

THESIS

INCREASING BUD COLD HARDINESS THROUGH FOLIAR APPLICATION OF
ABSCISIC ACID AND UREA ON FOUR CULTIVARS OF *V. VINIFERA* IN WESTERN
COLORADO

Submitted by

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ABSTRACT

INCREASING BUD COLD HARDINESS THROUGH FOLIAR APPLICATION OF ABSCISIC ACID AND UREA ON FOUR CULTIVARS OF *V. VINIFERA* IN WESTERN COLORADO

There is a lack of economic sustainability on the increasingly popular cold-sensitive *Vitis vinifera* cultivars due to cold damage, resulting in very low crop yields. Recent research to improve the cold hardiness of these cultivars to keep up with demands has shown that cold hardiness of grapevine buds can be increased through foliar applications of abscisic acid (ABA) and urea. Therefore, five different ABA treatments at 400 mg L⁻¹ each and one treatment of 40 g L⁻¹ urea were evaluated on Chardonnay and Syrah vines growing at the Western Colorado Research Center in Grand Junction, CO. The treatments were: veraison (V) which was applied at 50-75% veraison, 20 days post-veraison (V20), 40 days post-veraison (V40), double treatments at veraison plus 20 or 40 days post-veraison (V + V20 and V + V40, respectively) and a late season urea treatment. The treatments were evaluated against a control of 0.05% surfactant and water in Chardonnay and Syrah grapes. V and V20 were also evaluated against a control in Merlot and Cabernet Franc. Compound dormant buds were sampled monthly and primary bud survival was assessed. In the early part of the acclimation process, V, V20, V40, and V + V20 treatments showed significant improvements in bud cold hardiness Chardonnay and Syrah, as well as in Cabernet Franc. Bud cold hardiness was unaffected across all treatments in Merlot early on all the way past mid-winter. While no significant difference was detected after October between treatments and bud survivability compared to the control in all varieties, a few

treatments showed positive significant differences from month to month. Yield and basic fruit components were not affected. However, anthocyanin accumulation was significantly greatest in the V20 group for Cabernet Franc. Foliar applications of abscisic acid show potential as future cold hardiness methods and should be evaluated further over several growing seasons for potential prolonged increases in bud cold hardiness.

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1.0 INTRODUCTION

Globally, wine demand has been increasing especially for New World wine. In America, the top 5 wine-growing states, California, Washington, New York, Oregon, and Michigan all have seen increases in their vineyard production and wine sales and all five are still developing new cultivars to accommodate the changing climate in their states (Sommers, 2008). Several states have robust breeding programs including the Cornell-Geneva Breeding and Genetics program in upstate New York which recently released two new cultivars of wine grapes: Aromella, a Muscat-like super hardy cultivar and Arandell, a bluish red wine grape with high resistance to downy and powdery mildew diseases (Garris and Martinson, 2013).

Additionally, winemakers and grape growers are increasingly collaborating to create unique yet reasonably priced wines to highlight the terroir of their products in response to demands for local wines (Sommers, 2008). Terroir, the French word for ground or soil, defines more than just soil; the word is used to describe all the local characteristics of the environment and culture that influence the wine. It is commonly believed that the terroir can be tasted in the wine; in other words, the wine is a product of environmental forces and the decisions in vineyard management and the winemaking process that people employ in that environment. With terroir in mind, all of the 50 US states have established vineyards and wineries to display their unique characteristics and promote the local culture through wine (Sommers, 2008).

1.1 Winemaking in Colorado: The Grand Valley

While not one of the largest wine-producing states, Colorado is rapidly expanding its wine industry with more than 120 licensed wineries and 200 vineyards scattered all over the state, according to the Colorado Association for Viticulture and Enology (CAVE, 2014). 85% of

Colorado's grape production and 40% of wine production take place in the Grand Valley on the Western Slope, a region west of the Rocky Mountains and approximately 30 miles east from the Utah-Colorado border. One of the two federally designated 'American Viticultural Areas' in Colorado, with the West Elks region in Delta county being the other one, the Grand Valley expands from the mouth of the DeBeque Canyon all the way to the Colorado National Monument in Grand Junction, CO, running along the Colorado River (CAVE, 2014). With the Grand Mesa, the world's largest flattop mountain, on the east side and the Book cliffs on the north side, the wine region receives ample solar energy that reflects off the valley floor, providing excellent growing conditions for Syrah, Viognier, and other Rhone varietals. Bordeaux grapes especially Cabernet Franc, perform well on the slightly higher elevation and sloping of East Orchard Mesa in Grand Junction, CO. Both the DeBeque Canyon and the Colorado River provide breezes to cool the wine region down in the very hot, semi-arid summers and bring warmth in the winters that see harsh temperatures from time to time. Another characteristic of the Grand Valley that makes the region an excellent wine producing region is the extremely dry climate that keeps pest and disease numbers very low, so that applications of pesticides and other chemicals are almost unnecessary, unlike more humid climates (CAVE, 2014).

Average high temperatures are approximately 92.1 °F in July and 36.6 °F in January while average low temperatures are approximately 63 °F in July and 17 °F in January. Due to the unique climate characteristics, Grand Valley has just as many growing degree days (GDD) as the Napa Valley, Tuscany, and Bordeaux regions, over a shorter amount of time (CAVE, 2014). GDD reflects total heating over the growing season. The ideal GDD range is between 2,500 and 4,000 GDD, or a growing season with average temperatures between 73.4 and 79 °F (23-26 °C). The GDD concept provides an opportunity to compare the GDD of different wine producing

regions with the notion that areas with similar GDD should be able to produce similar wines (Sommers, 2008). However, other factors including climate, microclimate, altitude, and soil types, must be taken into consideration, as GDD alone does not define a wine growing region (Sommers, 2008). Finally, the climate and alkaline soil of the Grand Valley are very similar to the foothills of the Pyrenees Mountains in Spain, yielding somewhat successful results with growing Tempranillo, one of Spain's premier red wine grapes (CAVE, 2014).

1.2 Cold damage on the Western slope

As in any cool climate winegrowing regions, cold temperature injury to grapes especially in the buds is always a concern in the Grand Valley, as it is the main reason for low yields in Colorado (Caspari and Lumpkin, 2013). While the GDD in the Grand Valley is similar to the GDD in Napa Valley, Tuscany, and Bordeaux, the Grand Valley region's microclimate is different. The Grand Valley is not immune to the harsh Colorado winters and has occasionally seen temperatures below freezing and even below 0 °F especially in December and January (Caspari et al., 2014). From time to time, as in other parts of Colorado, the Grand Valley may observe warmer temperatures as high as 50 °F to 60 °F in the winter and some cultivars of grapes have a tendency to shed their mid-winter hardiness. Once temperatures return to the normal winter temperature range for that month, these grapes may suffer winter injuries as a result of loss of mid-winter hardiness and slow re-acclimation in the vines. Growers in the Grand Valley and the West Elk Valley have seen significant damage to Merlot and Syrah as well as to Tempranillo grapes, partly because of a prolonged exposure to warmer temperatures prior to a return to normal winter temperatures. In addition to winter events, Colorado is also susceptible to fall and spring frosts.

2.0 LITERATURE REVIEW

In order to build up ample protection against cold damage or to minimize the impact of cold damage, grapes must complete the plant physiological cycle from budbreak to dormancy and acclimate gradually immediately following the harvesting of grape berries. During the period of acclimation, after the accumulated sugars have been relocated to the plant tissues, the vine tissues begin to lignify (Davenport et al., 2008). The earlier the berries are harvested, the sooner the grapes are able to acclimate. Early season grape cultivars are much more likely to survive a cold event as they acclimate earlier. Both the fruit and wine quality and the vines' ability to acclimate and harden off properly are significantly affected by the length of the growing season, needing at least 180 days for successful results (Zabadal et al., 2007). Suckers and immature berries are removed to prevent the vine from remaining active late into the season and to jump start the acclimation process (Moyer et al., 2011). When vines do not reach maximum cold hardiness quickly or deeply enough to escape injury, these vines suffer winter injury. Growers of less cold-tolerant cultivars, including Merlot, often face this problem in the Grand Valley. Cold hardiness is influenced by many different factors including genetics, rootstocks, crop load and timing of harvesting, timing of acclimation, speed and magnitude of acclimation and de-acclimation, post-harvest conditions, vineyard weather conditions, and the duration of a cold event (Gu et al., 2001; Caspari et al., 2014).

2.1 Types of cold events

A cold event is when temperatures are low enough to cause injury or damage to the grapes, affecting crop productivity, quality, and survival of the vines themselves (Zhang and Dami, 2011). Beginning in the fall, and ending in late spring, cold events frequently happen with sub-freezing temperatures and winds with speeds that are enough to cause injury on every part of

the vine (Davenport et al, 2007). The abundance and duration of different types of cold events vary from season to season as do the resulting winter injuries. There are several different types of cold events: radiational frosts, freeze, and frost/freeze (Poling, 2008).

Radiational frost (hoar and black frosts) is caused by radiational cooling, a phenomena in which the heat radiates into the atmosphere. This type of frost often happens under clear skies and calm winds, allowing an inversion to develop and ground temperatures to drop below 32 °F. In an inversion, the temperature is proportional to the elevation, increasing with higher elevation to the top of an air layer, meaning the air at ground is the coldest compared to 5-10 feet above. (Zabadal et al., 2007). Drier air also leads to greater heat losses. Some areas observe frequent radiational frosts at low elevations and thus have ‘frost pockets’ of cold air stuck in place. As a result, vineyard topography must be evaluated prior to vineyard establishment (Moyer et al., 2011).

Hoar frost, or white frost, is the most common frost that occurs when water vapor freezes into small crystals on solid grounds. The water vapor initially forms as liquid dew then freezes in response to subzero temperatures. Super cooling, a phenomena in which the grape shoots can drop below their ‘normal’ freezing temperature range and not freeze, may happen at times (Zabadal et al., 2007; Poling, 2008). Another type of frost, black frost, happens when the humidity is too low for frost to form but temperatures fall so low that the water in the plant tissues freeze, damaging and killing the plant tissues. As a result, it is crucial to take the dew point into account when evaluating whether conditions are favorable for hoar or black frost and prepare accordingly. The dew point is the temperature at which the water vapor becomes saturated to the point when it then condenses into dew, fog, or frost (Poling, 2008). Hoar frost signals a high dew point while black frost is the result of a low dew point.

A freeze happens when a cold air mass move into the region via advection, through wind which mixes the air in the lower layers, leading to low day and night temperatures (Poling, 2007). A freeze warning from the National Weather service indicates that there are winds with speeds exceeding 4.5 m-s^{-1} and subfreezing temperatures. This type of event is usually associated with the passage of large frontal systems with cold air masses over an entire region, either with or without clouds (Poling, 2008). Timing is crucial when it comes to cold injury as spring freezes will result in major crop losses of up to 100% especially in the post-bud break stages in the shoot development process compared to mid-winter when the grapes are still in their dormant states and more hardy. Finally, a frost/freeze involves both radiational frost and freeze, and is characterized by winds with speeds typically in the range of 2.2 to 4.5 m-s^{-1} and temperatures below $32 \text{ }^{\circ}\text{F}$. However, losses from this kind of cold event can be quite extensive because of the long duration, which is typically more than 10 hours, plus the wind speed makes for a challenge when it comes to crop protection (Poling, 2008).

2.2 Current methods of cold damage remediation

Fortunately, there are ways to protect against these types of cold events. To start with, a site evaluation prior to a vineyard establishment is very critical, as there are many factors involved that affect crop production. First, as cold air is heavier than warm air, it sinks to the ground, forming an inversion, so that a minimum of 2 to 3% sloping is necessary to allow much of the cold air to move away and draw warmer air from higher layers (Moyer et al., 2011). Additionally, it is more common and preferable to establish a vineyard on a north-facing slope rather than a south-facing slope since the soil and vines on the south-facing soil may warm up due to being more exposed to the sun and thus reduce vine survival. (Zabadal et al., 2007). Vine survival also depends on the timing of a cultivar's bud break and the length of the season to get

full ripening. Vineyard owners must also avoid valleys and poorly drained sites as these may lead to frost pockets, or sinks of cold air that cannot be moved (Poling, 2008; Zabadal et al., 2007; Moyer et al., 2011). Grape cultivar selection when establishing a vineyard is also critical as the wrong cultivars, those that are unable to thrive in the prevailing weather conditions, will result in great economic losses; the grower must be aware of the macro- and microclimates of the site to be planted and select cultivars accordingly. Some cultivars do not ripen properly in certain regions as a result of these regions' short growing season, affecting not only the quality of fruit and wine but also the cold acclimation process and winter hardiness (Zabadal et al., 2007). Thus, an ideal grape cultivar has the capacity to acclimate early in the autumn and be able to reach maximum hardiness, have a slow response to temperature fluctuations in the winter, and de-acclimate late into the spring in order to survive (Howell, 2000; Dami and Zhang, 2011).

On the Western Slope, growers at some sites may benefit from selecting more cold-hardy grapes, typically those with an earlier bud break date such as Marquette, Cabernet Franc, and Pinot Blanc (Hamman and Dami, 1997). Some growers have had to learn this the hard way through trial and error especially with Merlot, resulting in a lessening of the acreage of Merlot being grown after seeing damage in the last 3 of 4 years in 2013 (Caspari et al., 2014). Other passive practices employed by vineyard owners include leaving extra buds to compensate for bud damage, retaining spare canes and renewing them frequently to maintain a full production status (Hamman and Dami, 1997; Keller and Mills, 2007). Cover cropping between rows of vines also significantly lowers the risk of mid-winter injury due to the cover crops' ability to insulate the lower parts of the grapes and reduce heat loss. In the spring, cover crops become active again and competition between the vines and cover crops is rekindled, causing buds to deacclimate at different rates due to the partitioning of the vines' energy between nutrient uptake

and deacclimation. Reduced vigor in the vines due to competition between the cover crops and the vines is associated with a greater cold hardiness as well.

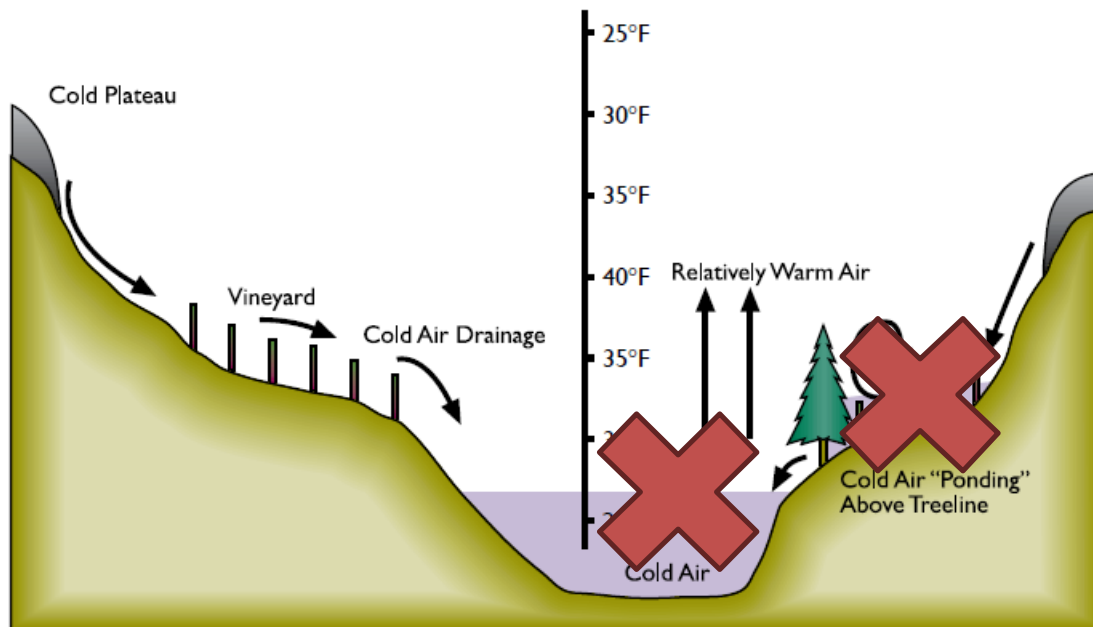


Figure 1: A good vineyard would have 2-3% slope and no frost pockets. (Vineyard site selection, Publication 463-020, Virginia Cooperative Extension)

Other cold protection methods include but are not limited to: wind machines which help mix up the cold air from the ground with the warmer air above, adding a few degrees to the ground temperature; overhead sprinkler systems; and hilling of the soil over the grafted areas for scion bud protection from fluctuating air temperatures. Wind machines can cover up to ten acres and gain 25-50% strength in thermal inversion, but they are expensive to purchase and to operate on fuel, at costs up to \$35,000 per wind machine (Davenport et al., 2008). Some vineyards also employ helicopters as helicopters can cover 25 to 60 acres of vineyards but again, cost is a major concern as it costs \$700-1600 per hour to operate a helicopter hovering at 5 to 10 mph. Overhead sprinkler systems can be used for late season irrigation to replenish the top 2-3 feet to field capacity as such soil moisture can act as a buffer to the low temperatures and reduce the depth of frost penetration (Moyer et al., 2011). Overhead sprinkler systems can also be of benefit during

spring freezes to protect the buds until the temperatures rise above freezing during the daytime (Davenport et al., 2008). This type of irrigation can also help in the repair of air bubbles in the xylem that form in the winter and lead to tissue recovery (Moyer et al., 2011). Propane heaters and return stack heaters both circulate hot air, mixing up the cold air near the ground, with the latter being able to warm up the ground temperature up to 21°F. Cryo-protectants are also another cold protection method used by some vineyard owners. Made with either ethylene glycol, surfactants, or potassium dextrolacetate, cryo-protectants protect plants by lowering the freezing point of tissues or surfaces on the plants, with limited efficiency. Other less common cold protection methods include using ice nucleation active bacteria, vegetable/mineral oil application to delay bud break by a few days, and SIS Frost protection systems (Davenport, 2008). Still other cold protection methods are currently being researched. In one study here at the WCRC, thick growth tubes are used as barriers for the Syrah block, extending 2 feet from the ground; these growth tubes are filled to the maximum with sawdust. This study was initiated to evaluate the potential of the growth tubes as a cold protection method for vine trunks. It also evaluated possible benefits of the growth tubes in protecting the suckers on the bottom of the vines which will give rise to replacement vines. Results were mixed.

2.3 Grapes and cold acclimation

As perennial and woody plants, grapes undergo an annual cycle with five main phases: maturation of the vine, sequestration of stored reserves to initiate and sustain spring growth until the new leaves can uptake carbohydrates to fulfill the nutrient requirements, the initiation of vine acclimation to cold, the maintenance of that cold hardy status and the slow loss of that hardiness through de-acclimation (Howell, 2000). The last three phases that are a part of the dormant season are the most critical components of the annual cycle as these phases determine the grape's

survivability and fruit production capacity in the active season. Grapes achieve cold hardiness during cold acclimation in the fall, reaching the maximum for cold hardiness in mid-winter. Once the maximum for cold hardiness has been reached for a given cultivar, no further increase will happen (Howell, 2000). On the other hand, if the maximum level of cold hardiness hasn't been reached, the grapes can continue to accumulate until the maximum level has been reached for that cultivar (Gu et al., 2001). The main mechanisms for grapes' cold hardiness are freeze avoidance and super-cooling for buds and freeze tolerance for canes and trunks. The speed and magnitude of the cold hardiness acclimation and de-acclimation processes determine the maximum tolerance to low temperatures during different periods of the dormant season (Gu et al., 2001).

Dormancy occurs once the grapes are exposed to decreasing day length and cooling temperatures in late summer following harvest. The first stage in the dormancy process, cold acclimation, in which the grapes respond and adjust to, the changing climate, consists of two parts. The first part, of cold acclimation, takes place in late summer/early Fall after the fruit has been harvested and shoot growth has ended. It is induced by low temperatures above 32 °F (Stafne, 2007). It isn't until the second part of cold acclimation that the grapes begin to achieve full cold hardiness upon exposure to temperatures below freezing (Jansson, 2013). During the cold acclimation process, cryo-protective compound mechanisms are developed, starch is converted to soluble sugars and the amount of free amino acids, lipids, nucleic acids, and proteins all increase. Sufficient stores of carbohydrates help grapes transit from a non-hardy state to a hardy state, with a direct link between stores of carbohydrates and the ability to acclimate to the cold (Howell, 2000; Gusta et al., 2013). If grapes have limited carbohydrate stores due to disease or improper

management practices during the growing season, these vines will not achieve full cold hardiness and will be more susceptible to winter injury.

In the middle of the winter, the grapes reach maximum hardiness (Zabadal et al., 2007). The second stage, or mid-winter hardiness, tests the grapes' abilities to withstand certain climatic adversities especially freezing events and low temperatures and may last until February or March in the Grand Valley (Gusta et al., 2005; CAVE, 2014). However, there is a maximum limit on how much cold grapes can tolerate until mortality, as prolonged exposure to temperatures at or below 0 °F are damaging to *Vitis vinifera* (Howell, 2000). The final stage in the dormancy process, or de-acclimation, is when grapes break their dormancy and readjust to warmer temperatures. De-acclimation occurs as a result of prolonged exposure to above freezing temperatures and normally happens in the spring (Gusta et al, 2013). However, periods of Spring-like temperatures can and do happen during the winter season, leading to severe cold damage. Following a warm spell and de-acclimation as a result, once the grapes re-acclimate in response to normal winter temperature changes, they may not reach their original cold hardiness levels prior to the warming spell (Gu et al., 2001; Stafne, 2007). Therefore, cold hardiness fluctuates from time to time throughout the dormant season (Keller and Mills, 2007).

2.4 Dormant buds and cold damage assessment

There are three main ways to evaluate for cold damage: bud, cane, and trunk. Buds are easier to evaluate for cold damage than either canes or trunks. However, buds do not always reflect the status of the vine and growers must be aware of that prior to making a cold damage assessment. Instead, these buds are viewed as a cold injury to the vines that affects production capacity and fruit quality (Howell, 2000). The vines are capable of producing new shoots in the coming growing seasons if they do not exhibit damage in the canes and/or trunks. Canes and

trunks are typically more hardy than buds during early and late winter but not in the middle of winter, a time when buds are more hardy (Howell, 2000). Xylem is always more hardy than either phloem or buds. Phloem damage can be repaired over time but xylem damage is more destructive and ultimately leads to vine death (Moyer et al., 2011). Tissue damage can be random and irregular throughout both the cane and trunk, which is why a large number of samples are necessary to properly evaluate both types of damage. Additionally, tissue damage can be done internally without any external signs that may lead to stunted growth; however tissue damage is more commonly accompanied by a splitting of the trunk or crown gall symptoms among other symptoms.

In order to evaluate cane and trunk damage, the phloem and xylem must be evaluated for signs of damage through thin longitudinal cuts cut parallel to the length of the cane or trunk. The first cut should remove only the thin cork layer that forms the outer bark; this cut will expose the phloem which should be bright green, if healthy (Goffinet, 2004). When a second cut is done, the xylem will be exposed and this should also be green if healthy. The color of damaged phloem will range from dark brown-green to completely brown. Damaged xylem will be milky white to brown. Trunk damage assessment is identical to the cane damage assessment with similar symptoms of both xylem and phloem damage (Moyer et al., 2011). Ultimately, assessments of cane and trunk damage should not be performed unless primary bud damage is at 50% or greater on a consistent basis. Additionally, since buds contain the fruit clusters for the next growing season, growers want to efficiently evaluate whether their vineyards will have the ability to produce fruit and plan accordingly. Therefore, assessing dormant buds is a very quick and efficient way to obtain answers.

Dormant buds are referred to as compound buds since each bud complex consists of three smaller buds, each representing a compressed shoot that is capable of growth. These buds are formed in the axils of foliar leaves (Goffinet, 2004). The primary bud is the largest and main bud as it contains the most preformed flower clusters. Preformed clusters are clusters that were formed in the previous year for the coming growing season (Goffinet, 2004). The secondary bud is not as developed and typically contains flower clusters which are smaller than primary flower clusters. The tertiary bud normally contains only a vegetative shoot. Following exposure to lethally low temperatures, primary buds are the first to be killed followed by the secondary and tertiary buds (Moyer et al., 2011). Overwintering compound buds begin their development in the spring and summer at the nodes of green shoots that are still growing while current clusters are flowering and developing fruit (Moyer et al., 2011). The compound buds initiate clusters for the next growing season (Goffinet, 2004). The compound buds form deep within the axil at which the leaf petiole and the main stem meet. This point of interest also bears summer lateral shoots that would later harden off or die, if underdeveloped.

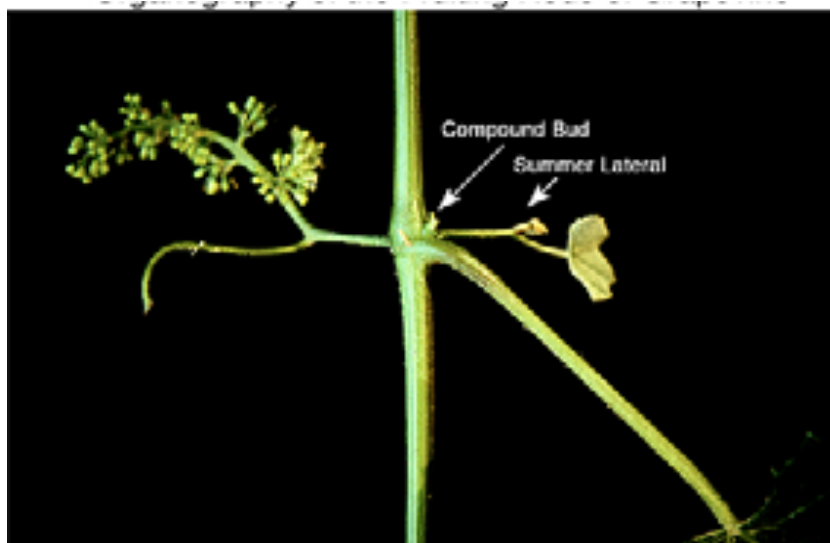


Figure 2: overview of the fruiting stem in midsummer with the compound bud at the axil (Zabadal et al., 2007).

Protected by internal wool and external rigid bud scales, dormant compound buds acclimate to the cold through several strategies (Andreini et al., 2009). A layer of dense lignified cells at the bud base is formed to inhibit perfusion of solutes and water. Additionally, a barrier zone is established just below the primary bud's stem and plays a role in isolating the compound buds from the cane. Finally, cells and tissues throughout the grape super-cool as sugars and solute increase in concentrations in buds. Cell dehydration, cold inducible protein accumulation, and membrane stabilization take place simultaneously (Gusta and Wisniewski, 2013).

In evaluating damage to dormant buds, there are several different techniques: cutting through buds to look for signs of oxidative browning or putting them into water to determine whether growth would occur following exposure to a damaging event (Caspari and Larson, 2006). The technique of cutting through buds, which is based on color change of buds following freezing, is the most common and inexpensive method in evaluating for bud damage (Zabadal et al., 2007). Healthy tissues are able to maintain its green color while injured tissues leak its cell contents particularly phenolic compounds and turn brown (Zabadal et al., 2007). Several cuts are made to the dormant buds and if green tissue is revealed across all three components of the compound bud, the bud is alive. If the primary bud was cut open and found to be brown while the other two are green, it indicates that the primary bud is dead but the secondary and tertiary buds are still alive (Figure 3). Again, secondary buds may produce fruit in smaller quantities and tertiary buds give rise to vegetative shoots only. However, if all three bud components are brown upon cutting, then that means all are dead and there will be no fruit in that compound bud for the next growing season. Growers then use this information to adjust their winter pruning, leaving more buds to compensate for lost buds and/or leaving more spurs/canes per vine (Moyer et al., 2011).

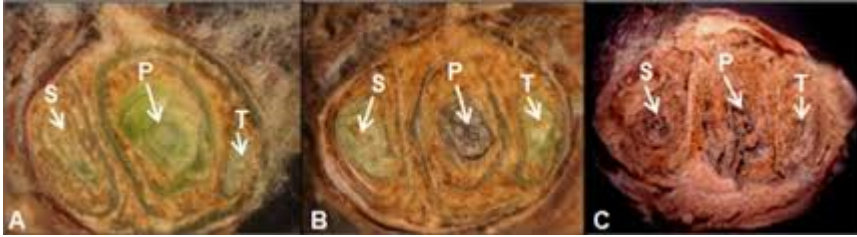


Figure 3: Cross sections of grape compound buds showing the location of primary (P), secondary (S), and tertiary (T) buds. A) alive P, S, and T buds B) dead P bud, alive S and T buds, C) dead P, S, and T buds (Moyer et al., 2011).

2.5 Future cold protection methods: urea and abscisic acid

There is growing research evaluating new cold protection methods for grapes. Two of the cold protection methods being evaluated, foliar applications of urea and abscisic acid, have had ambiguous results on stone fruits including cherries and apples (Zilkah et al., 1996; Ouzounis et al., 2011). With nitrogen as a limiting element in agricultural systems, the depletion of the soil organic matter content, leaching and denitrification often happen, despite the grapevine's moderate requirements for nitrogen (Metay et al., 2015). Hence, urea is a widely used nitrogen fertilizer that is normally applied through the soil but it can also be applied through the plant canopy, minimizing nitrogen losses to the environment (Zilkah et al., 1996; Ouzounis et al., 2011; Lasa et al., 2012). Urea's rapid absorption, low phytotoxicity, and high solubility make it an ideal foliar application product (Ouzounis et al., 2011).

Mixed results have been achieved with late season foliar urea application as a cold hardiness enhancement method. One study stated that foliar urea application had no effect on the cold tolerance of apple trees (Schupp et al., 2001). In another study, when only foliar urea was applied to peaches without any additional application of soil nitrogen, it was found that the weight per fruit and tree yields were significantly reduced compared to either soil application of nitrogen alone or a combination of foliar urea application and soil nitrogen application (Johnson

et al., 2001). However, in one study with avocados and peaches, three foliar applications of 2% low-biuret granular urea have temporarily led to 26% nitrogen enrichment in avocado leaves (Zilkah et al., 1996). As a result of this, leaf freezing hardiness was increased and senescence slowed down; urea-treated leaves were 2.5 times more tolerant to freezing than untreated leaves at the same senescence level. Additionally, a foliar application of 10% low-biuret urea has increased the freezing hardiness and survival of the reproductive organs in peaches before flowering, 3 days prior to a frost occurrence. Potted avocado plants treated with 2% low-biuret urea were exposed to -2 °C (28 °F) for 4 hours and found to be significantly hardier than control plants (Zilkah et al., 1996). In a study on cherries, the cold acclimation in urea-treated sweet cherry shoots were more cold-hardy than the control shoots and the greatest early cold acclimation happened with the earliest urea applications by 4.25 °C (7.65 °F). The late fall urea application samples have only slightly higher cold hardiness levels than the controls. However, despite the loss of hardiness in the control shoots, the cold hardiness of the urea-treated shoots was not affected during several warm spells (Ouzounis et al., 2011). Urea generally may have had a positive effect on cold acclimation, suggesting that higher storage N levels or forms of amino acids that are important for remobilization may lead to more rapid acclimation at moderate temperatures (Ouzounis et al., 2011). It was also discovered that foliar application of urea during early September not only inhibits late season growth but also was associated with the most rapid and long-lasting enhancement of cold hardiness (Ouzounis et al., 2011).

Several studies have demonstrated that nitrogen from urea was converted to amino acids in leaves after foliar application in the fall to apple trees and Concord grapes with the roots and bark being the main sinks for nitrogen (Dong et al., 2002; Cheng and Xia, 2004). Ample reserves of nitrogen from root uptake and the mobilization of nitrogen during leaf senescence, have been

identified as a potential key factor in increased cold tolerance in apple nursery trees as well as in Chardonnay grapes (Cheng, 2002; Tozzini et al., 2013). Nitrogen deficient apple trees have been shown to have a decreased tolerance to low temperatures and do not reach the same level of mid-winter hardiness as healthy apple trees. As a result, these trees are more susceptible to cold damage and poor tree growth. Fortunately, foliar application of urea has been found to enhance cold tolerance through nitrogen replenishment in apples (Schupp et al., 2001). However, freeze tolerance may be reduced if plants uptake too much nitrogen, along with a delay in the ripening process, poor fruit quality, and excessive vegetative growth. The vegetative growth then competes with sugar translocation and pigment accumulation in the grape (Lasa et al., 2012). Consequently, the flavors and berry aroma in wines are affected since the metabolic pathways involved are disrupted (Lasa et al., 2012).

Limited research is still being undertaken to evaluate the benefits of foliar application of urea to grapes, focusing mainly on grape quality through nitrogen management (Cheng and Xia, 2004; Lasa et al., 2012). In grapes, urea is taken up rapidly through the leaf cuticle and translocated back to storage tissues in the form of nitrogen (Cheng and Xia, 2004; Lasa et al., 2012). As a result of foliar application of urea and translocation of the nitrogen from the application to the storage tissues, Concord grapes had larger leaf area, a higher yield, and a higher total vine dry weight (Cheng and Xia, 2004). Fall foliar application of urea increased protein nitrogen and free amino acid nitrogen especially arginine, a good nitrogen source for the alcoholic fermentation of wine, while decreasing total nonstructural carbohydrates (starch and soluble sugars) in Concord grapes (Cheng and Xia, 2004). However, foliar application of urea has no effect on berry size but the timing of such application may be important in achieving optimal quality of grape berries (Lasa et al., 2012). Applications of urea during veraison in both

Merlot and Sauvignon Blanc also increased the abundance of many amino acids including arginine, glutamine which is involved with nitrogen metabolism, and finally, threonine, a major player in wine aroma composition (Lasa et al., 2012). In both Merlot and Sauvignon Blanc, urea applied 15 days after veraison, has been shown to increase the acidity of the resulting wine as well as limit the accumulation of sugars in the berry while maintaining fruit quality. In the second year of the study, both cultivars saw a great increase in the yeast assimilable nitrogen, a combined source of free amino nitrogen, ammonia, and ammonium, available for wine yeast to use during the wine fermentation process, with foliar urea application, especially late, high-dose applications, leading to greater better quality wines (Lasa et al., 2012).

Finally, one study evaluated varying concentrations of nitrogen fertilization in a mixture of 43.3% NH_4NO_3 , 35% urea, and 21.3 % water fed through the drip irrigation system and its role in bud cold hardiness and carbohydrate reserves in White Riesling grapes (Wample et al., 1993). The study has concluded that nitrogen applied in split applications of pre- and post-bloom but prior to veraison, and at rates up to 224 kg N/ha did not consistently reduce bud cold hardiness in White Riesling (Wample et al., 1993). There were specific dates in which high abundance of nitrogen fertilization had significant effects on cold hardiness of buds but these effects were not consistent enough to make a conclusion on whether nitrogen fertilization actually increases cold hardiness in Riesling grapes. Fertilizer applications took place at pre-bloom and again at post-bloom. There was no N application after veraison. On a positive note, the applications increased petiole nitrate-N at bloom and soluble sugars (Wample et al., 1993).

Currently, there is a lack of information on the role of late season foliar application of urea on bud cold hardiness in grapes. This current study applied a novel approach in evaluating foliar applications of urea and its role in bud cold hardiness in grapes based on mostly positive

results achieved with foliar urea in other woody fruit crops. Evaluation of foliar applied urea for cold tolerance also has potential in *V. vinifera* grapes based on results of successful accumulations of amino acids and sugars in grapes in response to nitrogen fertilization. In addition to evaluating late season foliar applications of urea, this study also evaluated foliar applications of 400 mg-L⁻¹ abscisic acid on four cultivars of *V. vinifera*: Chardonnay, Syrah, Merlot, and Cabernet Franc.

2.6 Abscisic acid and its role in bud cold hardiness

Phytohormones regulate many cellular activities from cell division and elongation to responses upon exposure to abiotic and biotic stress. There are 5 ‘classical’ phytohormones: auxins, cytokinins, ethylene, gibberellins, and finally, abscisic acid. Abscisic acid, or ABA, is involved with many processes from the regulation of potassium and sodium uptake in the guard cells to controlling environmental stress effects. Two genes are involved with ABA synthesis: 9-cis-epoxycarotenoid dioxygenase and zeaxanthin epoxidase (Wheeler, 2006). During the first part of berry development, ABA accumulation declines due to berry expansion. However, at veraison, a stage in when sugars and anthocyanins begin to accumulate, ABA has been found to play a role in the accumulation of anthocyanins in red skinned cultivars and berry ripening in all cultivars of grapes (Koyama et al., 2010; Ferrandino et al., 2014). Anthocyanins are secondary metabolites responsible for berry colors and are located in the cells of the skins of red grape cultivars within vacuoles with the greatest amount present in the epidermis and the first hypodermis layer (Mori et al., 2007; Koyama et al., 2010). During maceration, anthocyanins diffuse into the must and wine. Extractions of anthocyanins involve degradation of cell walls and components to allow the diffusion of the anthocyanins from the vacuoles (Romero-Cascales et

al., 2005). However, upon exposure to high temperatures, anthocyanin levels are significantly reduced and berry ripening efforts are negatively affected (Mori et al., 2007).

Finally, ABA plays many roles as the dormancy and senescence hormone, initiating bud dormancy and stimulating alteration of the last set of leaves into bud covers as well as prompting cold acclimation in plants (Zhang and Dami, 2011; Zhang and Dami, 2012a). ABA prevents bud growth during winter dormancy and slows down cellular and meristematic growth once shoots have begun producing mature leaves around harvest time (Gusta et al., 2005). Both ABA-dependent and ABA-independent pathways are involved in the cold acclimation process, demonstrating that ABA also interacts with sugars and nitrogen in the process. Another lesser known phytohormone, jasmonic acid is also involved with the cold acclimation process through a synergism with ABA (Gusta et al., 2005). In previous studies on *Arabidopsis*, it has been shown that both ABA deficient and insensitive mutants cannot acclimate to the cold and that ABA can activate genes for acclimation only upon exposure to low temperatures (Mäntylä et al., 1995; Howell, 2000). ABA was also proven to be ineffective in young grapevines in inducing growth cessation, leaf senescence and abscission, and dormancy. The opposite happened in older grapes; ABA was effective in all of these roles, confirming that ABA was age-induced. Therefore, an ABA-induced gene, RPK1 that encodes a kinase is expressed only at the mature stage of grape development (Lee et al., 2011; Zhang and Dami, 2012b).

During the first part of acclimation, ABA is translocated from leaves into the bark to induce cold acclimation in woody species including grapes. There are three main methods to apply ABA to plants: root drench, stem cuttings, and foliar application. The root drench and stem cutting applications of ABA in several plants, including cereal rye and potato plant cuttings, respectively, have demonstrated higher levels of freezing tolerance than the foliar application

method due to higher retention of ABA. In the foliar spray method of applying ABA, the leaf cuticle acts as a barrier that must be overcome through a high amount of application to force the guard cells to open up (Gusta et al., 2005). However, ABA induced a limited amount of freezing tolerance for a brief period, temporarily increasing freeze tolerance from -15 °F to -27 °F (Gusta et al., 2005).

Additionally, ABA has been identified to play a role in the development of cold acclimation and ultimately, freeze tolerance in silver birch, barley, cereal rye, common wheat, and potatoes as well as in *Arabidopsis* (Zhang and Dami, 2011). The speed and magnitude of cold hardiness acclimation/de-acclimation also determine maximum tolerance to low temperatures during different periods of the dormant season (Gu et al., 2001). On grapes, exogenous ABA applications have been evaluated to delay bud break for spring frost protection but the applications did not lead to consistent results in delaying budburst in field-grown Cabernet Sauvignon and Sangiovese grapes (Hellman et al., 2006). Only the soil applied container-grown grapevines have seen a consistent delay in bud break (Hellman et al., 2006). However, Zhang and Dami, 2012 has demonstrated that bud break of ABA-treated field grown vines at veraison and 20 days post-veraison have been delayed by an average of 6 days (Zhang and Dami, 2012a). Exogenous ABA application also increased the color quality and skin anthocyanins, leading to darker berries in table grapes (Peppi et al., 2006; Zhang and Dami, 2012). Additionally, it has been shown that the timing of the ABA application is important since the effectiveness of a fruit ripening and abscission regulator, ethephon, declines post veraison (Peppi et al., 2006). 20 to 30 days following 50-75% veraison has been proven to be optimal timing to apply ABA on field grown Chambourcin grapes, enhancing the periderm formation and anthocyanins (Zhang and Dami, 2012b). Peppi et al identified a reduction in ABA accumulation

in the berry skins, resulting in a decreased abundance of anthocyanin accumulation and color upon exposure to high temperatures (Peppi et al., 2006). Juice and yield components were not affected. In Cabernet Sauvignon, ABA application at between 80% berry softening and 10 days after 100% berry softening is the most effective for anthocyanin accumulation (Zhang and Dami, 2012a).

Zhang and Dami evaluated the influence of foliar application of ABA on grape dormancy and observed physiological and morphological changes in greenhouse grown grapes. Based on phytotoxicity results with deformed leaves and bud injury at concentrations higher than 400 mg-L⁻¹ up to 3200 mg-L⁻¹, the optimal concentration of ABA to apply was identified to be 400 mg-L⁻¹ (Zhang and Dami, 2011; Zhang and Dami, 2012b). Other evidence supporting this concentration selection is the successful periderm formation and shoot inhibition that took place in the greenhouse grown Cabernet Franc and Chambourcin grapes. Timing also plays a role in the successful periderm formation and shoot inhibition, confirming that ABA is optimally applied between fruit set and the post-veraison stage. However, optimal ABA concentrations depend on the cultivar, with 400 mg⁻¹L⁻¹ being the standard concentration based on successful results (Zhang and Dami, 2012b).

2.7 Objectives of this study

This study evaluated foliar application of 400 mg-L⁻¹ S-ABA on four cultivars of *Vitis vinifera* L. Cabernet Franc, Chardonnay, Merlot, and Syrah, to determine whether timing of application is a factor in achieving optimal bud cold hardiness. On the Chardonnay and Syrah blocks, this study also aimed to evaluate whether double applications of ABA would increase the bud cold hardiness compared to a single application. Additionally, this study also aimed to evaluate late Fall foliar application of 40 g-L⁻¹ urea on both the Chardonnay and Syrah blocks to

evaluate whether there is a marked improvement in cold tolerance by urea. Finally, this study examined the potential effects of both foliar applications on delaying bud break in all four cultivars.

Based on successful results with ABA in *V. vinifera* cultivars with similar development timings, foliar applications of ABA may increase bud cold hardiness in the Chardonnay, Syrah, Merlot, and Cabernet Franc (Zhang and Dami, 2012a/b). The timing of ABA foliar application may also positively influence bud cold hardiness. Double application of ABA may not improve the cold hardiness of either Chardonnay or Syrah because the grapes may have a limitation on how much ABA can be taken up. Urea application may also improve bud cold hardiness. Both urea and ABA foliar applications have the potential to delay de-acclimation, or bud break in all four cultivars being studied here.

3.0 MATERIALS AND METHODS

3.1 Site Description: Western Colorado Research Center- Orchard Mesa

The Western Colorado Research Center- Orchard Mesa (WCRC-OM) is part of a seven research center network throughout the state of Colorado, affiliated with Colorado State University's College of Agricultural Sciences. Located in the Grand Valley, WCRC-OM is located seven miles southeast of Grand Junction and houses a research greenhouse and labs, as well as Colorado State University's own teaching winery, Ram's Point Winery. The research center lies at an elevation of 4750 feet with the soil types being Gyprock mesa clay loam and Hinman clay loam. Average high temperatures are approximately 93.7 °F in July and 42 °F in January and average low temperatures are approximately 64.6 °F in July and 21.1 °F in January (Larsen, 2006). The eighty acre research center is home to twelve acres of experimental orchards, particularly apples, peaches, and pears as well as to approximately three acres of vineyards. The remaining acreage is devoted to an arboretum, other tree fruit experiments, and grass trials. The total vineyard acreage is 2.65 acres with 0.58 acres of Chardonnay grapes and 1.3 acres of Syrah grapes.

3.2 Site Description: Mesa Park Vineyard and Caspari Family Vineyard

Collaboration with both Mesa Park Vineyard (Site A), and Caspari Family Vineyard (Site B), provided an opportunity to evaluate Merlot and Cabernet Franc, respectively, in this study. Both vineyards are located two miles east of the WCRC-OM and therefore exhibit similar microclimates to the research vineyard blocks onsite. Established in 1996, on 7.65 acres of land with a 2% slope, Site A is home to not only the Merlot block that is used in this study but also Cabernet Franc and Cabernet Sauvignon plus a winery that specializes in Bordeaux style wines. Established in 2009, Site B consists of 3.0 acres of Albariño, Chambourcin, Souzão, and

Cabernet Franc grapes. Again, only the Merlot grapes from Site A and the Cabernet Franc grapes from Site B were used in this study in addition to the Chardonnay and Syrah at WCRC-OM.

3.3 Plant material and experimental design and treatments

Table 1: Management practices of the grapes being evaluated in this study *In the Syrah vineyard block, some vines have been pruned as low cordon and halbogen.

Variety	Vine x row spacing	Type of irrigation	Training system
Chardonnay (WCRC-OM)	5' x 10' or 1.5m x 3.05 m	Drip	Vertical shoot positioning
Syrah (WCRC-OM)	5' x 9' or 1.5m x 2.7 m	Sub-surface drip	Vertical shoot positioning*
Merlot (Site A)	5' x 9' or 1.5m x 2.7 m	Furrow	Vertical shoot positioning
Cabernet Franc (Site B)	4.9' x 6.6' or 1.5 m x 2 m	Furrow	Vertical shoot positioning

Vitis vinifera var. Cabernet Franc, Chardonnay, Merlot, and Syrah, were used in this study and all except Chardonnay were on own roots. The Chardonnay block consisted of vines grafted on *V. riparia* x *V. rupestris* 101-14, *V. berlanderi* Kober 420-A, *V. riparia* x *V. rupestris* Couderc 3309, and *V. berlanderi* x *V. riparia* Teleki 5C as well as vines on their own roots.

In 2014, seven treatments were randomly assigned to vines on the Chardonnay and Syrah blocks, using the Randomized Complete Block method: Control (0 mg/L and sprayed only with water and 0.05% surfactant), V (400 mg/L ABA sprayed at 50% veraison), V20 (400 mg/L ABA sprayed 20 days after 50% veraison), V40 (400 mg/L ABA sprayed 40 days after 50% veraison), V+ V20 (two applications of 400 mg/L ABA sprayed at veraison and 20 days after), V+ V40 (two applications of 400 mg/L ABA sprayed at veraison and 40 days after), and finally, a solution of urea at 200 g urea to 5 L water. On the Merlot and Cabernet Franc blocks, three

treatments were randomly assigned to vines: Control, V, and V+20. All blocks consisted of 10 replicates per treatment and each treated vine had a buffer vine on either side.

The ABA concentrate (ProTone SG; Valent Bioscience, Libertyville, IL), had an a.i. of 20.0 % (w/w) S-ABA and was then dissolved in water with 0.05% Tween-20 surfactant solution (Acros Organic, Hampton, NH). The final ABA solution was sprayed onto whole vine canopies (leaves and clusters) to runoff with a 20L backpack sprayer (Model SG20; STIHL, Inc., Norfolk, VA). The urea concentrate (46-0-0 urea fertilizer, Potash Corp Inc., Saskatoon, Saskatchewan) had an a.i. of 46.0 % of urea. The concentrate, at 40 g per 5 L of water, was dissolved in water and applied in the same behavior as the ABA solution had been. For both ABA and urea applications, each vine received an average of 0.4 and 0.5 L of spray solution, respectively.

Table 2: Treatment and sampling dates of foliar applications of both ABA and urea in Chardonnay and Syrah blocks.

3 Treatment	Treatment dates	Cold-hardiness sample dates
Control	13 August 2014	21 Oct. 2014, 17 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015,
V	13 August 2014	22 Oct. 2014, 17 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015
V20	02 September 2014	21 Oct. 2014, 17 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015,
V40	22 September 2014	21 Oct. 2014, 18 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015
V + V20	13 August 2014 and 02 September 2014	22 Oct. 2014, 17 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015
V + V40	13 August 2014 and 22 September 2014	22 Oct. 2014, 17 Nov. 2014, 5 Jan. 2015, 16 Feb. 2015, 24 Mar. 2015
Urea	02 October 2014	21 Oct. 2014, 18 Nov. 2014, 5 Jan. 2015 , 16 Feb. 2015, 24 Mar. 2015

Table 3: Treatment and sampling dates of foliar applications of ABA in Merlot and Cabernet Franc blocks.

Treatment	Treatment dates	Cold-hardiness sample dates
Control	15 August 2014	04 September 2014, 28 October 2014, 24 November 2014, 09 January 2015, 20 Feb. 2015, 23 Mar. 2015*
V	15 August 2014	27 Oct. 2014, 24 Nov. 2014, 09 Jan. 2015, 20 Feb. 2015, 23 Mar. 2015*
V20	09 September 2014	27 Oct. 2014, , 24 Nov. 2014, 09 Jan. 2015, 20 Feb. 2015, 23 Mar. 2015*

*Due to early pruning by cooperative grower in Site A, Merlot was not included in the study for March

3.4 Bud assessment for cold hardiness

With ten replicates for each treatment studied, five replicates with four buds per replicate per treatment, were randomly selected monthly at the time of bud sampling and cut into two-node sections for incubation in the Tenney temperature chamber (Caspari et al., 2014). The Tenney temperature chamber, a programmable freezer where the temperatures can be controlled, was initiated at the temperature that was equal to the outside temperature at the time of sampling. For instance, on 22 October 2014, the temperature at the time of the bud collection was approximately 48 °F or 9 °C, so the freezer was programmed to start at 48 °F or 9 °C. Regardless of the starting temperature, the freezer was programmed to reduce the temperature by 5 °F (3.2 °C) over a 30-minute interval, and then hold at that temperature for 30 minutes. This process was repeated until the target threshold temperature for a sample was reached. At the end of the holding period at a given temperature, a twenty-bud sample was removed for each treatment and the temperature continued to decrease by 5 °F (3.2 °C) over 30 minutes and held for 30 minutes, so on. Following removal from the freezers, the buds were left at room temperature for at least

24 hours prior to cutting. Afterwards, the buds were cut open to evaluate tissues, with vibrant green tissue identified as live and brown tissue as dead (Caspari et al., 2014). Live primary and secondary buds were evaluated to determine bud viability for each treatment in all blocks. This bud evaluation process was repeated monthly until bud break.

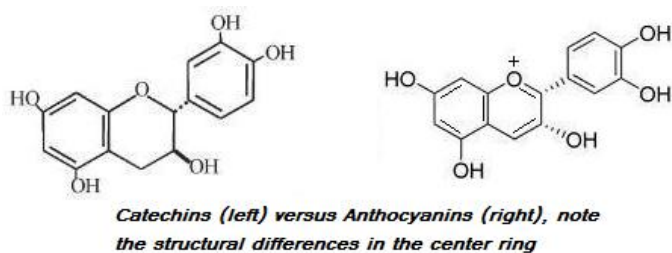
3.5 Yield components and basic juice analysis

Yield components including crop weight per vine, cluster number per vine and weight of 50 berry samples were collected for Cabernet Franc and Chardonnay. Pruning weights were collected from 26 March 2015 to 31 March 2015 for Chardonnay. Due to cold injury from a December 2013 event, there was no fruit on Merlot and Syrah. Cluster weight was calculated based on crop weight per vine divided by cluster number per vine. Following harvest for all blocks, a berry juice analysis was conducted to determine pH, soluble solids expressed in Brix, and titratable acidity (TA). The berry samples were weighed, then juiced at room temperature, and centrifuged for 30 seconds at 5000 rpm (International Clinical Centrifuge Model C., Boston, MA). Following centrifugation, soluble solids were measured with a digital refractometer and the pH was taken using a digital pH meter then 2.5 mL of juice samples were diluted with 50 mL of distilled H₂O for TA analysis (Atago USA Inc., Bellvue, WA; Mettler Toledo Inc., Columbus, OH; Model 720 Orion, Thermo Electron, Inc, Waltham, MA). The prepared juice samples were then titrated with 0.1 M NaOH to an endpoint of pH 8.2.

3.6 Anthocyanin analysis

Most anthocyanins, the color causing pigments, are present in middle lamella walls of the cells of the grape berry skins so the skins were separated and weighed to 1 gram per replicate and extracted with 5 mL of 10% formic in methanol twice on a shaker for one hour in the dark. The extracts were combined and centrifuged at 18,000 x g for 10 min and diluted to 1:20 with 0.2 M

sodium acetate hydrochloric acid buffer pH 1.0 prior to analysis at 530 nm on a spectrophotometer (Keller and Hrazdina, 1998; Jeong et al., 2004; Zhang and Dami, 2012a). The major anthocyanins of dark *V. vinifera* are the 3-glucosides of malvidin, delphinidin, peonidin, petunidin, and cyanidins, all of which are also phenolic compounds (Zhang and Dami, 2012a). Due to malvidin-3-glucoside standards not being available, a similar molecule, catechin as well as quercetin, a flavanol, were used as standards in concentrations of 1:10 and 1:50 in dimethyl sulfoxide since neither were able to dissolve in dH₂O and the anthocyanin content was corrected using the following: absorbance molar absorptivity (mol/L) * molecular weight (g/mol) * dilution ratio * 0.1 L * 1000 (mg/g)/sample weight (g) (Figure 4; Zhang and Dami, 2012).



(Modified from: Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr.* (2003) and Ferreira D, Coleman CM. Towards the synthesis of proanthocyanidins: half a century of innovation. *Planta Med.* (2011))

Figure 4: Structure of catechins versus anthocyanins

3.7 Statistical analysis

Each month, for each variety and treatment, a minimum of three temperature groups were evaluated and run in the programmable freezer. Based on the growth curves for all varieties, the temperatures that were evaluated change from month to month to test at the minimum and maximum threshold cold temperatures for all varieties (Figure 5). Again, ten replicates for each treatment were studied, five replicates with four buds per replicate per treatment, were randomly selected monthly at the time of bud sampling and cut into two-node sections, providing twenty buds per replicate per treatment for statistical analysis.

Fisher's Exact Test and t-tests were used to evaluate the differences between treatments in comparison to the control, using LT50 values. LT50, or lethal temperature thresholds were calculated at 50% bud survivability, using the bud survivability data from the stimulated freezing tests to compare at what temperatures 50 % of the buds from each replicate in each treatment group can survive each month (Figures 4-7). The null hypothesis is that "the treatment doesn't affect bud survivability" and the alternative is "the treatment positively affects bud survivability" and the null hypothesis is rejected at $p < 0.05$. LSD (least significant differences) and differences in means tests were also used to analyze how significantly different the treatments are from the control. In analyzing the harvest data, the mean of all 10 replicates per treatment group except for urea in Chardonnay and Cabernet Franc, were analyzed in the following categories: number of bunches, crop yield (kg), weight of 50-berry sample, pH, Brix (carbohydrates), titrable acidity, and finally, anthocyanins (pigment compounds in grape berry skins in Cabernet Franc, only). The standard deviations were calculated for the basic fruit and yield components as well to determine whether the replicates were close to the mean or further.

4.0 RESULTS & DISCUSSION

4.1 Effects of ABA and urea applications on fruit and yield components

Due to extensive cold damage in the winter of 2013-2014, the crop yields for both Merlot and Syrah were very low and no data has been collected. On the other hand, both the Cabernet Franc and Chardonnay blocks had nearly normal crop yields, however, there is no significance in the effects that the treatments have on the number of bunches, crop load, weight of 50-berry samples taken, pH, Brix, or titrable acidity in either Cabernet Franc or Chardonnay at $p < 0.05$ (Tables 4 and 5). Within the replicates, there were some moderately wide deviations from the mean value in the crop yield and the number of bunches, which is expected as the vines have variable numbers of shoots and therefore, fruit clusters. The 50-berry sample weights for all treatments in Chardonnay and the control group of Cabernet Franc also had wide deviations. This may be due to the fact that some of the fruit on the vines were at different stages of development, which may have caused variance in the weights of each replicate. In other words, some replicates may have contained a few unripe berries or underdeveloped berries in the 50-berry samples. Human errors may also have been present, which may have affected the harvest data information for both Chardonnay and Cabernet Franc. Nevertheless, the standard deviations for pH, Brix, titrable acidity, and anthocyanins (Cabernet Franc) were very small, which means that the replicates' values were close to the mean value for each group, with the exception of Chardonnay's control (Tables 4 and 5). The null hypothesis is that "the treatment hurts the harvest or does nothing" and the alternative is "the treatment helps the harvest" and the null hypothesis is rejected when the p-value is lower than 0.05 in a T test. Anthocyanin accumulations were found to have significantly increased with the V20 group for Cabernet Franc

at $p < 0.05$ for $p = 0.019$. The V group for Cabernet Franc has demonstrated a slight increase in anthocyanin accumulation, however it was not significant at $p = 0.104$.

Table 4: Average effects of ABA and urea on basic fruit and yield components in Cabernet Franc grapes

*Within rows, means followed by the letter b denote significant difference from a in anthocyanins

**standard deviations are in parentheses immediately below the mean values for each category

Harvest Date	Treatment	Number of bunches	Crop yield (kg)	weight of 50 berry sample	pH	Brix°	Titrable acidity (g/L)	Anthocyanins
								(mg/g skins)
6-Oct-14	Control	30 (21.13)	1.09 (0.72)	45.48 (24.41)	3.56 (0.10)	28.86 (0.94)	5.18 (0.69)	0.82a* (0.25)
	V	37.3 (20.45)	1.06 (0.61)	55.55 (7.48)	3.89 (0.97)	28.28 (0.76)	5.05 (0.71)	1.06b (0.51)
	V20	32.8 (20.53)	1.08 (1.08)	54.28 (2.43)	3.55 (0.08)	28.26 (1.04)	5.13 (0.44)	1.88b (1.37)
	Significance $p < 0.05$	ns	Ns	Ns	ns	ns	Ns	0.019

Table 5: Effects of ABA and urea on basic fruit and yield components in Chardonnay grapes

*no harvest data was available for urea treated vines as the vines were treated post-harvest

**standard deviations are in parentheses immediately below the mean values for each category

Harvest Date	Treatment	Number of bunches	Crop yield (kg)	Pruning weight (kg)	Weight of 50 berry sample (g)	pH	Brix°	Titrable acidity (g/L)
19-Sep-14	Control	16.1 (12.32)	1.02 (1.03)	0.30 (0.34)	41.82 (30.41)	3.07 (1.25)	25.49 (1.95)	6.87 (1.13)
	V	17.9 (11.02)	0.99 (0.69)	0.29 (0.23)	45.19 (24.55)	3.56 (0.14)	31.19 (11.19)	7.73 (1.17)
	V20	18.8 (9.90)	0.95 (0.54)	0.26 (0.24)	53.53 (22.19)	3.48 (0.12)	25.46 (1.87)	7.36 (2.00)
	V40	16.2 (11.03)	0.81 (0.57)	0.23 (0.17)	44.44 (24.48)	3.5 (0.20)	24.75 (1.29)	7.54 (1.39)
	V + V20	12.9 (9.94)	0.69 (0.68)	0.28 (0.22)	43.58 (31.13)	3.48 (0.18)	24.84 (1.34)	7.54 (1.96)
	V+ V40	27 (19.22)	1.72 (1.57)	0.24 (0.27)	46.13 (17.71)	3.51 (0.13)	26.21 (1.40)	6.24 (1.22)
	Urea	N/A*	N/A*	0.23 (0.17)	N/A*	N/A*	N/A*	N/A*
	Significance $P < 0.05$	Ns	Ns	Ns	ns	Ns	Ns	Ns
	Std. Dev.	12.89	0.94	0.23	24.7	0.523	5.06	1.51

4.2 Effects of ABA and urea on bud survivability and lethal temperature threshold

Though different treatments showed significant differences in primary bud survival compared to the control groups in all but the Merlot varieties, during each month of sampling, no consistent overall significant difference can be concluded at p levels of <0.05.

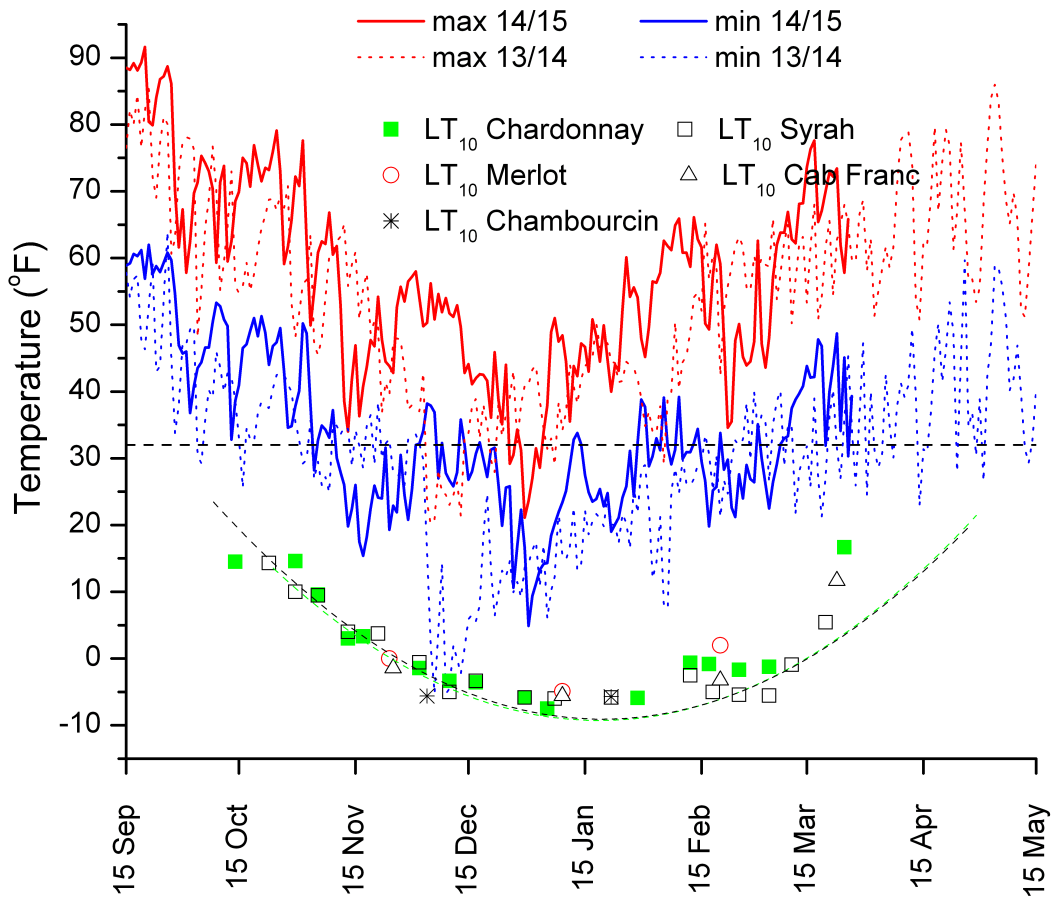


Figure 5: Grape buds' response to seasonal minimum and maximum temperatures at all experimental vineyards as reported by the Colorado Agricultural Meteorological network (Caspari et al., 2014)

Lower LT_{50} values mean the primary buds can survive at lower temperatures and thus, are more cold hardy. Additionally, grape buds tend to respond in accordance to daily temperatures and generally, cold hardiness should increase with decreasing temperature (Figure

5). In Chardonnay, in October, the V and V + V20 treatments demonstrated lower LT₅₀ than the control, meaning these two ABA treatments may have increased the bud cold hardiness of the buds for that month. These two treatments were significantly lower than the control for $p < 0.05$: V ($p = 0.001$) and V + V20 ($p = 0.001117$) (Table 6). In regards to LSD values, insufficient replicates were collected for three of the treatment groups in Chardonnay, so no LSD or differences in means can be calculated. The LSD and difference in means as well as LT₅₀ values were not significant for the other months, concluding that no treatments has demonstrated greater cold hardiness than the control group in November, January, February, or March (Table 6, Figure 6).

Table 6: Mean LT₅₀ values for all treatments at $p < 0.05$ in Chardonnay

*Within rows, means followed by the same letter (a) are not significantly different ($p < 0.05$).

**Differences in means are expressed for significantly different means in parentheses

Chardonnay	Control	V	V20	V40	V + V20	V + V40	Urea	LSD
October	13.2a	9.54b	11.67a	12.1a	9.3b	11.9a	12.3a	na
November	-5.5a	-4.5a	-6.11a	-5a	-6.5a	-4a	-4.67a	ns
January	-13.67a	-9.67a	-11.87a	-11.67a	-11.67a	-9.5a	-7.83a	ns
February	-6a	-2.33a	-2.5a	-6.875a	-3a	-5a	-1.875a	ns
March	11.53a	17.67a	15.5a	15.4a	14.33a	10.73a	14.73a	ns

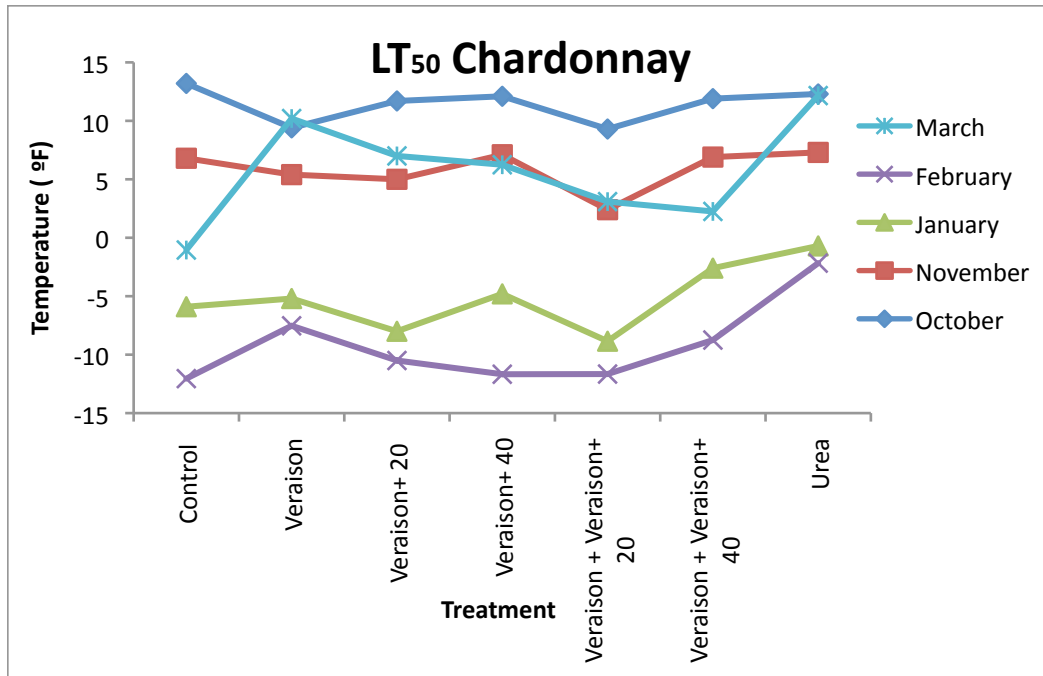


Figure 6: Lethal temperatures at which 50% of primary buds are killed across all treatments for Chardonnay.

Two treatments in the Syrah variety also demonstrated early significant increase in primary bud survival compared to control according to the LT₅₀ data: V40 ($p = 0.035$) and V + V20 ($p = 0.0218$) in October (Table 7). However, this was not confirmed by the LSD values and differences in means reported (V40 = 2.67 and V + V20 = 2.33) since if the differences in mean values are higher than LSD, then the results are significant at $p < 0.05$. November, for Syrah, urea showed the significantly lowest LT₅₀ of all treatments at -5.17 °F, $p = 0.043$ (Table 7). Again, the LSD = 9.58, difference in means = 2.33 did not confirm that urea absolutely demonstrated significance at $p < 0.05$ (Table 7). In January to March, the control group demonstrated the lowest LT₅₀ values, citing that the treatments may have had adversely affected bud cold hardiness (Table 7, Figure 7).

Table 7: Mean LT50 values for all treatments at $p < 0.05$ in Syrah

*Within rows, means followed by the same letter (a) are not significantly different and means followed by a different letter (b) are significantly different ($p < 0.05$).

**Differences in means are expressed for significantly different means in parentheses

Syrah	Control	V	V20	V40	V + V20	V + V40	Urea	LSD
October	11.17a	10.67a	11.83a	8.5b (2.67)	8.83b (2.33)	10a	10.9a	8.03
November	-1.7a	-2.17a	-3a	-3.17a	-4.17a	-1.7a	-5.17b (2.33)	9.58
January	-10.33a	-9.67	-8.17	-8.3	-8	-9.5a	-8.67a	ns
February	-7.22a	-7.2	-7.22	-7.36	-7.36	-7.22	-7.36a	ns
March	7a	11.4a	8.67a	8.78a	-7.83a	8.2a	11.67a	ns

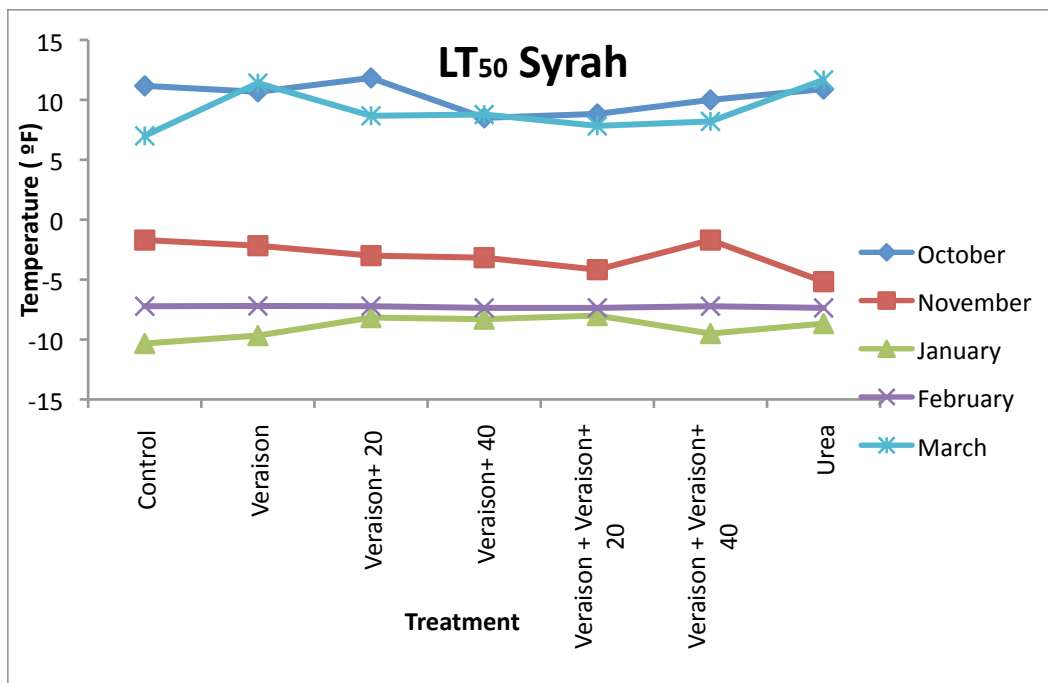


Figure 7: Lethal temperatures at which 50% of primary buds are killed across all treatments for Syrah.

There was no significant difference across all treatments in Merlot, however, both V and V20 for Cabernet Franc showed significantly lower LT50 temperatures than control at 0.05 and 0.015 in October, respectively (Tables 8 and 9). The LSD and differences in mean values did not support this conclusion, at LSD = 7.27, 2 and 3.16 in differences in mean for V and V20, respectively. Another treatment group (V) came close but was not significant, at -6.83 °F, $p = 0.113$ in November (Table 9). In January, February, and March, there were no significant

differences in the LT50 values across all treatments for all varieties. No significant LSD or difference in means has been reported for Merlot and following October for Cabernet Franc.

Table 8: Mean LT50 values for all treatments at $p < 0.05$ in Merlot

*Within rows, means followed by the same letter (a) are not significantly different ($p < 0.05$).

**Differences in means are expressed for significantly different means in parentheses

Merlot	Control	V	V20	LSD
October	15	13.83	15.63	ns
November	-4.67	-3.83	-4	ns
January	-8.5	-8.33	-6.5	ns
February	-4.33	-5.67	-4.5	ns

Table 9: Cabernet Franc LT50 values for all treatments

*Within rows, means followed by the same letter (a) are not significantly different ($p < 0.05$).

**Differences in means are expressed for significantly different means in parentheses

Cabernet Franc	Control	V	V20	LSD
October	13.2a	11.88b (2)	10.54b (3.16)	7.27
November	-6a	-6.83a	-5.83a	ns
January	-8.17a	-7.67a	-7.83a	ns
February	-5.33a	-5.33a	-5a	ns
March	7.05a	10a	7.05a	ns

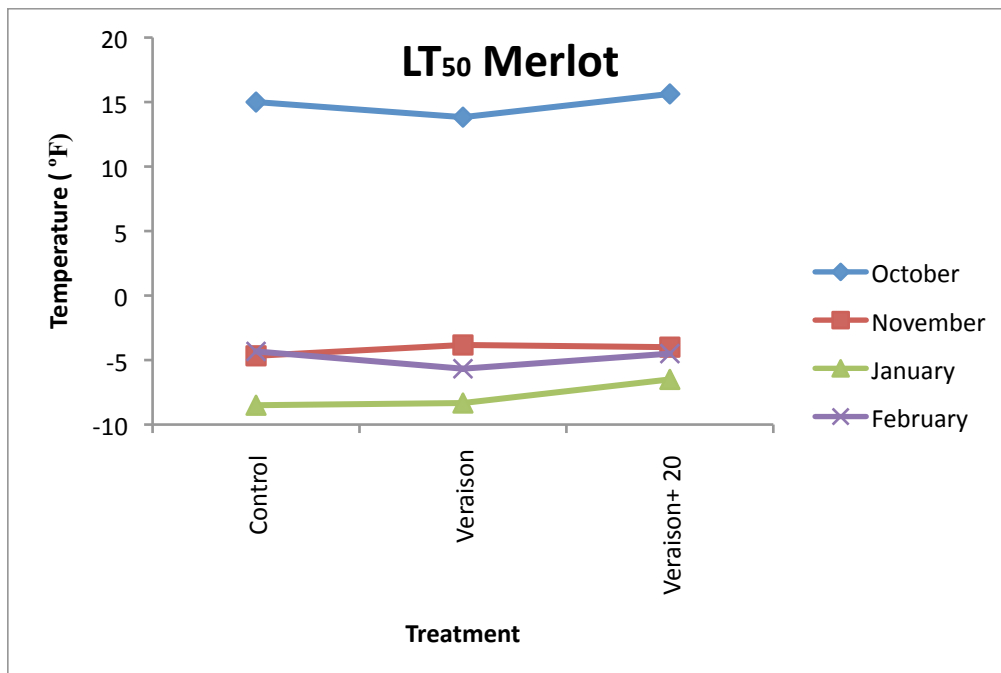


Figure 8: Lethal temperatures at which 50% of primary buds are killed across all treatments for Merlot.

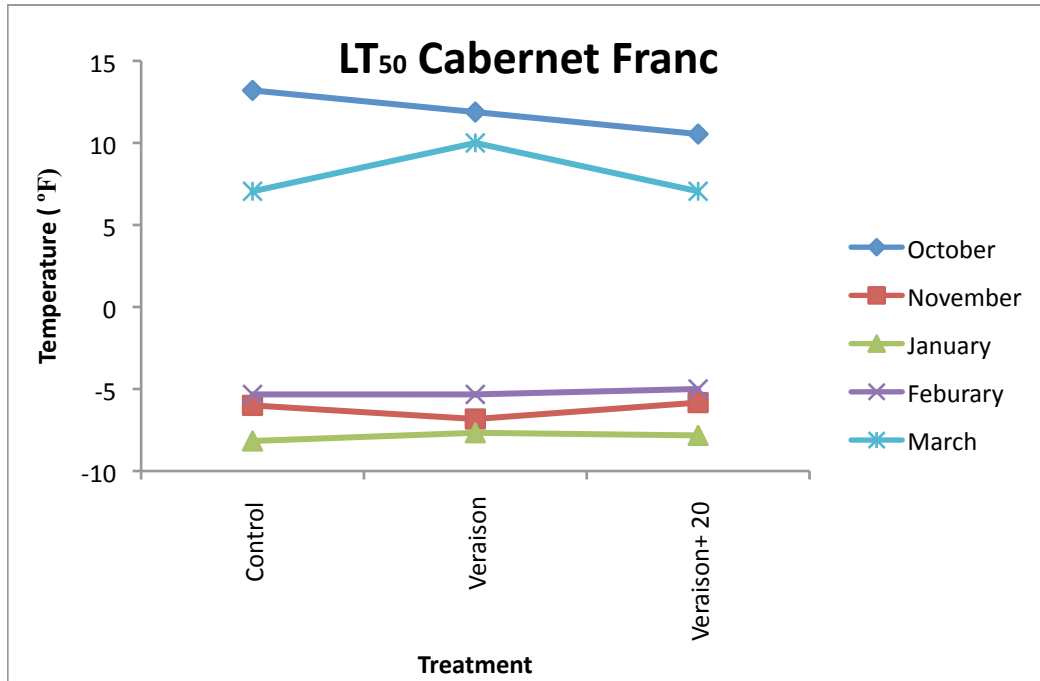


Figure 9: Lethal temperatures at which 50% of primary buds are killed across all treatments for Cabernet Franc.

Interestingly, during a cold spell in late February, the buds may have re-acclimated, a possible reason behind the greater bud survival in the C, V40, and V + V40 treatment groups. For these treatment groups, the temperature at sampling was at 20 °F. The temperature at sampling the previous day for V, V20, V + V20, and urea was 27 °F. Both days experienced lower temperatures following a prolonged warming spell with high temperatures in the high 50's-low 60's and low temperatures in the 30s'-40s' but it is possible that the buds in the C, V40, and V + V40 groups may have had one more day to re-acclimate to the lower temperatures than the other groups that were sampled the previous day (Figure 5).

In March, with an extended period of above normal temperatures, buds of the Chardonnay, Syrah, and Cabernet Franc blocks are coming out of dormancy and de-acclimating,

leading to mixed results between treatments in bud cold hardiness. Again, no treatment showed significantly lower bud cold hardiness than the control in all groups studied.

Without regards to LSD or the differences in means since the tests may not be accurate for a small sample size in this study, the LT_{50} values for Syrah yielded some interesting information: in October, all of the treatment groups except V20 may have demonstrated greater cold hardiness than the control group with only V40 and V + V20 demonstrating significantly greater cold hardiness levels through lower LT_{50} temperatures. In November, urea has significantly demonstrated the greatest cold hardiness, with the lowest LT_{50} temperature of all treatments while in January and February, no treatment groups achieved lower LT_{50} temperatures or greater cold hardiness than the control (Figure 7). As the bud survivability data has proven that the treatments are not significantly affecting the Merlot variety, there were no major increase or decrease in the temperatures or bud cold hardiness across the treatments (Figure 8). Data collection on Merlot has ceased following sampling in February due to early pruning by the cooperative grower of Site A and to the lack of effects of the ABA treatments conducted on increasing bud cold hardiness. As the buds from the Chardonnay, Syrah, and Cabernet Franc blocks have emerged from bud dormancy, sampling has ceased in March.

5.0 CONCLUSION

5.1 Conclusion and future studies

The main goal of the ABA treatments and urea were to evaluate whether the treatments would increase bud cold hardiness more quickly than nontreated control, during the fall acclimation process, as dictated by lower LT50 temperatures. Several treatments demonstrated this in October through significant LT50 temperature results: V, V20, V40, and V + V20. These treatments should be evaluated further, especially given that the majority of cold damage occurs in the fall (Tables 6, 7, 9). With successful LT50 results, V40 and V + V20 were also identified to significantly improve cold hardiness in Syrah in October. While the V + V20 treatment is promising, the V and V20 single treatments may be more cost-effective and demonstrated more significant results than V + V20 as the V and V20 treatments are applied once while V + V20 is applied twice. The significantly early acclimation differences in the treatments are promising as the cold acclimation phase of the dormancy period is much slower than the de-acclimation phase. Grapevines have different rates of acclimation, as dictated by the variety as well as by the timing of the harvest. Plus with the understanding that grape buds lag slightly behind in their response to changing temperatures in their environments, increasing cold hardiness following exposure to decreasing temperatures and vice versa, especially in the acclimation and midwinter stages, buds can get damaged when they have not acclimated to a level of cold hardiness that is sufficient to protect against winter injury (Figure 5) Some varieties may not reach maximum hardiness until the end of fall or even into the beginning of the winter season (Caspari et al, 2014). Therefore, these varieties are more susceptible to winter injury because of the slow acclimation made in comparison to more hardy varieties. The promising LT50 results of ABA treatments, especially the V and V20 treatments have demonstrated that the application of ABA may have played a role

in accelerating the acclimation process of the varieties studied in comparison to the control treatments.

On the other hand, there were no significant differences between treatments on all four varieties of grapes at the midwinter and de-acclimation stages between November and April following the acclimation stage in October with the exception of urea in Syrah in November (Table 7). Urea treatment should be evaluated further on Syrah since this treatment demonstrated the most significantly cold hardy temperature of all groups in November, at the beginning of the midwinter stage (Table 7). Nevertheless, it is possible that there was too much noise: the grapevines were of different ages and reacted differently to the treatments. Some of the buds were possibly hardier than other buds for all treatments due to varying levels of vigor. Other possible factors behind the mixed results achieved may be different rates of de- and re-acclimation in response to several warming periods in the first three months of 2015, or different rates of acclimation and uptake of ABA and urea. However, the timing and concentration as well as abundance of ABA and urea applied may have contributed to the mixed results achieved. It is also possible that some of the vines did not react upon exposure to exogenous ABA and urea when applied.

The V20 treatment group for Cabernet Franc demonstrated a significantly lower LT_{50} temperature than the Control and V in October in addition to having demonstrated significant accumulations of anthocyanins and should be investigated further. There is a possible link between anthocyanin accumulation and cold hardiness and should be evaluated further as growers seek out cost-effective methods to increase anthocyanins in their wine grapes for increased color and flavor contents as well as to increase the cold hardiness and ABA has the potential to perform both tasks (Zhang et al., 2011; Zhang et al., 2012ab).

Foliar application of urea and abscisic acid has potential as future cold protection methods for wine grapes and the latter have been researched intensively (Wample et al., 1993; Zhang and Dami, 2011; Zhang and Dami, 2012a/b). Again, the V20 application of ABA has the greatest potential and should be evaluated further as timing may be important when applying ABA. On the other hand, foliar applications of urea for cold hardiness have not been studied on grapes prior to this study and did not yield consistent results in this study. The timing of urea application may have played a role in its inefficiency so evaluating urea applications applied at harvest, during the dormant season, and just before bud break would be helpful to assess its role in bud cold hardiness. It would be also beneficial to repeat this study on the same varieties in different locations and over at least two growing seasons, as the microclimates and growing season conditions differ annually and may affect the results obtained. Again, the cold hardiness of different cultivars of grapevines is mainly determined by genetics, i.e. some varieties will acclimate earlier in the fall than other varieties while early bud-breaking varieties may de-acclimate earlier and are more susceptible to injury regardless of mid-winter hardiness. With that in mind, it would be beneficial to apply ABA or urea at the appropriate times at veraison, 20 days after veraison and at bud-break stage BBCH04, a stage in when buds are just opening, for specific cultivars individually, rather than spraying in the same period. Another potential study can be conducted on the benefits of ABA of cold hardiness enhancement on cane buds versus spur buds as cane buds being more fruitful than spur buds, may not be as cold hardy as spur buds.

Climate change is causing more extreme temperature fluctuations, especially with the increasing number of polar vortex events, and vineyard growers are looking to new methods to protect their grapes from extreme cold temperature fluctuations while satisfying increasing

demand for wine through production. According to the 2014 Grower survey, Colorado growers are reducing acreage of some cold tender *V. vinifera* cultivars acreage and replacing these cultivars with super cold hardy cultivars such as Aromella, Marquette, Valvin Muscat, among others bred specifically for their cold hardiness (Caspari and Lumpkin, 2013). Alternatively or in concert, urea and abscisic acid should be studied further as cold hardiness tolerance enhancement methods, based on promising results from this study and other studies.

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