AN ABSTRACT OF THE THESIS OF

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Title: Assessing the Impact of Temperature on Grape Phenolic Metabolism.

Abstract Approved:

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James A. Kennedy

Many climatic factors influence grape berry composition including nutrient

status, water availability, biotic stress, sun exposure and temperature. Previous

research examined the effects of many factors listed above and much progress has

been made. It is often difficult, however, to separate effects that typically confound

each other, such as sun exposure and temperature. Increasing exposure of a berry to

the sun will lead to some degree of heating unless the temperature is otherwise

maintained. In this study berry temperatures where manipulated independent of sun

exposure, necessarily separating the two effects.

The objective of this study was to assess the impact of fruit temperature on the

phenolic metabolism of grape berries (*Vitis vinifera* L. cv. Merlot) grown under field

conditions with controlled exposure to sunlight. While similar studies have focused

on production and accumulation of anthocyanins our primary focus was on

proanthocyanidins or 'tannins'. Here we report the effects of modulating daytime and

nighttime temperatures as well as damping the diurnal temperature range. Furthermore, research was broken into two phases: berry set to véraison (phase I) and véraison to commercial harvest (phase II). This was to assess the effects of treatments during two discrete phases of berry development characterized by accumulation of distinct phenolic metabolites.

Samples collected at véraison indicated that damping the diurnal temperature fluctuation advanced the onset of ripening. Those berries were larger (double-damped: 0.753 ± 0.015 vs control: 0.512 ± 0.034 g/berry) and more colored than all others. Phenolic material from grape seed and skin was quantified and characterized using three chromatography methods. Proanthocyanidin accumulation at véraison was linearly related to heat summation over the developmental period with nighttime heating yielding the highest concentration and daytime cooling yielding the lowest (night-heat: 1.46 ± 0.13 vs day-cool: 0.97 ± 0.09 mg/berry). Damping the diurnal temperature fluctuation reduced proanthocyanidin mDP (double-damped: 21.8 ± 1.0 vs control: 28.0 ± 1.7). Day-Cooling resulted in an increase in the concentration of flavonols at the end of phase I yet a decrease at the end of phase II. The goal of this work is to provide researchers with additional information regarding climatic factors influencing phenolic biosynthesis and to provide grape growers with tools to better manage their crop.

Assessing the Impact of Temperature on Grape Phenolic Metabolism

by
Seth D. Cohen

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I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Seth D. Cohen, Author

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CONTRIBUTION OF AUTHORS

Dr. Julie Tarara assisted with editing of the text in "Assessing the Impact of Temperature on Grape Phenolic Metabolism" and analysis of climate data associated with that research. Dr. James Kennedy assisted in the development of both documents.

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Introduction

Plant-based phenolic material plays numerous integral roles in nature. Plants utilize phenolic material to deter predators, attract pollinators and foragers that disseminate seeds, as well as for structural integrity. For animals, phenolics can be perceived as aromatic, bitter, astringent, and are thought to impact health both positively and negatively. Due to the importance in crop and food quality, there have been numerous studies on the effect of environment on the accumulation of phenolic material in plants. These studies include the effects of solar radiation, temperature, biotic stress, water and nutrient status. When viewed collectively, these studies exemplify the diverse roles phenolics play in nature and the variability of plant response among species. Information from these studies helps us understand the impact of growing conditions on crops and the potential to manipulate crop quality in the field.

For grape growers and winemakers, all characteristics of phenolics are important. In the vineyard, grape phenolics can aid in the protection of berries from fungal attack, sunburn and loss to predation. Once berries are harvested grape derived phenolics are an important component of finished wine. They afford protection from oxidation and contribute to the overall stability of wine during aging. Anthocyanins provide color to red wine while flavonols and flavanols can aid in stabilizing anthocyanin based pigments thorough co-pigmentation. Flavanols can polymerize into 'tannins', which provide both bitterness and astringency to wine. Smaller tannins can be bitter where larger tannin polymers are associated with astringency and 'mouthfeel' or 'texture' of wine. For all of these reasons phenolics

have been an important focus for scientific research in grapes and wine as well as many other food products.

In this study we assess the impact of temperature on phenolic metabolism in grapes ('Merlot') growing in an established vineyard. The effect of sunlight exposure and all foreseeable confounding factors were controlled for as to isolate temperature as a single variable. Climate control was achieved by monitoring *in situ* berry temperatures and making adjustments by heating and cooling clusters. Berries were analyzed for phenolic content and composition using three chromatography methods. Data regarding phenolic content of seeds and skins were considered with respect to climate data. Our goal was to determine the effects of cooling, heating and damping the diurnal temperature fluctuation in grape berries. Data presented here should provide insight into response of grapevines to temperature with regard to the accumulation of proanthocyanindins in grape berries. This should be useful to grape growers as well as those studying phenolic metabolism in grapes and other plant species.

Plant Metabolism and the Environment: Implications

for Managing Phenolics

Seth D. Cohen and James A. Kennedy

Abstract

The presence of phenolic compounds in food has garnered much attention over the years. The diversity of these compounds and their contribution to nutritive and sensory qualities has been investigated in depth to elucidate benefits and modes of action. This review is intended to provide information regarding the impact growing conditions have on accumulation of phenolics in plants. The aim is to provide knowledge of factors that influence phenolic biosynthesis in plants to help us manage them in the field. Through improving our understanding of plant responses to the environment, we may be able to manipulate growing parameters to suit the needs of the consumer. Research regarding plant responses to climate, farming practices, biotic and abiotic stresses will be presented here.

KEYWORDS: Plant Phenolics; Management; Preharvest; Development; Metabolism; Environment

Introduction

Product development is an important area of the food industry and ingredient sourcing and characterization are integral parts of this process. This review will focus on the significance of plant phenolics in food while making the food scientist aware that the management of phenolic compounds in foods begins prior to harvest. Understanding phenolic metabolism in plants is the first step in managing plant phenolics, regardless of the target variable (e.g. enhancing or reducing phenolic content).

Phenolics are essentially ubiquitous in the plant kingdom and serve a number of purposes. Likewise, phenolics are ubiquitous in the foods we eat. From a food science perspective, our concern regarding plant phenolics encompassess the realms of sensory, food stability and food functionality. Some of the more obvious and historically desirable aspects of phenolics relate to sensory perception. They provide the blue color to blueberries and the red to blood-oranges (Clifford, 2000). Phenolics contribute bitterness to grapefruits, hops and chocolate (Drewnowski, 2000; Lesschaeve, 2005). The astringency of unripe persimmon and the texture of red wine are both the result of plant phenolics (Joslyn, 1964; Gawel, 1998). Even honey and dairy products owe a portion of their unique aromas to volatile phenolics (Gil, 1995; O'Connel, 2001).

During plant growth, phenolic compounds also protect fruits and vegetables from microbial damage, Ultraviolet (UV) radiation, and predation among other environmental stresses (Swain, 1975; Stumpf and Conn, 1981; Bennett and Wallsgrove, 1994; Robards et al., 1997, 1999; Parr and Bolwell, 2000). Plant

phenolics are also thought to play roles in human health (Middleton and Kandaswami, 1994; Rice-Evans, 2001; Grotewold, 2006). A litany of literature reviews on the structure, function (in plants and animals), synthesis, and bio-availability reflect the interest and impact phenolics have had on the scientific community (Dixon and Paiva, 1995; Robards et al., 1997, 1999; Hammerschmidt, 1999; Parr and Bolwell, 2000; Winkel-Shirley, 2002). Historically, reviews have focused on the structure/function relationships, metabolism and evidence of health impacts. To date, there is less in review regarding cultural practices or field manipulation to affect yield of target plant phenolics though a wealth of research exists. Given the known activities of phenolics (both for plants and humans) and considering evidence of biological activity, it should be clear why managing phenolic development in plants is important from a food science perspective.

Phenolic Compounds

Phenolics are compounds that possess a benzene ring bearing a hydroxyl substituent, including functional derivatives (esters, methyl esters, glycosides, etc.) (Harborne, 1989). The simplest example is phenol (Fig.1.1.). The phenylpropanoid, consisting of a C6-C3-C6 conformation is another example (Fig.1.1., flavonoid backbone). To appreciate the diversity within the family of phenolic compounds the reader is referred to sources dealing with chemical structure (Robards and Antolovich, 1997; Khanbabaee and van Ree, 2001; Grotewold, 2006). Additional literature exists on distribution in nature, reactivity and bioavailability (Parr and

Bolwell, 2000; Rice-Evans, 2001; Pellegrini et al., 2003; Xie and Dixon, 2005;

Grotewold, 2006).

Within this paper a number of phenolic compounds will be discussed that are primarily phenylpropanoid in nature. This infers their biosynthesis stems from phenylalanine and continues down a complex biosynthetic path. The phenolic acids (Fig 1.1. e.g. ellagic acid, salicylic acid, coumaric acid) are simple phenolic compounds possessing a carboxyl group and are often precursors to a host of other compounds. Lignin is composed of derivatives from phenolic acids such as coumaryl, sinapic and coniferyl alcohol (Monties, 1989). Stilbenes, such as resveratrol, are characterized by having two benzene rings linked by ethane or an ethane bridge (Gorham, 1989). Examples of flavonols, anthocyanins and flavan-3-ols are given in Figure 1.1. and show the common structure of the flavonoid class of compounds. These are C6-C3-C6 structures that can polymerize into proanthocyanidins (tannins) or other polymers (Fig. 1.1.). Often, the reactivity of the compound determines its propensity to polymerize (generally oxidative in nature) and can be an important aspect in many food applications. It is also common to find sugar moieties (such as glucose) attached to flavonoids, which alters their stability, reactivity and functionality (e.g. anthocyanin, Fig. 1.1.). The composition and variability of phenolic compounds is far more complex than presented here, this is only intended to familiarize the reader with the class of compounds in discussion. Familiarity with chemical structure and functionality will certainly make the information presented here more coherent and may provide explanation to observations not provided by the authors.

Biosynthesis

As mentioned above, the majority of compounds discussed here stem from a common precursor; L-phenylalanine. Synthesis of phenylalanine begins with erythrose-4-phosphate (pentose phosphate pathway) and phosphoenolpyruvate (glycolysis) entering into the Shikimate metabolic pathway (Strack, 1997). From there, metabolites can yield simple phenolics (phenol, benzoic acids, chlorogenic acid etc.) or proceed to either L-tryptophan or L-arogenate. The metabolism of L-arogenate can yield L-tyrosine or L-phenylalanine, the latter of which is the main building block for plant phenolics or phenylpropanoids. It should be noted that this pathway is utilized for protein synthesis and is theorized to compete with phenolic biosynthesis under some circumstances (Haukioja et al., 1998). Phenylalanine proceeds through the phenylpropanoid pathway to yield cinnamic acids (and derivatives), which can be further modified to produce hydroxycinnamoyl CoA's (e.g. p-coumaryl CoA). The flavonoid class of phenolic compounds, characterized by the C6-C3-C6 backbone, result from condensation reactions between hydroxycinnamoyl CoA's and malonyl-CoA's (Krebs cycle). A generalized biosynthetic pathway to flavonoid synthesis is shown in Figure 1.2. with some key enzymes present. There are many branch-points during this synthetic pathway, all leading to different compounds with different functions in the plant. Some of these will be discussed here. Although, as with structure, there are numerous literature sources providing much more discussion and depth (Heller and Forkmann, 1994; Dixon and Paiva, 1995; Koes et al., 1994; Marles et al., 2003; Xie and Dixon, 2005).

Plants and Environment

As growers and producers it is important to understand how plants respond to their environment. Here we will examine common environmental factors that influence phenolic development in plants. For the sake of simplification, these will include cultivar and seasonal variability, sunlight and temperature, irrigation/water relations, pests/microbial stress and fertilization/nutrient status.

Cultivar and Seasonal Variability

Before examining direct environmental influences it is important to note the variation in phenolic development due to season and cultivar. In nearly all multi-year studies considerable variation exists in phenolic development and accumulation. The same can be said for multi-cultivar studies of the same species. Evidence in this review will exhibit this type of variation, which is important to consider especially when conducting agricultural experiments. (Hartley et al., 2000; Esteban et al., 2001; Spayd et al., 2002; Bennett et al., 2004; Downey et al., 2004; Giorgi et al., 2005)

Solar Effects

Sun exposure is credited with being a major element relating to plant metabolism (Kliewer and Lider, 1968; Winkler et al, 1974; Coombe, 1987; Jackson and Lombard, 1993). Literature suggests these light related changes are generally hormonal in nature (Zucker et al., 1967). It was also found that phenylalanine ammonia lyase (PAL), an important enzyme in chlorogenic acid biosynthesis, was

inducible by white light (Zucker, 1965). This could be considered one of the first large steps in understanding the influence of light on phenolic metabolism.

A major challenge studying sun exposure and plants is the dual effect of UV radiation. As the amount of sun exposure increases there is a concomitant rise in temperature (Kliewer and Lider, 1968; Smart, 1985; Bergqvist et al., 2001; Spayd et al., 2002). This phenomenon makes it difficult to assess whether physiological changes are related UV exposure or temperature variations.   Due to radiant heating from the sun, critical temperature thresholds can be breached, representing confounding factors when trying to understand developmental changes in plants.

Over the years researchers have devised methods to better understand and separate these confounding effects. One way is to use controlled-climate glass-houses or growth chambers (Kliewer and Torres, 1972; Kliewer, 1977; Li et al., 1993; Pinto et al., 1999; Wang et al., 2001; Bradfield and Stamp, 2004; Mori et al., 2005; Burchard et al., 2000). A second approach has been the placement of physical barriers in the field to screen out one factor, generally sunlight. In some cases, UV absorbing/reflecting synthetic material is used (Mazza et al., 2000; Zavala et al., 2001; Bureau et al., 2000a, 2000b; Kolb et al, 2001; Spayd et al., 2002; Downey et al, 2004). In other cases, natural shading techniques have been implemented (Price et al., 1995; Bureau et al., 2000a, 2000b; Bergqvist et al., 2001; Spayd et al, 2002). In a novel approach, a method allowing for temperature control of plant parts within a field environment was developed (Tarara et al., 2000; Spayd et al., 2002). The benefit of this is affording manipulation of temperature and shading treatments independent of each other while working plant material in a 'native' environment.

This is arguably a first approach at effectively separating light and temperature effects in a field environment.

UV

In a 2-year vineyard study Spayd et al. (2002) imposed UV barriers over the canopy and fruiting zone. Flavonols (glucosides of quercetin, myricetin and kaempferol) all showed large significant increases with sun exposure. From their study it was determined that sunlight increased accumulation of total skin monomeric anthocyanins (TSMA) and flavonols. Sun exposure resulting in excessive berry temperatures inhibited anthocyanin production and may have contributed to degradation. UV exposure, while not requisite, is found to be significant in the accumulation of flavonols such as quercetin-3-glucoside. Use of UV barriers did not effectively reduce the TSMA concentration but did significantly reduce accumulation of all flavonols monitored.

Alenius et al. (1995) examined the relationship between UV- B radiation and production of chlorophyll and UV screening metabolites (quercetin, kaempferol and hydroxycinnamic acid derivatives) in *Brassica napus* L. cv. Ceres (rape or canola). Increasing UV-B exposure elevated both chlorophyll and screening compounds. It was also noted that increased UV-B exposure dramatically increased the ratio of quercetin to kaempferol suggesting quercetin is a preferred metabolite for UV defense.

In a mutant barley strain (*Hordeum vulgare* L.), UV radiation was correlated with an overall decline in plant status (leaf weight, rigidity, vigor) and a lower

accumulation of flavones and HCAs (Reuber et al., 1996). In a field grown 'non-mutant' barley strain, UV radiation led to an increase in HCAs and two main flavones saponarin (26%) and lutonarin (500%). This increase is in accord with Mazza et al. (1999) and Alenius et al. (1995). With a decrease in UV screening compounds, the mutant strain showed higher light penetration (310nm) into the ad axial leaf tissue and lower quantum yield (photosynthetic).

In a separate experiment, exposing barley plants to UV-B radiation simulating 5% and 25% ozone depletion resulted in dramatic increases in rate and total accumulation of the barley polyphenols, saponarin and lutonarin (Liu et al., 1995a, 1995b). UV-A exposure resulted in smaller increases in both phenolic compounds. Soluble and insoluble ferulic acid declined in final concentration with response to UV-B exposure. Examination of enzymes related to phenolic metabolism showed similar initial PAL activity in all treatments and higher sustained levels with increasing irradiation levels. Chalcone-flavanone Isomerase (CFI) levels did not differ with exposure levels while peroxidase (involved in phenolic degradation) activity was lowest under UV-B and highest under UV-A exposure. It is suggested that prolonged PAL activity from UV-B exposure contributes to the increased accumulation of phenolics while decreased peroxidase activity is *not* an indicator of reduced degradation.

UV-B exposure has also been shown to increase flavonoids and cinnamoyl esters in the primary leaves of rye seedlings (*Secale cereale* L. var. Kustro) (Tevini et al., 1991). In this case phenolic, accumulation correlated with an increase in photosynthetic activity. Booij-James et al. (2000) found that Arabidopsis plants

capable of synthesizing some sinapic acid derivatives were afforded protection and lower levels of protein damage compared to mutant lines deficient in this ability. From these and other studies it is clear that with UV exposure, plant phenolics can positively affect plant photosynthesis and assimilation of carbon required for growth and development (Tevini et al., 1991; Alenius et al., 1995; Pinto et al., 1999; Booij-James et al., 2000).

Temperature

In a greenhouse study, phenolics increased when strawberries (*Fragaria x ananassa* Duch.) were subjected to elevated temperature regimes (Wang et al., 2001). As seen in species of *Vitis*, temperature can be positively correlated to phenolic accumulation (e.g. anthocyanins) but breaching a critical limit (high and low) has detrimental effects (Kliewer and Torres, 1972; Spayd et al., 2002; Bradfield and Stamp, 2004). It is likely that all plants exhibit this temperature threshold that relates largely to the environment they in which evolved.

Based upon global warming models and their predicted affects, Bradfield and Stamp (2004) studied the effect of increasing night temperatures on the production of phenolic compounds in tomato (*Lycopersicon esculentum* Mill.*)*. Overall total plant phenolics showed no effect due to variations in nighttime temperature. However, when each phenolic compound was monitored in different plant parts (root, stem and leaf) trends were present. Catecholic phenolics showed a peak in leaf material at 17°C, a minimum in stem material at 18°C and downward trend in roots with increasing temperature. Chlorogenic acid increased in the leaf with temperature,

peaked in stems at 17°C (similar to catecholic phenolics) and was relatively stable in roots.

Rosmarinic acid, a polyphenol found in Spearmint (*Mentha spicita* L.), has known antioxidant activity and many other biological functions. The concentration in spearmint leaves, which can account for nearly 30% dry weight, was shown to decrease over time under heat stress conditions (Fletcher et al., 2005). The same trend was shown in total antioxidant capacity with 5 of 7 clones responding negatively to heat. It was shown that a temperature of 30°C can have a detrimental effect on phenolic accumulation in spearmint leaves which correlates strongly to their antioxidant capacity. The authors hypothesized that the production of prenylquinones gave the plant protection from reactive oxygen species thereby diverting products from the rosmarinic acid pathway (Fletcher et al., 2005). Results from grape studies also indicate that high night temperatures and high constant temperatures (30°C) will impede phenolic synthesis (Kliewer and Torres, 1972; Mori et al., 2005; Yamane et al., 2006).

In a series of experiments studying grape anthocyanins and flavonols response to temperature, plants grown under higher temperatures (30-35°C) yielded significantly lower anthocyanin concentrations (Mori et al., 2005). It was also found that plants grown at a constant 30°C accumulated less anthocyanins than those grown at 30°C/15°C (day/night). Temperature did not have an effect on flavonol accumulation. Phenylalanine ammonia-lyase (PAL) activity was similar approaching 30 days post-veraison, after which activity in high temperature berries showed a dramatic decline over cooler temperatures. UDP glucose flavonoid-

glucosyltransferase (UFGT) activity was lower at 15 days post-véraison and remained significantly lower through 45 days post-véraison. While PAL activity did not correlate strongly with anthocyanin accumulation, UFGT activity did correlate strongly. At véraison, expression levels of all genes monitored were higher with cooler night temperatures. At 15 days post véraison, only UFGT expression was significantly higher in cooler night temperatures. These results suggest UFGT activity is the most import parameter relating to anthocyanin accumulation and is most sensitive to temperature variations shortly after véraison.

A similar study was conducted where temperature treatments were applied at various stages of grape berry development (Yamane et al., 2006). In this study mRNA and abscisic acid levels were monitored in conjunction with anthocyanins. It was found that a two week 20°C growing temperature resulted in higher anthocyanins than 30°C during growing stages II-IV, in agreement with previous data (Kliewer and Torres, 1972; Spayd et al., 2002; Mori et al., 2005). Furthermore, temperature variation during stage III (1-3 weeks post veraison) had the largest effect on anthocyanin accumulation. Holding the temperature at 20°C resulted in a doubling in anthocyanin concentration while 30°C resulted in considerable (2-3 fold) reduction at harvest. Results from the study show that intermittent 2-week periods of 30°C temperatures generally inhibit accumulation of anthocyanins in grape berries, regardless of developmental stage. Two-week periods of cooler (20°C) temperatures increase accumulation and are most affective during stage III of development. Abscisic acid values were also higher after 20°C treatments in stage III and lower after 30°C, supporting a positive correlation with anthocyanin accumulation. In all

cases, mRNA values were lowest in 30°C treatments after stage III. Higher temperatures as explained by the authors, likely resulted in inhibition *and* degradation of anthocyanins as well as possibly diverting substrate into competitive reactions (Fletcher et al., 2005). It is clear that high temperatures can impede accumulation of anthocyanins in some fruit via reduction in synthesis, degradation or competitive inhibition (Kliewer and Lider, 1968; Spayd et al., 2002).

Temperature stress was investigated in tomato and watermelon by evaluating shoot dry weight, accumulation of phenolics and enzyme activity (Rivero et al., 2001) Tomato plants at 35°C showed signs of heat stress and had the highest levels of phenolics, PAL activity and the lowest oxidase activity. The data suggests tomato plants have an optimal growth temperature of ca. 25°C. Watermelon had an optimal growth temperature of 35°C with lower PAL and phenolic concentrations. Oxidase activity and shoot weight decreased with decreasing growth temperatures resulting in higher total phenolic concentrations and concomitant higher PAL activity. It would appear that plants encourage the accumulation of phenolic material at the expense of accumulating plant material (dry weight). In this case, heat and cold stress can negatively affect plant yield or productivity but may also result in plants being more resistant to some environmental stresses.

Exposure

In two independent studies the effect of sun exposure on grape berries (*Vitis vinifera L.*) was investigated (Crippen and Morrison., 1986; Bergqvist et al., 2001). In the first, it was found that berries from exposed clusters were significantly heavier

at harvest than from shaded clusters. Sun exposed berries had higher total soluble

phenols *per berry* but there was no difference in terms of concentration (by weight).

Anthocyanin concentration was higher in exposed berries from their inception close

to harvest date on both a *per berr*y and on a weight basis. They were found to be not

different at harvest on a *per berry* basis and actually decreased significantly on a

weight basis. Polymerized polyphenol concentrations were found to be higher in

shaded berries through most of development and at harvest. This is slightly

contradictory to work done by Price et al. (1995) where cv. Pinot noir showed higher

polymerized phenolics in exposed berries. As the authors point out, this study shows

the impact berry size has on concentration. Results suggest that on a *per berry* basis

accumulation of total phenols and anthocyanins occurs faster in exposed fruit. On a

weight basis, total phenols do not appear to differ while anthocyanins still are

significantly higher in exposed berries, until the last 2 weeks of development. In any

case, there is clearly a reduction in anthocyanin concentration on a weight basis late

in berry development.

In the second study two grape varieties (Cabernet Sauvignon (CS) and

Grenache (G)) were harvested from one block in a vineyard (Bergqvist et al., 2001).

Clusters were harvested based on: i) exposure levels from 'full exposure' to 'shaded'

and ii) temperature. Berries on the North facing canopy were generally cooler than

those facing South. As found by others (Kliewer and Lider, 1968; Smart, 1985),

berry temperature increased with photosynthetically active radiation (PAR). In both

varieties berries from a northern aspect showed a positive, linear correlation between

phenolic accumulation (anthocyanins and total phenolics) and PAR. Phenolic

concentration in Northerly oriented berries was also higher than south facing berries in nearly all cases. South facing berries showed a maximum in phenolic accumulation occurring between a PAR of 50 and 200 (μmol m^{-2} sec^{-1}) followed by a decline (or decline in rate). While the total concentration of phenolics is generally higher in CS, the response to exposure was shown to be nearly identical between varieties. The results imply that excessive solar radiation (>200 µmol m^{-2} sec^{-1}) coupled with concomitant high temperatures can significantly reduce phenolic accumulation in berries.

In a 3-year study on grapes, Downey et al. (2004) deployed light exclusion cluster boxes. There was seasonal variability over the course of study though several trends emerged independent of year. In year one, berry weight was found to be higher in exposed clusters while years two and three showed little treatment effect. As expected, chlorophyll amounts were significantly lower in shaded fruit. While anthocyanin accumulation was shown to be slightly higher in shaded fruit on a weight basis, it was slightly lower than exposed fruit on a *per berry* basis. In years two and three, anthocyanin accumulation was generally higher in exposed fruit. Overall, year two yielded berries with the lowest concentration of anthocyanins, which may be attributed to higher mean temperatures over the course of the experiment. One consistent trend over three years was an increase in peonidin and cyanidin over malvidin, petunidin and delphinidin due to shading. This is seen as an increase in anthocyanins with B ring di-substitution compared to tri-substitution. The higher temperatures in year two were thought to increase the amount of coumaroyl glucosides, possibly due to higher stability or resistance to degradation. These same

effects were seen when comparing substitution patterns of tannin (proanthocyanidin) extension units; shaded treatments favoring di-substitution. In year one the rate and maximum level of tannin accumulation was generally higher *per berry* in exposed fruit though final content was similar. It also was found that skin and seed tannins had a higher mean degree of polymerization (mDP, polymer length) coming from exposed fruit. Similar results have been observed in another study (Cortell et al., 2005). This confirms that sunlight or exposure can have an effect on phenolic chemistry (polymer size and substitution patterns) (Crippen and Morrison, 1986; Price et al., 1995; Reuber et al., 1996). In agreement with other studies, light exclusion significantly reduced or inhibited flavonol accumulation in grape skins and tissue (Price et al., 1995; Spayd et al., 2002).

Work done by Price et al. (1995) investigated the effect of natural canopy shading on phenolic development in Pinot noir berries (*Vitis vinifera*). Analysis of skin disk extracts showed little difference in anthocyanins but nearly 10-fold increase in flavonols (quercetin glycosides) and resveratrol with exposure. Wine analysis showed higher total phenolics, monomeric anthocyanins, caffeic acid, quercetin, polymeric phenolics and anthocyanins from exposed fruit. Shaded fruit had higher concentrations of caftaric acid and catechin only.

Biotic Stress

It is possible that phenolic compounds have been selected as a result of predatory pressure as many are shown to be an effective form of defense (Herms et al., 1992; Bennett and Wallsgrove, 1994; Matsuki, 1996; Close and McArthur, 2002).

Furthermore, research has identified many metabolites that are inducible by biotic pressures. There are a number of resources discussing the evolution and beneficial functions of plant phenolics (Levin, 1971; Koes et al., 1994; Matsuki, 1996; Robards et al., 1997; Hammerschmidt, 1999; Parr and Bolwell, 2000; Close and McArthur, 2002). While there is agreement as to the efficacy of phenolic compounds, there is still uncertainty as to response factors. It is useful to mention the difference between two types of resistance; constitutive and induced. Constitutive resistance is attributed to compounds present prior to infection or stress while induced resistance is a *response* to a pressure (Levin, 1971). Antimicrobial compounds produced via induced resistance are often called phytoalexins and have been investigated for some time (Hammerschmidt, 1999; Parr and Bolwell, 2000). As suggested by Hammerschmidt (1999) there are a series of criteria that are helpful in evaluating the utility of plant phytoalexins or other defense chemistry.

Wheat infected with wheat streak mosaic virus was found to accumulate higher amounts (1.5 fold) of phenolics and different compounds than non-infected plants (Kofalvi et al., 1995). Here the infected plants accumulated cinnamyl alcohols suggesting that the plants are producing lignin in response to infection. This hypothesis is supported by greater cinnamyl alcohol dehydrogenase (CAD) activity in infected plants while PAL and TAL (tyrosine ammonia-lyase) activity are similar between groups. Quantification of lignin, however, shows no difference attributed to infection and is supported by a decrease in peroxidase (POD) activity (involved in lignin polymerization) in infected plants. From this study it is clear that wheat

responds to viral attack by increasing phenolic accumulation but the exact defense mechanism is not known.

Lauvergeat et al., (2001) identified two genes in *Arabidopsis thaliana* L. responsible for encoding cinnamoyl-CoA reductase (CCR), an enzyme involved in synthesizing lignin precursors. Of the two genes, *At*CCR1 is involved in lignin synthesis during development while *At*CCR2 is primarily induced in response to a pathogen and not involved in lignin synthesis. Following inoculation with *Xanthomonas campestris* pv. c*ampestris*, *At*CCR1 transcription level was not induced but *At*CCR2 accumulated in infected leaves. The authors suggest that lignification is a late response to infection whereas *At*CCR2 activity is likely associated with rapid production of phenolics with phytoalexin activity or to strengthen the cell wall as supported by others (Barber et al., 2000; de Ascensao and Dubery, 2003). Applying this theory to wheat, it would appear that increased phenolic accumulation and CAD activity were intended to combat infection although not by synthesis of lignin *per se*.

Evaluation of banana roots (*Musa acuminata)* infected with an elicitor from a common pathogen yielded similar results as presented above (de Ascensao and Dubery, 2003). The authors reinforce the idea that cell-wall-bound esters (e.g. esterified cinnamic acids) may be synthesized rapidly compared to lignin and have improved efficacy as antibacterial agents or enhancing cell-wall integrity (Barber et al., 2000; Lauvergeat et al., 2001; de Ascensao and Dubery, 2003).

Response by grape berries infected with powdery mildew (*Uncinula nectar*) was found to include induction of phenolic metabolism but only as a secondary response (Ficke et al., 2004). It was found that within 24 hours post-infection older

plants had an ontogenic resistance to the pathogen which was effective before induction of phenolic metabolism. Phenolic response was found to be highest in younger, more susceptible plants suggesting it is not the cause of ontogenic resistance in the older, resistant plants. It is thought that phenolics play a role reducing the survival of hyphae and may prevent subsequent disease but are not a primary agent in inoculation against the pathogen.

For *Vitis vinifera*, and many other plant species, *Botrytis cinerea* is a common fungus which can cause damage. In winemaking, infection from this pathogen is known as 'noble-rot' and is responsible for the production of many dessert wines (e.g. Sauternes). When unwanted, this pathogen can render fruit unfit for wine production. In response to fungal infection, various phenolic compounds (e.g stilbenes) can be produced. Resveratrol, a specific stilbene, has garnered much attention as a therapeutic agent following presentation of the French paradox, encouraging investigation into other crops that may yield significant quantities (Renaud and Lorgeril, 1992; Kopp, 1998; Sun et al., 2002; Renaud et al., 2004; Rimando et al., 2004). It was determined that resveratrol production responded positively to UV radiation and was considerably higher in leaves than fruit, where it is localized in the skin (Jeandet et al., 1991; Jeandet et al., 1995a; Douillet-Breuil et al., 1999). One survey of French wines from a 12 year span was intended to correlate Resveratrol levels to fungal pressures during vintage (Jeandet et al, 1995a). It was found that years of high fungal pressure resulted in lower resveratrol levels in wine. It is noted that resveratrol was found in wines over 12 years old, implying chemical stability. The rationale for this discovery is that the fungus produces an exo-enzyme (laccase-

type) capable of oxidizing resveratrol in self-defense. This rationale is supported by evidence of Landrault et al. that shows general temporal increases in stilbenes in response to infection followed by a decline towards maturity (Landrault et al., 2002). Analysis of grapes exposed to *Botrytis cinerea* shows accumulation of resveratrol in areas surrounding fungal infection suggesting a localized effect of the antimicrobial compound (Jeandet et al., 1995b).

While many bacteria are problematic for plant growth there are examples of symbiotic relationships between plants and bacteria (Mishra et al., 2006). In one case, rhizobia were effective in inducing accumulation of phenolics in rice (*Oryza sativa* L.). *Rhizoctonia solani* is responsible for causing sheath blight in rice and significantly reducing yields. Phenolic acids are the major phenolics in rice and are thought to inhibit infection through their protein-binding capacity. In rice, rhizobium-inoculated plants infected with *Rhizoctonia solani* show an even greater induction of phenolic accumulation. By colonizing in the root interiors of the plant, rhizobia are effective in stimulating plant defenses and overall plant growth. A similar situation was observed when betelvine (*Piper betel* L.) was inoculated with a rhizobium (Lavania et al., 2006). However, when non-inoculated plants were infected with *Phytophthora nicotianae* there was a marked decrease in phenolic acids, plant status (length and weights) and enzyme activity implicated in plant defense (PAL, POD, PPO). The results suggest chlorogenic acid, which always increased in response to infection or inoculation, plays a crucial role in induced defense. In this case the rhizobium and pathogen not only induced changes in metabolic rates but in end-product synthesis as well.

Leaves of the tea plant, *Camellia sinensis* L., are a rich source of phenolic compounds and have a history of human health benefits (McKay and Blumberg, 2002; Hernandez et al., 2006). Punyasiri et al. (2005) studied the effect of infection on tea plants to determine the roles the chemicals have in plant defense. Cultivars resistant to *Exobasidium vexans* tended to be higher in (-)-epicatechin (EC) and lower in epigallocatechin gallate (EGCG) than susceptible cultivars. EC and epigallocatechin (EGC) both decreased significantly upon infection while epicatechin gallate (ECG) increased. The increase in ECG could result from esterification of EC with gallic acid, having higher antibacterial, antiviral and antioxidant capacity (Punyasiri et al., 2005). The authors also report the formation of proanthocyanidins (tannins) upon infection. In turn, the variety with highest resistance was found be high in anthocyanins.

Common plant hormones or growth substances such as gibberellic acid (GA) and ethylene can have varied effects on phenolic metabolism (McClure, 1975). In some plants GA promotes anthocyanin accumulation where in others it is inhibitory. In fact, some phenolic compounds act as signaling compounds and can affect plant metabolism (Parr and Bolwell, 2000). In *Arabidopsis* salicylic acid induced transcription of the *At*CCR2 enzyme and accumulation of related defense phenolics (Lauvergeat et al., 2001). Likewise, infection of cucumber (*Cucumis sativus* L.) with a *Pseudomonas* pathogen resulted in accumulation of salicylic acid as a signaling compound for defense (Rasmussen et al., 1991). The authors note that salicylic acid was not the translocated signal but was important in inducing systematic resistance and peroxidase activity.

Water Stress

Irrigation is a management tool used in plant production in arid and semi-arid parts of the world and has also been found to influence phenolic production. Wine grape irrigation is achieved by a number of methods including furrow or basin, sprinkler and drip-type systems (Winkler et al., 1974). As an increasing popular system, drip irrigation is intended to supply water to the root system with minimal evaporative loss and high efficiency (Gladstones, 1992). Santos et al. (2005) reports on the effects of partial root-zone drying (PRD) irrigation compared to fully irrigated (FI) and deficit-irrigated (DI) systems. PRD grown berries were intermediate in weight although total yield parameters were not significantly different between FI, PRD and DI. PRD grown berries were also found to yield the highest concentration of anthocyanins and total phenolics as well as significantly higher water use efficiency over FI. While the greater size of FI berries may account for some concentration differences, they do not account for all. As the authors point out, there was more vegetative growth associated with FI vines resulting in higher light interception and lower photosynthetic light flux. They suggest this (increased cluster exposure) may be one of the more profound factors related to phenolic accumulation, a point iterated by others (Gladstones, 1992; Ginestar et al., 1998; Santos et al., 2005).

In work by Roby et al. (2004a, 2004b), irrigation treatments were imposed when vines experienced roughly -1.0 MPa mid-day leaf water potential; control (C) and high (H), twice the volume of C. A low (L) irrigated treatment delivered the same rate of water as C once the water potential reached -1.5 MPa. In two years of

data, berry weight was the same for C and H and always significantly lower for L

berries. The group compared berries of the same size to reduce the effect of dilution.

It was found that skin tannin and anthocyanin concentrations were nearly always

higher in L and lowest in H berries. Seed tannin concentration was generally

unaffected by irrigation.

In another study on *Vitis vinifera*, Esteban et al. (2001) studied cv.

Tempranillo in a two year study in which a non-irrigated (*NI*) treatment was

compared to an irrigated (*I*) treatment intended to replace water lost to

evapotranspiration. In both years *NI* berries had a higher concentration of total

phenolics evaluated on a *per gram* basis but on a *per berry* basis the opposite was

true. For anthocyanins, the first year showed significantly higher *per gram*

concentrations for *NI* until harvest, at which point the difference was not significant.

Per berry concentrations were slightly higher in *I* berries until harvest, at which point

the difference became significantly larger. In year two, anthocyanins were slightly

elevated in *I* berries on a *per gram* basis and significantly higher on a *per berry* basis,

indicating irrigation led to greater accumulation of anthocyanins in year two,

independent of berry size. Quantification of total tannins showed a similar trend with

NI treatments yielding higher concentrations on *per gram* basis, differences that were

significant at nearly all points in both years. Tannin values on a *per gram* basis

favored *I* treatment although the difference was only significant in year two from

mid-season to harvest. Analysis of individual anthocyanins showed variability in

rates of accumulation, by harvest *I* treatments resulted in the highest concentration in

every case. This difference was found on both a *per gram* and *per berry* basis and

was statistically significant in most cases. Skin from *NI* treatment tended to be more concentrated in tannins while anthocyanins were favored by the *I* treatment, at least by harvest date. As discussed by the author, some differences are likely related to berry size (concentration/dilution) although it is not the only factor involved. This is an important issue to consider when evaluating these and other studies as there lies an important distinction between concentration and biosynthetic responses to environment (Deloire et al., 2004).

Sivilotti et al. (2005) examined the effects of various water stresses ranging from (C) 80% of available water (aw), (M) 30% aw to (S) 15% aw. They also investigated the efficiency of various solvents in extracting phenolic material from grapes; an aqueous tartaric acid solution (TB), EtOH (only solvent in year 2) and MeOH. On a weight basis, total seed polyphenols were significantly higher in S berries using MeOH as a solvent. In year one, EtOH seed extracts from C were highest, in year two seed extracts from M were highest. Year one total skin polyphenols (weight basis) where highest in C following TB and EtOH extraction while MeOH extracted the most from S berries. In year two, highest skin polyphenols were found in S berries. Anthocyanins were highest in S (weight basis) in both years and with all solvents except TB, where differences were not significant. From this data, it appears that water deficits resulted in higher total phenolics in both seeds and skins of cv. Merlot berries when MeOH was used for extraction. As discussed by the authors, berry size does play a role in influencing concentration while structural differences (e.g. mDP) play a role in compound extractability.

Despite this, it is important to realize that growing crops to a target composition is only practical if one can extract or make use of the metabolites of interest.

It was recently found that major flavan-3-ol monomers in tea leaves *(Camellia sinensis),* (-) epicatechin (EC) and (-) epigallocatechin gallate (EGCG) show protective functions for plants in vivo (Hernandez et al., 2006). A study of tea plants during a drought period determined the in vivo existence of EC-quinone and EGCG-quinone, both flavan-3-ol monomers oxidation species. The group also monitored levels of malondialdehyde (MDA), a marker of lipid peroxidation, and found it inversely correlated with significant accumulation of the quinone species. At the same time, EC and EGCG levels remained relatively unchanged, suggesting their synthesis was immediately followed by oxidation; this implies a protective response by the plant to fight oxidative stresses associated with drought. It is noted that EC-Q and EGCG-Q were nearly undetectable prior to drought status and later showed a measurable decrease corresponding to low rainfall. Another discovery was the increase in proanthocyanidins (PA) prior to the accumulation of the quinone species. The study supports a valid mechanism by which plants may protect themselves from oxidative stress during drought and offers some explanation why others observe an increase in grape PA mDP during water stress (Ojeda et al., 2002; Sivilotti et al., 2005; Kennedy et al., 2002).

As outlined by Deloire et al. (2004) and addressed by others, the timing of irrigation is a critical factor in vine management (Winkler et al., 1974; Gladstones et al., 1992). Timing depends on parameters such as rainfall, cultivar, rootstock, soil type and composition, site exposure (temperature and rate of evaporation), aspect and,

most important, the rationale for watering. As seen above and numerous other studies, irrigation directly effects vine vigor and yield, which are shown to influence berry composition (Roby et al, 2004a, 2004b; Esteban et al, 2001; Santos et al., 2004; Ojeda et al., 2001, 2002; Petrie et al., 2004; Sivilotti et al., 2005; Kennedy et al., 2002; Cortell et al. 2005).

In work conducted by Ojeda et al. (2001, 2002) water deficit during various stages of growth were imposed. Overall, deficits correlated with higher skin flavonol concentrations on a *per berry* basis in all cases. Highest values were typically found following late season deficit compared to early season deficit. Also, proanthocyanidins (PA) were influenced by water stress suggesting this played a significant role in tannin polymerization. The underlying conclusion from this study was that post-veraison water deficit was effective at increasing flavonols, anthocyanins, PA's and PA mDP without a dramatic loss in berry size or yield. Similar results have been observed by others (Salon et al., 2005; Koundouras et al., 2006; Kennedy et al., 2002 ; Petrie et al., 2004).

In a study of olive trees, *Olea europaea* L., a linear irrigation strategy was used to determine the effects of increasing water application while monitoring fruit during ripening (Tovar et al., 2002). In all cases PAL activity was positively correlated with polyphenol and *o*-diphenol content, all of which decreased over the course of maturity, regardless of treatment. Highest levels of PAL activity and phenolic material resulted from treatments with the least water applied. Irrigation had a significant, and relatively linear, effect on these parameters over the course of the experiment while fruit weight and fat content were not affected. As observed by

Tovar et al., trees supplied the least water yielded fruit with the highest level of phenolic material on a fruit-weight basis while no correlation was found between irrigation and oil content (Tovar et al, 2002; Marsilio et al., 2006). The control treatment (T_0) received no additional water and had nearly seven-fold increases in several phenolic compounds. It was noted that the compounds tyrosol, hydroxytyrosol and oleuropein are known *in vivo* antioxidants capable of protecting against oxidative stress (Marsilio et al., 2006). Treatments that received the same water levels (T_{66p} and T_{66w}) at different timing showed very little difference with respect to phenolic content, suggesting water stress can be compensated for during the second stage of fruit growth or phenolic development is most sensitive during the second. Sensory analysis of experimental fruit revealed reduced water application (T_0) increased the bitterness, acidity and firmness resulting in an overall decrease in perceived quality (Marsilio et al., 2006). Judging from knowledge of the sensory attributes of phenolic material, it would reason that increased concentrations could certainly lead to increased bitterness and could de responsible for an increased perception of astringency (puckering) (Gawel , 1998; Vidal et al., 2003).

As found by Glynn et al. (2004), differences in genotype can result in different responses to environment such as water stress. They found that willow (*Salix spp*. L.) genotype had a large influence on phenolic accumulation and response to drought. In the case of salicylate and cinnamic acid concentrations, some genotypes showed no response to drought, some showed an increase in content and others showed a decrease. Overall there was little effect of irrigation except for PAs and total phenolics, which were greater in well-irrigated plants.

Research with four soy bean (*Glycine max.* L.) cultivars showed definite variations with respect to composition including isoflavones (Bennett et al., 2004). Each cultivar was planted early in the season and again at traditional timing, and treated by irrigation and non-irrigation. Isoflavone content was variable in some cultivars depending on the time of planting (early or late) while irrigation led to an increase in all cultivars at all planting times. Irrigation had a positive effect on oil content in two of four cultivars while protein content was not affected. Overall, cultivar played a large role in compositional differences while irrigation was most effective in altering the isoflavone content of soy bean; increased water yielded higher levels. Similar observations have been made by Dumas et al. (2003) with regard to tomato plants (*Lycopersicon spp.*) and accumulation of lycopene and carotenoids. They report conflicting studies in which water deficit has opposing effects on metabolite accumulation and seems to be an effect of cultivar or experimental design.

In other studies, the effect of rootstock on fruit composition was found to be significant. Grafting of V*itis vinifera* scion onto rootstocks has been used for disease resistance, nutritional needs and vigor control (Deloire et al., 2004; Sampaio et al., 2006). Work by Sampaio et al. (2006) has revealed information regarding phenolic accumulation in cv. Pinot noir due to rootstock. Data shows considerable variation in tannin and anthocyanin content of fruit and resulting wine. There does not seem be a relationship between vine vigor or yield and phenolic status imposed by rootstock. Furthermore, consideration of phenolic extractability suggests compositional differences as well; an important factor to consider as total berry content does not

necessitate total content extracted into wine. Similar work has shown variations in phenolic content of peach fruit, *Prunus persica* L., as a function of rootstock (Giorgi et al., 2005). Peach cultivars have traditionally been grafted onto various rootstocks with the intent of improving adaptability to growing conditions and allow fruit growth in sub-optimal areas. The results from this study imply that plant vigor and yield may influence phenolic accumulation in peaches.

Exogenous Factors

The final focus of this review is on crop nutrition and the effects of supplemental application on phenolic development. Fertilization of agricultural fields is fairly commonplace as a means to condition or amend soil and to encourage crop vigor, yield and quality. As with irrigation, application of fertilizers or nutrients can be costly and results should be evaluated to assure results are in concert with expectations. As shown above, much experimentation with *Vitis vinifera* has included assessing impact on phenolics due to their importance in wine quality. Pirie and Mullins.(1976) studied the effect of treating grape leaf and fruit tissue with sucrose, nitrate and abscisic acid (ABA). In old leaf, new leaf and skin tissue, anthocyanin content was highest from treatment with sucrose and ABA together. Total phenolics also responded positively to sucrose and ABA. Nitrate appeared to have an inhibitory effect on accumulation of both anthocyanins and total phenolics. In all cases moderately high levels of sucrose and ABA induced phenolic accumulation especially when used in combination. Some related observations are

noted in studies such as Spayd et al. (1994) working with cv White Riesling and nitrogen fertilization. Total phenols were found be to lower as nitrogen levels increased, a difference the authors attribute to vine vigor. Low nitrogen resulted in lighter canopy and better light exposure was cited as a reason for increased phenolic accumulation.

Keller and Hrazdina (1998) found a fair correlation between soluble solids content and phenolic accumulation, suggesting at least anthocyanins and total phenols were related to accumulation of reducing sugars in the berry. Total flavonols responded negatively to increases in nitrogen while anthocyanins and total phenols were more variable. At high sunlight exposure, increases in nitrogen did not always result in reduced anthocyanins or total phenols, in some cases content increased slightly. At lower sunlight levels, increases in nitrogen nearly always reduced anthocyanins and total phenols. It is evident that nitrogen and light variations can influence composition of phenolics as seen with anthocyanins. Nitrogen has also been associated with a reduction in phenolic accumulation by Kliewer (1977).

Delgado et al. (2004) applied various amounts of nitrogen and potassium to cv. Tempranillo grapes and saw a delay in ripening with increasing nitrogen. At veraison, moderate nitrogen application showed the highest total tannins and high nitrogen showed the highest color density. Between veraison and harvest, increased nitrogen generally resulted in lower tannins and anthocyanins although differences between treatments were typically narrow at harvest. Potassium application appeared to reduce the effects of high nitrogen to some extent and there were interaction effects from the two variables. At high nitrogen applications, tannin mDP was slightly

lowered, an effect countered by potassium increases. The highest mDP was at zero

nitrogen and the highest potassium applied. Overall, low to moderate nitrogen levels

tend to favor phenolic accumulation and the negative effects of nitrogen can be

somewhat dissuaded by application of potassium. Regardless of the exact mechanism

involved, excessive nitrogen can impede berry ripening in *Vitis vinifera* and can be

detrimental to phenolic biosynthesis.

Application of nutrients and exogenous growth factors have been studied in

other crops, for many of which plant phenolics play a large role in viability and

quality. In strawberries (*Fragaria x ananassa* Duch.), the effects of a multi-

component fertilizer among other growth factors was studied (Anttonen et al., 2006).

It was found that the lowest rate of fertilization resulted in the highest content of

quercetin, kaempferol and ellagic acid. Other factors such as mulch color, fruiting

order and cultivar had large effects as well.

Calcium (Ca^{2+}), an essential plant nutrient, is a common component in

fertilizers and plays a role in many signaling processes (Ruiz et al., 2003). Castaneda

and Perez (1996) found that *Citrus limon* L. responded to wounding and fungal

elicitors (FE) by elevating levels of PAL activity Application of Ca (as $CaCl_2$ and an

ionophore) was found to induce a more rapid increase in PAL in response to

wounding and FE. Application of a chelator (ethyleneglycol-bis(2-aminoethyl) -

tetraacetic acid, EGTA) and a Ca channel blocker (Verapamil ™) both resulted in

inhibited responses to stress. This suggests Ca^{2+} is, indeed, involved in PAL

induction and exogenous Ca^{2+} is effective at promoting PAL activity. The same

induction of PAL activity was seen when Ca was applied to tobacco (*Nicotiana*

tabacum L.) although there was a concomitant rise in PPO and POD (Ruiz et al., 2003). As total calcium increased, enzyme activity increased and the amount of total leaf phenolics actually decreased. The authors suggest that while PAL activity may be increasing, the rate of enzymatic oxidation is increasing enough to cause significant declines in total phenolics.

Ruiz et al. (1998) also found that Boron (B), another essential nutrient, was effective in altering the yield of phenolics in tobacco plants. As expected, B deficiency led to increased phenolic content accompanied by high PAL activity and low PPO and POD activity. Moderate levels of B resulted in lower levels of PAL activity and higher levels of the oxidizing enzymes (PPO and POD) with a net decrease in phenolic content. When excessive dosages of B was applied PAL activity increased, PPO and POD decreased and phenolic content was the highest observed. The authors explain that B has the ability to form complexes with phenols and pectins, resulting in stable forms which are less susceptible to oxidation. At low and excessive B levels, the bound form of B dominates, resulting in more, stable phenols. At moderate levels, free B promotes activity of PPO and POD resulting in increased oxidation of phenols not bound to B.

Copper, Cu^{2+}, is another cation with metabolic consequences in plants. In Spinach (*Spinacea oleracea* L.) low levels have been shown to increase content of some phenolic compounds where high levels generally inhibit accumulation of all phenolics (Caldwell, 2002). The effects of Cu appear to be compound specific and independent of treatment pH as the same trends appeared at all pH levels with Cu

level held constant. The author notes, however, that higher pH levels in the soil would inhibit Cu uptake by the plant and allow for higher accumulation of phenolics.

As mentioned previously, tomato plants subjected to increasing CO_2 levels showed variable response and accumulation of phenolics (Bradfield and Stamp, 2004). On a whole plant basis, increases in CO_2 levels resulted in decreases in phenolic concentrations. The data infers this response is specific to the various plant parts (leaf, stem and root) and shows variability depending on the compound of interest; accumulation of chlorogenic acid and rutin following different trends. Elevated CO_2 levels in poison ivy (*Toxicodendron radicans* L.), in contrast, resulted in increases in biomass as well as favoring accumulation of the more toxic congener of the compound phenolic compound, urushiol (Mohan et al., 2006). In this case, the plant appears to produce more of the unsaturated triene of urushiol which is a more potent toxin to humans. This is another case where environmental factors can alter plant metabolism and favor accumulation of metabolites based on relatively minor structural differences. Although the exact mechanism or rationale is unclear it is likely a direct attempt to combat levels of stress imposed by the environment.

Hartley et al. (2000) also studied the impact of CO_2 on phenolic biosynthesis in different plant species over several seasons. In generations one and two, elevated CO_2 generally resulted in increased PAL activity which was not correlated similar increases in total phenolics or lignin content. Generation 3 revealed the significant influence or interaction that soil status has on these parameters; comparing soil after supporting 2 previous generations to fresh soil. The effect of CO_2 was variable depending on the nutrient content of the soil as well as the species of consideration.

Haukioja et al. (1998) hypothesize that phenolic accumulation is compound dependent and that only compounds stemming from phenylalanine should be directly affected by fertilization. Their hypothesis was supported by a meta-analysis of relevant studies. They found that phenolics in general (phenylpropanoids) were negatively affected by fertilization; likely due to competition for phenylalanine, a precursor in protein synthesis. Terpenoids and some other compounds *not* requiring phenylalanine were generally unaffected by fertilization. For this and reasons discussed within it is stressed that assessment of phenolic metabolism in pants is limited to the experimental design and type of analysis. One should always bear in mind what compounds are of primary interest and what factors, direct or indirect, may influence their presence. Compositional analysis should capture all information that may provide clues relating a plants response to its environment.

Conclusion

The presence of phenolic compounds in food has garnered much attention in recent years. The primary reason relates to the antioxidant properties of phenolics as well as proposed health benefits such as inhibiting certain cancers and promoting cardiovascular health. This is evident in the increasing use of product labels highlighting their phenolic content. While this marketing strategy may be relatively new the contribution of phenolics to food quality has been known for some time. They have obvious sensory impacts ranging from color to bitterness and aroma. These compounds also offer protection to foods in terms of food stability: protecting against oxidation and microbial spoilage. For these reasons, the management of

phenolics in food is an important aspect of product development. The information presented here should provide insight into factors that influence phenolic accumulation in plants and what we can do to manipulate the composition of food ingredients.

As seen here, many factors influence phenolic accumulation in plants although some are more significant then others. Application of water or exogenous growth factors, exposures to various light sources and the presence of fungal or predatory pressures are shown to alter plant biosynthesis. While many of these parameters are controlled by the grower, they are also impacted by the climate. Current climate data shows short and long term trends that present considerable changes in growing conditions in many areas. As growers or consumers searching for product sources, we should pay attention to these parameters and use them as management tools whenever possible. It is feasible to alter standard growing practices and yield products with higher nutritive, sensory or stability attributes. While many of the studies presented have monitored changes in phenolic compounds over time, not all provide an exact mechanism or rationale for their findings. There are also discrepancies that arise due to extraction, isolation and detection of these compounds that can result in conflicting data. Despite some of these limitations the implications of the research presented here should be evident. The data should provide the reader with tools needed to move forward in finding ways to elevate the quality of their product, starting in the field.

List of Figures

Figure 1.1. General Phenolic Compounds and Oxidation Mechanism

Phenol	Malonyl- CoA (intermediate)	*p*-Coumaryl-CoA

Hydroxycinnamic acids

Hydroxycinnamic acids	R1	R2	R3
p-Coumaric acid	H	H	H
Caffeic acid	OH	H	H
Ferulic acid	O-Me	H	H
Caftaric acid	OH	H	Tart

General Flavonoid Backbone

Flavonols

Flavonols	R1	R2	R3
Quercetin	H	OH	H
Myricetin	OH	OH	H
Kaempferol 3- O- Glucoside	H	H	Glc

Anthocyanins

Anthocyanins	R1	R2	R3
Cyanidin-3-glucoside	H	OH	H
Malvidin-3-glucoside	O-Me	O-Me	H
Delphinidn-3-(6''-acetyl)glucoside	OH	OH	Ace

Flavan-3-ols

Flavan-3-ols	R1
(+)- Catechin	A
(-)-Epicatechin	B
Epigallocatechin gallate	Gall

trans-Resveratrol (Stilbene)

Salicylic acid   Ellagic acid

Phenolic oxidative mechanism leading to quinone species

Me = ~CH$_3$
Glc = ~Glucose
Ace = ~COCH$_3$

Tart =

Gall =

Figure 1.2. Simplified Flavonoid Biosynthetic Pathway via Phenylalanine

Enzyme Abbreviations:
PAL - phenylalanine ammonia lyase
C4H - cinnamate-4-hydroxylase
C3H - *p*-coumarate-3-hydroxylase
StSy - stilbene synthase
CHS - chalcone synthase
CHI - chalcone isomerase
F3H - flavanone-3-hydroxylase
F3'H - flavonoid-3'-hydroxylase
FLS - flavonol synthase
DFR - dihydroflavonol-4-reductase
LAR - leucoanthocyanidin reductase
LDOX - leucoanthocyanidin dioxygenase
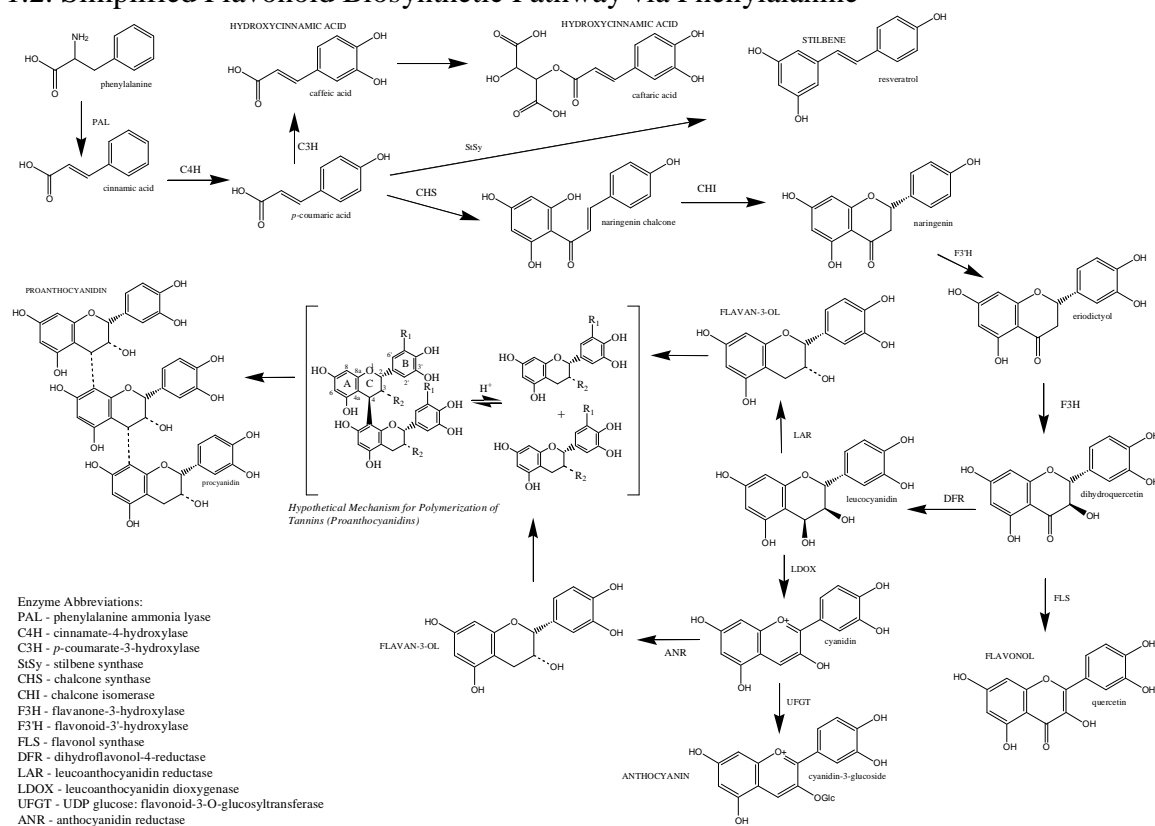UFGT - UDP glucose: flavonoid-3-O-glucosyltransferase
ANR - anthocyanidin reductase

Table 1.1. List of Environmental Factors and Associate Plant Response

Class	Compound	Plant	Environmental Factor	Response	Reference
Anthocyanin	-	*Vitis vinifera* (Grape; Agiorgitiko)	Irrigation	Water deficit increased concentration.	Koundouras et al., 2006
	-	*Vitis vinifera* (Grape; Syrah)	Irrigation	Highest in Early deficit, followed by Late deficit and continual irrigation.	Matthews and Anderson, 1988
	-	*Vitis vinifera* (Grape; Shiraz)	Irrigation	Late season irrigation highest; early season, severe deficit lowest.	Ojeda et al., 2002
	-	*Vitis vinifera* (Grape; Merlot)	Irrigation	Strong deficit highest content *per gram*.	Sivilotti et al., 2005
	-	*Vitis vinifera* (Grape; Tempranillo)	Irrigation (all season)	Irrigated had highest *per-berry* at harvest.	Esteban et al., 2001
	-	*Vitis vinifera* (Grape; Caberney sauvignon)	Irrigation (all season)	Decreased irrigation increased skin anthocyanins.	Roby et al., 2004b
	-	*Vitis vinifera* (Grape; Castelao)	Irrigation (all season)	Partial Root-zone Drying increased anthocyanins.	Santos et al., 2005
	-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Irrigation (Post veraison)	Water deficit increased concentration (moderate).	Kennedy et al., 2002
	-	*Vitis vinifera* (Grape; Syrah)	Irrigation/Pruning	Aggressive Pruning increased concentration. First-Year deficit irrigation increased second-year concentration.	Petrie et al., 2004
	-	*Vitis vinifera* (Grape; Tempranillo)	Nitrogen and Potassium	Nitrogen generally reduced anthocyanins; potassium countered some effects of nitrogen. Low to moderate N favored accumulation.	Delgado et al., 2004
	-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Nitrogen/Light exposure	Low light plus increased nitrogen reduced anthocyanins, influenced composition.	Keller and Hrazdina, 1998
	-	*Vitis vinifera* (Grape; Emperor)	Nitrogen/Light exposure	Low light and increased nitrogen reduced anthocyanins.	Kliewer, 1977
	-	*Vitis vinifera* (leaf/skin tissue)	Nutrient (ABA, sucrose, nitrate)	Increased content from treatment with sucrose plus ABA; nitrate inhibitory.	Pirie and Mullins, 1976
	-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Sun exposure	General increase *per berry* content.	Crippen and Morrison., 1986
	-	*Vitis vinifera* (Grape; Shiraz)	Sun exposure (shade/exposed)	Exposed; increase in *per berry* content. Shade; increase di-substitution:tri-substitution.	Downey et al., 2004

	-	*Vitis vinifera x Vitis labrusca* (Grape; Darkridge)	Temperature	Decrease at high temps. (30-35°C)	Mori et al., 2005
	-	*Vitis vinifera* (Grape)	Temperature	20°C had higher anthocyanins than 30°C; growth stage III was most sensitive period.	Yamane et al., 2006
	delphinidin, cyanidin, petunidin, peonidin	*Vitis vinifera* (Grape; Merlot)	Temperature	Cooler temp. increased all.	Spayd et al., 2002
	TSMA	*Vitis vinifera* (Grape; Merlot)	Temperature	Cooler temp. increased TSMA.	Spayd et al., 2002
	-	*Vitis vinifera* (Grape)	Temperature, above 35°C	Decrease accumulation.	Kliewer and Torres, 1972; Spayd et al., 2002
Catechol	Urushiol	*Toxicodendron radicans* (Poison ivy)	Carbon dioxide (CO_2)	Increased CO_2 increased more-toxic congener (unsaturated triene).	Mohan et al., 2006
	catecholic phenolics	*Lycopersicon esculentum (Tomato)*	Temperature	Variable between plant parts; decrease in roots with increased temp., general maximum approx. 17-18°C.	Bradfield and Stamp, 2004
Cinnamic acid	ellagic acid	*Fragaria x ananassa* Duch. (Strawberry)	Fertilizer	Increased fertilizer decreased content.	Anttonen et al., 2006
	ferulic acid, caffeic acid	*Musa acuminata* (Banana)	Pathogen infection	Ferulic increased two fold. Caffeic (and others) accumulate only in response to pathogen.	de Ascensao and Dubery, 2003
	salicylic acid	*Arabidopsis thaliana*	Pathogen infection	Increased Slicylic acid, induced *At*CCR2 transcription and phenolic synthesis.	Lauvergeat et al., 2001
	salicylic acid	*Cucumis sativus* L. (Cucumber)	Pathogen infection (*Pseudomonas*)	Increased accumulatin of Salicylic acid.	Rasmussen et al., 1991
	rosmarinic acid	*Mentha spicita (Spearmint)*	Temperature	Decrease under heat stress (+30°C).	Fletcher et al., 2005
	chlorogenic acid	*Lycopersicon esculentum (Tomato)*	Temperature	Variable between plant parts; increase in leaf with increased temp., general maximum approx. 17-18°C.	Bradfield and Stamp, 2004
	-	*Hordeum vulgare (Barley)*	UV-B exposure	Increase with exposure.	Reuber et al., 1996
	ferulic acids	*Hordeum vulgare (Barley)*	UV-B exposure	Variable, lower final content with exposure.	Liu et al., 1995a, 1995b
	chlorogenic acid	*Solanum tuberosum* (Potato)	White light	Increase synthesis.	Zucker, 1965

Dihydroflavonol	astilbin	*Vitis vinifera* (Sauvignon blanc, Semillon)	Pathogen infection (*Botrytis cinerea*)	Increased in Sauvignon; lower levels in Semillon, less response to infection.	Landrault et al., 2002
Enzymes	PAL, PPO, POD	*Nicotiana tabacum* L. (Tobacco)	Boron	Variable: boron deficiency and excess increased PAL, decreased PPO and POD; moderate boron reduced PAL, increased PPO and POD.	Ruiz et al., 1998
	PAL	*Citrus limon* (Lemon)	Calcium	Calcium induced PAL activity in response to fungal elicitor.	Castaneda et al., 1996
	PAL, PPO, POD	*Nicotiana tabacum* L. (Tobacco)	Calcium	Calcium induced PAL, POD and PPO activity.	Ruiz et al., 2003
	PAL	*Olea europaea* (Olive)	Irrigation	Water deficit increased activity.	Tovar et al., 2002
	PAL, POD, PPO	*Piper betel* L. (Betelvine)	Pathogen infection (*Phytophthora nicotianae*) and Rhizobia inoculation	Increase in response to infection and rhizobia.	Lavania et al., 2006
	PAL, PPO, POD	*Citrullus lanatus (Watermelon)*	Temperature	35°C; lowest PAL, POD decreased with decreasing temp.	Rivero et al., 2001
	PAL, PPO, POD	*Lycopersicon esculentum (Tomato)*	Temperature	25°C; lowest PAL, highest PPO and POD. 35°C; highest PAL, lowest PPO, POD.	Rivero et al., 2001
	PAL, UFGT	*Vitis vinifera x Vitis labrusca* (Grape; Darkridge)	Temperature	PAL decrease with increased temp.; UFGT seasonal variability, heat retard activity until 15 days post-veraison.	Mori et al., 2005
	PAL, CFI, POD	*Hordeum vulgare (Barley)*	UV-A, UV-B	UV-B increase PAL, CFI no change, POD decrease; UV-A increase POD activity.	Liu et al., 1995a, 1995b
	CAD, PAL, TAL, POD	*Triticum aestivum* (Wheat)	Virus infection (wheat stroke mosaic)	Increased CAD, decrease POD, no change in PAL or TAL.	Kofalvi et al., 1995
	PAL	*Solanum tuberosum* (Potato)	White light	Induced increase in activity.	Zucker, 1965
Flavan-3-ols	-	*Vitis vinifera* (Grape; Shiraz)	Irrigation	Irrigation and Late season deficit highest concentration.	Ojeda et al., 2002
	EC,EGCG, quinone species	*Camellia sinensis (Tea)*	Irrigation	During drought EC and EGCG unchanged, accumulation of quinones.	Hernandez et al., 2006
	catechin	*Vitis vinifera* (Grape; Cabernet	Irrigation (Post veraison)	Irrigation highest *per berry;* deficit highest *per gram.*	Kennedy et al., 2002

				sauvignon)	
	EC, ECG, EGCG	*Camellia sinensis* (Tea)	Pathogen infection (Exobasidium vexans)	Resistant lines higher in EC, lower in EGCG. Infection decreased EC, EGC; ECG increased.	Punyasiri et al., 2005
Flavones	saponarin, lutonarin	*Hordeum vulgare (Barley)*	UV-B exposure	Increase UV-B, increase ratio lutonarin:saponarin.	Reuber et al., 1996; Liu et al., 1995a, 1995b
Flavonoids, cinnamoyl esters	-	*Secale cereale* (Rye)	UV-B	Increase with exposure.	Booij-James et al., 2000
Flavonols	quercetin, kaempferol	*Fragaria x ananassa* Duch. (Strawberry)	Fertilizer	Increased fertilizer decreased content.	Anttonen et al., 2006
	-	*Vitis vinifera* (Grape; Shiraz)	Irrigation	Water deficit increased concentration.	Ojeda et al., 2002
	-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Irrigation (Post veraison)	Irrigation highest *per berry*, Deficit highest *per gram*.	Kennedy et al., 2002
	-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Nitrogen	Lower content with increased nitrogen.	Keller and Hrazdina, 1998
	quercetin, myricetin, kaempferol	*Vitis vinifera* (Grape; Merlot)	Sun exposure	Increase with exposure.	Spayd et al., 2002
	quercetin glycosides	*Vitis vinifera* (Grape, Pinot noir)	Sun exposure (shade/exposed)	Increase with exposure.	Price et al., 1995
	-	*Vitis vinifera x Vitis labrusca* (Grape; Darkridge)	Temperature	No effect.	Mori et al., 2005
	quercetin, kaempferol	*Brassica napus* (Rape)	UV exposure	Increased quercetin:kaempferol ratio.	Alenius et al., 1995
	UV-B screening compounds (280-320nm)	*Brassica napus* (Rape)	UV exposure	Increase with exposure.	Alenius et al., 1995
Genetics	*At*CCR1, *At*CCR2	*Arabidopsis thaliana*	pathogen infection	*At*CCR2 induced by pathogen; *At*CCR1, for lignin synthesis, was not.	Lauvergeat et al., 2001
	*Vv*FLS1, *Vv*UFGT	*Vitis vinifera* (Grape; Shiraz)	Sun exposure (shade/exposed)	Decrease light, decrease *Vv*FLS1, decrease flavonols; *Vv*UFGT highest in exposed fruit, increased anthocyanins.	Downey et al., 2004

Isoflavones	genistein and daidzein	*Glycine max.* (Soy)	Irrigation	Increased water increased concentrations.	Bennett et al., 2004
Phenolic acids	total phenolic acids and chlorogenic acid	*Piper betel* L. (Betelvine)	Pathogen infection (*Phytophthora nicotianae*) and Rhizobia inoculation	Infection decreased acids; rhizobia increased acids; infection plus rhizobia highest; chlorogenic acid only present from infection and inoculation.	Lavania et al., 2006
	-	*Oryza sativa* L. (Rice)	Pathogen infection (*Rhizoctonia solani*) and Rhizobia inoculation	Increase in response to infection and rhizobia; infection plus rhizobia increase phenolic acids 4-fold.	Mishra et al., 2006
Phenolic polymers	phenolic polymers, lignin	*Musa acuminata* (Banana)	Pathogen infection	Increased in response to pathogen, increase in esterified cinnamic esters and cell-wall phenolics.	de Ascensao and Dubery, 2004
Proanthocyanidins	-	*Vitis vinifera* (Grape; Agiorgitiko)	Irrigation	Water deficit increased concentration.	Koundouras et al., 2006
	-	*Vitis vinifera* (Grape; Shiraz)	Irrigation	Late season irrigation highest, early season severe deficit lowest. mDP highest in deficit treatments.	Ojeda et al., 2002
	condensed tannins	*Salix spp*(Willow)	Irrigation	Water deficit increased concentration.	Glynn et al., 2004
	tannins	*Camellia sinensis (Tea)*	Irrigation	Increased during drought, prior to accumulation of quinones.	Hernandez et al., 2006
	skin tannin	*Vitis vinifera* (Grape; Cabernet sauvignon)	Irrigation (all season)	Decreased irrigation increased skin tannin.	Roby et al., 2004b
	total tannins	*Vitis vinifera* (Grape; Tempranillo)	Irrigation (all season)	Non-irrigated had highest *per-gram*, lowest *per-berry*.	Esteban et al., 2001
	total tannins	*Vitis vinifera* (Grape; Cabernet sauvignon)	Irrigation (Post veraison)	Water deficit increased concentration (moderate), increased mDP.	Kennedy et al., 2002
	toal tannins	*Vitis vinifera* (Grape; Tempranillo)	Nitrogen and Potassium	Nitrogen generally reduced tannins, reduced mDP; potassium countered some effects of nitrogen, resulted in highest mDP. Low to moderate N favored accumulation.	Delgado et al., 2004
	tannins	*Camellia sinensis* (Tea)	Pathogen infection (Exobasidium vexans)	Resistant line higher in anthocyanins; infection increased	Punyasiri et al., 2005

				tannins.	
	-	*Vitis vinifera* (Grape; Shiraz)	Sun exposure (shade/exposed)	Exposed; general increase in per berry content. Shade; increase di-substitution:tri-substitution.	Downey et al., 2004
	skin and seed	*Vitis vinifera* (Grape; Shiraz)	Sun exposure (shade/exposed)	Exposed; increase mDP.	Downey et al., 2004
Stilbenes	resveratrol	*Vitis vinifera* (Grape)	Pathogen infection (*Botrytis cinerea*)	Increase with infection, followed by decline due to fungal exo-enzyme activity.	Jeandet et al., 1995b
	t-resveratrol, *t*-astringen, *t*-piceid, viniferin	*Vitis vinifera* (Grape; Sauvignon blanc, Semillon)	Pathogen infection (*Botrytis cinerea*)	Viniferin increase in both cultivars; others variable between cultivars, relatively low concentration.	Landrault et al., 2002
	resveratrol	*Vitis vinifera* (Grape)	UV exposure	Increased; higher in leaves than berries.	Jeandet et al., 1991; Jeandet et al., 1995b; Douillet-Breuil et al., 1999
Total Phenolics	-	*Nicotiana tabacum* L. (Tobacco)	Boron	Variable: boron deficiency and excess increased phenolics; moderate boron reduced phenolics.	Ruiz et al., 1998
	-	*Nicotiana tabacum* L. (Tobacco)	Calcium	Increased calcium decreased content.	Ruiz et al., 2003
	-	*Lycopersicon esculentum (Tomato)*	Carbon dioxide	Tissue specific: general reduction in whole plant with increased CO_2.	Bradfield and Stamp,, 2004
	Various	*Spinacea oleracea* L. (Spinach)	Copper	Compound specific: general decrease in accumulation due to copper.	Caldwell, 2002
	-	*Salix spp*(Willow)	Irrigation	Water deficit increased concentration.	Glynn et al., 2004
	-	*Vitis vinifera* (Grape; Agiorgitiko)	Irrigation	Water deficit increased concentration.	Koundouras et al., 2006
	-	*Vitis vinifera* (Grape; Cabernet franc)	Irrigation	Water deficit increased concentration.	Matthews and Anderson, 1988
	-	*Vitis vinifera* (Grape; Syrah)	Irrigation	Water deficit increased concentration in first year and carry-over to second year.	Petrie et al., 2004
	-	*Olea europaea* (Olive)	Irrigation	Water deficit increased concentration.	Tovar et al., 2002
	seed and skin (MeOH extract)	*Vitis vinifera* (Grape; Merlot)	Irrigation	Water deficit increased concentration.	Sivilotti et al., 2005

tyrosol , hydroxytyrosol and oleuropein	*Olea europaea* (Olive)	Irrigation	Water deficit increased concentration.	Marsilio et al., 2006
-	*Vitis vinifera* (Grape; Tempranillo)	Irrigation (all season)	Non-irrigated had highest *per-gram*, lowest *per-berry*.	Esteban et al., 2001
-	*Vitis vinifera* (Grape; Castelao)	Irrigation (all season)	Partial Root-zone Drying increased total phenolics.	Santos et al., 2005
-	*Sorghum bicolor* (Sorghum)	Nitrogen	Increased nitrogen availability increased content.	Sene et al., 2001
-	*Vitis vinifera* (Grape; Riesling)	Nitrogen	Lower content with increased nitrogen.	Spayd et al., 1994
-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Nitrogen/Light exposure	Low light plus increased nitrogen reduced phenolics.	Keller and Hrazdina, 2000
-	*Vitis vinifera* (leaf/skin tissue)	Nutrient (ABA, sucrose, nitrate)	Increased content from treatment with sucrose plus ABA; nitrate inhibitory.	Pirie and Mullins, 1976
-	*Vitis vinifera* (Grape; Chardonnay)	Pathogen infection (powdery mildew)	Increased in younger plants.	Ficke et al., 2004
-	*Prunus persica* (Peach)	Rootstock	Varibility in accumulation imposed by rootstock.	Giorgi et al., 2005
tannins and anthocyanins	V*itis vinifera* (Grape; Pinot noir)	Rootstock	Variability in content and composition imposed by rootstock.	Sampaio et al., 2006
-	*Vitis vinifera* (Grape; Cabernet sauvignon)	Sun exposure	Increased *per berry* content, polymeric phenolics higher in shade.	Crippen and Morrison., 1987
-	*Vitis vinifera* (Grape; Cabernet sauvignon, Grenache)	Sun exposure (north/south)	North facing grapes highest total phenolics.	Bergqvist et al., 2001
caftaric acid, resveratrol, total phenolics	*Vitis vinifera* (Grape, Pinot noir)	Sun exposure (shade/exposed)	Exposed; increase in all. Increase in polymeric phenolics extracted.	Price et al., 1995
-	*Vitis vinifera* (Grape; Cabernet sauvignon, Grenache)	Temperature	High temperatures reduce total phenolics.	Bergqvist et al., 2001
-	*Citrullus lanatus (Watermelon)*	Temperature	35°C; lowest total phenolics.	Rivero et al., 2001
-	*Lycopersicon esculentum (Tomato)*	Temperature	25°C; lowest total phenolics. 35°C; heat stress, highest phenolics.	Rivero et al., 2001
phenolic aids, flavonols, anthocyanins	*Fragaria x ananassa* (Strawberry)	Temperature	Increase temp, increase phenolics.	Wang et al., 2001
phenolic glycosides, cinnamyl alcohols	*Triticum aestivum* (Wheat)	Virus infection (wheat stroke mosaic)	Infection increased accumulation, increased hydrophobic phenolics.	Kofalvi et al., 1995

Volatile Phenolics	aroma Compounds	*Vitis vinifera* (Grape; Agiorgitiko)	Irrigation	Water deficit increased concentration.	Koundouras et al., 2006

Assessing the Impact of Temperature on Grape Phenolic Metabolism.

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Abstract

This study assessed the impact of fruit temperature on the phenolic metabolism of grape berries (*Vitis vinifera* L. cv. Merlot) grown under field conditions with controlled exposure to sunlight. Individual cluster temperatures were manipulated in situ. Diurnal temperature fluctuation was damped by daytime cooling and nighttime heating of clusters. Daytime-only and nighttime-only temperature controls were applied for comparison. Berry temperatures were recorded continuously to compare to chemical data. Samples collected at véraison indicated that damping the diurnal temperature fluctuation advanced the onset of ripening. Those berries were larger (double-damped: 0.753±0.015 vs control: 0.512±0.034 g/berry) and more colored than all others. Development of phenolic metabolites was followed by two reversed-phase HPLC methods and gel permeation chromatography. These methods provided information on anthocyanins, proanthocyanidins, flavonols, flavan-3-ol monomers, and polymeric material. Damping the diurnal temperature fluctuation reduced proanthocyanidin mDP (double-damped: 21.8±1.0 vs control: 28.0±1.7). Proanthocyanidin accumulation at véraison was linearly related to heat summation over the developmental period with nighttime heating yielding the highest concentration and daytime cooling yielding the lowest (night-heat: 1.46±0.13 vs day-cool: 0.97±0.09 mg/berry). Damping the diurnal temperature fluctuation had a marked effect on the rate of fruit development whereas total heat summation had more of an effect on phenolic metabolism alone. The results provide insight on the direct effect of temperature on phenolic metabolism.

1. Introduction

Grape-derived phenolics are essential components of wine quality, contributing to its aroma, color, bitterness, and mouth-feel properties. Grape phenolics can be broken into two groups: flavonoids and non-flavonoids. In studying grape seeds and skin the flavonoids are most relevant to wine quality. Seeds are dominated by flavan-3-ols while skins contain flavonols, flavan-3-ols, and anthocyanins, all sharing a similar biosynthetic pathway (Fig. 2.1.) (Koes, Quattrocchio et al. 1994; Waterhouse 2002). While anthocyanins are responsible for the color of red wine, various interactions occur among the three flavonoid classes in wine, resulting in a complex matrix of pigmented polymers (Boulton 2001; Kennedy and Hayasaka 2004; Saucier, Lopes et al. 2004; Wang, Race et al. 2004). Proanthocyanidins (PAs) or condensed tannins are composed of flavan-3-ol subunits, and are primarily responsible for the astringency of wine and contribute to its bitterness (Fischer and Noble 1994; Gawel 1998; Drewnowski and Gomez-Carneros 2000; Vidal, Francis et al. 2003; Lesschaeve and Noble 2005). Collectively, these phenolic compounds are also thought to impact human health (Renaud and de Lorgeril 1992; Robards, Prenzler et al. 1999; Parr and Bolwell 2000; Rice-Evans 2001; Beecher 2004).

On the vine, grape flavonoids are thought to serve multiple functions. They protect against predation and UV damage, and serve as attractants for pollinators or foraging animals to aid in seed dispersal (Parr and Bolwell 2000). Due to the myriad roles of flavonoids for plants and humans there have been numerous studies to

determine factors influencing their metabolism. Within winegrape research, the primary focus has been on the production of anthocyanins, seen as critical to wine quality. Research has helped uncover effects of environmental factors such as sunlight exposure and temperature on the accumulation of these compounds as well as the biosynthetic pathways involved in their biosynthesis (Kliewer and Torres 1972; Kliewer 1977; Dokoozlian and Kliewer 1996; Bergqvist, Dokoozlian et al. 2001; Spayd, Tarara et al. 2002; Downey, Harvey et al. 2004; Yamane, Jeong et al. 2006; Mori, Goto-Yamamoto et al. 2007). With respect to PA accumulation, research also has elucidated elements of genetic regulation, climate, and other factors found to influence berry composition (Spayd, Tarara et al. 2002; Xie, Sharma et al. 2003; Downey, Harvey et al. 2004; Bogs, Downey et al. 2005; Cortell, Halbleib et al. 2005; Bogs, Jaffe et al. 2007; Fujita, Soma et al. 2007). Still, there are many areas of uncertainty regarding PA biosynthesis and the effect of climate during development.

There is no element of climate that has been proven scientifically to pose *no* impact on the development of grape berries. On the contrary, many use the term "terroir" to encompass the culmination of all environmental factors into one word. Of the elements encompassed by that term, temperature has the most marked effect on berry development. Because of this, classifications of growing degree days or heat summation have been developed to demarcate where certain varieties of grapes can be cultivated successfully (Amerine and Winkler 1963; Gladstones 1992). These are based on the requirements of each cultivar to ripen satisfactorily including accumulation of sugars, acids, and various secondary metabolites. Moreover, it has been shown that grape berry metabolism is sensitive to variations in both day and

night temperatures as well as the magnitude of diurnal temperature variation (Kliewer and Torres 1972; Gladstones 1992; Mori, Sugaya et al. 2005). More recent meteorological studies show trends in climate change that ultimately may affect the cultivation of winegrapes worldwide (Karl, Jones et al. 1993; Easterling, Evans et al. 2000; Jones, White et al. 2005; White, Diffenbaugh et al. 2006). Those findings indicate that most temperature fluctuations are the result of warmer nights (higher minima) resulting in a reduction in the amplitude of diurnal temperature fluctuation. For this reason it is important to improve our understanding of the effect of day and night temperatures as well as variations in the diurnal temperature range on the metabolism of secondary metabolites in grapes. This would allow grape growers and winemakers to be well equipped when faced with cultivation decisions in the years ahead.

The current study was designed to assess the impact of varying day and night temperatures as well as modulating the diurnal temperature range on the development of grape berries (*Vitis vinifera* L. cv. Merlot) with particular regard to accumulation of PAs. Established methodologies were used to exclude the effects of solar radiation from confounding the assessment of those related to temperature alone (Tarara, Ferguson et al. 2000; Spayd, Tarara et al. 2002). Furthermore, previous work has shown that accumulation of PAs in skins and seeds occurs predominantly before véraison with little production between then and commercial ripening (Kennedy, Matthews et al. 2000; Downey, Harvey et al. 2004). We investigated this observation further. Three complimentary chromatographic methods were used to provide

detailed information regarding phenolic content and composition (Lamuela-Raventos and Waterhouse 1994; Kennedy and Jones 2001; Kennedy and Taylor 2003).

2. Experimental

2.1. *Field Procedure*

The study was conducted during 2006 at the Irrigated Agriculture Research and Extension Center in Prosser, WA, USA (46.30º N, 119.75º W) in a block of own-rooted 'Merlot' planted in 1999. Rows were oriented north-south. Vines were trained to a bilateral cordon at 1.2 m above ground and spur-pruned annually. Fruit clusters were exposed to incident solar radiation on the east aspect of the canopy by tucking shoots under a catch wire that was parallel to the cordon at 1.5 m above ground. Treatments were applied to individual clusters, and each cluster was treated as a replicate ($n = 4$). Six temperature-control regimens were applied during each of two experimental phases, where 'Phase I' was defined as comprising the period between E-L developmental stage 27-28 (berry diameter 2 to 4 mm) and véraison; and 'Phase II' was defined as comprising the period between véraison and commercial ripeness (Coombe 1995). Temperature classifications were as follows: (1) ambient, (2) convective control (*Blower*), (3) daytime heated (*Heat*), (4) nighttime cooled (*Cool*), (5) reduced diurnal temperature range (*Damped*), and (6) twice-reduced diurnal temperature range (*Double-damped*). All *in situ* temperature control was accomplished by forced-air delivery across the treated cluster (Tarara, Ferguson et al. 2000). The temperature of ambient clusters was not manipulated. Convective

control refers to ambient air delivered to a cluster at the same rate that heated or cooled air was delivered to the temperature-controlled clusters, to account for the effects of heat transfer by forced convection. Target temperature differences above or below ambient clusters were 5 °C. *Damped* clusters were cooled during the day and heated at night to achieve this magnitude difference. *Double-damped* clusters were cooled and heated likewise, with a target temperature difference from ambient clusters of approximately 8 °C. Exemplary temperature profiles for the treatments are shown for phase I (Table 2.1. and Fig. 2.2.a) and phase II (Table 2.2. and Fig. 2.2.b). Phase I clusters were collected at véraison and Phase II clusters were collected at commercial ripeness. Berries were excised from the rachis, counted, weighed, and snap-frozen in liquid nitrogen then stored at -80 °C pending chemical analyses.

2.2. *Equipment and Chemicals*

All HPLC analysis was performed on a Hewlett-Packard model 1100 (Palo Alto,
CA, USA). The instrument was equipped with diode array (DAD) and fluorescence detectors (FLD) and an external column oven when required (Eppendorf; Westbury, N.Y., USA). All data were analyzed using Agilent chemstation software. Acetonitrile, acetone, methanol, and glacial acetic acid were purchased from J. T. Baker (Phillipsburg, NJ, USA). *N,N*-dimethylformamide (DMF), ammonium phosphate monobasic, ortho-phosphoric acid, and lithium chloride were purchased from Fisher Scientific (Santa Clara, CA, USA). All solvents were HPLC grade.

Quercetin, phloroglucinol, (+)-catechin, and (-)-epicatechin were purchased from Sigma (St. Louis, MO, USA) and malvidin-3-O-glucoside from Extrasynthèse (Genay, France). Hydrochloric acid was purchased from E. M. Science (Gibbstown, NJ, USA) and sodium acetate anhydrous from Mallinckrodt (Phillipsburg, NJ, USA). All water was treated by reverse osmosis and purified using a Millipore Milli-Q filtration system (Bedford, MA, USA).

2.3. *Chemical Analysis*

Using a hand-held digital refractometer (WM-7, Atago, Tokyo, Japan), soluble solids concentration was measured on 15- to 20-berry samples. Samples of 100 berries were used to estimate average berry volume based on H_2O displacement (Kennedy, Matthews et al. 2000). Berry skins and seeds we separated, freeze dried, and the dry mass of these components ascertained prior to extraction as described elsewhere (Kennedy, Matthews et al. 2000). Following removal of acetone, samples were brought to 100 ml volume with Milli-Q H_2O and kept at -30 °C until further analysis.

2.4. *Analysis of flavonols and anthocyanins*

Analysis of monomeric phenolics was performed following a previously described method (Lamuela-Raventos and Waterhouse 1994). Prior to analysis, aqueous extracts were filtered to 0.45µm using a syringe filter. Quercetin and malvidin-3-O-glucoside were used as quantitative standards for flavonols and anthocyanins, respectively.

2.5. Analysis of PAs

Analysis of PAs was carried out following acid-catalyzed cleavage in the presence of phloroglucinol to elucidate detailed compositional information as described previously (Kennedy and Jones 2001). Appropriate aliquots of aqueous extracts were lyophilized and dissolved in MeOH prior to reacting with phloroglucinol reagent as previously described (Kennedy and Taylor 2003; Cortell, Halbleib et al. 2005). After quenching the reaction with aqueous sodium acetate, samples were immediately analyzed as described earlier (Kennedy and Taylor 2003). Quantification of PA subunits was calculated as described earlier using a (+)-catechin quantitative standard (Kennedy and Jones 2001).

Analysis of PAs was also carried out while PAs were still intact. Gel permeation chromatography (GPC) was performed following the methodology detailed by Kennedy and Taylor to determine the size distribution of the phenolic extract (Kennedy and Taylor 2003). Separations were performed on two PL_{Gel} columns (100 Å and 500 Å) in series protected by a guard column containing the same material (Polymer labs; Amherst, MA, USA). Aliquots of aqueous extract were lyophilized and dissolved in mobile phase (0.15M LiCl in DMF containing 1% and 5% (v/v) acetic acid and water, respectively).

2.6. Statistical Analyses

Berry temperature data were summarized over time and by treatment in SAS (ver 9.1, SAS Institute, Cary, NC, USA) using the MEANS procedure. Thermal time or "heat

accumulation" was computed using a trapezoidal method of integration of temperature over time (Tobin, Nagarkatti et al. 2001).

3. Results and Discussion

3.1. *Berry Physiology*

Berry mass during ripening followed a typical double-sigmoidal growth curve. Berry weights and soluble solids content are shown for samples collected at véraison and harvest (Table 2.3.). Berry weights were highest in temperature-damped treatments followed by night-heated, day-cooled, and ambient. Differences among treatments were significant ($P<0.05$). Sugar accumulation followed the same trend as berry weights and volumes with diurnal temperature damping leading to the highest sugar concentration at the end of phase I. Berry coloration also was significantly higher in damped treatments than in all others. The combination of these observations implies that damping diurnal temperature fluctuations substantially hastened berry ripening. This is in general agreement with existing knowledge on the influence of temperature on ripening: higher temperatures are associated with higher rates of ripening (Winkler 1974; Gladstones 1992). It appears that both day-cooling and night-heating resulted in slight increases in the rate of ripening, while combining the two treatments by damping the entire diurnal temperature range dramatically increases that rate. It should be noted that there were no excessively high temperatures (i.e. > 40 °C) during 2006 for any considerable length of time (Kliewer 1977; Spayd, Tarara et al. 2002).

3.2. Seed Phenolics

In general, there was no significant effect of treatment on the physiological development of seeds or the number of seeds per berry. At véraison all treatments showed a slight increase in PA concentration *per berry*, where night-heated and damped fruit had significantly more PA than ambient fruit (Fig. 2.3.a). On a *per seed* basis (Fig. 2.3.b) damping tended to increase concentrations but differences were not statistically significant. The composition of PAs (Table 2.4.) also showed no significant differences between treatments. These results are consistent with research that investigated the effects of shading, which showed minimal variation in seed chemistry (Downey, Harvey et al. 2004; Fujita, Soma et al. 2007). It should be noted that our temperature measurements were made under the berry skin and should approximate seed temperature.

Seeds collected at harvest also showed little variation with respect to heating or cooling of berries. Daytime cooling did result in a reduction in total PA concentration on both a *per berry* and *per seed* basis but was only significantly different from other treatments in the latter (data not shown). Proanthocyanidin composition of seeds showed no significant differences resulting from treatments (Table 2.5.).

3.3. Skin Phenolics: Anthocyanins and Flavonols

At véraison we observed obvious coloration differences among treatments. Analysis of anthocyanins validated these observations with damp and double-damp

fruit having 13.1 and 7.7 µg berry^{-1} total anthocyanins, respectively. By contrast, other treatments had only trace amounts of anthocyanins: only one heated cluster had noticeable color (< 10% of berries) and less than 3% of day-cooled berries were colored. Flavonol content was highest in day-cooled berries (30 µg berry^{-1}), but otherwise there were no significant differences (ca. 23 µg berry^{-1}) among treatments in flavonol composition.

Anthocyanins showed significant variation at harvest with temperature-damped treatments resulting in higher total concentration (Fig. 2.4.a). Day-cooled and damped treatments had a higher proportion of dioxygenated (cyanindin, peonidin) than trioxygenated (delphinidin, petunidin, malvidin) anthocyanins (data not shown). Furthermore, those treatments resulted in considerably lower proportions of acylated anthocyanins and slightly lower proportions of those with acetyl- and coumaroyl-glucosides. Of all treatments, day-cooling varied most from ambient. These results do not appear to be directly related to heat accumulation because ambient and damped treatments accumulated similar thermal time during the study period. On the contrary, it would appear that cooling during the day has the most marked affect on the accumulation and composition of anthocyanins. These results agree with other published data and exemplify the effect of temperature as opposed to sunlight exposure (Spayd, Tarara et al. 2002; Downey, Harvey et al. 2004).

At commercial harvest there was a lower concentration of total flavonols following daytime cooling (Fig. 2.4.b) and a slightly higher proportion of flavonols with di-hydroxylation in cooled and double-damped treatments (data not shown). These differences were consistent with differences in anthocyanin composition as

well as to data from a similar study (Spayd, Tarara et al. 2002). The effect of daytime cooling differed by phase of berry growth, suggesting multiple roles for flavonols during development.

3.4. Skin Phenolics: Proanthocyanidins at Véraison

Grape skin total PAs were linearly related to heat accumulation during phase I of the experiment (Fig. 2.5.a). Night-heating produced the highest amounts of skin PA at véraison. Similar results can be inferred from other studies where exposure to solar radiation apparently increased the concentration of PAs or total phenolics (Crippen and Morrison 1986; Fujita, Soma et al. 2007).

Heating and cooling slightly altered PA composition at véraison (Table 2.6.). The concentration of extension subunits was highest with night-heating, repeating the trend observed for total PAs. The composition of PA extension subunits was consistent although day-cooling resulted in a slight increase in the proportion of EGC (tri-hydroxylated), and could be related to variable flavonoid hydroxylase activity (Mori, Goto-Yamamoto et al. 2007). Terminal subunit concentrations were proportionally highest in the damped treatments. Although treatment differences were small, they do indicate trends related to berry temperature. Increased PA biosynthesis with increasing temperature was evident in the production of extension subunits or leucocyanidin / leucodelphinidin equivalents, which are biosynthetic presursors to terminal subunits. It is reasonable to propose that the biosynthetic mechanism yielding terminal subunits was minimally affected by temperature while overall flux through the pathway was measurably affected (i.e. increased with

heating). Fruit with the highest heat accumulation also had the highest PA mDP calculated by phloroglucinol analysis (Fig. 2.5.b). A similar result was found in Shiraz grapes and attributed to increased sun exposure (Downey, Harvey et al. 2004). Conversely, cooling slightly decreased mDP and damping significantly decreased mDP. In this case, the effect of damping had a significant effect over heating or cooling alone.

Gel permeation chromatography allowed us to assess the size distribution of skin extracts. Chromatographs of ambient samples from phase I show a pronounced peak at approximately 10 minutes (Fig. 2.6.a). Based upon previous studies, this peak is consistent with material that exceeded the exclusion limit of the columns. The proportion of this material was high before véraison and lower at commercial ripeness (Fig. 2.6.b). There was a marked reduction in material eluting at 10 minutes (Fig. 2.6.c), resulting from day-cooling and damping, which correlates well with mDP's calculated from phloroglucinol analysis.

3.5. Skin Phenolics: Proanthocyanidins at Harvest

The trends in skin proanthocyanidin concentration at harvest as a function of berry temperature were similar to those for flavonols. Day-cooling generally resulted in less total PAs; otherwise, no treatment differences were observed (Fig. 2.7.a). There were minor differences among treatments in PA subunit composition (Table 2.7.). Calculation of PA mDP revealed no trend with respect to heating or cooling treatments (Fig. 2.7.b).

There was little difference among treatments in the size distribution of skin phenolic polymers at harvest (data not shown). The amount of excluded material was negligible. There does not appear to be a trend between skin phenolic polymer size distribution and berry heat accumulation. These results also correlate well with PA mDP's calculated from phloroglucinol analysis. Combined results (GPC and phloroglucinol analysis) suggest that moderate temperature variations between day and night have little impact on the development of skin PAs after véraison.

4. Concluding Remarks

We report the first data on the effects of damping the diurnal temperature range of field-grown grape berries with a focus on proanthocyanidin composition and accumulation. Results provide information on the effect of berry temperature without confounding factors such as solar radiation, irrigation, or vine vigor. The data differentiate between the effects of heat accumulation and the amplitude of the daily range in temperature. There was little difference in seed phenolic material yet considerable variation in skin material within the temperature regimens produced. Damping the diurnal temperature fluctuation of grape berries significantly increased the rate of ripening, exemplified by higher sugar content, berry weight, and anthocyanin concentrations at harvest. Damping berry temperature decreased the mDP of skin PAs at véraison. At harvest, anthocyanin concentration was also higher with temperature-damping. Heating berries during the night increased the mDP and amount of PAs accumulated at véraison, which is correlated with heat accumulation

over time. Cooling berries during the day led to higher flavonol content at véraison but a lower flavonol and PA concentration at harvest.

Recent studies on the biosynthesis of grape flavonoids have focused primarily on the accumulation and composition of anthocyanins. Anthocyanins represent the final step in the flavonoid biosynthetic pathway, which yields flavonols and flavan-3-ols at intermediate steps (Fig. 2.1.). While these flavonoid classes share a common biosynthetic pathway they appear to be under different regulation, as demonstrated in our data. Higher temperature can result in less accumulation of anthocyanins and variations in composition (Kliewer and Torres 1972; Mori, Sugaya et al. 2005; Yamane, Jeong et al. 2006; Mori, Goto-Yamamoto et al. 2007; Walker, Lee et al. 2007) due to plant growth regulator and gene regulation (e.g.: abscisic acid and *VvMYBA1/VvMYBA2*) causing variation in the expression of genes encoding enzymes such as phenylalanine ammonia-lyase (PAL), flavonoid-3′-hydroxylase(F3′H), flavonoid-3′,5′-hydroxylase (F3′5′H), and leucoanthocyanidin dioxygenase (LDOX) (Mori, Sugaya et al. 2005; Yamane, Jeong et al. 2006; Mori, Goto-Yamamoto et al. 2007; Walker, Lee et al. 2007). Because these or analogous genes are involved in PA biosynthesis, that process also may be temperature sensitive. Recently, a transcription factor, *VvMYBPA1*, was found to control genes involved in the PA biosynthetic pathway (*VvLAR* and *VvANR*) in grapes, while not influencing *UFGT* required for subsequent anthocyanin synthesis (Bogs, Jaffe et al. 2007). *VvMYBPA1* affects the production of flavan-3-ols subunits by promoting *VvLAR* / *VvANR* activity. However, there is still conjecture about the mechanism by which extension subunits (flavan-3,4-diol equivalents) are introduced to yield PAs of varying size. From our

study, there appears to be an increase in accumulation of extension as opposed to terminal subunits at véraison with heat accumulation. Treatments involving night-heating had slightly higher concentration of terminal subunits; it is possible that the rate of synthesis of terminal subunits reaches an apparent maximum while the flux of flavan-3,4-diols is proportional to heat accumulated over time. Fujita et al. (2007) recently reported that shading berries resulted in reductions in PA content and mRNA levels of *VvANR, VvLAR1 and VvLAR2* in 'Cabernet Sauvignon.' This correlates well with our observations on heated and cooled berries but does not fully explain responses in the temperature-damped fruit. The latter may be explained by more rapid berry ripening and a shift in metabolism away from PA synthesis to accumulation of anthocyanins. It is likely that the effects of damping diurnal temperature ranges are a combination of influencing the onset of ripening related processes in addition to the kinetics of biosynthesis (Castellarin, Matthews et al. 2007). Investigating gene expression during the manipulation of berry temperature could provide insight into the mechanism behind PA biosynthesis.

The methodologies employed in this study allow detailed information to be collected on the composition and accumulation of secondary metabolites in grape berries. These methods could be used to screen phenolic metabolites to elucidate complex biosynthetic pathways or to study other environmental influences on berry development. Moderate fluctuations in temperature had distinct effects on accumulation of various phenolic compounds and influenced ripening, which may prove to be a valuable knowledge to many in marginal climates.

Acknowledgements

List of Tables

Table 2.1.

Summary statistics for treatments during Phase I

Treatment	Thermal Time[a]	Mean	Max	Min	>40[b]	>35	>30	>25
Ambient	605±7	23.7	40.8	6.4	2.2	49	235	480
Double-Damped	583±5	23.3	35.4	11.3	0	1.4	62	341
Damped	587±6	23.3	36.7	9.4	0	9.6	120	400
Day-Cool	487±36	21.1	35.2	6.5	0	1	50	278
Night-Heat	685±18	25.6	40.1	11.3	0.2	35	235	536

[a] Expressed as degree-days (C) above base 10 ℃, accumulated during Phase I
[b] # hours treatment mean was above 40, 35, 30, 25 ℃ during Phase I

Table 2.2.
Summary statistics for treatments during Phase II

Treatment	Thermal Time[a]	Mean	Max	Min	>40[b]	>35	>30	>25
Ambient	411±9	19.8	40.6	2.5	1.2	39	171	295
Double-Damped	419±15	20.0	39.6	7.3	0	11	57	193
Damped	412±3	19.8	39.9	5.8	0	11	89	240
Day-Cool	322±27	17.6	37.4	3.1	0	7	46	172
Night-Heat	493±13	21.8	39.9	7.0	0	31	164	306

[a] Expressed as degree-days (C) above base 10 ºC, accumulated during Phase II
[b] # hours treatment mean was above 40, 35, 30, 25 ºC during Phase II

Table 2.3.
Berry weights and soluble solids

Sample Date	Ambient weight (g)	ºBrix	Cool weight (g)	ºBrix	Heat weight (g)	ºBrix	Damp weight (g)	ºBrix	Double-Damp weight (g)	ºBrix
12 July 2007	0.36 ± 0.03									
1 August 2007	0.48 ± 0.02									
11 August 2007[a]	0.50 ± 0.03	5.0	0.60 ± 0.03	5.0	0.61 ± 0.03	6.8	0.71 ± 0.11	10.6	0.75 ± 0.01	10.2
24 August 2007	0.61 ± 0.06	15.7								
8 September 2007	0.94 ± 0.06									
27 September 2007[b]	0.98 ± 0.11	22.9	0.95 ± 0.04	21.90	1.06 ± 0.07	23.6	1.07 ± 0.11	23.1	1.04 ± 0.05	23.3

Values expressed as average (n = 4) ± standard error of mean; [a]denotes véraison; [c]denotes "commercial" harvest. Ambient time-point samples: 12 July 2007 = phase I, time1. 1 August 2007 = phase I, time2. 24 August 2007 = phase II, time1. 8 September 2007 = phase II, time2.

Table 2.4.
Seed proanthocyanidin composition, Phase I

| Treatment | Extension Subunits | | | | Flavan-3-ol Monomer and Terminal Subunits | | | | |
	C[a]	EC	ECG	Total[b]	C	EC	ECG	Total[b]	TOTAL[c]
Ambient	508 ± 21	5184 ± 50	1164 ± 27	6856 ± 98	2480 ± 87	1195 ± 64	966 ± 17	4641 ± 168	11497 ± 266
Cool	607 ± 30	5275 ± 232	1178 ± 44	7060 ± 306	2604 ± 208	1708 ± 221	872 ± 77	5185 ± 506	12245 ± 812
Heat	594 ± 41	5420 ± 226	1194 ± 76	7208 ± 343	3005 ± 103	1444 ± 39	1003 ± 49	5453 ± 191	12661 ± 534
Damp	656 ± 24	5790 ± 539	1261 ± 134	7707 ± 697	3479 ± 490	1736 ± 256	945 ± 94	6161 ± 840	13868 ± 1537
Double-Damp	714 ± 24	5370 ± 238	1180 ± 68	7264 ± 330	3263 ± 163	1637 ± 68	867 ± 83	5768 ± 314	13032 ± 644

[a]Values expressed as average (n = 4) $\pm$ standard error of mean in nmol berry^{-1} and with the following subunit abbreviations: C (+)-catechin, EC (-)-epicatechin, ECG (-)-epicatechin-3-O-gallate; [b] Sum total of extension or flavan-3-ol monomer and terminal subunits; [c] Grand total of all flavan-3-ol units.

Table 2.5.
Seed proanthocyanidin composition, Phase II

Treatment	Extension Subunits				Flavan-3-ol Monomers and Terminal Subunits				TOTAL[c]
	C[a]	EC	ECG	Total[b]	C	EC	ECG	Total[b]	
Ambient	384 ± 44	3364 ± 281	783 ± 55	4532 ± 380	735 ± 142	512 ± 101	358 ± 45	1606 ± 288	6138 ± 678
Cool	298 ± 31	2938 ± 185	671 ± 52	3909 ± 268	692 ± 73	483 ± 38	314 ± 22	1490 ± 133	5400 ± 411
Heat	356 ± 28	3286 ± 241	747 ± 97	4389 ± 366	724 ± 42	558 ± 36	369 ± 42	1652 ± 120	6042 ± 503
Damp	398 ± 49	3431 ± 187	806 ± 52	4636 ± 288	770 ± 67	536 ± 23	394 ± 20	1701 ± 110	6337 ± 418
Double-Damp	340 ± 36	3021 ± 68	698 ± 33	4059 ± 137	628 ± 33	466 ± 21	307 ± 10	1402 ± 64	5462 ± 254

[a]Values expressed as average (n = 4) ± standard error of mean in nmol berry^{-1} and with the following subunit abbreviations: C (+)-catechin, EC (-)-epicatechin, ECG (-)-epicatechin-3-O-gallate; [b] Sum total of extension or flavan-3-ol monomer and terminal subunits; [c] Grand total of all flavan-3-ol units.

Table 2.6.
Skin proanthocyanidin composition, Phase I

Treatment	C[a]	EC	ECG	EGC	Total[b]	C	EC	ECG	Total[b]	TOTAL[c]
		Extension Subunits				**Flavan-3-ol Monomers and Terminal Subunits**				
Ambient	38 ± 3	1390 ± 141	53 ± 4	1139 ± 96	2621 ± 244	118 ± 16	21 ± 3	10 ± 1	150 ± 20	2772 ± 266
Cool	33 ± 4	1045 ± 104	38 ± 2	1073 ± 97	2190 ± 207	109 ± 8	15 ± 1	8 ± 1	133 ± 10	2323 ± 220
Heat	45 ± 5	1823 ± 169	59 ± 6	1381 ± 136	3309 ± 316	157 ± 10	15 ± 5	11 ± 0	184 ± 15	3493 ± 334
Damp	35 ± 2	1225 ± 57	46 ± 1	1060 ± 34	2367 ± 94	137 ± 12	16 ± 2	7 ± 2	162 ± 16	2529 ± 113
Double-Damp	35 ± 1	1204 ± 46	40 ± 3	1041 ± 39	2321 ± 89	141 ± 5	17 ± 2	8 ± 1	167 ± 8	2488 ± 100

[a]Values expressed as average (n = 4) $\pm$ standard error of mean in nmol berry^{-1} and with the following subunit abbreviations: C (+)-catechin, EC (-)-epicatechin, ECG (-)-epicatechin-3-O-gallate, EGC (-)-epigallocatechin; [b] Sum total of extension or flavan-3-ol monomer and terminal units; [c] Grand total of all flavan-3-ol subunits.

Table 2.7.
Skin proanthocyanidin composition, Phase II

| Treatment | Extension Subunits | | | | | Flavan-3-ol Monomers and Terminal Subunits | | | | TOTAL[c] |
	C[a]	EC	ECG	EGC	Total[b]	C	EC	ECG	Total[b]	
Ambient	40 ± 5	1645 ± 234	46 ± 5	1300 ± 141	3034 ± 385	152 ± 21	19 ± 4	0 ± 0	172 ± 25	3206 ± 411
Cool	36 ± 2	1203 ± 162	34 ± 3	1106 ± 136	2381 ± 303	117 ± 9	17 ± 0	3 ± 3	137 ± 12	2519 ± 317
Heat	36 ± 5	1477 ± 268	37 ± 5	1298 ± 170	2850 ± 448	135 ± 24	18 ± 2	0 ± 0	154 ± 26	3005 ± 477
Damp	35 ± 2	1384 ± 61	39 ± 2	1284 ± 67	2744 ± 132	123 ± 8	17 ± 6	7 ± 4	149 ± 18	2893 ± 153
Double-Damp	41 ± 7	1571 ± 273	40 ± 7	1319 ± 107	2973 ± 394	153 ± 24	22 ± 1	3 ± 3	179 ± 28	3152 ± 427

[a]Values expressed as average (n = 4) ± standard error of mean in nmol berry^{-1} and with the following subunit abbreviations: C (+)-catechin, EC (-)-epicatechin, ECG (-)-epicatechin-3-O-gallate, EGC (-)-epigallocatechin; [b] Sum total of extension or flavan-3-ol monomer and terminal units; [c] Grand total of all flavan-3-ol subunits.
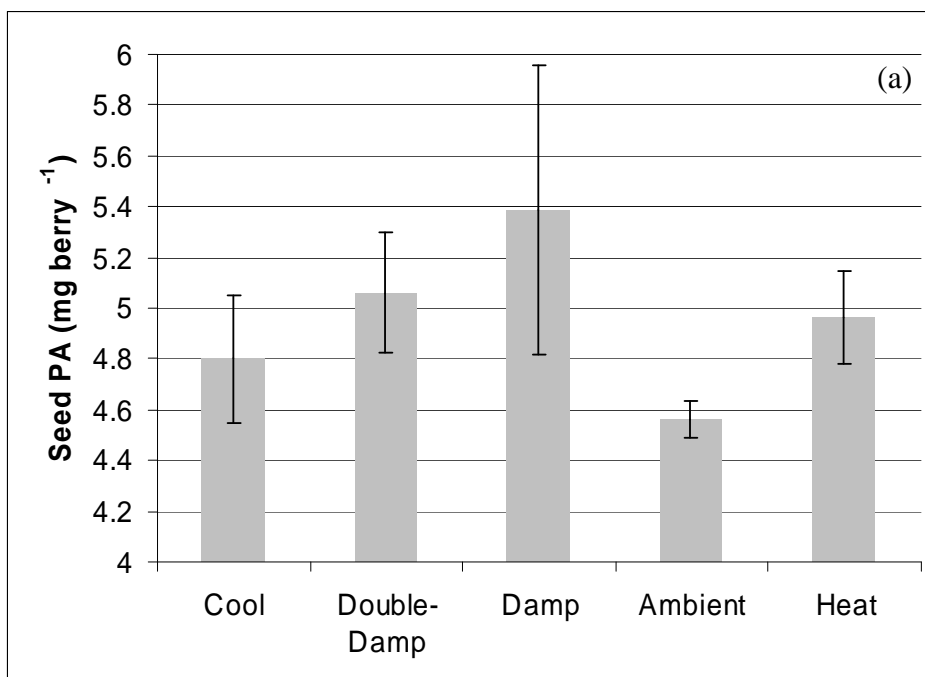
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Figure 2.1.

Figure 2.2.
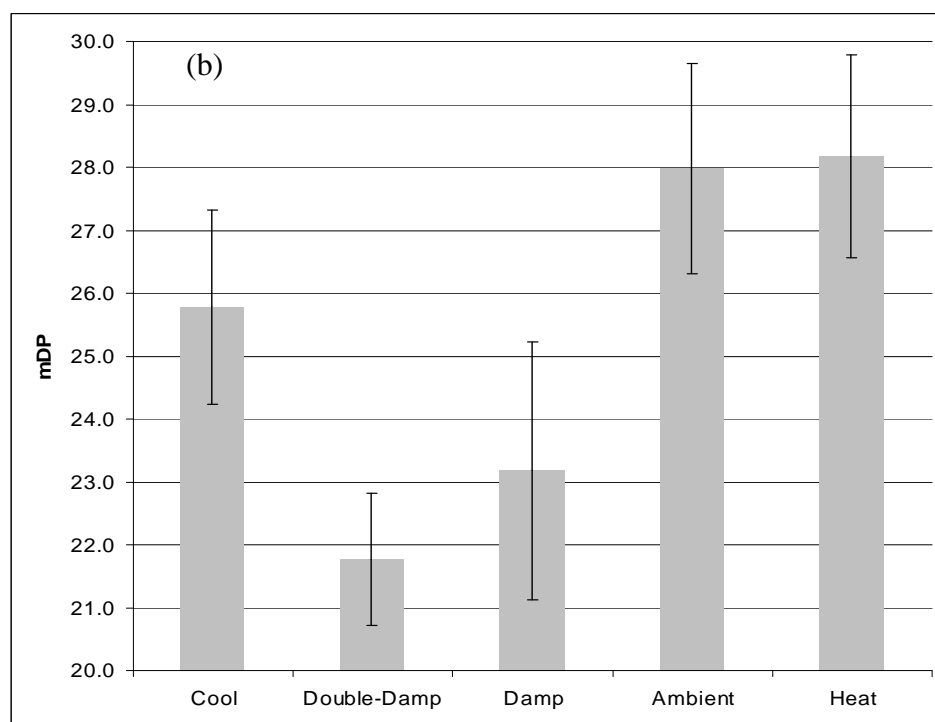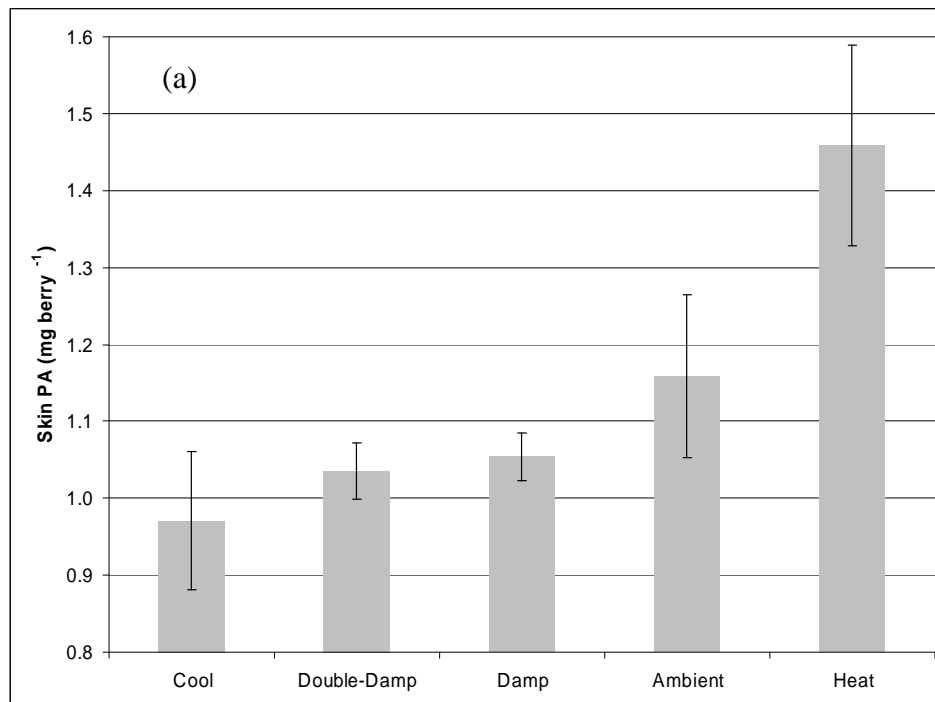

(a)

(b)

Figure 2.3.

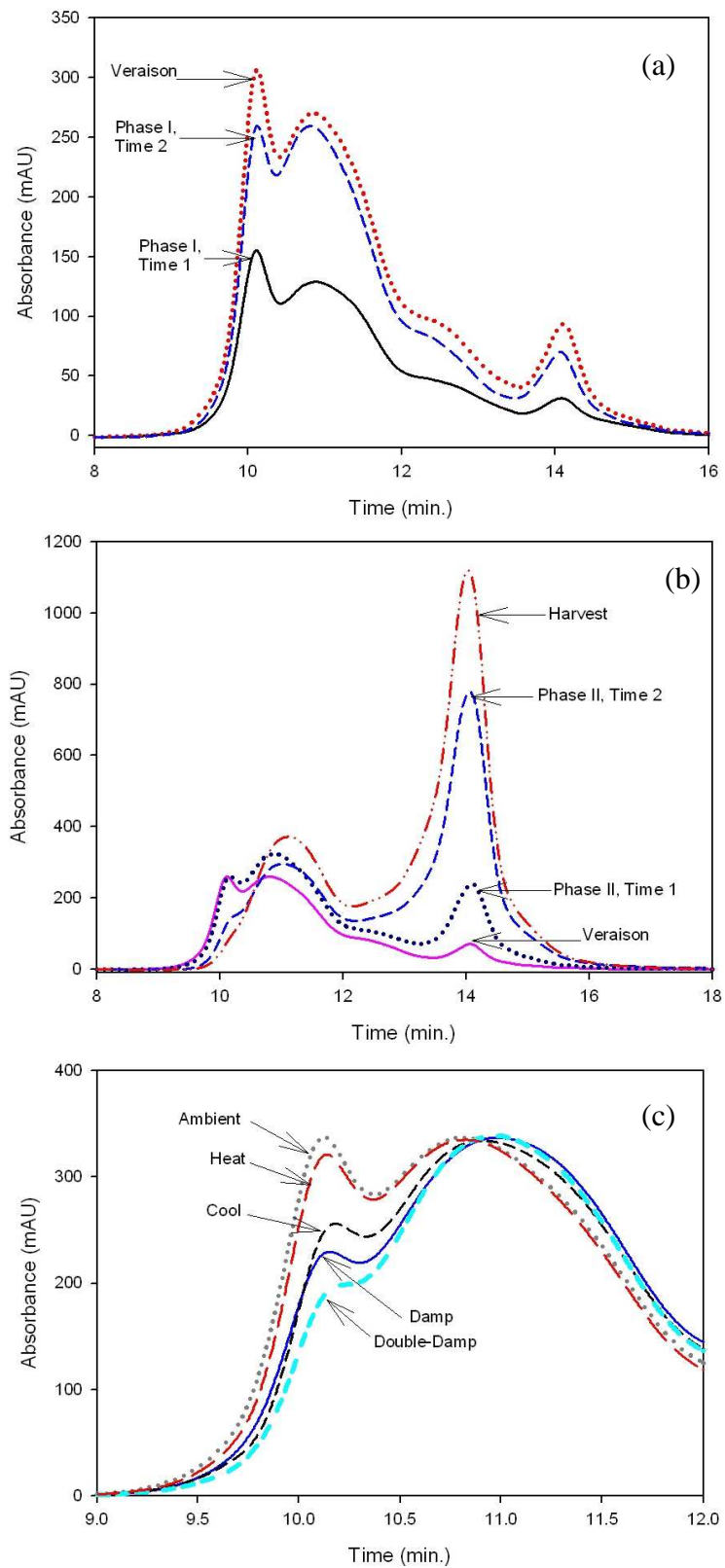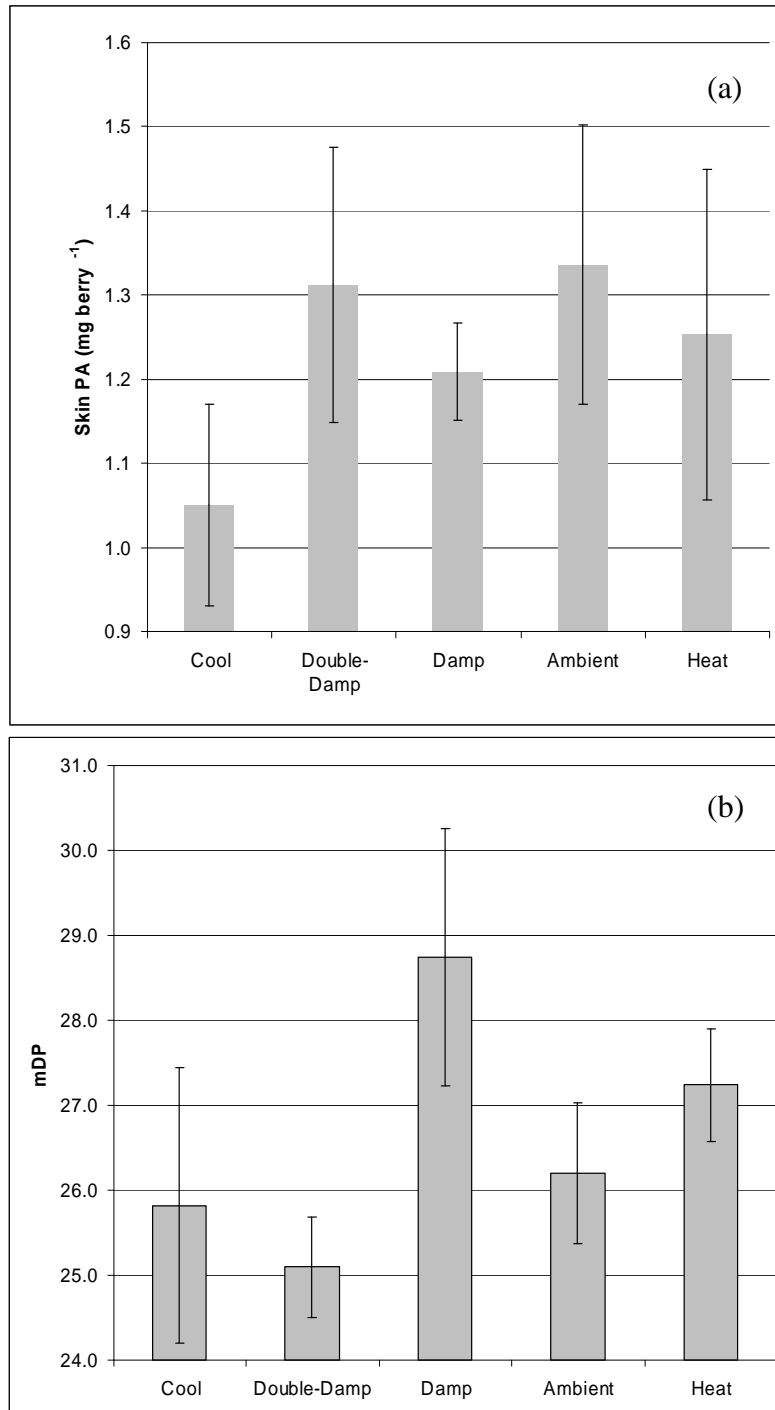Figure 2.4.

Figure 2.5.

Figure 2.6.

Figure 2.7.

Conclusion

The objective of this research was to assess the impact of temperature on phenolic metabolism in grape berries growing in a established vineyard. Novel technological implements allowed us to successfully study the effect of temperature without the confounding effect of sunlight exposure. Precision climate monitoring techniques and detailed chemical analysis was used to determine subtle differences in berry composition due to our treatments. This is the first example of this type of research in grape berries known by the author.

Data from this study shows the effects of day-cooling, night-heating and damping the diurnal temperature fluctuation on grape berries of the type 'Merlot'. The concentration and composition of proanthocyanidins (PA) was most sensitive to treatments when imposed between fruit set and véraison (phase I) whereas anthocyanins were most sensitive to treatment imposed between véraison and commercial harvest (phase II). Flavonol accumulation was effected in both phases of the study although in an opposing manner. Total PA concentration was linearly correlated with total heat accumulation during phase I of the study while PA mean degree of polymerization was decreased by damping the diurnal temperature fluctuation. The number of days to véraison was effectively reduced following damping of diurnal temperature fluctuation suggesting the treatment hastened the ripening process during phase I of the study. These results suggest differences related to timing of ripening as well as differences related to shifts in metabolic rates.

This study should provide information to grape growers faced with decisions regarding planting and cultivation of winegrapes in various climates. It is possible to not only manipulate the composition of a crop but also the time required to ripen. Both of these factors should have obvious implications to grape growers as well as those farming comparable crops. Furthermore, this information should provide insight to those studying phenolic metabolism from a genetic perspective. Combing detailed studies of plant genetics with those of plant metabolomics will hopefully help elucidate biochemical mechanisms not currently understood. These types of studies could prove to be beneficial for improving the disease or pest resistance of plants as well as improving the nutrition and sensory aspects of many important food crops.

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