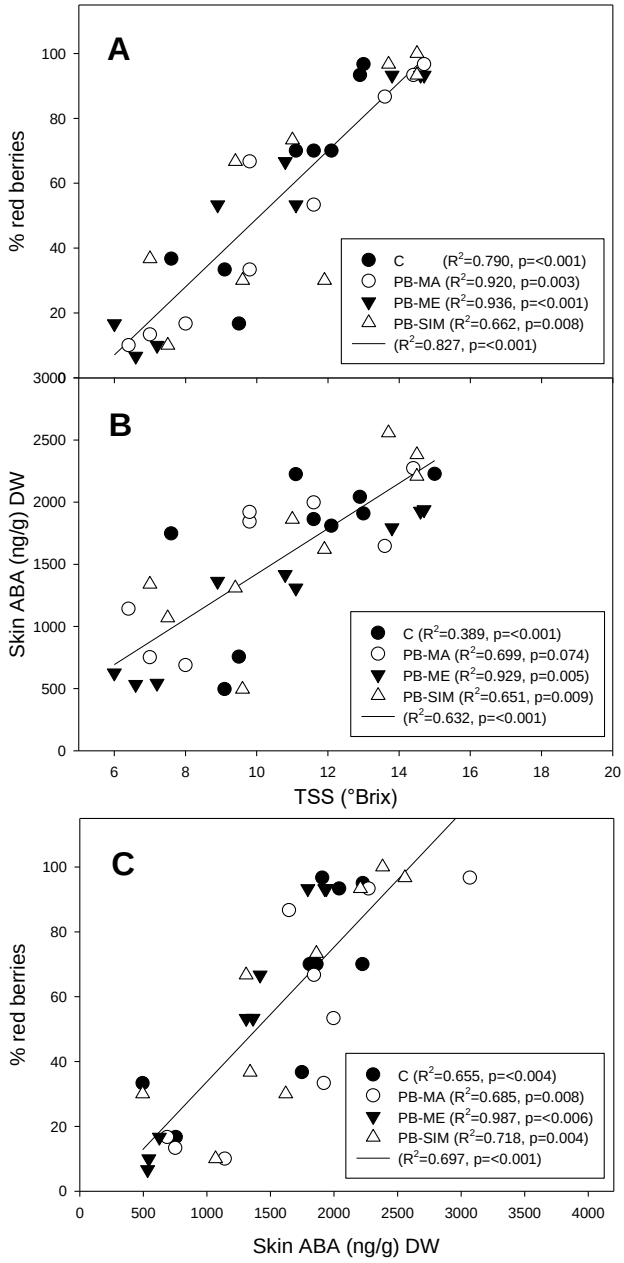

Figure 5. Correlation between A) fruit total soluble solids (TSS) and the percentage of red berries, B) fruit TSS and skin ABA concentration, and C) skin ABA concentration and the percentage of red berries during veraison (50-82 DAB). C, control; PB-MA, pre-bloom manual leaf removal of 5 leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal.

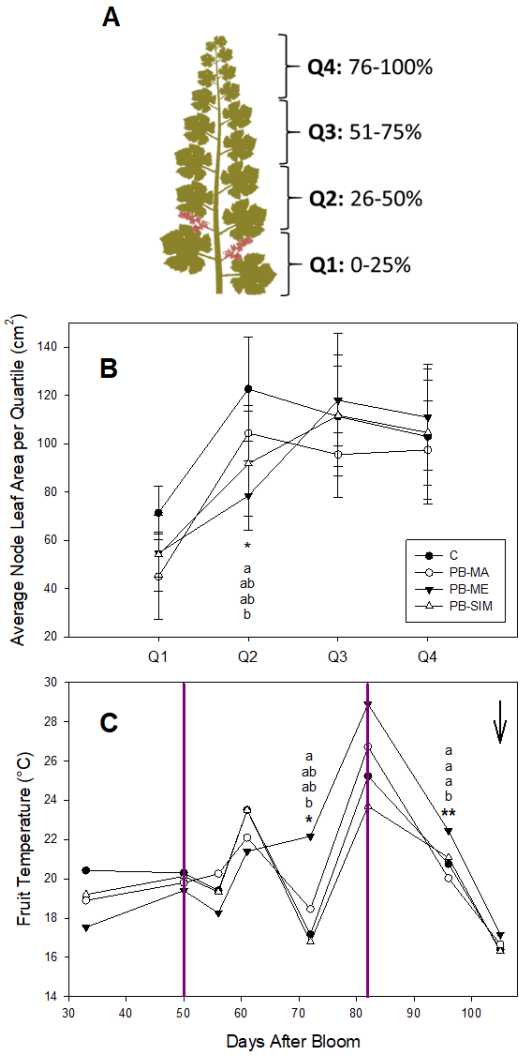

Figure 6. A) Visual illustration of the quartile (Q1-Q4) calculation, B) the average node leaf area on each quarter (Q) of canopy, and C) fruit temperature during ripening. Purple lines at 50 and 82 DAB represent the start and end of berry color change, and the arrow marks the date of harvest. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test (p<0.05). Different letters identify significantly different means at each timepoint. Q, quartile; C, control; PB-MA, pre-bloom manual leaf removal of 5 leaves; PB-ME, pre-bloom mechanical leaf removal; PB-SIM, pre-bloom simulated leaf removal. *, p<0.05; **, p<0.01.

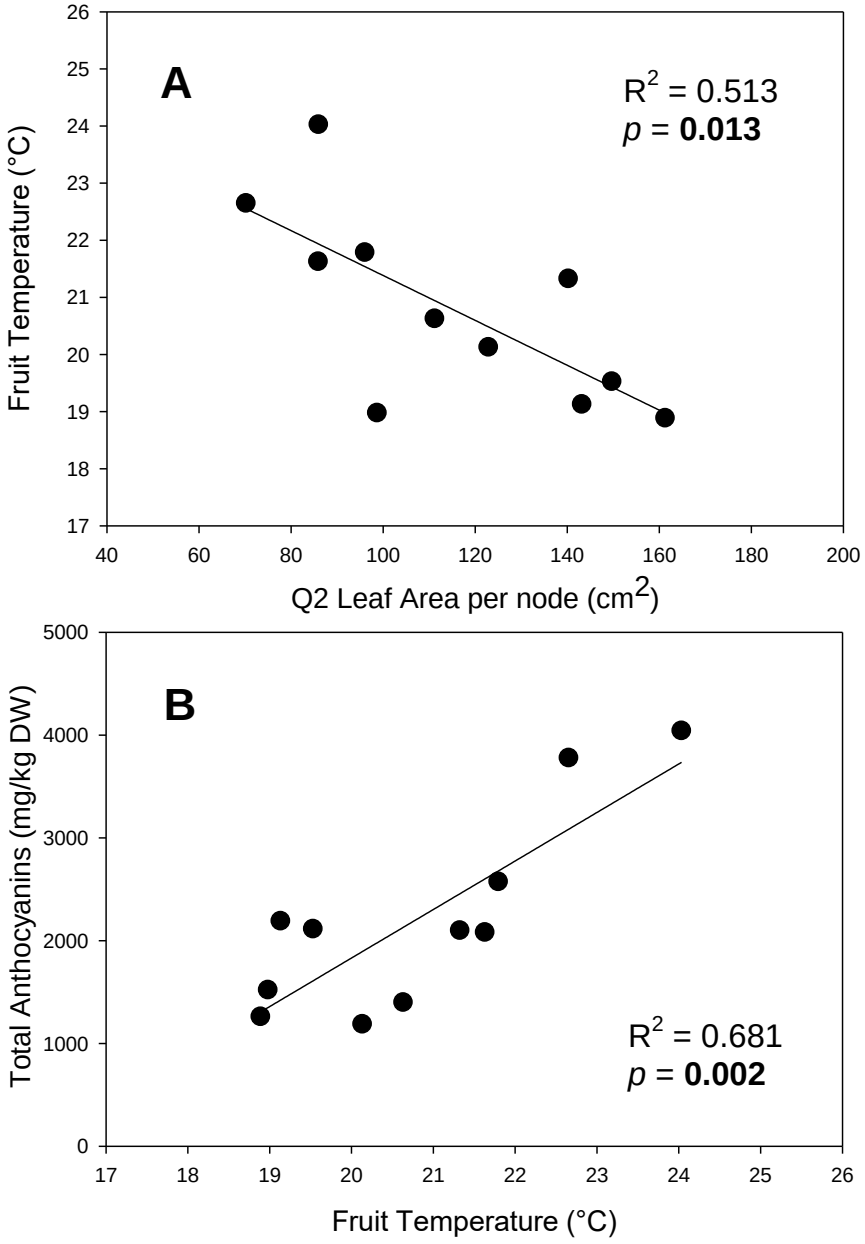

Figure 7. Correlation between the average leaf area per node in the second quartile (Q2) of vine canopy with A) total anthocyanins at harvest and B) post-veraison fruit temperature. Q2, leaf area from nodes 6-10.

CHAPTER 6

Radiation has a greater influence than vine balance on flavonoid biosynthesis in Merlot (*Vitis vinifera* L.)

ABSTRACT

The factors controlling flavonoid biosynthesis in *Vitis vinifera* are of upmost importance to elucidate given the contribution of anthocyanins, flavonols, and flavan-3-ols to red wine quality. Previous research has established the importance of fruit exposure to light in determining total anthocyanin and flavonol concentrations as well as their distinct patterning. Likewise, the source-to-sink balance has been implicated in influencing this pathway regardless of light exposure. Despite this, these factors have not been evaluated in tandem. Here, three levels of leaf removal (0LR (C), 5LR, 8LR) was performed at fruit-set in order to create vines with varying levels of light exposure and contrasting source-to-sink balances. Fruit technological maturity analysis, as well as hexoses analysis indicated that treatments had no effect on primary metabolism, with the exception of titratable acidity at harvest being lowest in LR8 in both locations. However, flavonoid biosynthesis was significantly altered by light exposure, especially MI, indicating the importance of this factor on fruit quality. This was supported by RT-qPCR analysis of select genes involved in flavonoid biosynthesis. Specifically, the expression of *F3'5'Hh* and *F3'5'Hi* were enhanced at veraison with increasing light exposure, while *UFGT* saw an opposite trend at the same time. The results indicate that enhanced light exposure is conducive for increase flavonoid concentration in fruit, especially in cool climates. In conclusion, fruit exposure to light, rather than source-to-sink balance has a greater influence on flavonoid biosynthesis in grape berries.

5.1 INTRODUCTION

Fruit quality perception is generally subjective to an individual's genetic makeup, however, dozens of primary and secondary metabolites are known to represent grape quality. The primary metabolites of importance in grape are sugars and organic acids. Sugars accumulate at the onset of veraison and increase steadily until harvest where they are stored in the vacuoles of mesocarp cells. The most prominent sugars are hexoses (glucose and fructose), followed by trace amounts of sucrose (Lecourieux et al. 2014). Sugars have a large influence on fruit quality as their concentration is representative of total soluble solids (°Brix) (Kliewer 1966) and predict the final alcohol content in wines (Degu et al. 2016). In addition, they influence the biosynthesis of many secondary metabolites (Castellarin et al. 2011; Lecourieux et al. 2014). The organic acids of relative abundance in grape include tartaric and malic, each of which greatly contribute to pH, and the organoleptic properties important to fruit quality (Kliewer 1966). In contrast to sugar, organic acids are present throughout cell division, and decrease sharply as berries expand (Sweetman et al. 2009).

Secondary metabolites stem from primary metabolic pathways and play important roles in self-defense against abiotic and/ or biotic stresses (Lemut et al. 2013), which can be inherited in subsequent seasons (Marfil et al. 2019). Flavonoids represent the largest group of secondary metabolites found in grape, and are synthesized from the shikimic and phenylpropanoid pathways (Ali et al. 2010). In red grapes, approximately 80 to 90% of the total phenolic content stem from three classes of flavonoid compounds – flavan-3-ols, anthocyanins, flavonols (Jeong et al. 2008). Flavan-3-ols, also known as procyanidins, or condensed tannins, are the largest class of phenolics in red grapes; found in both peels and seeds (Yilmaz and Toledo 2004). The major flavan-3-ols include: catechin, epicatechin, epigallocatechin and epicatechin gallate, which contribute to

bitterness in fruit (Adams 2006). Flavan-3-ols are present in high amounts before the onset of ripening, whereby they slowly polymerize during ripening to form more astringent procyanidins (Wollmann and Hofmann 2013). Of the flavonoids found in grape, anthocyanin compounds are among those most studied, due to their role in grape color (Mazza and Francis 1995). Anthocyanins start to accumulate in grape skins after the onset of the veraison stage, when sugar is initially transported to berries (Gambetta et al. 2010). The transportation of sugar regulates specific genes involved in the anthocyanin biosynthesis pathway (Lecourieux et al. 2014), with sucrose being the most related to anthocyanin accumulation, followed by glucose, and fructose (Larronde et al. 1998). Five main anthocyanins are found in red grapes (cyanidin, peonidin, delphinidin, petunidin, malvidin), but acylation, hydroxylation, and methylation reactions produce over a dozen unique compounds or varying hue and stability (Ferrandino and Guidoni 2010, He et al. 2010a, Mazza and Francis 1995). Flavanols are another class of flavonoids synthesized in skins that are important to fruit quality. They play an important role as protection from UV radiation, due to their ability to absorb both UV-A (325-400 nm) and UV-B (280-325 nm) wavelengths (Downey, Harvey, and Robinson 2003). They also contribute to wine color through the co-pigmentation process with anthocyanins (Boulton 2001). The most common flavonols found in grape are the 3-O-glucosides and 3-O-glucuronides of kaempferol, quercetin, and myricetin (Downey, Harvey, and Robinson 2003; Mattivi et al. 2006).

Vine balance, or the ratio of vine leaf area to vine crop load, has long been regarded as a parameter playing a central role in fruit quality metabolic pathways (Poni et al. 2018). Although fruit develops later than other sinks, competition for assimilates still exists, and sink demand determines prioritization of sucrose allocation (Lemoine et al. 2013). Kliewer and Dokoozlian (2005) inspected vines with ranging source-sink balances, and showed that the optimal source-

sink balance for most wine grape cultivars is 0.8 to 1.2 m^2/kg. More recently Parker et al. showed that increasing the source-sink balance by cluster thinning in Syrah vines not only improved the ripening rate (Parker et al. 2014), but also the homogeneity of berry ripening (Parker et al. 2015).

Radiation, in particular UV-A and UV-B wavelength ranges, are known to upregulate phenylpropanoid biosynthesis in grape, including phenolic acids, flavonols, and anthocyanins (Zhang et al. 2012; Martínez-Lüscher et al. 2014; Song et al. 2015). Some of this increase is due to the ability of these compounds to absorb UV wavelengths, while part may also be indirectly due to the subsequent increase in fruit temperature that accompanies the absorption of UV light (Spayd et al. 2002). UV radiation is been shown to increase the expression of particular genes controlling key branch points of this pathway. Chalcone synthase (*CHS*) and flavonol synthase 4 (*FLS4*) were shown to be significantly increased by light (Azuma et al. 2015). Most recently, Sun et al. described the upregulation of the expression of genes coding phenylalanine ammonia-lyase (*PAL*), 4-coumarate: CoA ligase (*4CL*), flavanone 3-hydroxylase (*F3H*) and flavonol synthase (*FLS*) in response to sunlight-exposed fruit (Sun et al. 2017).

A goal of cool-climate viticulture involves the manipulation both source-sink balance and light availability to fruit in order to stimulate biosynthetic pathways involved in fruit quality. Among them is cluster thinning, whereby a specific percentage of fruit are removed prior to or during ripening. This increases the sink strength of remaining clusters and therefore, primary and secondary metabolite accumulation. Improving source-sink balance via cluster thinning performed at pea-size berry has been shown to promote anthocyanin biosynthesis in some studies (Pastore et al. 2011, Xi et al. 2018), while not altering concentrations in others (Wang et al. 2018; Frioni et al. 2017b).

Leaf removal is a cultural practice that involves the removal of select leaves along shoots. The position and number of leaves removed, as well as the timing of removal are dependent on the specific goals of a growing region. This promotes an increase in fruit exposure to radiation and heat during the maturation process. In cool climates, the goal of leaf removal in red cultivars is to enhance fruit technological maturity and phenylpropanoid metabolism. Previous research has shown that performing leaf removal at the after-bloom phenological stage is largely ineffective at altering °Brix concentrations (Sivilotti et al. 2016; Lee and Skinkis 2013; VanderWeide et al. 2018), while leaf removal often improves phenylpropanoids concentration (Acimovic et al. 2016, Feng et al. 2015).

Research conducted in warm climates showed that leaf removal can be more effective at improving fruit quality than cluster thinning in Malvasia (Bubola et al. 2019), Teran (Gatti et al. 2012), and Sangiovese (Bubola et al. 2017). While Frioni et al. indicated that both practices were required to improve anthocyanins and phenolics in Cabernet Franc in a cool season (Frioni et al. 2017b). Despite this, no research has been conducted in cool climates to disseminate the importance of both parameters. Here, this work provides insight into the ability of source-sink balance and light exposure to enhance phenylpropanoid biosynthesis in (*Vitis vinifera* L.) Merlot grown in Michigan, USA and Udine, Italy in the 2018 season. The results of this work have important implications for the management of red wine grape cultivars in cool climates.

5.2 MATERIALS AND METHODS

Vineyard sites and plant material

The experiment was conducted at a commercial vineyard in two locations during the 2018 growing season. The first site was in Michigan (MI), Midwest USA (41°96’ N; 86°44’ W; x m a.s.l.) on 8-

year-old grapevines cv. Merlot, clone 181, grafted on 101-14 rootstock, 1.83 m × x 2.44 m spacing (2252 vines per hectare). While the second was in Udine (UD), Friuli Venezia Giulia region, Northeastern Italy (45°55′ N, 12°42′ E; 20 m a.s.l.) on 15-years-old grapevines cv. Merlot, clone 181, grafted on 3309 C rootstock, 1.6 m × 0.8 m spacing (7812 vines per hectare). In both vineyards, vines were trained to a bilateral vertically shoot positioned (VSP) training system. No irrigation was used, and standard vineyard practices were applied, including mechanical topping and chemicals sprays during the growing season. During the winter period, vines in MI and UD were cane-pruned to 20 buds per vine and spur-pruned to 8-10 buds per vine, respectively. At both locations, no irrigation was used, and standard vineyard practices were utilized during the growing season, including mechanical hedging and chemicals sprays.

Experimental design

At both locations, a completely randomized block design was set up with 3 treatments and 4 replicates of 10 vines. In MI, these replications were established across four vineyard rows, while in UD, they were arranged within two rows. To standardize the vines, the shoot number was adjusted to approximately 20 per vine, with each shoot having one cluster. In detail, the treatments were imposed as follows: C, control with no leaf removal; AB5, leaf removal applied after bloom removing 5 leaves/shoot, and; AB8, leaf removal applied after bloom removing 8 leaves/shoot. The treatments were applied on the 2nd of July and the 7th of June in MI and UD, respectively.

Leaf-area measurements and cluster morphology

Within each plot, three target vines were randomly selected, and three representative shoots on each vine were tagged for tracking detailed measurements of leaf area. Leaf area was determined before and after leaf removal, before and after shoot trimming, and at harvest. In MI, leaf area was analyzed with a leaf area meter (LI-3050AHS, Lambda Instruments Corporation, Nebraska, USA).

In UD, leaf area was calculated by measuring the main vein length of all the leaves in the target shoots, and using a regression between leaf length and leaf area previously determined from a selected leaves from main and lateral shoots using a leaf area meter (LI-3100C, Lambda Instruments Corporation, Nebraska, USA). The total leaf area (TLA) was obtained by multiplying the shoot leaf area by the number of shoots/vine. At harvest, yield and number of clusters per vine were collected. The TLA and yield (Y) per vine were used to calculate the leaf area-to-yield ratio (LA/Y) at harvest. On a subsample of 10 clusters/plot measurements of rachis length, number of berries and berry weight were performed. Moreover, cluster compactness index (CCI) was computed by rating the number of berries/cluster and the rachis length, according to Acimovic et al. (Acimovic et al. 2016).

Berry sampling and basic analysis fruit quality analysis

From approximately 20 days after bloom (DAB) (bloom: MI, 07 June; UD, 27 May) to harvest, 80-berry samples were collected with pedicels approximately every 10 days from each plot, immediately frozen in liquid nitrogen, and stored in dry ice until reaching the laboratory. A subsample of 40 berries was weighed and manually pressed at room temperature. Total soluble solids (°Brix) and pH were measured using a manual refractometer (MI: ATA-3810 PAL-1, Pulse, Inc.; UD: ATC-1, Atago, Tokyo, Japan) and a pH meter (MI: 370 Thermo Orion pH meter, Thermo Fisher Scientific, Inc. ; UD: HI2211, Hanna Instruments, Woonsocket, RI), respectively. Titratable acidity (expressed as g/L tartaric acid equivalents) was determined by titration of the juice with NaOH 0.1 N until a pH 8.2. The second set of 40 berries was weighed and immediately stored at −80 °C. Subsequently, skin and seeds were separated from the frozen berries using a scalpel. After separation, berry tissues were immediately dropped into liquid nitrogen, weighed, and ground to a

fine powder using a mortar and pestle under liquid nitrogen. Powder was then immediately refrozen at -80 °C.

Sugars analysis

For both locations, concentrations of glucose, fructose, and sucrose were analyzed in fruit skins and juice. Briefly, 20 pulp sections from peeled berries were placed in a plastic bag and allowed to reach room temperature, where after they were thoroughly crushed, and the juice placed in 15 mL centrifuge tubes. Samples were centrifuged for 5 minutes at 15 °C and 2,000 rpm, and 1 mL of supernatant was filtered using a 0.45 um syringe filter. 50 µL of filtrate was diluted 1:10 with distilled water, and samples were freeze-dried. Samples were reconstituted with distilled water on a 1:10 basis of the dry weight of each sample on an individual basis.

At both locations, a Quatro Premier XE LC-MS/MS was used to measure glucose, fructose, and sucrose concentrations. 2 mL of juice used for analysis of °Brix, pH, and TA was filtered using a 0.45 um PES syringe filter. 50 µL of filtered juice was diluted 10:1 with distilled water in a 1.7 µL centrifuge tube and freeze-dried. Material was reconstituted with HPLC-grade water at a ratio of 10-times the total weight of the freeze-dried material. 10 µL of this filtrate was diluted in 90 µL of 90% acetonitrile containing 100 µM D-Glucose-13C6 internal standard to precipitate protein from samples. The mixture was vortexed and centrifuged for 5 m at 2000 rpm. This process was repeated using 90% HPLC-grade acetonitrile to create a final 1:1000 dilution from the original juice. The instrumentation consisted of an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA) connected to an Acquity BEH Amide column (2.1 mm x 100 mm; Waters, Milford, MA) held at 40°C. A gradient of 10 mM NH4Ac (solvent A) and acetonitrile (solvent B) was used with a mobile phase flow rate of 0.4 mL/m and run time of 10 m. The UPLC was connected to a Quattro Premier XE tandem quadrupole mass spectrometer

(Waters Corporation, Milford, MA), which was equipped with electrospray ionization and operated in negative ion mode. A standard curve containing glucose, fructose, and sucrose was utilized to quantify compounds based on integrated peak areas. Data was processed using Masslynx 4.1 software (Waters, Milford, MA).

Flavonoid analysis

Flavonoids were extracted according to Vrhovsek (2012) and quantified with the method by Rossetto (Rossetto et al. 2004). Briefly, 1 g of ground skins was placed in a 15 mL centrifuge tube, followed by 2.5 mL of water/methanol/chloroform (40:40:20). Samples were vortexed for 30 seconds and mixed on an orbital shaker for 15 m at room temperature. Following this, vials were centrifuged at 4 °C for 10 m at 1,000 g. The upper methanol phase was removed from each tube and placed in another tube, and 1.5 mL of water/methanol (1:2) was added to the remaining pellet. The samples were vortexed, mixed, and centrifuged under the same conditions, and the resulting methanolic phase was removed and combined in the supernatant tube. The combined methanol supernatants were brought to a total volume of 5 mL with HPLC grade water using a graduated cylinder.

RNA extraction and RT-qPCR.

RT-qPCR was utilized for key flavonoid biosynthesis genes. RNA was extracted using the Spectrum™ Plant Total RNA Kit, with slight modification. The volume Lysis Solution + mercaptoethanol was increase from 500 µL to 700 µL to improve extraction. Additionally, a 20 mg/mL solution of polyvinylpolypyrrolidone (PVPP) was added to the Lysis Solution in order to precipitate phenylpropanoid compounds. The QuantiTect Reverse Transcription Kit was used for gDNA removal and cDNA synthesis. RNA was multiplied with a thermocycler with conditions: xx. SSOFAST™EVAGREEN® SUPERMIX was used for RNA quantification with a Bio-rad

CFX96 - C1000™ thermal cycler. Ubiquitin served as the housekeeping gene. Primers were ordered from Sigma-Aldrich.

Statistical analysis

Data were analyzed by a one-way (treatment) ANOVA using IBM SPSS software (IBM, Armonk, NY, USA), and means were separated by Tukey's HSD test. Regression analyses was performed using Sigma Plot ver. 11.0 (Systat software Inc., San Josè, CA, USA).

RESULTS AND DISCUSSION

Comparison of cluster morphology and vine balance between locations.

Leaf removal treatments were carried out at fruit-set in order prevent an alteration of cluster morphology, and potentially, influence of environment (VanderWeide et al. 2018). Indeed, the removal of either 0, 5, or 8 leaves did not alter cluster weight in either location (Figure 9). Additionally, other morphological characteristics, such as berry number, cluster compactness, and cluster length, were not changed by treatments in either location (Table 3, 4). Interestingly, berry size was reduced by AB8 in Michigan. The Merlot vineyard in Michigan received much less solar radiation during the growing season, and during fruit set (June) compared to the Udine vineyard, as quantified by the Hargreaves Model (Figure 8). This suggests that the removal of eight leaves at fruit set likely restricted carbohydrate translocation to fruit at this time, limiting berry growth. This result is similar to what has been observed in experiments where leaves were manually removed at fruit set (Bubola et al. 2019).

Rather than change vine balance through a decrease in cluster number or size, removing leaves at fruit set – when vegetative growth is largely complete – significantly changed vine leaf area, and therefore, vine balance (Figure 9). Leaf area per yield in AB5 was 34% and 55%, and in

AB8, 20% and 44% of C in Michigan and Udine, respectively. Thus, the treatment with the greatest vine balance (C) also offered the least light exposure to fruit during ripening. In contrast, AB8, having the lowest vine balance, allowed the greatest light penetration to fruit.

Impact of vine balance and fruit-zone microclimate on basic fruit quality.

Figure 3 displays the evolution of basic fruit quality components in Michigan and Udine. Neither location saw a change to total soluble solids (°Brix) or pH in response to treatments, also observed previously (Sivilotti et al. 2016). In addition, quantification of glucose, fructose, and sucrose yielded no significant differences in induvial sugars between treatments (Tables 5, 6).

Despite this, titratable acidity (TA) was significantly reduced by AB8 in both locations at harvest (Figure 10). The organic acid content in grape is primarily comprised of tartaric acid, with a small percentage being malic acid. Tartaric acid content on a per berry basis does not change during ripening, however, malic acid is susceptible to degradation relative to fruit exposure to radiation (Ruffner 1982, Sweetman et al. 2009). Given the greatest exposure of AB8 fruit to light during ripening, the significant decrease in TA is in alignment with the literature.

Impact of experimental parameters on phenylpropanoid biosynthesis.

The expression of genes controlling phenylpropanoid and flavonoid biosynthesis were profiled during fruit development in both locations. Four genes (F3’5’Hh, F3’5’Hi, LAR2, LDOX2) saw an increasing pattern of expression with increasing degree of leaf removal (and light exposure), with the former two seeing a significant increase in AB8 (Figure 12). In contrast, four genes (F3’Ha, UFGT, AOMT1, F3’5’Hp) saw an increase in expression with increasing vine balance, with only UFGT seeing a significant decrease in AB8 from C (Figure 12). Metabolomics were utilized to quantify phenylpropanoid classes (hydroxybenzoic acids, hydroxycinnamic acids, flavonols, flavan-3-ols, procyanidins, anthocyanins) were profiled via HPLC in the fruit skins at

both locations. Despite the significant change in expression of select genes, only the flavonols class of compounds was significantly enhanced during ripening and at harvest in Udine (Figure 11). Table 7 displays the concentration of all compounds detected in skin samples at both locations. All but one flavonol was significantly increased in AB8 from C in Udine, while quercetin 3-glu was the only compound significantly increased also by AB5 in Udine and MI. Previous work on Merlot in Michigan also showed little alteration of both flavonol and anthocyanin profiles in response to early leaf removal (VanderWeide et al. 2018). Flavonols play an important role as protection from UV radiation, due to their ability to absorb both UV-A (325-400 nm) and UV-B (280-325 nm) wavelengths (Downey, Harvey, and Robinson 2003). The greater response in fruit in Udine may be related to the higher light conditions during ripening (Figure 8) (Del-Castillo-Alonso et al. 2016; Martínez-Lüscher, Brillante, and Kurtural 2019). Besides their role in protecting fruit from high radiation levels, flavonols are known to contribute to wine color through the co-pigmentation process with anthocyanins (Baranac et al. 1997, Boulton 2001). This increase in flavonol concentration implies an enhancement of wine quality through higher co-pigmentation.

Relationship between light exposure and flavonoid B-ring substitution pattern.

With the gene expression data from the Udine location, a significant correlation was observed between the ratio of both 4’ to 3’4’ and 4’ to 3’4’5’ substituted anthocyanins and flavonols at harvest and the ratio of the sum of flavonol synthase 5 (FLS5) and flavanone 3-hydroxylase a (F3’H a) expression during fruit maturation (Figure 13). This suggests that increasing radiation favors the biosynthesis of 4’-substituted compounds, followed by 3’4’-substituted and 3’4’5’-substituted compounds. Martínez-Lüscher et al. (Martínez-Lüscher, Brillante, and Kurtural 2019) recently observed this same phenomenon, and suggested that the

proportion of the flavonol profile comprised of the 4'-substituted flavonol kaempferol could be used as a marker for fruit exposure to radiation.

CONCLUSION

In conclusion, while treatments significantly altered both vine balance and light exposure to fruit, neither total soluble solids nor hexoses were changed. Titratable acidity was decreased by LR8 having the greatest light exposure to fruit. Additionally, flavonoid biosynthesis was significantly altered by light exposure, especially MI, indicating the importance of this factor on fruit quality. This was supported by RT-qPCR analysis of select genes involved in flavonoid biosynthesis. The results indicate that enhanced light exposure is conducive for increasing flavonoid concentration in fruit, especially in cool climates. In conclusion, fruit exposure to light, rather than source-to-sink balance has a greater influence on flavonoid biosynthesis in grape berries.

Table 3. Cluster morphology parameters at harvest in Michigan.

Treatment	Cluster weight (g)	Berry Number	Berry weight (g)	Rachis Length (cm)	Cluster Compactness[c]
C[b]	119	84.2	1.35 a[a]	17.0	4.86
AB5	121	89.2	1.27 a	16.8	5.28
AB8	110	89.2	1.15 b	17.0	5.19
trt	0.501	0.788	0.003	0.916	0.692

[a]Data were analyzed by one-way ANOVA with treatment as the fixed factor, and when the differences were significant, means were separated with Tukey's HSD test ($p < 0.05$).

[b]Different letters identify significantly different means. C = no leaf removal; AB5 = after-bloom manual leaf removal, 5 leaves; AB8 = after-bloom manual leaf removal, 8 leaves.

[c]Calculated as: (berry number per cluster / rachis length).

Table 4. Cluster morphology parameters at harvest in Udine.

Treatment	Cluster weight (g)	Berry Number	Berry weight (g)	Rachis Length (cm)	Cluster Compactness[b]
C[a]	148	83.6	1.75	11.3	7.36
AB5	132	79.3	1.65	11.3	6.50
AB8	134	81.6	1.63	11.5	6.22
trt	0.425	0.842	0.426	0.530	0.194

[a]C = no leaf removal; AB5 = after-bloom manual leaf removal, 5 leaves; AB8 = after-bloom manual leaf removal, 8 leaves.

[b]Calculated as: (berry number per cluster*individual berry weight / rachis length).

Table 5. Juice hexoses concentration in Michigan at harvest.

Treatment	Glucose (g/L FW)	Fructose (g/L FW)	Sucrose (g/L FW)	Total Sugars (g/L FW)
C[a]	136	81.2	0.08	217
AB5	133	78.8	0.09	212
AB8	114	67.6	0.07	182
p value (trt)	0.209	0.262	0.561	0.224

[a]C = no leaf removal; AB5 = after-bloom manual leaf removal, 5 leaves; AB8 = after-bloom manual leaf removal, 8 leaves.

Table 6. Juice hexoses concentration in Udine at harvest.

Treatment	Glucose (g/L FW)	Fructose (g/L FW)	Sucrose (g/L FW)	Total Sugars (g/L FW)
C[a]	111	89.5	0.16	201
AB5	113	91.3	0.15	204
AB8	118	92.6	0.17	211
p value (trt)	0.741	0.929	0.813	0.840

[a]C = no leaf removal; AB5 = after-bloom manual leaf removal, 5 leaves; AB8 = after-bloom manual leaf removal, 8 leaves.

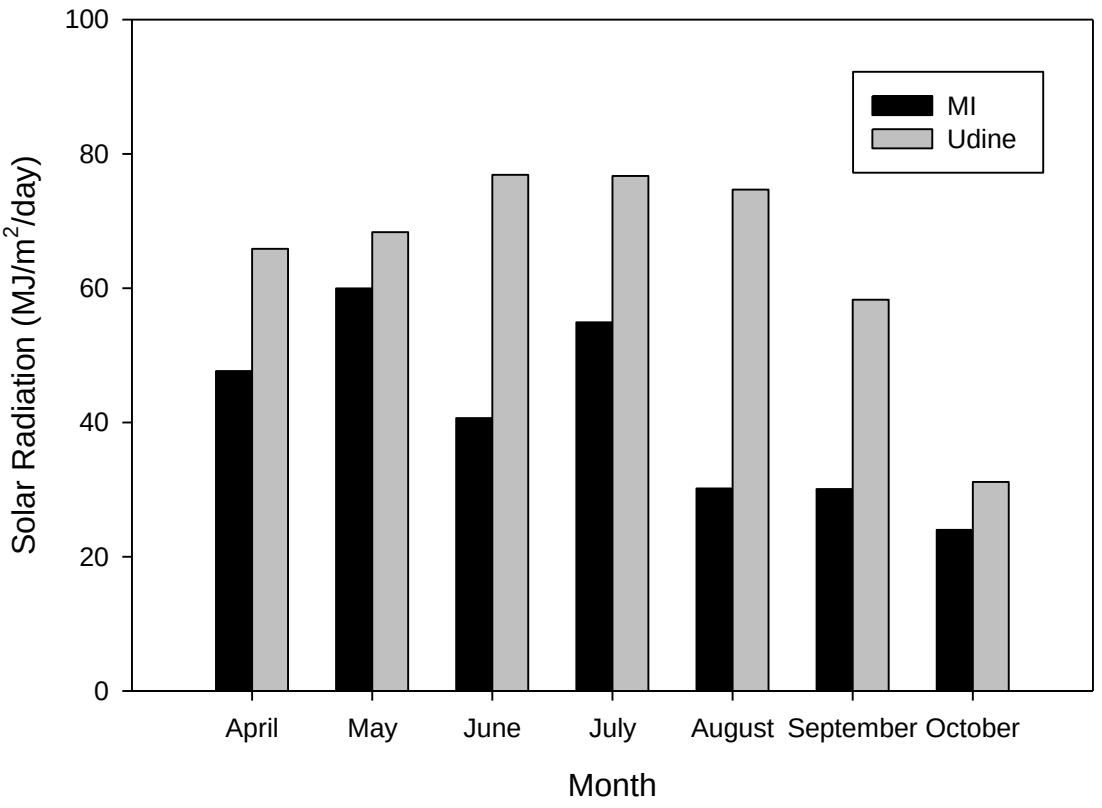

Figure 8. Estimation of monthly solar radiation averages in Michigan (MI) and Udine using Hargreaves Model.

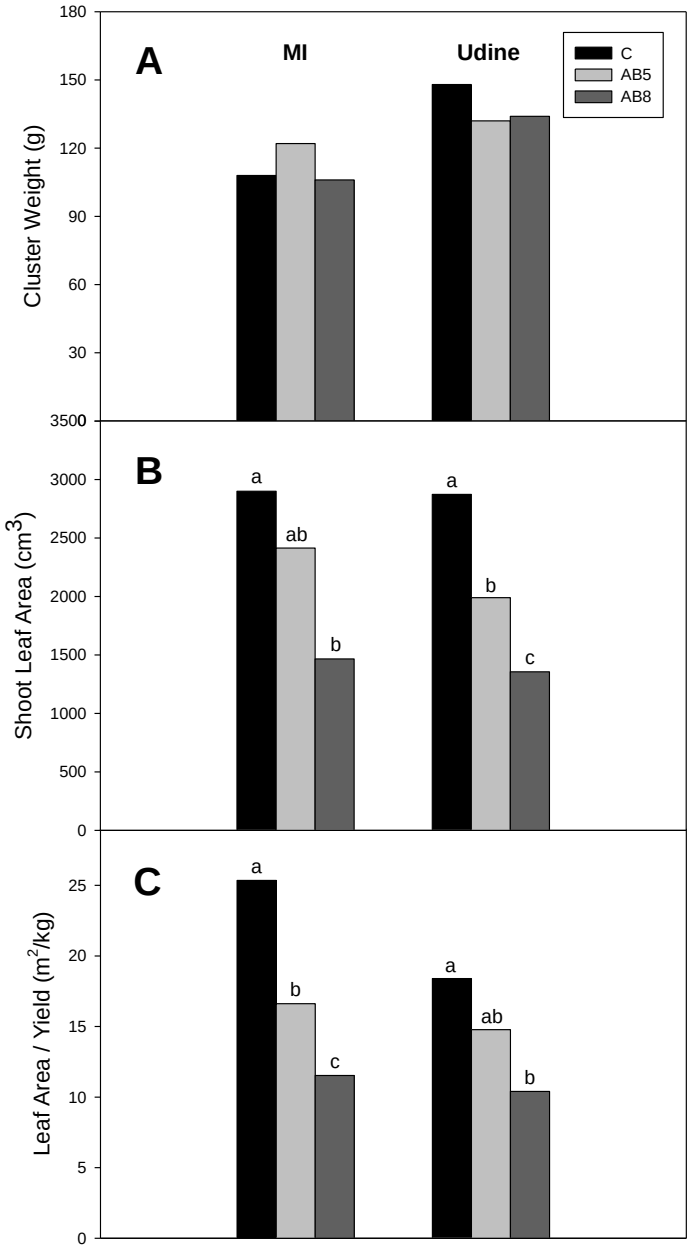

Figure 9. A) Vine yield, B) shoot leaf area, and C) vine source-sink balance at harvest in Michigan and Udine. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. *, $p < 0.05$; **, $p < 0.01$. C, untreated control; AB5, removal of leaves from nodes 1-5 at after-bloom; AB8, removal of leaves from nodes 1-8 at after bloom.

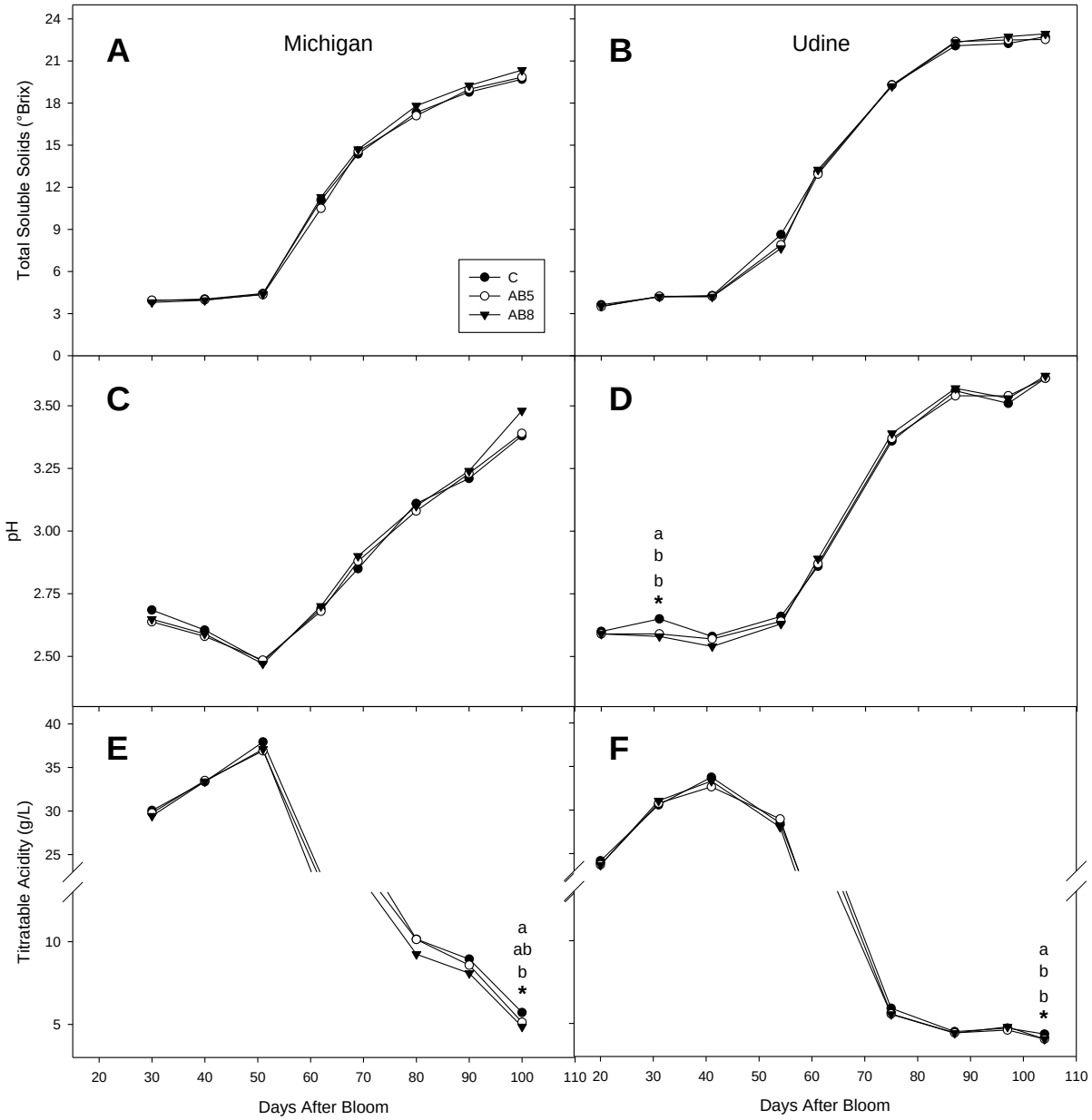

Figure 10. Evolution of total soluble solids (°Brix), pH, and titratable acidity in Michigan (A, C, E) and Udine (B, D, F). Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. *, $p < 0.05$; **, $p < 0.01$. C, untreated control; AB5, removal of leaves from nodes 1-5 at after-bloom; AB8, removal of leaves from nodes 1-8 at after-bloom.

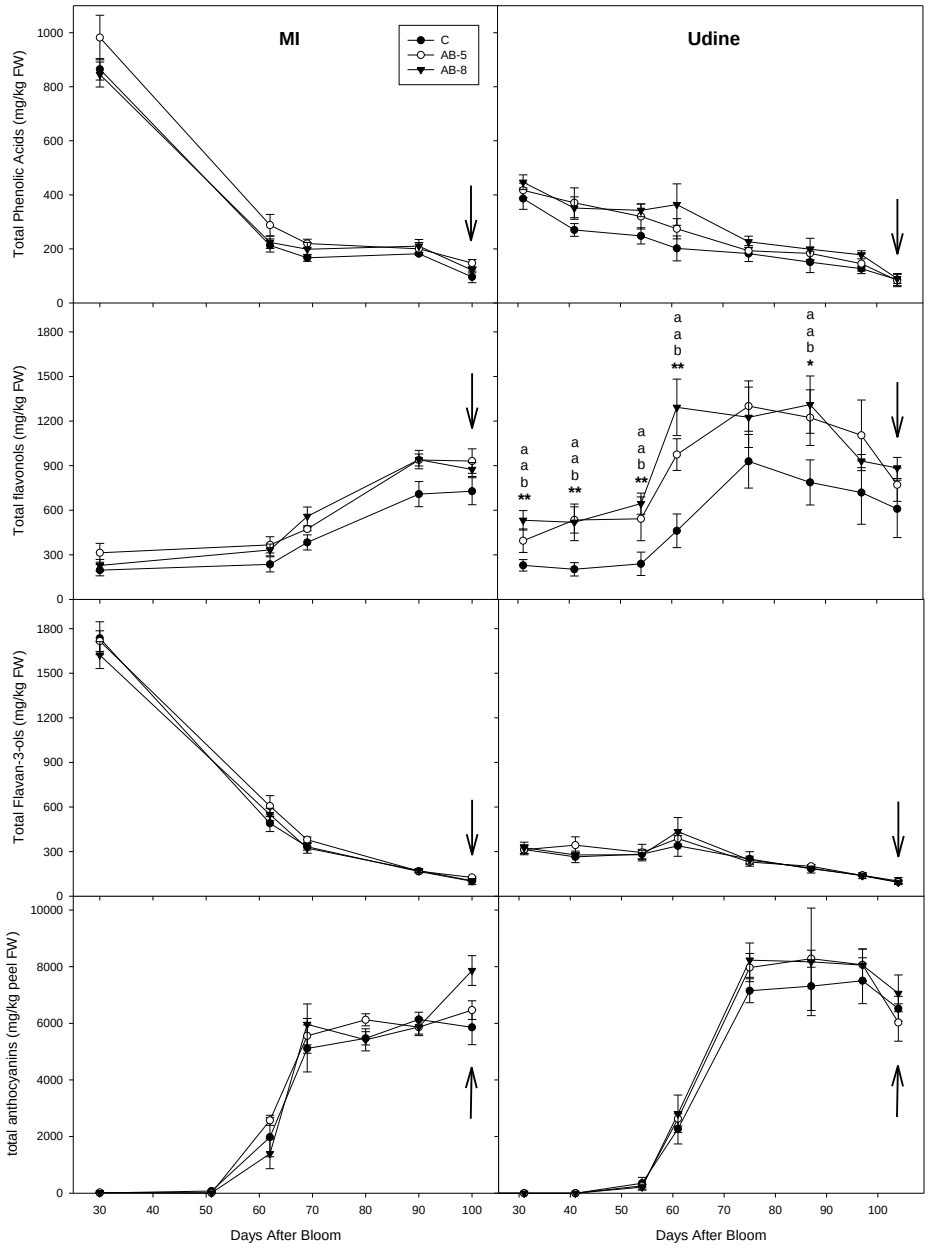

Figure 11. Evolution of phenylpropanoid and flavonoid classes during fruit ripening in Michigan and Udine. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. *, $p < 0.05$; **, $p < 0.01$. C, untreated control; AB5, removal of leaves from nodes 1-5 at after-bloom; AB8, removal of leaves from nodes 1-8 at after-bloom.

Table 7. Targeted metabolomics profile of phenolic acids and flavonoids in Merlot peels at harvest in response to leaf removal treatments.

	Michigan				Udine			
Compound (mg/kg peel) FW	**C**	**AB5**	**AB8**	**p-value**	**C**	**AB5**	**AB8**	**p-value**
Total phenols	6797	7752	8996	*0.330*	7195	6827	8019	*0.121*
Phenolic acids								
total phenolic acids	96.4	148	122	*0.193*	86.2	83.2	90.1	*0.924*
gallic acid	0.88	0.77	1.60	*0.704*	5.79	3.81	5.45	*0.133*
procatechuic acid	0.14	0.35	0.27	*0.132*	2.10	1.21	1.80	*0.126*
fertaric acid	11.0	13.7	12.3	*0.368*	3.79	3.37	3.85	*0.454*
caftaric acid	82.1	129	104	*0.189*	74.5	74.8	79.0	*0.955*
Flavonols								
total flavonols	907	1235	1118	*0.180*	492 b	624 b	781 a	***0.001***
kaempferol 3-glu	125	192	175	*0.337*	76.8 b	110 ab	142 a	***0.002***
kaempferol 3-glc	3.61	6.52	5.28	*0.127*	2.69 b	4.12 ab	5.67 a	***0.002***
quercetin 3-glu	245	269	262	*0.779*	193 b	238 ab	287 a	***0.003***
quercetin 3-glc	221 b	345 a	284 ab	***0.047***	116 c	161 b	213 a	***0.000***
isorhamnetin 3-glu	117	161	145	*0.121*	60.3 b	73.3 ab	89.7 a	***0.007***
syringetin 3-glu	196	260	246	*0.189*	39.8	33.1	38.3	*0.292*
Flavan-3-ols								
total catechins	100	126	103	*0.511*	102.2	95.0	93.1	*0.792*
(+)- catechin	45.6	59.1	48.3	*0.585*	41.7	40.3	38.0	*0.786*
(-)- epicatechin	54.5	66.4	54.9	*0.540*	19.0	17.5	16.4	*0.673*
epi/gallocatechin	3.99	5.76	4.42	*0.661*	40.5	36.5	37.7	*0.793*
epi/catechin gallate	0.00	0.40	0.40	*0.200*	2.61	1.98	2.71	*0.653*
Anthocyanins								
total anthocyanins	5856	6467	7865	*0.340*	6516	6029	7059	*0.159*
cyanidin 3-glu	254	266	317	*0.675*	189	194	248	*0.127*
peonidin 3-glu	518	502	649	*0.541*	640	593	670	*0.411*
delphinidin 3-glu	422	479	455	*0.815*	514	537	679	*0.077*

Table 7 (cont'd)

petunidin 3-glu	399	442	542	*0.291*	448	452	562	*0.064*
malvidin 3-glu	989	1078	1417	*0.298*	2185	2017	2344	*0.202*
cyanidin 3-acyl-glu	109	107	128	*0.647*	28.3	27.9	36.5	*0,062*
peonidin 3- acyl-glu	298	287	379	*0.502*	160	144	166	*0.108*
delphinidin 3- acyl-glu	123	132	163	*0.407*	83.1	85.1	106	*0.093*
petunidin 3- acyl-glu	209	229	291	*0.265*	105	107	127	*0.114*
malvidin 3- acyl-glu	849	915	1231	*0.257*	844	721	823	*0.220*
cyanidin 3-p-coumaryl-glu	108	132	139	*0.523*	61.3	54.3	58.9	*0.325*
peonidin 3-p-coumaryl-glu	446	506	555	*0.549*	221	199	228	*0.271*
delphinidin 3-p-coumaryl-glu	82.4	112	121	*0.300*	110	107	123	*0.281*
petunidin 3-p-coumaryl-glu	201	249	281	*0.296*	108	102	115	*0.386*
malvidin 3-p-coumaryl-glu	844	1027	1193	*0.305*	819	688	772	*0.213*

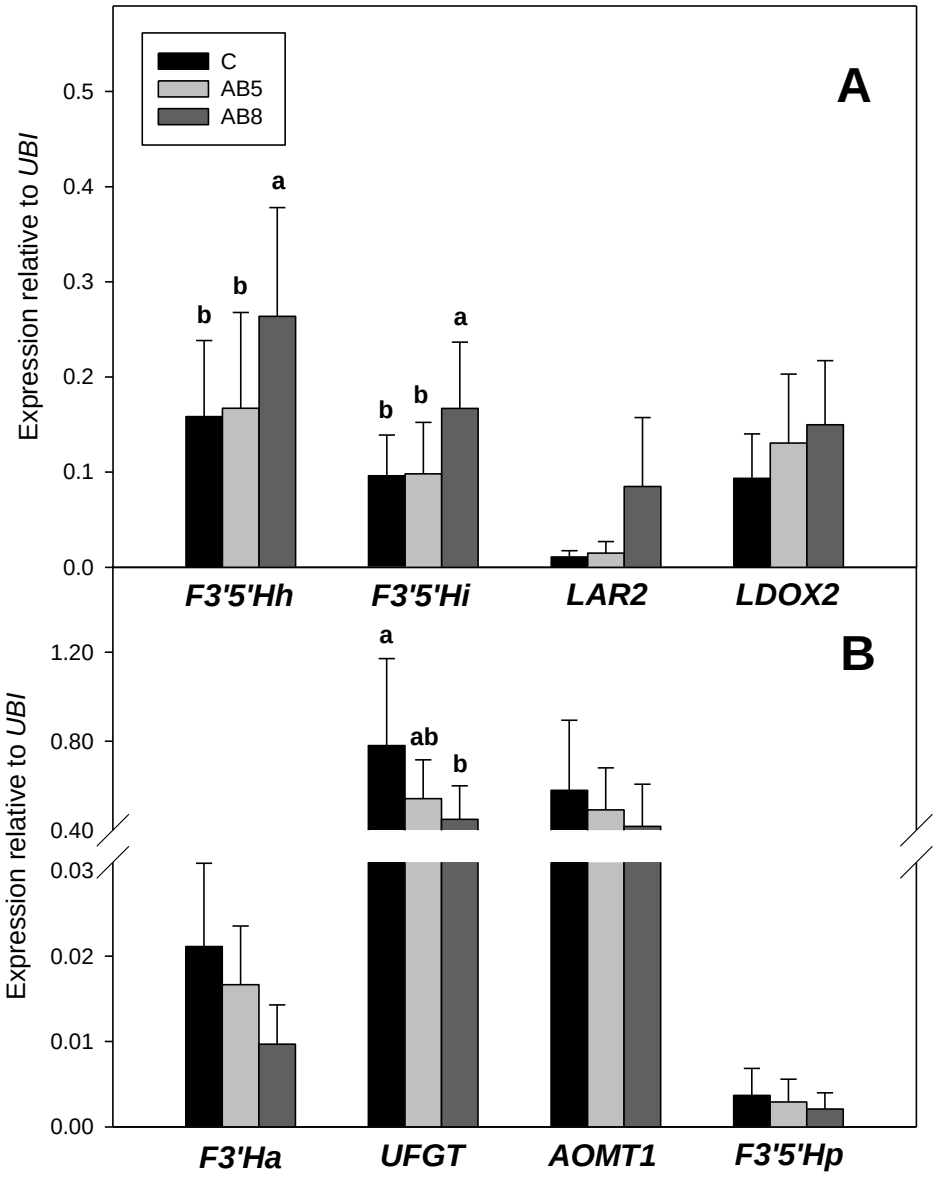

Figure 12. Expression of phenylpropanoid biosynthetic genes in Udine that A) increase with increasing light exposure (and decreasing source-sink balance), and B) decrease with increasing light exposure (increasing source-sink balance). Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. *, $p < 0.05$; **, $p < 0.01$. C, untreated control; AB5, removal of leaves from nodes 1-5 at after-bloom; AB8, removal of leaves from nodes 1-8 at after-bloom.

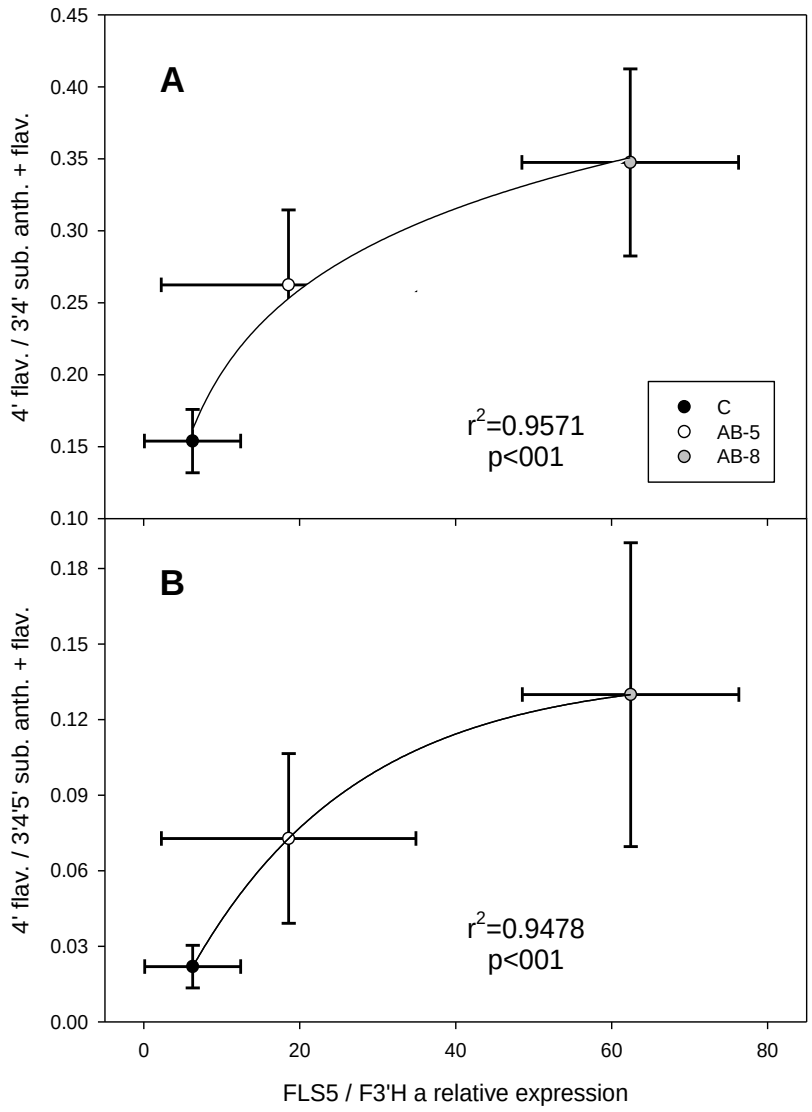

Figure 13. Relationship between the ratio of A) 4' to 3'4' and B) 4' to 3'4'5' substituted anthocyanins and flavonols at harvest in Udine and the ratio of the sum of flavonol synthase 5 (FLS5) and flavanone 3-hydroxylase a (F3'H a) expression during fruit maturation. Data were analyzed by one-way ANOVA with treatment as a fixed factor, and when the differences were statistically significant, means were separated with Tukey's HSD test ($p<0.05$). Different letters identify significantly different means at each timepoint. *, $p < 0.05$; **, $p < 0.01$. C, untreated control; AB5, removal of leaves from nodes 1-5 at after-bloom; AB8, removal of leaves from nodes 1-8 at after-bloom.

GENERAL CONCLUSIONS

The research outlined in this dissertation adds to body of knowledge on early leaf removal, and the potential of this practice to improve fruit quality while reducing bunch rot disease. The first chapter focused on understanding the role of early manual and mechanical leaf removal on fruit technological maturity and bunch rot disease in (*Vitis vinifera* L.) Pinot Grigio. The loss of fruit to gray mold was lowered by all leaf removal treatments in the drier 2017 season, but only manual treatments mitigated loss from sour rot in that year. This indicates that a clear fruit zone and reduced cluster compactness are both needed to lower the effect of cluster rot disease. Only pre-bloom treatments enhanced fruit quality, likely driven by a similar reduction in cluster compactness. The results suggest that mechanical removal at pre-bloom may be used to decrease fruit loss to gray mold in dry seasons and enhance fruit total soluble solids. Nevertheless, pre-bloom manual removal can be an effective means to reduce fruit loss to sour rot in drier seasons and enhance ripening in years with high precipitation during veraison. This information provides a single approach to alleviate two prominent issues facing seasonal management strategies in cool climate viticulture.

The third and fourth chapters of this dissertation studied the impact of early leaf removal on fruit quality in (*Vitis vinifera* L.) Merlot. Similar to the results from Pinot Grigio, berry sugar concentration was highest with the pre-bloom mechanical treatment (PB-ME). Furthermore, metabolomics analysis revealed that PB-ME favored the accumulation of significantly more disubstituted anthocyanins and flavonols and OH-substituted anthocyanins compared with manual application. Given that vine balance was similar between treatments, increased ripening with PB-ME is likely due to enhanced microclimate conditions and higher carbon partitioning through a younger canopy containing basal leaf fragments proximal to fruit. This information provides an important strategy for consistently ripening red *Vitis vinifera* cultivars in cool climates.

The fifth and sixth chapters aimed at elucidating the factors impacting improved phenylpropanoid concentration in Merlot fruit at harvest. Chapter five suggested that early leaf removal produced a younger, more photosynthetically active canopy during the ripening phase. Analysis of shoot leaf area at harvest revealed that while leaf removal treatments had less leaf area compared to the control in the bottom 50% of the shoot, a strong compensation in lateral leaf area led to no differences in total leaf area in the bottom quartile of the shoot. Instead, PB-ME had significantly less total leaf area in the zone of leaves above fruit (~nodes 6-10), leading to higher fruit temperature (+2.8°C) in this treatment during veraison. As a result, PB-ME produced significantly greater concentrations of phenolic acids, flavonols, flavan-3-ols, and anthocyanins, but not procyanidins in fruit skin. This work revealed that enhanced fruit-zone microclimate conditions, not ripening-related phytohormones, improved phenylpropanoid biosynthesis in vines subjected to early mechanical leaf removal. This research also suggests the importance of light interception angle to fruit in dictating phenylpropanoid biosynthesis. In coordination with this work, chapter six compared the influence of vine balance and light exposure on fruit quality parameters at two locations (Michigan, Italy). Primary metabolism was not significantly altered with the excepting of titratable acidity being decreased by LR-8 having the greatest light exposure during ripening. Flavonoid biosynthesis was significantly altered by light exposure, but not by vine balance. This was supported by RT-qPCR analysis of the expression of *UFGT*, *FAOMT1*/*2*, *F3'5'H*, and *3-AT*, which were enhanced at veraison with increasing light exposure. The results indicate that fruit exposure to light, rather than source-to-sink balance has a greater influence on flavonoid biosynthesis in grape berries.

Together, our studies highlight that pre-bloom mechanical leaf removal is an effective strategy to improve multiple aspects of fruit quality in both white and red wine grapes. This strategy was effective at enhancing total soluble solids across two growing seasons in both (*Vitis vinifera*) Pinot Grigio and Merlot, two cultivars with contrasting morphology and ripening characteristics. Additionally, in Merlot, phenylpropanoids such as anthocyanins and flavonols were increased in all three years of experimentation by pre-bloom mechanical leaf removal. From our last experiment, it is clear that the location of leaves removed plays a large role in improving fruit quality. The leaf area on nodes directly above the fruiting zone, rather than in front of fruit, were shown to negatively correlate with fruit temperature and anthocyanin concentration. This information provides greater detail for growers to improve fruit quality in cool climate viticulture regions.

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