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Cold Climate Grape Cultivar`s Physiological and Gene Expression Responses to Low and Freezing Temperatures

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COLD CLIMATE GRAPE CULTIVAR`S PHYSIOLOGICAL AND GENE EXPRESSION RESPONSES TO LOW AND FREEZING TEMPERATURES

BY

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DISSERTATION ACCEPTANCE PAGE

Turhan Yilmaz

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

-	Subtraction
%	Percentage
* or ×	Multiplication
+	Addition
^{0}C	Degree Celsius
Ch	Chilling hours
DAS	Data acquisition system
DTA	Differential thermal analysis
df	Degrees of freedom
DEG	Differentially expressed genes
DGEA	Differential Gene Expression Analysis
GSEA	Gene Set Enrichment Analysis
HTEs	High temperature exotherms
h	Hours
LTEs	Low temperature exotherms
Mean Sq	Mean sums of square
ns	Not significant
p-value	Probability value
PCA	Principal Component Analysis
R	R statistical software
Sum Sq	Sums of squares
SP	Spur pruning

SCP	Short cane pruning
SPSC	Spur plus short cane
SE	Standard error
TEM	Thermoelectric modules
VIVC	*Vitis* International Variety Catalogue

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ABSTRACT

COLD CLIMATE GRAPE CULTIVARS' PHYSIOLOGICAL AND GENE EXPRESSION RESPONSES TO LOW AND FREEZING TEMPERATURES

TURHAN YILMAZ

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Grapevine (*Vitis Vinifera*), widely cultivated in the world and USA, is a significant and valuable fruit crop. After cold climate grapevine cultivars were released by breeding programs in the 1990s, the production of grapes expanded in the Northern cold climate region of the US. The objectives of this study were to test 1) freezing tolerance and chilling fulfillment, 2) the effect of pruning methods on yield and winter survival, and 3) transcriptomic changes in natural and controlled chilling conditions during chilling requirement fulfillment in cold climate grapevine cultivars.

Pruning methods, spur (SP), short cane (SC), and spur plus short cane (SPSC), were tested on Brianna, Frontenac, La Crescent, and Marquette in three growing seasons. Bud viability, total cluster number, cluster weight, yield, and fruit quality (soluble solids, pH, and total acid) were evaluated on pruning treatments. Yield in all cultivars was lower in 2019 and 2020 than in 2018 due to severe winter cold. Results of this study indicate different pruning techniques in consideration with winter injury have a role to optimize each grape cultivar's yield and fruit quality.

Freezing tolerance was assessed by low temperature exotherms on dormant grapevine buds for three winter seasons. The correlation between freezing tolerance and the seven-day minimum temperatures preceding the freezing test was found significantly correlated. The freezing tolerance of Brianna, Frontenac gris, La Crescent, and Marquette

was analyzed monthly from November to April. Marquette was the most freezing tolerant cultivar to extreme cold temperatures across three years. Chilling fulfillment was evaluated in natural and controlled chilling conditions for Brianna, Frontenac gris, La Crescent, and Marquette. Chilling fulfillment was monitored at 200-500, 501-700, 701-900, and > 901 chilling hour periods in controlled and natural conditions. There were no differences in bud break status between natural and controlled (4 ^{0}C) conditions at the same chilling hours. Brianna which has been reported to be a slow acclimating cultivar exhibited faster deacclimation.

Bud transcriptome changes were assessed during the transition from dormancy to ecodormancy in Marquette and Brianna during controlled (constant 4 ^{0}C in the dark) and natural field chilling. There were a greater number of differentially expressed genes at 1000 chilling hours in both controlled and natural chilling conditions. Auxin signaling and cell wall pathways were enriched in controlled chilling conditions while ethylene and jasmonate signaling pathways were enriched in natural field chilling conditions. Phenylpropanoid biosynthesis, photosynthesis, and plant-pathogen interaction pathways were enriched in both controlled and natural chilling conditions. There were more enriched pathways in natural field chilling than controlled chilling, which may have been influenced by natural light and fluctuating temperatures in the field.

1. Chapter 1 Introduction and literature review

1.1 The importance of grapevine

The world grape production is nearly 77.6 million tons for wine (57%), table grape (36%), and dried production (7%). China has the highest production of grapes with around 14 million tons, and Italy, the USA, France, Spain, and Turkey had nearly 8, 7, 6, 5, and 4 million tons production, respectively (ATLAS, 2020; USDAStat, 2021). Grapes are the highest value fruit crops of $6.5 billion in the US which has nearly 1 million bearing vineyard acres (USDAStat, 2021; WINE, 2020). After the release of cold hybrid cold-hardy cultivars, grape production has increased in Midwest. Cold-hardy grapes provide $16.8 million in economic activity to North and South Dakota (Extension, 2014).

1.2 Cold climate grapevine cultivars

Vitis vinifera cultivars have an ability to survive temperatures from −10 to −20ºC while *Vitis riparia* can survive − 40 ^{0}C; therefore, grapes for the Midwest are predominantly hybrid cultivars (Fennell, 2004). These hybrid cultivars are generally crossed with *Vitis riparia* and *Vitis vinifera* (Goldsmith, 2009). North American *Vitis* spp. are suitable species to grow as table and wine grape cultivars because of their greater freezing tolerance (Hemstad & Luby, 1998). The life and fruitfulness of the grapevine are dependent on the minimum temperatures in winter. The freezing tolerance is impacted by local temperatures and other physiological factors (Ahmedullah, 1985; Fennell, 2004; Levitt, 1980; Londo & Kovaleski, 2017; Zabadal et al., 2007). Brianna, Frontenac, La Crescent, Frontenac gris, and Marquette (Maul 2014) are the cultivars commonly grown

in South Dakota. Grapevine cultivars need greater freezing tolerance to survive in South Dakota than in major grape production regions (Fennell, 2004). The temperatures in a region can vary from year to year and features such as slope, altitude, and windbreak may modify the temperature and provide protection (Wolf, 2008) so that grapevines may be able to tolerate the minimum temperatures to maintain economic fruitfulness.

1.3 Freezing tolerance and chilling fulfillment on cold climate grapevine cultivars

1.3.1 Definition and utilization of freezing tolerance

Freezing tolerance in grapevines is the capability of tolerating exposure to temperatures below zero during autumn and winter. Freezing tolerance is generally identified as temperatures at which 50% of buds are killed, which is called lethal temperature 50 or LT50 (Andrews e al., 1984; Fennell, 2004; Levitt, 1980). Freezing tolerance in dormant grapevine season can be divided into three stages which are cold acclimation (September to December), maximum hardiness (December to February), and deacclimation (February to April) (Londo & Kovaleski, 2017). Controlled temperature freeze testing in the lab and natural analyses are used to determine freezing tolerance for different cultivars (Fennell, 2004; Zabadal et al., 2007). There are several laboratory methods, for example, electrolyte leakage, tissue viability, chlorophyll fluorescence, oxidative browning, and differentially thermal analysis. In a differential thermal analysis, as bud temperatures drop below 0 ^{0}C supercooled grapevine buds are nucleated to form ice which releases heats (Andrews et al., 1984; Fennell, 2004; Kaya & Köse, 2017; Keller, 2020; Londo & Kovaleski, 2017; Zabadal et al., 2007). Freezing stress can occur at temperatures below 0 ºC (Fennell, 2004; Levitt, 1980). The temperature at which freeze injury occurs is influenced by physiological changes in water and solute

concentrations and membrane chemistry (Olien & Smith, 1977). In addition, the species, cultivar, level of maturity of the plant, duration of freezing event, and level of acclimation or deacclimation have a role in the level of freezing injury (Fennell, 2004).

Maximum cold hardiness is generally related to the deepest of endodormancy and occurs during December, January, and February (Zabadal et al., 2007). The onset of the low temperatures and short day lengths initiate acclimation; this leads to leaf senescence and physiological changes prepare the grapevines to tolerate temperatures below 0 ^{0}C in the winter (Fennell, 2004). The effect of freezing temperature has been tested in many studies as it has a role in the yield and winter survival of the grapevine. (Fennell, 2004; Fennell & Hoover, 1991; Kovaleski & Londo, 2019; Londo & Kovaleski, 2017; Zabadal et al., 2007). It is reported that laboratory freeze testing of buds from naturally grown vines shows a similar level of freezing tolerance as found in vines subjected to freezing in the natural (Howell & Shaulis, 1980). Dormant buds have three meristem tissues which are primary, secondary, and tertiary buds. Primary buds generally have less freezing tolerance than secondary and tertiary, and secondary buds have less freezing tolerance than tertiary buds. Therefore, tertiary buds have a greater ability to survive low temperatures, but they generally have no flower clusters. Choosing cultivars with greater primary bud freezing tolerance is the best way to limit freezing damage (Fennell, 2004). There are many methods to test bud freezing tolerance, but the most common one is differential thermal analyses (DTA) to monitor lethal temperature exotherms in the lab (Ferguson et al. 2011; Londo & Kovaleski, 2017; Mills et al. 2006). The changes in cell physiology during acclimation promote supercooling of water in the cells (Fennell, 2004; Mills et al., 2006). Extracellular water typically freezes at temperatures between -2 to -10

^{0}C and the heat that is released is termed a high-temperature exotherm (Mills et al., 2006). Supercooled intracellular water occurs at temperature <-10 ^{0}C and the heat is released is called by low-temperature exotherm (LTE). The freezing of supercooled water at temperatures below -10 ^{0}C occurs intracellularly and is typically lethal thus the temperature at which the LTE occurs is used to identify the bud-killing temperature (Fennell, 2004; Fennell & Mathiason, 2002; Mills et al., 2006).

1.3.2 Definition of dormancy and chilling fulfillment

Dormancy is a period of growth suspension and promotes winter survival (Arora et al., 2003). Dormancy is divided into paradormancy, endodormacy, and ecodormancy (Lang et al. 1987). Paradormancy is an inhibition driven by physiology during the growing season. Endodormancy is a stage controlled by both physiology and biochemical factors internal to the bud. Accumulation of chilling hours at 0 to 7 transitions the grapevine buds to ecodormancy. Ecodormancy is the stage when the chilling requirement has been fulfilled but local temperature conditions limit growth (temperature <10 ^{0}C) (Anzanello et al., 2018; Horvath et al., 2003; Lang et al., 1987; Londo & Johnson, 2014). Chilling is defined as the necessary specific number of low temperature hours between 0 and 7 ^{0}C to break dormancy (Dokoozlian, 1999). Chilling requirement is one of the main factors impacting bud break as inadequate chilling causes delayed and nonuniform bud break and flowering (Mathiason et al., 2009). Bud break forcing in controlled conditions (growth chambers) is used to measure chilling fulfillment (Kovaleski & Londo, 2019; Londo & Johnson, 2014). Bud break is defined when the green tip is visible bud scales (Coombe, 1995). There is a relationship between bud break and an amount of chilling. When chilling is increased, bud break is more rapid (Dokoozlian, 1999). *V.*

vinifera cultivars generally require 50–400 chilling hours (0 to 7 °C) while *V riparia* cultivars needed lower number chilling to the break of buds; however, other species ranges between 250–2250 h (Londo & Johnson, 2014). Buds start to break when chilling is fulfilled, and suitable temperature conditions have happened.

1.4 Pruning treatments on cold climate grapevine cultivars

Pruning is used to balance vine vegetative growth and yield. Controlling the loading of the crops by pruning is important for grape production as it can impact vine carbohydrate storage and winter survival (Bravdo et al., 1984). Carbohydrates are needed for shoot lignification during acclimation and for next year's growth (Dami, 2005). Overcropping can cause uneven ripening and poor fruit quality and decrease vine vigor and winter hardiness (Buttrose, 1966). In the upper Midwest, cold-hardy wine grapes often have inconsistent yield, low fruit quality, and high vegetative vigor (Riesterer-Loper et al., 2019). Spur pruning and cane pruning methods have been tested on *V. vinifera* cultivars and have shown impacts on vine vigor and yield (Rosner & Cook, 1983), fruit phenolic content and quality, and starch in overwintering wood (Jones et al., 2018), bud viability (Kaya & Köse, 2017; May, 2004), and shoot growth pattern (Bernizzoni et al., 2009). However, these same methods have not been thoroughly tested in the new cold-hardy grapevine cultivars.

1.5 Transcriptome analyses on cold climate grapevine cultivars

The role of metabolic pathways, gene networks, cell division and growth, and carbohydrate metabolism impacting grapevine dormancy can be shown by transcriptome

analyses (Khalil-Ur-Rehman et al., 2017; Mathiason et al., 2008; Mathiason et al., 2009; Min et al., 2017; Noriega & Pérez, 2017). Understanding the genes and metabolic pathways involved in the chilling fulfillment process can be used for improving cultural practices and selecting grapevine cultivars suitable for a region (Mathiason et al., 2008). Natural and controlled conditions have been tested in blueberry by transcript profiles. More up-regulate transcripts were found under controlled conditions than natural conditions. Genes related with stress tolerance and protein synthesis machinery were found just in cold room conditions while the genes related to light stress were found under natural conditions (Dhanaraj et al., 2007). Another study comparing biochemical changes in kiwi during chilling showed total phenol, radical scavenging, polyphenol oxidase, and phenylalanine ammonia-lyase activity were lower in controlled chilling than natural chilling (Gheshlaghi et al., 2018). Comparison of controlled and natural chilling conditions in cold-hardy grapevine cultivars have not been tested yet; Therefore, the transcriptomic analysis will be used to determine whether there are differences in gene expression in natural and controlled chilling treatments.

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2 Chapter 2 Spur and short cane pruning influence bud viability, yield, and fruit quality

2.1 Abstract

Balanced pruning is used to manage vegetative vigor and fruit load to optimize yield and fruit quality in most the fruit species. The objective of this study was to determine the bud viability, yield, and fruit quality potential of four grapevine cultivars using three pruning strategies. Four cold climate grapevine cultivars--Brianna, Frontenac, La Crescent, and Marquette--were tested with spur (SP), short cane (SC), and spur plus short cane (SPSC) pruning treatments in 2018, 2019, and 2020. The SP treatment was 10 two-bud spurs per vine, SC was five four-bud short canes and SPSC vines had four SP and three SC. Soluble solids, pH, and total acid were measured for individual bud positions on all spurs, canes or spurs, and canes on each treated vine. Yield in all cultivars was lower in 2019 and 2020 due to severe winter cold. The greatest bud viability across the three years in each cultivar was achieved in Frontenac and Marquette with SP, followed by Brianna with SP and SC and La Crescent with SPSC pruning treatments. The highest yield for pruning treatments was Brianna with SC, Frontenac with SPSC, La Crescent with SC and SPSC, and Marquette with SP and SC pruning treatments. Brianna had the greatest fruit SS and pH in SC pruning treatment. In contrast, Frontenac and La Crescent had greatest fruit soluble solids and lowest total acid with SP pruning treatment. Marquette showed similar soluble solids across all pruning treatments; however, pH was greatest in SC and total acid was lower in SP and SC than in SPSC. The pruning strategy impacted bud viability, yield, and fruit quality measures most differently in Brianna and La Crescent; however, with these vigorous vines, the SC could provide a greater yield. In

contrast, in Marquette and Frontenac bud viability, yield, and fruit quality were generally favored with SP. Results of this study indicate different pruning techniques, which are taken into consideration with winter injury, can be used to optimize each grape cultivar's yield and fruit quality.

2.2 Introduction

The development of complex hybrids with *Vitis riparia* in their pedigree has enabled grape production in regions of the United States with extremely low winter temperatures (Atucha et al., 2018; Rice et al., 2017; Riesterer-Loper et al., 2019). Different pruning strategies in these grape cultivars are used to manage vine vigor, crop load, yield, and fruit quality (Jones et al., 2018). However, inconsistent yield, low fruit quality, high vegetative vigor, and insufficient fruit ripening are issues frequently reported in cold-hardy wine grapes grown in the upper Midwest (Atucha et al., 2018). Spur pruning has been reported to result in balanced vigor, yield, and uniform bud break in Cabernet Sauvignon (Rosner & Cook, 1983). The use of spur (SP) and short cane (SC) pruning is well adapted to mechanization (Poni et al., 2004) that produces a more standardized shoot growth pattern (Bernizzoni et al., 2009).

Balancing vegetative and fruit-bearing shoots (balanced pruning) is important as increasing bud number per vine does not always give a linear yield response (Wolpert et al., 1983). It is also important to consider that the vine can compensate for unbalanced pruning or injury by regulating the flower cluster numbers and average cluster weight (Heazlewood et al., 2006). Bud viability varies based on node position in the cane and has a role in yield (May, 2004). Spur pruning in contrast to cane pruning showed greater

fruit phenolic content quality and starch in overwintering wood in Pinot noir and Chardonnay (Jones et al., 2018). However, there is limited information on the effect of pruning on bud viability and yield on cold-hardy grapevine cultivars managed with SP and SC pruning. The main aim of this study was to identify how different pruning methods (spur and cane) affect bud viability, yield, and fruit quality in a high cordon training system. Therefore, the objective of this study was to determine the effect of three different pruning strategies in four cold-hardy wine cultivars (*Vitis* hybrid) to provide growers information for vine management with high cordon training.

2.3 Material and Methods

This study was performed in 2018, 2019, and 2020 with four cold-hardy grapevine cultivars (Brianna, Frontenac, La Crescent, and Marquette) (Maul 2014) growing in the Hansen Research Center, Brookings, SD (lat. 44° 18' 40.8816" N, long. 96° 47' 54.1896" W) in USDA Plant Hardiness Zone 4b (USDA, 2021). The vineyard was planted in a randomized complete block design with six vine replicates in each block. All vines were trained to a high cordon under non-irrigated conditions. The study had three pruning treatments: SP (10 two-bud spurs), SC (five four-bud canes), and SPSC (three four-bud canes + four two-bud spurs) (Figure 2-1). Thus, each pruning treatment resulted in 20 buds per vine. Three replicates were used for each treatment (vine = experimental unit) with each replicate from a separate block.

The position of the buds on spurs (one and two) and canes (one, two, three, and four) were each monitored separately, with position number one being the basal or closest to cordon and number two through four away from the cordon. Bud viability was

determined after bud break by checking for an actively growing shoot (viable) or no bud break (dead) at each bud position. Harvest timing was determined when the field measure of soluble solid was estimated at 18% to 20% for Brianna (Okie, 2004) and 22% to 24% for Frontenac, La Crescent, and Marquette (Dharmadhikari, 2001). A cluster for each bud position was collected separately, and then total yield (grams), total cluster number, and cluster weight were recorded for each bud position in the spurs or canes for each replicate vine. Clusters were collected for each bud position separately, maintaining the vine replicate, and the bud position identity in each spur or cane on the vine replicate. Data for each bud position and spur or cane number on each vine was tracked throughout harvest, extraction, and analysis. Therefore, although one to two cluster (s) were collected from a single shoot arising from one bud resulting in 20 to 40 clusters per vine, all clusters were kept separate by bud position on a spur or short cane. After recording cluster weight, twenty-five random berries from all berries from an individual bud/shoot were frozen and maintained at -20 ^{0}C until tested for soluble solids, pH, and total acid. Thawed but cold berry samples were pressed using a Stomacher 400 circulator (Cole-Parmer, Vernon Hills, IL) for five minutes to produce juice. The juice samples were centrifuged in 1.5 ml tubes to remove particles. Finally, soluble solids, pH, and total acid were measured using an OenoFOSS, which uses near- infrared and standard curves for each parameter to determine concentrations (FOSS, Hillerød, Denmark).

Bud viability, yield, cluster number, and fruit quality parameters were analyzed using the statistical package in R (R, 2020). The effect of pruning treatment (n=three), cultivar (n=four), year (n=three), bud position (four), and factor interactions on viability, yield, cluster number, cluster weight, and fruit quality (soluble solids, pH, and total acid)

were assessed by ANOVA. Mean separations were performed using Tukey's HSD ($P < 0.05$) for treatment, cultivar, and bud position.

2.4 Results

Bud viability varied by cultivar and pruning treatment. Frontenac had the greatest bud viability across treatments, followed by Marquette, Brianna, and La Crescent, respectively. Brianna with SP and SC had more viable buds than SPSC. Frontenac and Marquette had the greatest bud viability with the SP treatment and La Crescent with the SPSC pruning treatment (Figure 2-2).

The yield was affected by treatment, cultivar, years, positions, and interactions between treatment by cultivar, treatment by year, and cultivar by year (Table 2-1). All cultivars had the highest yield in 2018 and the lowest in 2020. Brianna had a similar yield in 2018 and 2019. Winter injury in dormant seasons prior to the 2019 growing seasons impacted the yield for the other three cultivars. Brianna had the greatest yield across all years followed by Frontenac, Marquette, and La Crescent, respectively (Table 2-2, Figure 2-3). The greatest vine yield occurred with SC in Brianna, SPSC in Frontenac, SC in La Crescent, and SP and SC in Marquette. Total cluster number and cluster weight results corresponded with the yield results (Table 2-2).

Grape soluble solids were affected by treatment, cultivar, year, and their interactions (Table 2-1). Soluble solids were greater for Brianna in SC compared to the other pruning methods. Frontenac soluble solids were greatest in SP and lowest in SC pruning treatment. La Crescent had the greatest soluble solids with SP and did not differ

between SC and SPSC. Marquette had similar soluble solids across all pruning methods (Table 2-3). Grapevine pH was affected by treatment, cultivar, year, bud position, and their interactions (Table 2-1). The pH was highest in SC in Brianna, Frontenac, and Marquette and was not significantly different between SC and SPSC for Brianna and Frontenac. In contrast, the pH was highest with SPSC in La Crescent (Table 2-3). Grapevine total acid was also affected by treatment, cultivar, year, bud position, and their interactions (Table 2-1). Total acid was greatest with SPSC in Brianna and Marquette, and SC and SPSC in Frontenac and SC in La Crescent (Table 2-3). Recommended pruning treatments for Brianna, Frontenac, La Crescent, and Marquette are summarized in Table 2-4. SC is recommended for Brianna as SC provided greater yield, soluble solids. SP and/or SPSC are recommended for Frontenac and La Crescent as they are coordinated with greater bud viability, increased pH, and decreased total acid. SP is recommended for Marquette as SP provided greater bud viability and yield and decreased TA (Table 2-4).

2.5 Discussion

In Iowa, Marquette was the top-performing cold-hardy cultivar when yield, total number clusters, and fruit quality were considered (Schrader et al., 2020). Frontenac was also one of the highest yielding red cultivars in Iowa (Schrader et al., 2020) and Frontenac and Marquette were the highest yielding cultivars in this study. In our findings, all cultivars had similar yield across all treatments in 2018, but winter injury in 2019 and 2020 reduced yield in all cultivars. Early low temperatures in November in 2019 damaged primary buds. The sequential winter damage of 2019 and 2020 resulted in

greater yield reduction in the 2020 growing season (Yilmaz et al. 2021). Cultivars fruiting from secondary buds after winter damage have less yield compared with primary buds (Fennell, 2004; Keller, 2020). Spur pruning in a high cordon training system provides good light exposure to the developing buds, and in this study, the spur pruning treatment resulted in greater bud viability across all cultivars except for La Crescent. Other training systems such as low cordon training (Scott Henry, Vertical shoot positioning) or high cordon double curtain (Geneva double curtain) have been shown to increase yield in comparison to the single high cordon; however, further comparisons would need to be made under critical winter temperatures (Bavougian et al., 2013; Luby, 2012; Wimmer et al., 2018).

Previous comparison of fruit quality in Chile with vines pruned with spurs or long canes (eight buds) has shown no differences in yield or fruit soluble solids and pH (Peppi & Kania, 2013). However, three-node spurs had higher soluble solids and vine vigor compared with a short cane (six-node) even though there were no differences in pH and yield (Morris & Main, 2010). In contrast, Chardonnay vines had higher soluble solids and pH in one-year comparison of spur pruned than long cane pruned vines (Jones et al., 2018). Although fruit quality (chemically) of cold-hardy grapevine cultivars is still under research (Riesterer-Loper et al., 2019), the quality of harvested berries, 21% to 22% soluble solids, 3.2 to 3.4 pH for white cultivars, and 22 % to 24% soluble solids, 3.3 to 3.5 pH for red cultivars are standard target values for wine grapes (Dharmadhikari, 2001). In the white cultivars, La Crescent met the standards on soluble solids with SP and SPSC pruning. Brianna is typically collected at lower soluble solids as pH begins increasing at lower soluble solids than the other cultivars. Brianna and La Crescent reached the

recommended pH level under all pruning strategies. In our trial, Marquette fruit reached recommended soluble solids and pH target values under all pruning methods; however, Frontenac's soluble solids and pH was lower for all pruning methods. The Marquette and La Crescent soluble solids values were lower than shown in Iowa and western Vermont studies (Schrader et al., 2020) and fruit quality results in Wisconsin studies (Wimmer et al., 2018). The current study indicates that the pruning method does impact soluble solids, pH, and total acid differently in the cultivars tested and should be considered when choosing a pruning strategy. It should be noted that training systems other than the high cordon were not tested in this study, and bud number was maintained at 20 buds per vine in coordination with pruning weight. Studies in other states have shown increased yield with different training systems (Aipperspach et al., 2020; Bavougian et al., 2013; Wimmer et al., 2018); however, all training decisions will need to consider local winter injury and vine vigor to determine optimal training and pruning strategies.

2.6 Conclusion

The bud viability, yield, and fruit quality results indicated SC is a good pruning strategy for Brianna with a high cordon training system. In Frontenac SP provided the greatest viability; however, good yield and fruit quality can be achieved with either SP or SPSC. SPSC resulted in the greatest bud viability in La Crescent, but SP provided the best fruit quality. For Marquette, SP pruning resulted in greater bud viability, yield, and fruit quality. Therefore, growers can adopt a pruning strategy to vigor and bud viability if winter injury is a common problem, whereas SP pruning can be utilized in most cultivars to optimize fruit quality.

2.7 References

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Table 2-1. ANOVA results of pruning treatment, cultivar, year, bud position, and their interactions on yield, total cluster number, cluster weight and, fruit quality (soluble solids, pH, and total acid) in 2018, 2019, and 2020 growing seasons. ANOVA based on three replicate vines for each cultivar in each treatment and year.

	Yield(g)/Vine (P^z-value)	Total cluster number/vine (*P*-value)	Cluster Weight (*P*-value)	SS (%) (*P*-value)	pH (*P*-value)	Total acid (*P*-value)
Treatment (T)	0.000	0.000	0.000	0.000	0.000	0.000
Cultivar (C)	0.000	0.000	0.000	0.000	0.000	0.000
Y (Year)	0.000	0.000	0.000	0.000	0.000	0.000
P (Position)	0.027	0.016	0.000	ns	0.000	0.007
T x C	0.000	0.000	0.000	0.000	0.000	0.000
T x Y	0.000	0.000	0.075	0.000	0.000	0.000
C x Y	0.000	0.000	0.000	0.000	0.000	0.000
T x P	ns	ns	0.001	ns	0.000	ns
C x P	ns	0.000	0.000	0.004	0.002	0.000
Y x P	ns	ns	0.000	ns	0.001	0.022
T x C x Y	0.000	0.000	0.000	0.000	0.000	0.000
T x C x P	ns	0.037	0.002	0.000	0.008	0.000
T x Y x P	ns	ns	0.002	0.000	0.000	0.003
C x Y x P	ns	ns	0.000	ns	0.000	0.000
T x C x Y x P	ns	ns	0.001	0.000	0.000	0.000

Table 2-2. Main effects of pruning treatments for each cultivar on yield, total cluster number, and cluster weight evaluated in Brookings in 2018, 2019, and 2020 growing seasons. Values for treatments for each cultivar are means across years and positions of three replicate vines for each treatment in each year.

	Yield(g) /Vine (mean ± SE)	Total cluster number /Vine (mean ± SE)	Cluster Weight (mean ± SE)
Brianna			
SP[Z]	1715 ± 59.7 b	16.5 ± 0.5 a	113 ± 4.8 ab
SC	2007 ± 33.8 a	17.3 ± 0.3 a	116 ± 2.7 a
SPSC	1411 ± 46.7 c	13.1 ± 0.4 b	104 ± 3.7 b
Frontenac			
SP	1513 ± 49.0 b	15.6 ± 0.2 b	101.4 ± 3.4 a
SC	1099 ± 48.0 c	11.8 ± 0.2 c	89.1 ± 3.4 b
SPSC	1915 ± 43.0 a	18.0 ± 0.2 a	98.5 ± 3.0 a
La Crescent			
SP	1049 ± 70.3 b	14.1 ± 0.6[ns]	56.5 ± 4.1 b
SC	1323 ± 55.3 a	13.0 ± 0.5	81.9 ± 3.2 a
SPSC	1199 ± 43.1 ab	13.9 ± 0.3	77.0 ± 2.5 a
Marquette			
SP	1613 ± 23.2 a	23.6 ± 0.2 a	76.2 ± 2.3 a
SC	1651 ± 26.4 a	23.8 ± 0.3 a	67.2 ± 2.6 b
SPSC	1419 ± 21.7 b	17.6 ± 0.2 b	75.4 ± 2.2 a

[Z]Statistical analysis was made by ANOVA with the main effect of treatments throughout the 3-year evaluation. If important main effects were detected among treatments, mean values were separated by Tukey's HSD with $P < 0.05$. [ns]; not significant.

Table 2-3. Main effects of spur (SP), short cane (SC), and spur plus short cane (SPSC) pruning treatments for each cultivar on soluble solids, pH, and total acid evaluated in Brookings in 2018, 2019, and 2020 growing seasons. Values for three treatments for each cultivar are means across years and bud position.

	Soluble solids (%) (mean ± SE)	pH (mean ± SE)	Total acid (mean ± SE)
Brianna			
SP[z]	14.8 ± 0.1 b	3.19 ± 0.0 b	11.3 ± 0.1 b
SC	15.8 ± 0.1 a	3.26 ± 0.0 a	11.5 ± 0.0 b
SPSC	14.7 ± 0.1 b	3.28 ± 0.0 a	12.0 ± 0.1 a
Frontenac			
SP	22.1 ± 0.1 a	3.08 ± 0.0 ab	11.4 ± 0.7 b
SC	20.7 ± 0.1 c	3.10 ± 0.0 a	11.8 ± 0.8 a
SPSC	21.6 ± 0.1 b	3.06 ± 0.0 b	11.9 ± 0.8 a
La Crescent			
SP	21.9 ± 0.1 a	3.16 ± 0.0 ab	11.4 ± 0.1 a
SC	20.7 ± 0.1 b	3.11 ± 0.0 b	12.8 ± 0.1 a
SPSC	21.0 ± 0.1 b	3.22 ± 0.0 a	11.4 ± 0.0 b
Marquette			
SP	22.4 ± 0.1[ns]	3.35 ± 0.0 b	9.43 ± 0.0 b
SC	22.7 ± 0.1	3.41 ± 0.0 a	9.19 ± 0.1 b
SPSC	22.5 ± 0.1	3.31 ± 0.0 c	10.13 ± 0.0 a

[z]Statistical analysis was made by ANOVA with the main effect of treatments throughout the 3-year evaluation. If important main effects were detected among treatments, mean values were separated by Tukey's HSD test. Different letters demonstrate significant differences at $P < 0.05$, n=3. [ns]; not significant.

Table 2-4. Recommended pruning treatments associated with significant positive traits for yield and fruit quality as identified in Table 2-2 and 2-3. Bold and outline indicates recommended pruning treatment.

	Bud viability	Yield	Cluster number	Cluster weight	Soluble solids	pH	Total Acid
Brianna							
SP	X		X	X			X
SC	**X**	**X**	**X**	**X**	**X**	**X**	**X**
SPSC						X	
Frontenac							
SP	**X**		**X**	**X**	**X**	**X**	**X**
SC							
SPSC		**X**	**X**	**X**		**X**	
La Crescent							
SP					**X**	**X**	**X**
SC		X		X			
SPSC	**X**			**X**		**X**	**X**
Marquette							
SP	**X**	**X**	**X**	**X**	**X**		**X**
SC		X	X		X	X	
SPSC				X	X		X

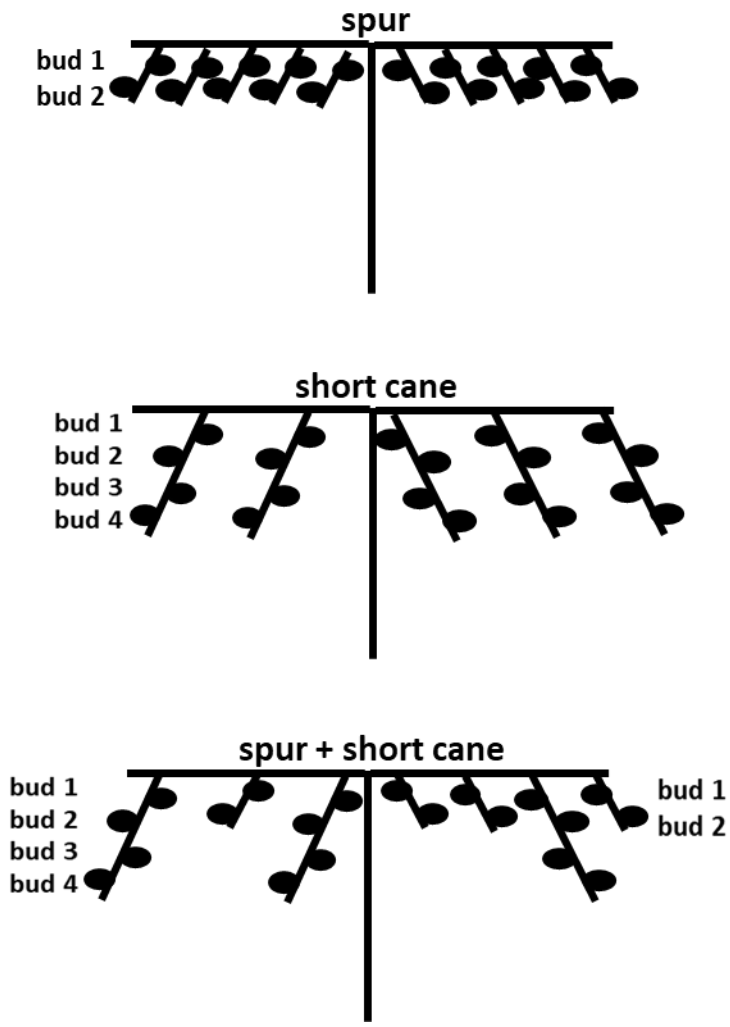

Figure 2-1. Pruning treatments were applied to 3 replicate vines for each treatment, cultivar, and year.

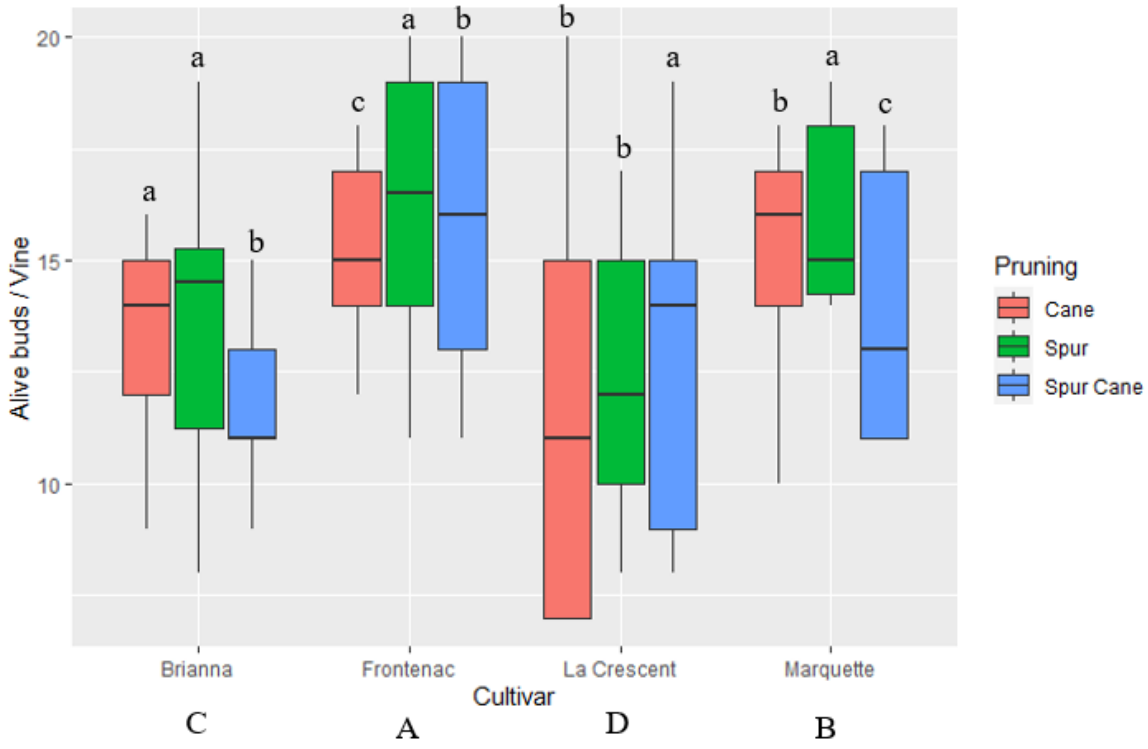

Figure 2-2. Bud viability in each cultivar under different pruning strategies. Distribution and mean of live buds are shown for each pruning treatment across three years in Brianna, Frontenac, La Crescent, and Marquette. Lower case letters represent a significant difference between pruning treatment within a cultivar. Upper case letters show significant differences in bud viability among cultivars across all treatments. Significance determined by Tukey's HSD with a $P < 0.05$ $n=3$.

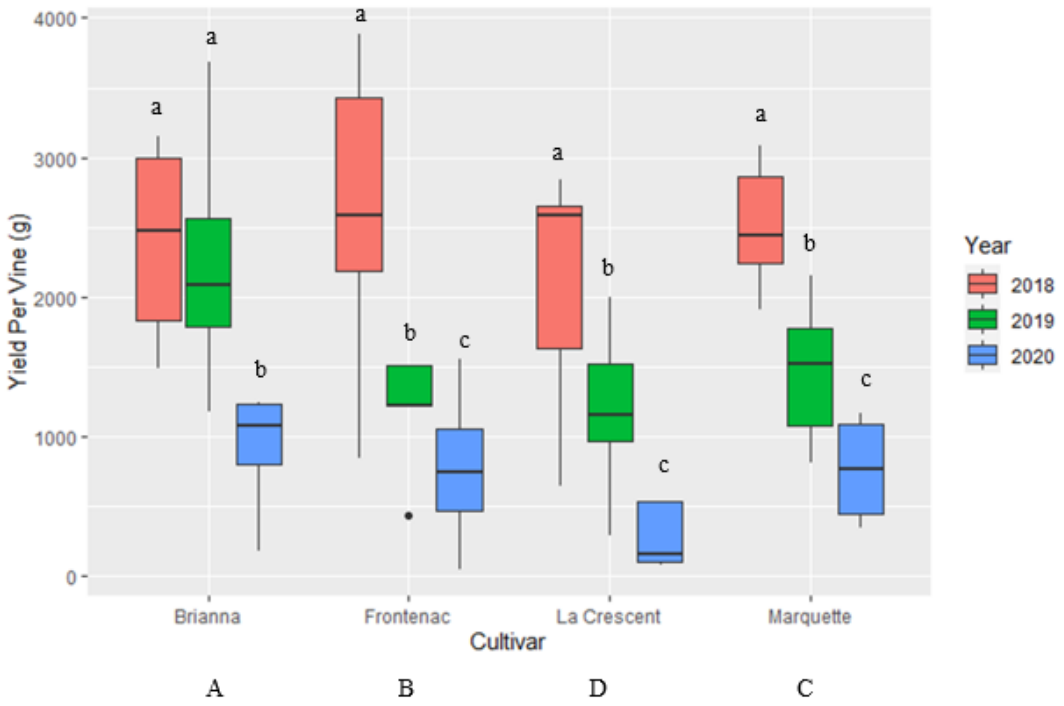

Figure 2-3. Yield for Brianna, Frontenac, La Crescent, and Marquette across three years. Lower case letters represent a significant difference in yield among years within a cultivar. Upper case letters show significant differences in yield between cultivars across all years. Significance determined by Tukey's HSD with a $P < 0.05$.

3 Chapter 3 Freezing Tolerance and Chilling Fulfillment Differences in Cold Climate Grapevine Cultivars

3.1 Abstract

Grapevine sustainability is impacted by the timing of dormancy initiation and freezing tolerance in fall and winter and chilling fulfillment and bud break in the spring. These traits have genetic and local temperature contributing factors; therefore, this study was undertaken to develop an understanding of these characteristics in four recently developed cold climate cultivars. The cold hardiness and chilling fulfillment profiles were monitored in Brianna, Frontenac gris, La Crescent, and Marquette using differential thermal analyses and bud break assays. Bud cold hardiness of all cultivars increased with the declining temperatures from November through February, after which the buds began to lose freezing tolerance. There were significant differences in cold hardiness and chilling fulfillment between cultivars during the endodormant and ecodormant periods of winter. Marquette had the greatest freezing tolerance from early November through midwinter suggesting it has potential as a sentinel cultivar for comparisons of new cold climate selections. Brianna was slower to acclimate and deacclimated more rapidly than the other cultivars. Chilling fulfillment under natural conditions or constant 4℃ in the dark showed no main effect differences for chilling accumulation condition; however, there were significant cultivar, condition, and time point interactions, indicating the cultivars differed in chilling fulfillment responses.

3.2 Introduction

Freezing injury is one of the most problematic issues impacting production of grapevine in the Northern regions of the United States (Fennell, 2004; Svyantek et al., 2020; T. Zabadal, 2015). The freezing tolerance of grapevine species and cultivars vary considerably, with *Vitis riparia* having the greatest reported tolerance of -40℃ (Patrick et al., 1980; Pierquet et al., 1977). The cultivars belonging to *V. vinifera* have high grape quality; however, their winter freezing tolerance is reported to range between -10℃ and -26℃ (Fennell, 2004; Lipe et al., 1992; Mills et al., 2006). Introduction of new cultivars developed from complex interspecific hybrids of *V. vinifera*, *V. riparia*, and *V. labrusca* since the 1980s has resulted in new grape and wine production in the regions of the North Central and North Eastern states in the USA and Southern Canada (Londo & Kovaleski, 2017; Reynolds, 2015). These cold-hardy wine grapes have been reported to survive temperatures from -25℃ to -38℃ in these regions; however, other reports indicate freezing injury can occur under less severe temperatures depending on the timing of the freeze event and the dormancy status of the vines (Hemstad & Luby, 1998). South Dakota has winter temperatures that can reach -30℃ in some years (Universty, 2019); however, it is noted that freezing injury can also occur in years with warmer winter temperatures. Typically, as temperatures decrease in fall and winter, the dormant buds survive increasingly negative temperatures, maintaining freezing tolerance at low mid-winter temperatures and then deacclimate and lose freezing tolerance with increasing temperatures and chilling fulfillment (Ferguson et al., 2014; Ferguson et al., 2011; Mills et al., 2006). However, temperature conditions can fluctuate widely on a daily and weekly basis in a continental climate with potential sudden temperature drops after

warming periods, which may contribute to freezing injury early or late in the winter season.

Freezing tolerance is dynamic, rather than a fixed character in each cultivar and is affected by temperature fluctuations and bud dormancy status during the winter season (Londo & Johnson, 2014; Londo & Kovaleski, 2017). Bud dormancy is typically divided into three stages with internal and external factors controlling the stages, paradormant (correlative inhibition during the growing season), endodormant (growth restriction within the bud), and ecodormancy (chilling fulfilled but growth limited by environmental conditions) (Lang et al., 1987). The timing of subzero events, high temperatures, and the dormancy status of the buds may affect the potential bud freezing damage. Subzero temperature drops in early fall as buds are entering dormancy or in the spring when bud chilling requirement is fulfilled can be damaging (Londo & Kovaleski, 2019; Londo & Kovaleski, 2017). The transition from endodormancy to ecodormancy in preparation for grapevine growth resumption is driven by a genotype-specific amount of exposure to hours of low temperature (0 to 7℃) needed to achieve chilling fulfillment (Fuchigami et al., 1982), and transition the vine to ecodormancy followed by bud break with the increasing spring temperature (Lang et al., 1987). Bud break assays can be used to estimate chilling requirements; however, these measures are frequently confounded with winter injury in grapevines (Fennell, 2004). Under non-injurious conditions, *V. vinifera* cultivars typically require 50-400 chilling hours (0 to 7℃) while other species range between 250-2250 hours (Londo & Johnson, 2014). To select cultivars suited for a region’s climatic conditions, it is important to understand the interaction of chilling fulfillment and the rate of bud break (Londo & Johnson, 2014). In regions with early

warming periods, it is important to maintain dormancy to avoid frost damage in the spring (Londo & Johnson, 2014; Meier et al., 2018). Cultivars with greater chilling fulfillment and slower deacclimation rates would be useful for avoiding spring freezes in a changing climate (Londo & Kovaleski, 2019). The sustainability of grapevines is dependent on the interaction of the grapevine's response to local temperatures during acclimation and deacclimation periods, as well as the extreme winter low temperatures in a year. The objective of this study was to provide baseline information on four interspecific grape cultivars' freezing tolerance and chilling fulfillment patterns throughout the dormancy cycle in South Dakota, USA.

3.3 Materials and Methods

3.3.1 Plant Materials

Four cultivars with complex interspecific pedigrees were examined (*Vitis* International Variety Catalogue (VIVC) variety number is listed in parenthesis: Brianna (VIVC 23260) (Okie, 2004), Frontenac gris (VIVC 23928) (Luby & Hemstad, 2006), La Crescent (VIVC 17632) (Okie, 2002), and Marquette (VIVC 22714) (Peter Hemstad & Luby, 2008). Samples of the grape cultivars were collected from bearing vines trained with bilateral low cordons and vertical shoot positioning at Tucker's Walk commercial vineyard in Garretson, SD (lat. 43°43'2.901" N, long. 96°30'10.155" W) in USDA Plant Hardiness Zone 4b (USDA, 2020). Canes were collected bi-weekly from November 2, 2017, to March 23, 2018 (year 1) and November 7, 2018, to April 3, 2019 (year 2) and November 12, 2019, to March 11, 2020 (year 3). Sample days are in Julian days for each dormancy season starting from January 1 of a given year through the next spring (next

calendar year). Vines were sampled randomly across the cultivar block each sample time. For each cultivar, a random cane (containing nodes 5-10 numbered from cane origin/base) was collected from each of five vines for one replicate. A total of five replicates were tested for freezing tolerance and dormancy status at each sampling time. Vines were sampled across the vineyard blocks for each cultivar. To monitor controlled chilling fulfillment, 45 additional canes (one per vine, containing nodes 5-10 from cane origin/base) were collected from vines distributed across each cultivar block, on the first field sample date in November. Controlled chilling canes were cut into single nodes and nodes from each cane were placed in Ziplock bags at 4℃ to fulfill the chilling requirements.

3.3.2 Low temperature exotherms

Bud low temperature exotherms (LTEs) were determined using differential thermal analysis (DTA) with a Keithley Multimeter Data Acquisition System (model 2700-DAQ-40; Keithley Instruments, Cleveland, OH), a programmable freezer (Tenney Environmental Test Chamber, model T2C, Thermal Product Solutions, Williamsport, PA) and thermoelectric modules (TEM) constructed as previously described by Mills et al. (2006). Five buds (one from each of the individual canes) were placed in a TEM and five replicates (five buds in each of five TEMs) were used for each cultivar. The temperature program was as described by Mills et al. (2006). (1 hour at 4℃, followed by 4℃/hour temperature decline to -40℃). LTEs representing the bud killing temperature were identified for each replicate (Ferguson et al., 2011; Mills et al., 2006).

3.3.3 Dormancy Status

Dormancy status was monitored for field-collected and control chilled buds at two-week intervals using forcing assays. Dormancy status/bud break capacity was determined by placing a five cm long node section (sixth node from cane origin/base) in water at 22℃ and 24-hour day length (n=5). Bud phenological stage was monitored weekly using the modified E-L grapevine growth stage system and E-L stage 4 (green tip visible) was considered bud break (Coombe, 1995). Chilling was considered fulfilled when 50% of buds reached E-L stage 4 within 4 weeks (Londo & Johnson, 2014). After four weeks, buds that did not break were cut longitudinally to determine viability (bud interior was brown). Chilling hours were calculated as hours of exposure to temperatures between 0 and 7℃ in the field or controlled conditions (Dokoozlian, 1999). Chilling hour accumulation for the field condition was calculated from October 1 to the sample time by using hourly temperature data from the Garretson station of South Dakota Mesonet (University, 2019). Chilling accumulation for the controlled chilling treatment was calculated by adding the field chilling hours from October 1 to the collection date for controlled treatment and adding hours accumulated in a 4℃ controlled refrigeration cooler (24 chilling hours/day) until the sample date of bud break assay. The buds in controlled chilling treatment accumulated chilling hours more quickly than under field conditions, four chilling hour periods (200-500, 501-700, 701-900 and >901 (922 to 1538 and 917 to 1629 chilling fours in the field and controlled conditions, respectively) were used to compare the field and controlled condition responses. The resulting experimental design was a three-way factorial with two chilling treatments, four cultivars, and four chilling periods.

3.3.4 Statistical Analysis

3.3.4.1 Freezing

Descriptive analysis was done using the psych library in R (Revelle, 2020). Correlation analysis was performed between cultivar LTEs and mean minimum temperature of the week prior to sample collection using stats library in R (Team, 2013). Influence of seasons, cultivar, sampling time (in Julian days), cultivar*sampling time, and cultivar*season interaction on LTEs were assessed by a linear model (lm function) applied in the stats package in R software (Team, 2013). Seven models (one model for each of the three seasons, two models for the first two seasons, two models for all seasons) were built to check cultivar, sampling time, season main effect, and cultivar by environment (sample time, season, or both) interactions. The most appropriate model to describe the current experimental data was selected by model adequacy. In addition, each model's residual was checked for normality assumptions. Freezing tolerance plots were plotted using ggplot2 in R (Wickham, 2016).

3.3.4.2 Dormancy status

Chilling fulfillment descriptive analysis was performed using psych library in R (Revelle, 2020). The effect of chilling accumulation method (natural or controlled), cultivar (4), chilling hour accumulation group (200-500, 501-700, 701-900, and >901 chilling hours), season (3), treatment by cultivar, treatment by chilling group, treatment by season, and cultivar by season interactions relative to bud break growth stages were assessed by ANOVA using stats package in R (Team, 2013). A model that included all main effects and interaction effects was tested for normality assumptions.

3.4 Results

3.4.1 Dormant season temperature variation 2017-2020

The three winter seasons had different low temperature severity (Figure 3-1). The 2017/18 to 2018/19 dormant seasons show wide fluctuation in minimum hourly temperatures in Garretson, SD. The 2017/18 and 2018/19 winters were similar with the exception that the lowest temperatures occurred later in 2018/19. Temperatures below -15℃ typically do not occur until late November or early December in South Dakota, as noted by the first temperature below -15℃ in 2017/18 and 2018/19 temperatures (December 7, 2017, Julian day 342, and December 29, 2019, Julian day 364). However, in 2019/2020 a -18℃ occurred very early (November 7, 2019, Julian day 312). In most winters, the lowest temperatures occur in January and the March temperatures were the most variable ranging from -11 to -29℃ in this three-year period. Mean monthly temperatures were similar for the three seasons, emphasizing the need to track daily temperatures (Supplementary Table 3-1).

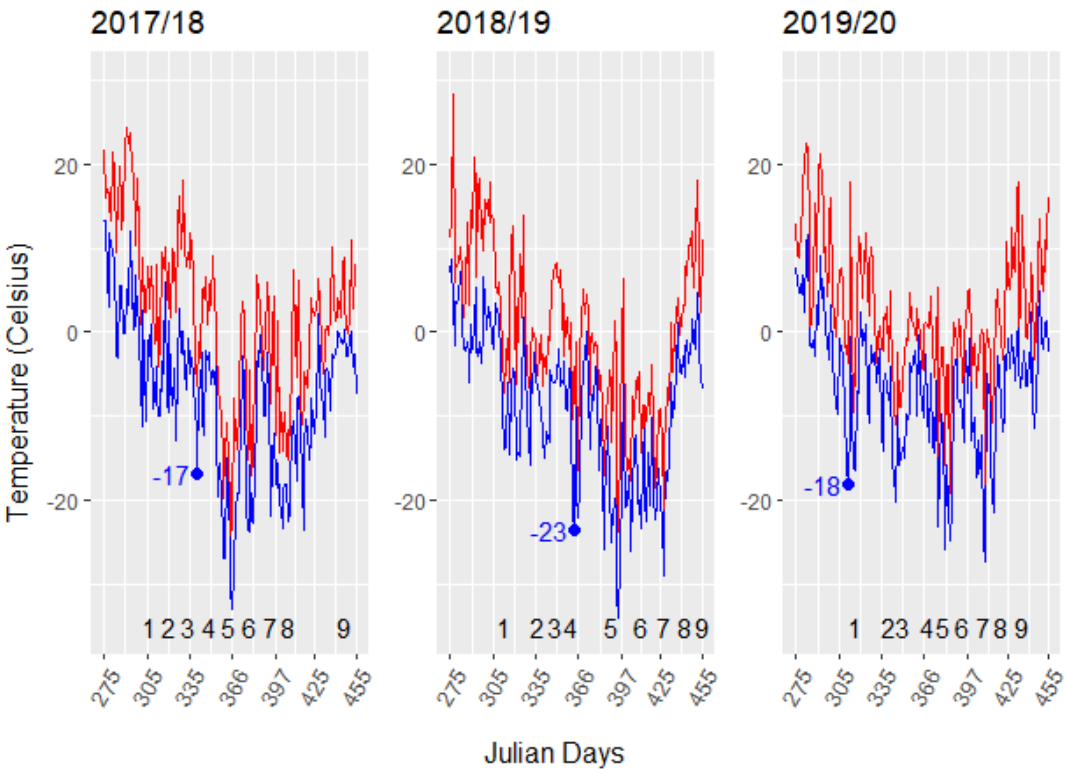

Figure 3-1. Dormant season temperatures 2017-2020. Daily maximum and minimum temperature are indicated by red and blue, respectively. Numbers from 1 to 9 indicate tissue sampling time each year. The blue dot indicates the first date that the minimum temperature was below -15℃ in the respective dormant season. The first day of each month for a dormant season (November through April) are 305, 335, 366, 398, 426, and 457 Julian days, respectively.

3.4.2 Bud freezing tolerance differs between seasons and cultivars

The cultivar LTEs were lower in 2017/18 than the 2018/19 and 2019/20 dormant seasons (Figure 3-2). Freezing tolerance was significantly different by cultivar, season, sampling time, and cultivar by temperature interaction effects. The earlier colder temperatures in 2017/18 winter season are reflected in lower temperature LTEs in all cultivars (Supplementary Table 3-2). Minimum LTEs varied by the winter season, occurring January 29 in 2017/18, March 3 in 2018/19, and February 28 in 2019/20. Buds began to deacclimate after January 2017/18, March 2018/19, and February 2019/20 (Fig. 3-2, Supplementary Table 3-2). Brianna and Marquette had consistently lower LTEs in mid-winter than other cultivars; however, Brianna appeared to deacclimate more rapidly with higher LTEs in March and April (Supplementary Table 3-2). Across the three years, Marquette had greater overall freezing tolerance showing a consistently lower mean LTEs in November and March than the other cultivars (Fig. 3-2, Supplementary Table 3-2).

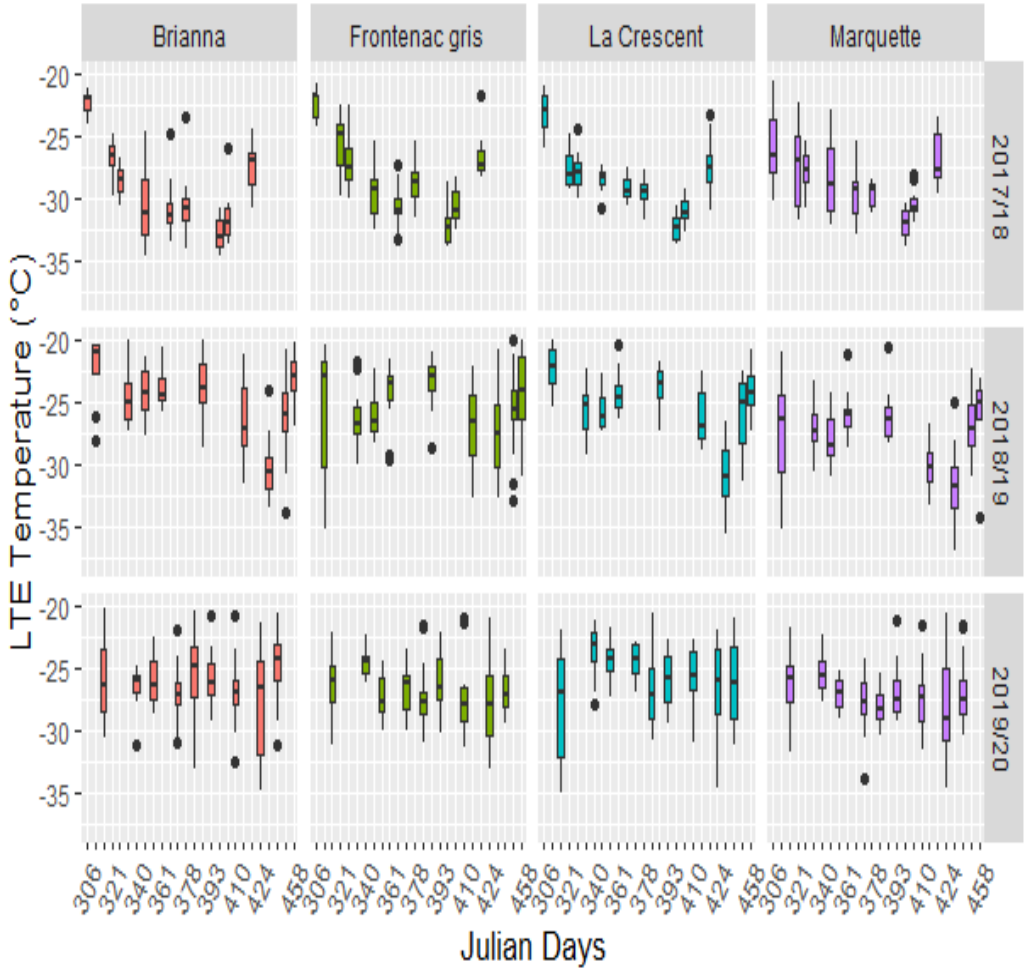

Figure 3-2. Low temperature exotherms for Brianna, Frontenac gris, La Crescent, and Marquette across the dormant season for 2017/18, 2018/19, and 2019/20.

The LTEs temperatures paralleled the minimum temperature in 2017/18 and 2018/19, with the lowest temperatures and lowest LTEs being skewed towards February in 2018/19 (Fig. 3-1 and 3-2). There was little change in LTEs throughout 2019/20 after the early -18℃ freezing temperature. There were significant correlations between cultivar LTEs and the mean minimum temperature of the seven days prior to sampling in 2017/18 and 2018/19 (Table 3-1). There were no significant correlations between temperature and

LTEs in the 2019/20 season (Table 3-1). The early -18℃ temperature in the 2018/19 season before sample collection started resulted in bud damage limiting cultivar LTE fluctuation with local temperature in 2019/20. Modeling the contribution of cultivar, season and, sample time indicated the complexity of grapevine bud freezing tolerance and its interactions with environmental changes (Table 3-2 All models showed significant environment (season or sample time) main effects. Complex models showed significant cultivar environment interactions and increased model complexity did not violate normality assumptions). The cultivar was a significant contributor to LTEs in 2017/18 and 2018/19; but not in 2019/20 (Table 3-2, models 1-3), suggesting the bud damage occurred with the extreme early low temperature in 2019/2020. Further comparison of the full model (cultivar, sample time, season, and interactions) for the first two seasons with that for all three seasons further supports this as cultivar was not a significant contributor when all three seasons were included (Table 3-2, bottom row). This indicates that the timing of acclimation induction and extremely low temperatures in the early season are both important factors in freezing tolerance.

Table 3-1. Pearson correlation coefficient between cultivar low temperature exotherm and mean seven-day minimum temperature prior to sampling in 2017/18, 2018/19, and 2019/20 winter seasons.

Cultivar	**All seasons**	**2017/18**	**2018/19**	**2019/20**
Mean across cultivars	0.23*	0.46*	0.28*	0.05
Brianna	0.27*	0.45*	0.45*	0.06
Frontenac gris	0.18*	0.52*	0.08*	0.11
La Crescent	0.25*	0.45*	0.40*	0.01
Marquette	0.25*	0.43*	0.28*	0.05

*, Significant at p-value <0.05

Table 3-2. Modeling contribution of cultivar, season, and sample time and their interactions to the low temperature exotherms.

	Factors in ANOVA				
Season(s)	**Season**	**Cultivar**	**Sampling time**	**Cultivar*Sampling time**	**Cultivar*Season**
2017/18	NA	*	*	*	NA
2018/19	NA	*	*	*	NA
2019/20	NA	NS	*	*	NA
2017/18+2018/19	*	*	*	*	NA
2017/18+2018/19	*	*	*	*	*
2017/18+2018/19+2019/20	*	*	*	*	NA
2017/18+2018/19+2019/20	*	NS	*	*	*

*, Significant at p-value <0.05; NA, not included in the model; NS, not significant at p-value <0.05; bold indicates best model

3.4.3 Bud dormancy release showed significant cultivar by treatment or season interactions

The controlled and natural field chilling conditions showed similar bud break phenology across cultivars at each chilling hour accumulation group (Fig. 3-3). Both controlled and natural conditions resulted in the bud break phenology stage that increased similarly with greater chilling hours as the major effect of chilling treatment (controlled or natural) was not significant (Supplementary Table 3-2). Cultivar differences in the relationship between chilling and bud break stage are noted for the four chilling periods (200-500, 501-700, 701-900, >901). The main effects for cultivar, chilling accumulation group, and season were significant. The two-way interaction effects of cultivar, chilling hour group, and season with chilling treatment were significant and cultivar and season interaction was significant indicating cultivar and seasonal components (Supplementary Table 3-3). Cultivars demonstrated differences in the bud break phenology stage as chilling hours accumulated. Brianna responded to chilling at lower chilling hours as evidenced by the greater E-L phenology stage (Table 3-3). Frontenac gris and La Crescent chilling fulfillment response was similar and intermediate to Brianna and Marquette. Freezing injury to the primary bud meristem can cause a delay in bud break and it is noted that La Crescent had a lower bud break phenology stage under natural conditions (Table 3-3) and higher LTEs (Supplementary Table 3-2), suggesting potential for a delayed break in response to freezing injury; however, in this study, the potential impact of prior freezing damage to primary buds resulting in delays in bud break could not be determined, as emerging shoots were not differentiated as arising from the primary or secondary bud meristems in this assay.

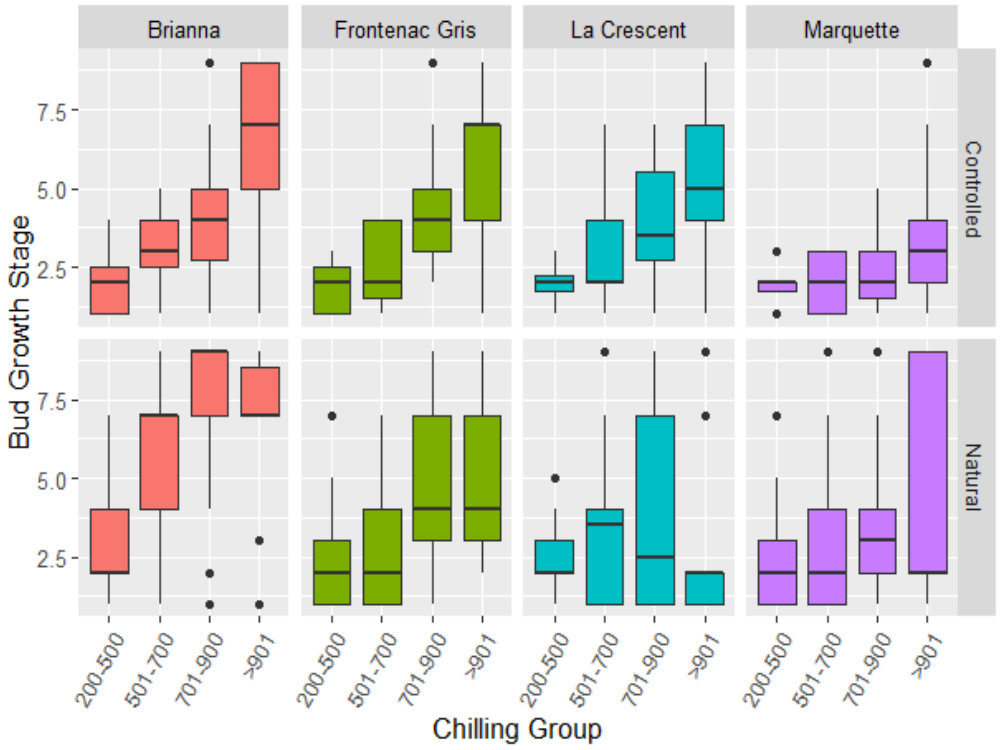

Figure 3-3. Cultivar bud break changes in response to similar chilling hour groups in controlled and natural chilling hour accumulation in 2017/18, 2018/19, and 2019/20 dormant seasons.

Table 3-3. Bud phenology stage determined after 4 weeks forcing for Brianna, Frontenac gris, La Crescent, and Marquette for 200-500, 501-700, 701-900, or >901 chilling hour accumulation in controlled (4 ^{0}C) and natural field conditions.

		Mean bud phenology stage (standard deviation) in chilling groups			
Cultivar	**Condition**	**200-500**	**501-700**	**701-900**	**>901**
Brianna[x] (a)	Natural	2.8 ± 1.6	5.9 ± 2.4	7.7 ± 2.3	6.6 ± 2.5
	Controlled	2.0 ± 1.2	3.3 ± 0.7	4.2 ± 2.2	7.2 ± 2.2
Frontenac gris (b)	Natural	2.3 ± 1.6	3.0 ± 2.2	5.1 ± 2.8	4.9 ± 2.6
	Controlled	2.1 ± 0.9	2.2 ± 1.3	4.4 ± 2.5	6.0 ± 2.5
La Crescent (b)	Natural	2.4 ± 1.1	3.5 ± 2.4	3.9 ± 3.1	2.7 ± 2.7
	Controlled	2.1 ± 0.8	2.8 ± 1.9	4.2 ± 2.2	5.6 ± 2.1
Marquette (c)	Natural	2.6 ± 1.6	2.7 ± 2.0	3.4 ± 2.4	4.6 ± 3.6
	Controlled	2.0 ± 0.7	1.9 ± 1.0	2.2 ± 1.2	3.8 ± 2.3

Mean phenology stage ± standard error in columns for three years of measure. Differing letters in parenthesis after cultivar indicate phenology stage in response to chilling hour accumulation was significantly different between cultivars.

3.5 Discussion

Grapevine bud freezing tolerance increases from October to February with decreasing temperatures especially sub-freezing temperatures (Wolf & Cook, 1992). Typically, the maximum freezing tolerance occurs in January and begins to decrease with increasing temperatures in February or March (Bourne & Moore, 1991; Bourne et al., 1991; Wolf & Cook, 1994). Interspecific cultivars are reported to have a wide range of inherent cold hardiness and winter survival characteristics (Wolf & Cook, 1994). The results in this study showed the complex interactions of the grapevine freezing tolerance and bud dormancy phenotype with changing temperatures. Interspecific cultivars had substantial interactions with the environment. Sampling time and season influence bud freezing tolerance in the interspecific cultivars. In addition, data modeling helps to predict the most influencing factors for bud freezing tolerance across years as field conditions vary each year. Differing field temperature conditions impacted LTE values (temperatures of bud injury due to intracellular freezing) at the various sample times; however, cultivar comparative differences were consistent from year to year. As shown for other grape cultivars, in mild winters LTEs are less negative than in colder winters (Ferguson et al., 2011; Londo & Kovaleski, 2017). This can be seen in the LTEs for Brianna averaging -24.7℃ and -26℃ in 2018/19 and 2019/20, respectfully. January is frequently considered midwinter and when vines will be at their maximum freezing tolerance. The muscadine cultivars Carlos and Summit were maximally hardy in January (Clark et al., 1996). Similarly, in *V. vinifera* cultivars and hybrids Vignole and St. Vincent, bud cold hardiness correlates with the recent cold temperatures (Sanliang et al., 2001). In this study, the lowest cultivar LTEs occurred in January in 2018 and 2020;

however, in 2019 the lowest LTEs occurred in March in conjunction with field temperatures approaching -30℃. In March 2019, the cultivars are ecodormant having received enough chilling hours for rapid bud break upon exposure to warm temperatures. Of the cultivars tested here, Brianna is at more potential risk for bud injury under such conditions than is Marquette.

The influence of local temperatures at different times in the winter have been reported for several cultivars (Gu et al., 2001). Similar responses are apparent in this study, including inherent cultivar differences in freezing tolerance. For example, Chardonnay was found to be more freezing tolerant than Cabernet Sauvignon; however, Chardonnay is noted to transition to ecodormancy and break bud earlier than Cabernet Sauvignon (Cragin et al., 2017). Marquette was more freezing tolerant than Brianna in 2018/19 and 2019/20. In contrast, in 2017/2018 Marquette and Brianna showed similar freezing tolerance over the dormant season. The weather patterns shown in this three year period emphasizes the need for cultivars that acclimate quickly and have a moderate to slow deacclimation characteristics (Gu et al., 2001). In 2019/20, there was an early low temperature in November that appeared to damage primary buds resulting in little change in freezing tolerance during the rest of the season. In this study, Brianna appears to deacclimate more rapidly, whereas Marquette deacclimation is more moderate. The bud break phenology at different chilling hours suggests that Brianna requires lower chilling than the other three cultivars, which indicate that although it is a cold-hardy grape it may be susceptible to injury in late winter due to rapid bud break (Londo & Johnson, 2014).

While bud freezing tolerance is a critical factor in sustainable grape production, these results indicate the importance of determining both freezing tolerance and chilling

requirements of new cultivars to identify their potential success in northern cold climate regions (Londo & Kovaleski, 2017). It is of note that the controlled chilling and natural field chilling produced a similar bud break phenology. This makes it possible to avoid the confounding factor of primary bud injury on bud break phenology. Damage of the primary bud frequently delays bud break with the secondary bud emerging more slowly than a healthy primary bud. In addition, collection of materials shortly after leaf drop and testing bud break phenology over a series of chilling hours makes it possible to accurately determine the chilling requirement of new cultivars.

Long term sustainability of cultivars is influenced by their ability to acclimate with changing dormant season temperatures. La Crescent and Marquette were identified as suitable cultivars for Wisconsin (Atucha et al., 2018). In contrast, La Crescent had a higher survival rate than Marquette in Vermont (Berkett et al., 2008). Marquette had more than 90% bud survival six years in Iowa and yearly trial performance in the primary bud injury was lower than Brianna, Frontenac gris and, La Crescent (Domoto et al., 2011). Brianna had greater primary bud kill than La Crescent and Marquette (Domoto et al., 2013). In this study, Brianna is noted as a cold-hardy cultivar, with the potential risk of early bud break due to a lower chilling requirement. Marquette was a superior cultivar in South Dakota for freezing tolerance and slow bud burst (deacclimation) ability compared to Brianna, Frontenac gris, and La Crescent. Marquette had maximum freezing tolerance in most years and was less affected by warming temperatures in early spring with a higher chilling hours requirement.

A relationship between the loss of freezing tolerance and greater chilling accumulation in grapevines has been reported (Kovaleski et al., 2018) Thus potential

differences in cultivar chilling fulfillment requirements should be considered when choosing cultivars for a specific areas as it may contribute to long term sustainability (Fennell, 2004). Increasing temperatures or warming periods in late winter and early spring can trigger deacclimation and promote bud break; therefore, early chilling fulfillment could contribute to early break, putting cultivars at risk of freezing stress (Lipe et al., 1992; Meier et al., 2018). In this study, Brianna showed increased bud break at lower chilling hour accumulation than other cultivars and in some cases, Brianna also had a higher LTE in corresponding timeframes suggesting there may be a potential interaction that may influence long term sustainability. It is not possible to separate prior freezing damage in natural conditions on the rate of bud break without destructively viewing the bud. Additional study of controlled chilling and controlled non-lethal freezing acclimation conditions would be needed to determine specific interactions between freezing tolerance and chilling accumulation.

3.6 Conclusions

All cultivars showed distinct acclimation and chilling fulfillment characteristics with Marquette showing the greatest freezing tolerance in early and mid-winter. There is a correlation between the 7-day temperature average and LTEs in 2017/18 and 2018/19. Modeling the potential components (cultivar, season, and sample time) contributing to LTEs indicates that in seasons with a gradual decrease in temperatures (2017/18 and 2018/19) all main factors and their interactions contribute to LTEs. In 2019/20 an early low temperature of -18℃ resulted in major bud damage apparent in lack of freezing tolerance change in response to low temperature in mid-winter. This early low temperature injury indicates the strong need for early acclimation and that very early low temperature extremes can cause damage in these interspecific cultivars. There is also a risk associated with early deacclimation as noted in Brianna due to its lower chilling requirement than other cold-hardy cultivars. Marquette had a greater chilling requirement than Brianna, La Crescent and, Frontenac gris. The chilling fulfillment studies indicated that the main effect of natural accruing and constantly controlled environment temperatures showed similar bud break stage results; however, there were significant interactions between cultivar and treatment and season indicating greater complexity to the chilling fulfillment trait.

Supplementary Materials: Table 3-1. Mean monthly maximum and minimum temperatures during the dormant seasons of 2017/18, 2018/19 and, 2019/20. Table 2-2. Mean bud LTE for Brianna, Frontenac gris, La Crescent, and Marquette during the dormant seasons of 2017/18, 2018/19 and, 2019/20. Table 3-3. ANOVA results for controlled and natural chilling on grapevine bud break across three seasons.

3.7 References

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Supplementary Table 3-1. Mean monthly minimum and maximum temperature during the dormant seasons of 2017/18, 2018/19, and 2019/20.

	2017/18 mean maximum (℃)	2018/19 mean maximum (℃)	2019/20 mean maximum (℃)	2017/18 mean minimum (℃)	2018/19 mean minimum (℃)	2019/20 mean minimum (℃)
October	15.0	11.8	10.7	3.1	1.3	1.4
November	6.7	1.9	4.0	-4.6	-7.0	-5.9
December	-2.6	0.1	-1.7	-11.5	-8.8	-9.4
January	-4.5	-5.5	-3.2	-14.1	-14.8	-12.3
February	-5.5	-9.4	-1.4	-15.6	-17.5	-11.6
March	3.2	1.6	7.0	-3.8	-7.0	-2.7

Supplementary Table 3-2. Mean Bud LTE for Brianna, Frontenac gris, La Crescent, and Marquette during the dormant seasons of 2017/18, 2018/19, and 2019/20.

	Brianna	**Frontenac gris**	**La Crescent**	**Marquette**
Mean (all years)	**26.6**[w]	**26.7**	**26.5**	**27.8**
2017-2018	**LTE**[X]	**LTE**	**LTE**	**LTE**
11.02.17	-22.3[Y]	-21.8	-22.5	-25.8
11.17.17	-26.8	-25.4	-27.6	-27.3
11.30.17	-28.7	-27.1	-27.8	-27.7
12.15.17	-30.6	-29.4	-28.5	-28.3
12.29.17	-30.8	-30.7	-29.2	-29.6
01.13.18	-30.4	-28.7	-29.5	-29.6
01.29.18	-32.8	-32.0	-32.2	-32.0
02.10.18	-31.4	-30.5	-31.0	-30.5
03.23.18	-27.5	-26.7	-27.5	-27.0
Mean 2017-2018	**-29.1**	**-28.1**	**-28.4**	**-28.7**
2018-2019	**LTE**	**LTE**	**LTE**	**LTE**
11.09.18	-20.5	-25.7	-21.5	-27.8
12.03.18	-24.5	-26.2	-25.6	-27.1
12.15.18	-24.1	-26.1	-25.6	-27.9
12.27.18	-23.5	-24.2	-24.5	-26.0
01.25.19	-23.4	-22.9	-23.8	-26.2
02.15.19	-26.3	-26.9	-26.2	-30.2
03.03.19	-30.3	-27.4	-31.0	-31.6
03.18.19	-25.9	-25.7	-26.1	-26.8
04.03.19	-22.8	-23.9	-24.1	-23.9
Mean 2018-2019	**-24.7**	**-25.4**	**-25.5**	**-27.8**
2019-2020	**LTE**	**LTE**	**LTE**	**LTE**
11.12.19	-25.3	-26.4	-27.0	-25.3
12.06.19	-26.4	-24.5	-23.6	-25.4
12.16.19	-25.9	-27.2	-24.5	-27.1
01.03.20	-27.0	-26.6	-24.4	-27.6
01.14.20	-25.6	-27.2	-26.8	-28.2
01.28.20	-26.1	-25.6	-25.8	-26.9
02.14.20	-26.5	-27.3	-25.8	-27.6
02.28.20	-27.5	-27.7	-26.4	-28.2
03.11.20	-24.7	-26.5	-26.1	-26.9
Mean 2019-2020	-26.0	-26.6	-25.7	-27.0

w= mean of all time points across all years; x= mean cultivar LTE at given year; y = mean cultivar LTE at given time points (n=5).

Supplementary Table 3-3. ANOVA results for controlled and natural chilling on grapevine bud break across three seasons.

Terms in the model	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	1	9	8.8	1.888	0.1697
Cultivar	3	984	328	70.641	< 2e-16
Chilling Group	3	1364	454.8	97.959	< 2e-16
Season	2	255	127.7	27.497	2.39e-12
Treatment: Cultivar	3	119	39.7	8.560	1.29e-05
Treatment: Chilling Group	3	182	60.7	13.071	2.24e-08
Treatment: Season	2	158	78.9	16.989	5.56e-08
Cultivar: Season	6	76	12.6	2.715	0.0128
Residuals	992	4606	4.6		

Df, degrees of freedom; Sum Sq, sums of squares; Mean Sq, mean sums of square

4 Chapter 4 Comparative transcriptome investigation of grapevine bud transition during natural and controlled chilling

4.1 Abstract

Dormant grapevines require chilling temperatures (0 to 7 °C) for transition to ecodormancy, to allow growth resumption in response to increasing temperatures in the spring. Understanding dormancy control and release are important as extended or too little chilling may result in delayed bud break, weak growth, and decline in vine vigor. Response to controlled (4 °C) and field chilling fulfillment may differ in different cultivars. Therefore, a transcriptomic investigation using RNA-Seq was performed to determine the potential molecular mechanisms (pathways) involved in chilling fulfillment in Marquette and Brianna under controlled (constant 4 °C in the dark) and natural field chilling conditions. Principal components analysis of all expressed genes indicated that gene expression differed in the natural field and controlled for both cultivars. In controlled and field chilling conditions, there were 4571 differentially expressed genes (2076 up-regulated in controlled and 2495 up-regulated in natural) with increased chilling from 450 to 1000 chilling hours. Phenylpropanoid biosynthesis, photosynthesis antenna proteins, and plant-pathogen interaction pathways were significantly enriched in controlled and natural chilling conditions. Cell wall and auxin signaling pathways were significantly enriched in controlled chilling, while jasmonate and ethylene signaling pathways were significantly enriched in natural field chilling. The results suggest that the fluctuating temperatures in the field promote different metabolic processes in contrast with controlled chilling.

4.2 Introduction

Grapevines typically have met their chilling requirement during February and start to blossom at the end of spring (Khalil-Ur-Rehman et al., 2017). Insufficient chilling may lead to non-uniform or delayed bud break (Mathiason et al., 2009). Under natural and controlled conditions in cold-climate wine grapes, physiological assays indicate that there was no major effect between controlled and natural chilling treatments, but there was a significant genotype by chilling treatment interaction effect (Yilmaz et al., 2021). Dormancy processes in grapevine were shown to activate cell division and cell growth metabolic pathways (Mathiason et al., 2009), and carbohydrate metabolism (Min et al., 2017). A study conducted during chilling fulfillment in grapevine showed that dormancy transition is associated with antioxidant systems, secondary metabolism, cell cycle and division, cell wall metabolism, as well as carbohydrates metabolism. In particular, gibberellin catabolism and sucrose synthase genes were up-regulated just before bud break (Shangguan et al., 2020). Understanding the genes and pathways involved in chilling fulfillment is important for developing improved cultural management and selecting suitable grapevines for specific regions (Mathiason et al., 2008). More transcription factors were up-regulated in natural than controlled (4 ^{0}C) chilling in blueberry; in addition to genes related to stress tolerance (Dhanaraj et al., 2007). A cold acclimation study on wild grapevine identified plant hormone biosynthesis (ABA biosynthesis, ethylene, jasmonate, gibberellin, and cytokinin synthesis), starch synthesis, and photosynthesis pathways enriched in response to low temperatures). Many studies have been conducted to monitor molecular changes during the induction of dormancy and have identified gibberellin metabolism, carbohydrate metabolism, cell division, and

growth related to different phases of dormancy (Díaz-Riquelme et al., 2012; Khalil-Ur-Rehman et al., 2017; Min et al., 2017; Shangguan et al., 2020). However, most of these studies on the transition from endodormancy to ecodormancy in response to chilling have occurred in *V. vinifera* cultivars. Therefore, this study used transcriptomic analysis to determine whether there are differences in gene expression relative to the field and constant 4 °C chilling treatments using two-hybrid wine cultivars (Marquette and Brianna).

4.3 Materials and methods

4.3.1 Plant material

Two cultivars, Marquette and Brianna, with complex interspecific pedigrees were used for this study (Vitis International Variety Catalogue (VIVC) (Maul et al. 2014). Canes were sampled from bearing vines trained to a bilateral low cordon and vertical shoot positioning at Tucker's Walk commercial vineyard in Garretson, SD (lat. 43°43'2.901" N, long. 96°30'10.155" W) in USDA Plant Hardiness Zone 4b (USDA, 2021) bi-weekly from November to March. Vines were sampled randomly across the vineyard for each cultivar block for each sampling time. A random cane (containing nodes 5–10 numbered from cane origin/base) was collected from each of six vines for one replicate. Two chilling conditions were used in this study field chilling (natural conditions) and constant 4 °C in the laboratory. Transcriptomic profiles were determined at 450, 650, 750, 950, and 1000 chilling hours under natural field conditions during the dormant season. For this purpose, the cane collection times were in November, December, January, February, and March. Buds were excised into liquid nitrogen and stored at a -80 ^{0}C freezer for each sampling time. Three replicates with 6 buds/replicate

were collected for each chilling hour accumulation. When all samples were collected, they were sent for RNA-Seq analysis at USDA/ARS Geneva, NY. Chilling hour accumulation for the natural condition was calculated from 1 October to the sample time by using hourly temperature data from the Garretson station of South Dakota Mesonet (Mesonet, 2021). Transcriptomic profiles were determined of 450, 750, 1100, 1400, and 1700 chilling hours for the constant 4 °C chilling study. For this purpose, canes were collected on November 1, 2018, from the field. Canes were cut into single nodes and nodes from each cane were be placed into ziplock bags per cultivar and placed in a cooler (4 0C) for chilling treatment. A total of three replicates with 6 buds/replication were collected from the cooler at 450, 750, 1100, 1400, and 1700 chilling hours for the controlled treatment. Chilling hour accumulation for the controlled chilling treatment was calculated by adding the field chilling hours from 1 October to the collection date for constant 4 °C treatment and adding hours accumulated in 4 ^{0}C cooler (24 chilling hours/day) until sample date.

4.3.2 Experimental units

Two studies were conducted using Marquette and Brianna buds that received controlled (constant 4 °C) or natural field chilling. The controlled and natural field were compared at the same chilling hours (450, 650, and 1000). In the second study increasing chilling hour pairwise comparisons were made for Marquette and Brianna in the controlled (450/750, 750/1000, 1000/1400, and 1400/17000) or natural (450/650, 650/750, 750/950, and 950/1000) chilling hours.

4.3.3 RNA extraction

Buds were excised at different time points as described above, immediately put in liquid nitrogen, and stored in the freezer (−80°C) for both natural and controlled chilling (Fennell & Mathiason, 2002). Total RNA was extracted from bud tissues by utilizing Sigma Spectrum kits (Sigma-Aldrich, St. Louis, MO, USA) (Borodina et al., 2011). RNA quality and quantity were verified with an Agilent (Santa Clara, CA, USA) 2100 Bioanalyzer RNA6000 nanochip. RNA-seq libraries for natural and controlled bud transcriptomes were prepared and sequenced by Illumina HiScanSQ (100 bp, single strand) at the Cornell University Institute of Biotechnology Genome Facility (Ithaca, NY, USA).

4.3.4 Read count determination and visualization technique

Raw sample read quality was checked by Fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and data trimmed by tool Trimmomatic (Bolger et al., 2014). Trimmed quality reads were aligned with the V. vinifera 12X V2 genome using HISAT2 (ftp://ftp.ensemblgenomes.org/pub/release-23/plants/gtf/vitis_vinifera/) at the same time with HISAT2 (Kim et al., 2015). Counts of mapped reads for each gene were determined for all samples using featureCounts (Liao et al., 2014). A gene count matrix was constructed for all 60 samples. Principal components analysis was conducted for field vs constant 4 °C, Marquette vs Brianna in constant 4 °C, and Marquette vs Brianna in field chilling using IRIS-EDA (Monier et al., 2019). Differential gene expression (DEG) analysis was conducted using DESeq2 with a p-value of 0.05 and minimum fold change of 1 in IRIS-EDA. The constant 4 °C and field chilling DEG analysis was conducted across cultivars in the two conditions. For the increasing

chill duration study, DEGs were determined for pairwise comparisons within a cultivar and chilling treatment. Venn diagrams of controlled (constant 4 °C) vs natural field across both cultivars in chilling comparison were created in OmicsBox (https://www.biobam.com/venn-diagram/).

4.3.5 Gene set enrichment analyses and Vitis Pathway

Gene Set Enrichment Analysis (GSEA) was conducted utilizing normalized read count data with GSEA-P 2.0 (https://www.gsea-msigdb.org/gsea/index.jsp) and custom gene pathway set VitisNet (Grimplet et al 2012). Controlled (constant 4 °C) and natural field chilling conditions were tested across cultivars. Increased chilling duration in the field or constant 4 °C were tested separately by cultivar in each condition. The recommended GSEA-P 2.0 default parameters of 1000 permutations, nominal p-value < 0.05 was used to identify enriched VitisNet molecular networks.

4.4 Results

4.4.1 Data exploration

4.4.1.1 Principal Component Analysis (PCA).

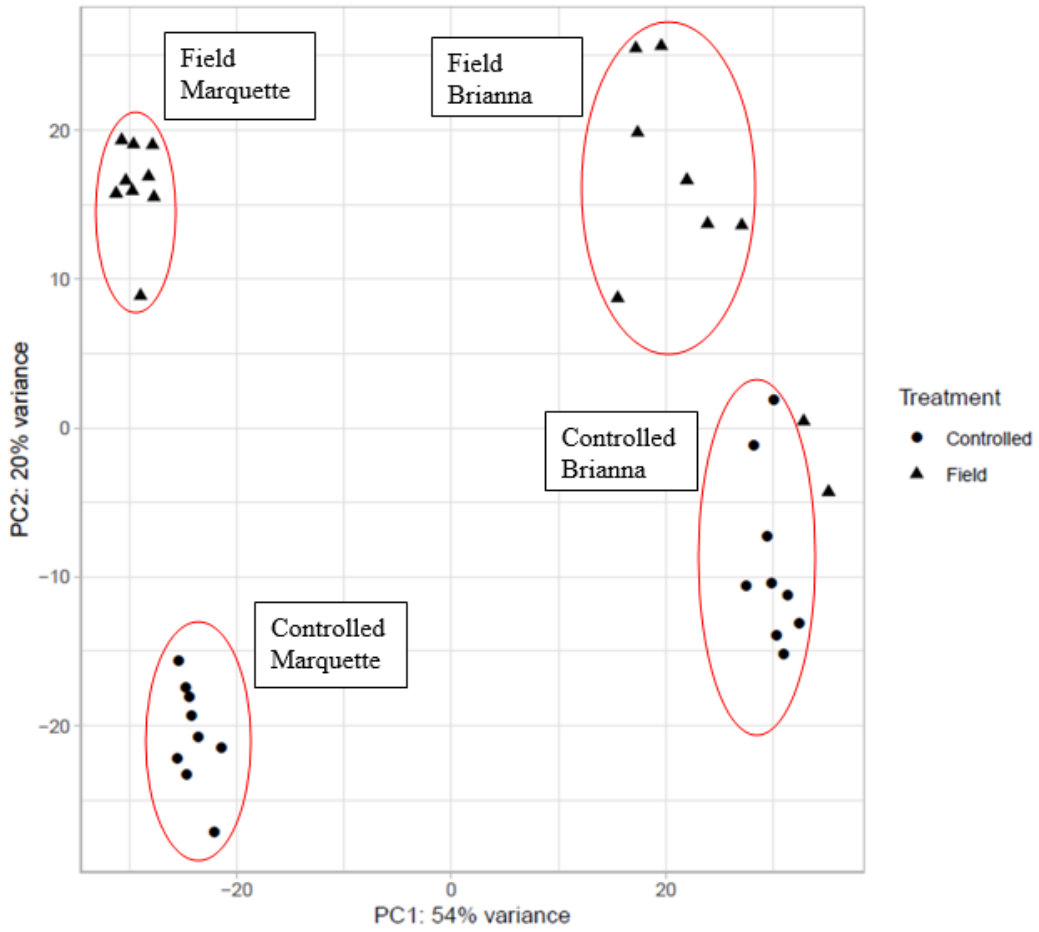

Figure 4-1. Principal component analysis (PCA) for controlled and natural field and Brianna and Marquette. Dots represent controlled (constant 4 °C in the dark) chilling and triangles represent natural field chilling for Marquette and Brianna (n=3).

The PCA showed differences between the chilling treatments and cultivars in response to chilling. Marquette and Brianna showed four distinctly separate clusters for controlled and natural conditions in the PCA. Two Brianna 450 chilling hour natural field samples were more like the controlled chilling than the other field chilling samples.

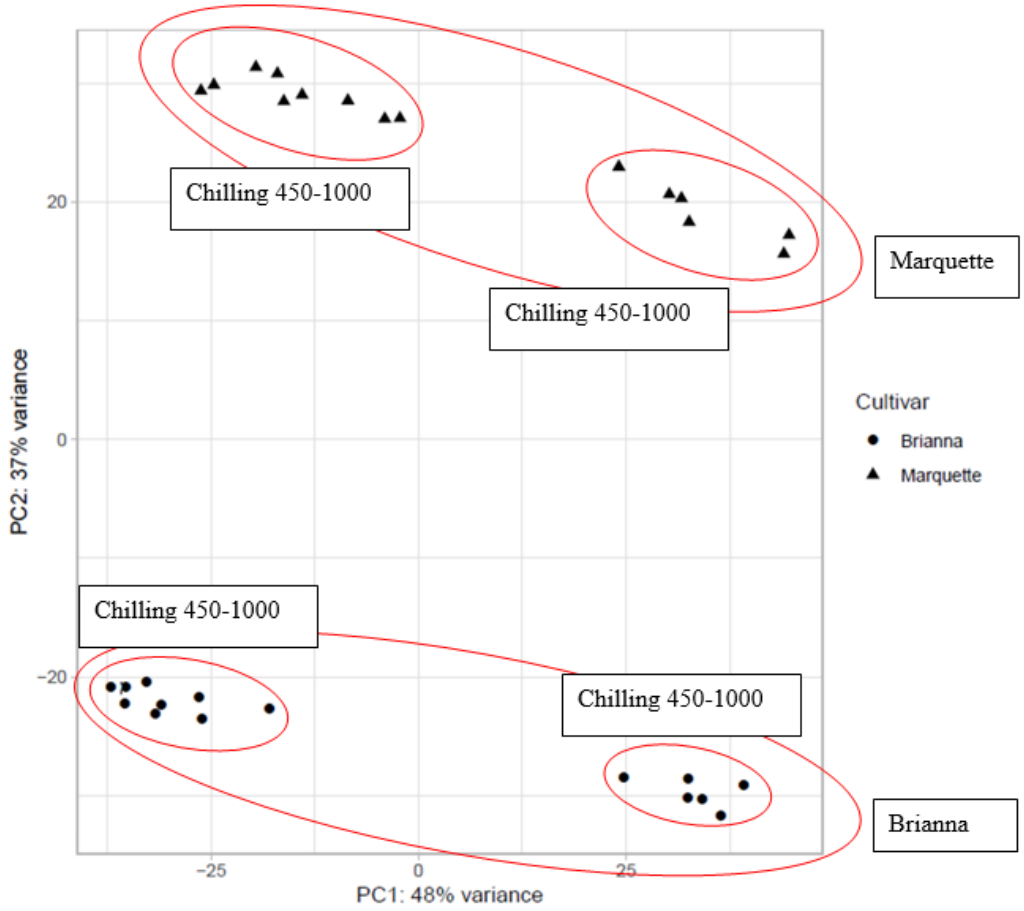

Figure 4-2. Principal component analysis (PCA) of Marquette and Brianna for controlled chilling condition. Dots represent Brianna, and triangles represent Marquette at 450, 750, 1000, 1400 and, 1700 chilling hours in controlled (constant 4 °C) treatment (n=3).

Marquette and Brianna were separated at all chilling hours in PC2. Additionally, lower and greater chilling hours are grouped separately from each other (PC1). The data points on the left side of the graph represent the lower chilling hours (450 to 1000) and the greater chilling hours (1400 to 1700) are found on the right side of the graph for each cultivar (Figure 4-2).

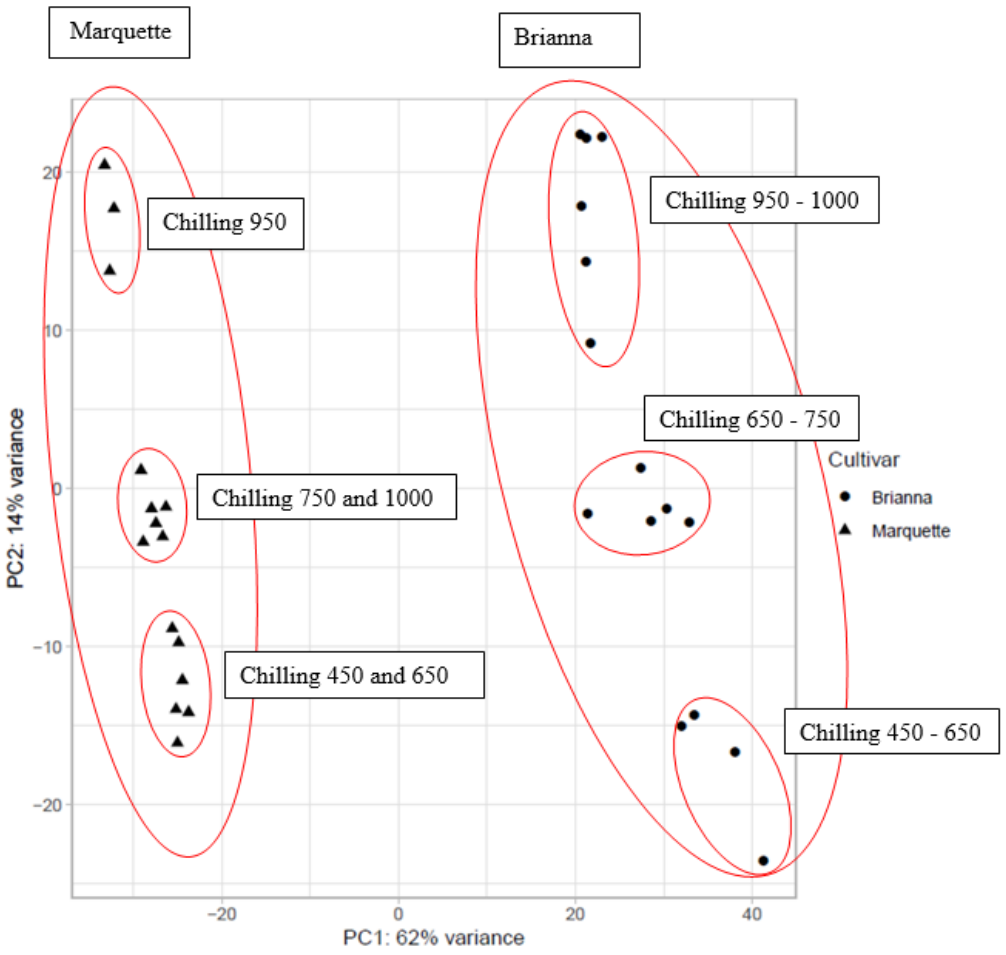

Figure 4-3. Principal component analysis of Marquette and Brianna in field chilling conditions. Dots represent Brianna, and triangles represent Marquette at 450, 650, 750, 950, and 1000 chilling in field treatment (n=3).

The PCA showed distinct differences between Marquetta and Brianna in field chilling (PC1). The distribution of the samples for Marquette was tighter than Brianna within chilling groups (PC2) (Figure 4-3).

4.4.1.2 Differentially gene expression comparison and Venn diagrams.

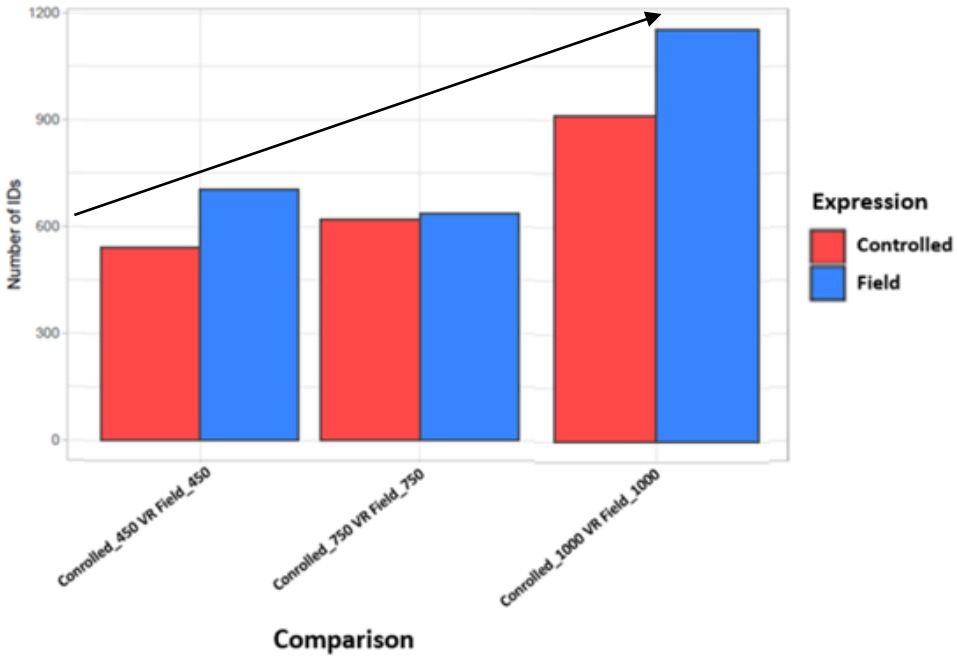

Figure 4-4. Differentially expressed genes in controlled (constant 4 °C) vs natural field in chilling conditions (450, 750 and 1000). Red boxes represent constant 4 °C and blue boxes represent field chilling conditions.

The number of differentially expressed genes (DEG) in controlled (constant 4 °C) relative to field conditions increased with increased chilling. In this comparison genes up-regulated in controlled are by inference down-regulated in the field and if they are down-regulated in constant 4 °C they are up-regulated in field; therefore, this graph presents total DEG up-regulated in controlled (constant 4 °C) and DEG up-regulated in the field to show the difference between controlled (constant 4 °C) and natural field. There is an increasing number of DEG from 450 to 1000 chilling hours (Figure 4-4).

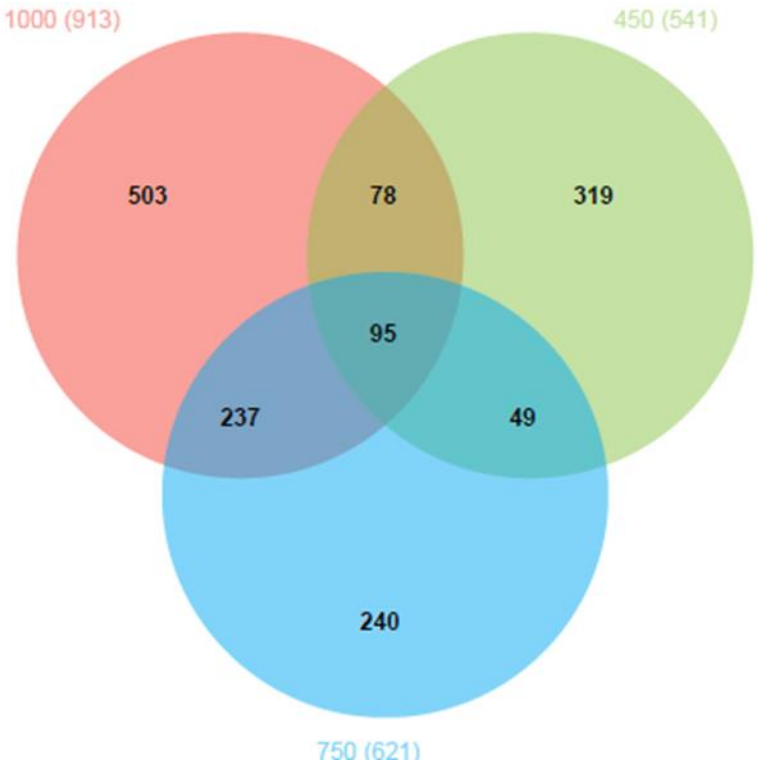

Figure 4-5. Differentially expressed genes up-regulated genes in controlled (constant 4 °C) relative to natural field chilling.

There is an increasing number of up-regulated DEG from 450 to 1000 chilling hours in controlled temperature conditions. There are 95 genes in common to 450, 750, and 1000 chilling hours. The 750 and 1000 share the most DEG in common (Figure 4-5).

Figure 4-6. Differentially expressed genes downregulated in controlled (constant 4 °C) relative to natural field chilling.

In the field, there were more down-regulated DEG (175) in common to all chilling hours (450, 750 and, 1000). As found in the controlled chilling condition, the 750 and 1000 had the greatest number of DEG in common in field chilling conditions (Figure 4-6).

Table 4-1. Enriched pathways in controlled (constant 4 °C) and natural field chilling across all cultivars for 450, 750, and 1000 chilling hours.

Enriched pathways	**NOM p-value**
Pathways enriched in controlled chilling	**Nom p-value**
VV10190OXIDATIVE_PHOSPHORYLATION	0.004
VV10530AMINOSUGARS_METABOLISM	0.004
VV10592ALPHA-LINOLENIC_ACID_METABOLISM	0.032
VV10900TERPENOID_BIOSYNTHESIS	0.028
VV60048PHD	0.012
Pathways enriched in field chilling	**Nom p-value**
VV10480GLUTATHIONE_METABOLISM	0.004
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.002
VV23010RIBOSOME	0.000
VV23020RNA_POLYMERASE	0.016
VV23022BASAL_TRANSCRIPTION_FACTORS	0.042
VV23430MISMATCH_REPAIR	0.011
VV50112NUCLEAR_PORE_COMPLEX	0.000
VV50113THYLAKOID_TARGETING_PATHWAY	0.049
VV50133PRIMARY_ACTIVE_TRANSPORTER_CAT_A9_TO_A18	0.006
VV52010ABC_TRANSPORTERS	0.004
VV60073ORPHANS_ZF-B_BOX	0.032

There were a greater number of enriched pathways in field chilling than in controlled chilling conditions. Transport pathways were enriched in the field (thylakoid targeting, transporter category A9 to A18, and ABC transporters). Controlled chilling was enriched in oxidative phosphorylation, fatty acid, and terpenoid biosynthesis pathways.

4.4.1.3 Differentially gene expression comparison in controlled condition.

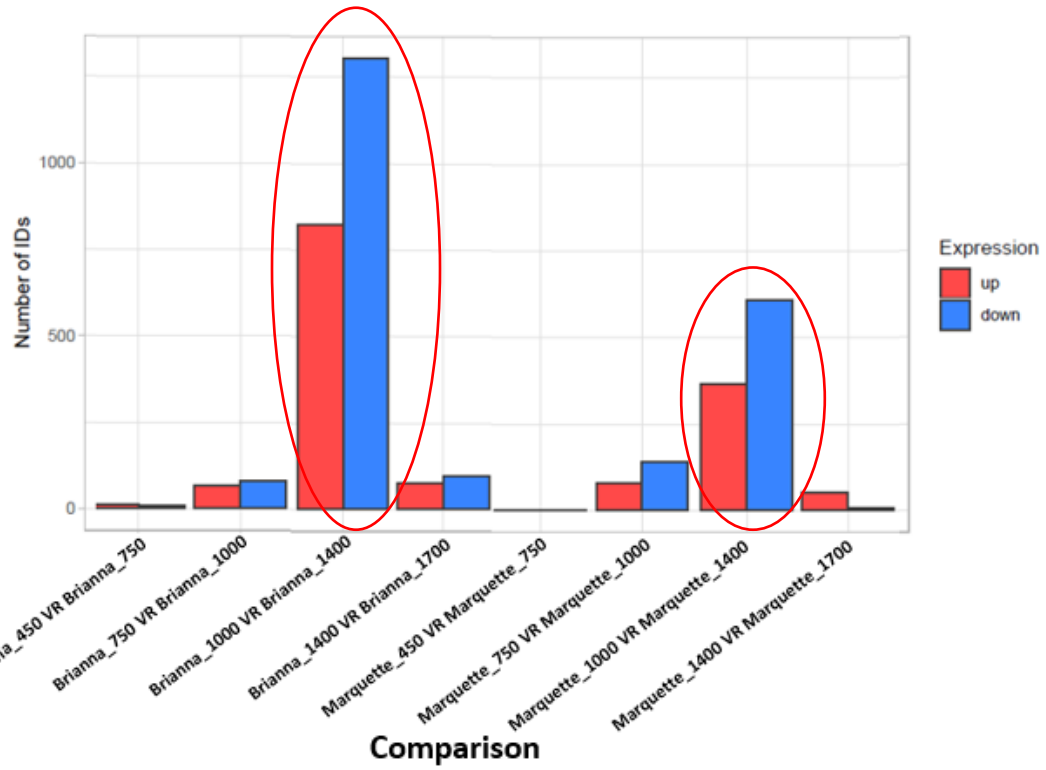

Figure 4-7. Differentially expressed genes for Marquette and Brianna during increased chilling in controlled (constant 4 °C) condition. Red boxes represent up and blue boxes represent down-regulated genes in each pairwise comparison (450/750, 750/1000, 1000/1400, and 1400/1700).

The greatest number of DEGs were found in the 1000/1400 chilling hour comparison for each cultivar in controlled chilling conditions. There were more down-regulated DEG in each cultivar than up-regulated DEG (Figure 4-7).

4.4.1.4 Differentially gene expression comparison in natural condition.

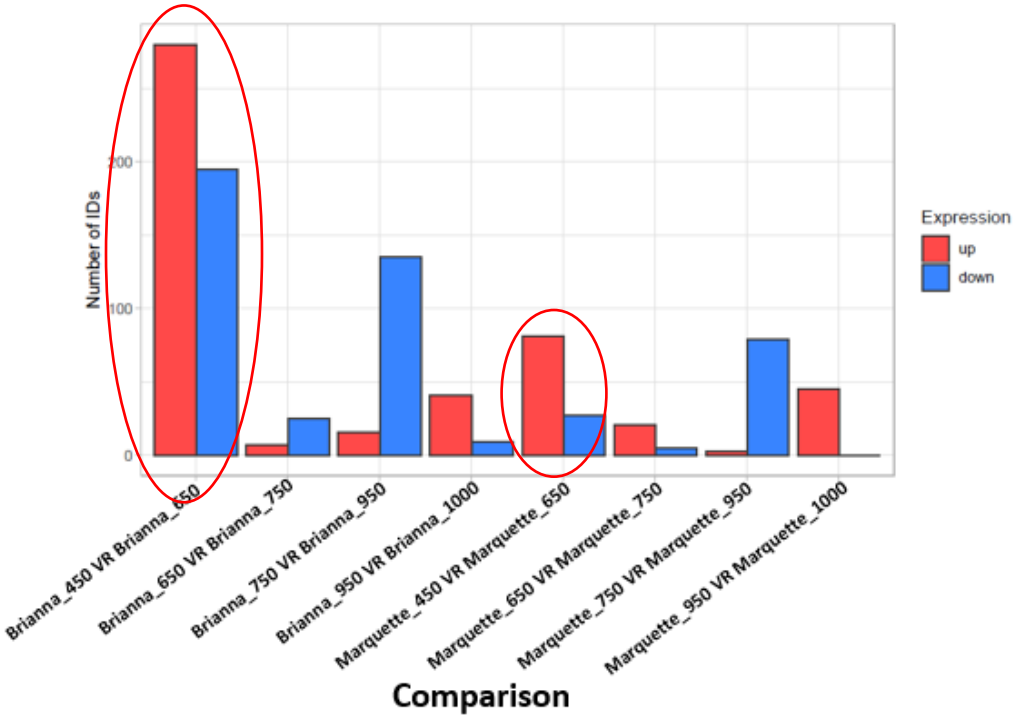

Figure 4-8. Differentially expressed genes for Marquette and Brianna during increased chilling in natural field condition. Red boxes represent up and blue boxes represent down-regulated genes in each pairwise comparison (450/650, 650/750, 750/950, and 950/1000).

The greatest number of DEGs were found in the 450/650 chilling hour comparison for each cultivar. In contrast to the controlled chilling, there were more up-regulated than down-regulated DEG in both cultivars. There were a greater number of down-regulated DEG in the 750/950 chilling hour time point (Figure 4-8).

4.4.2 VitisNet Gene set enrichment analysis (GSEA)

Table 4-2. Enriched pathways in Marquette during increased controlled chilling hours. Pairwise comparisons of increased chilling (450 h/750 h, 750 h/1000 h, 1000 h/1400h, 1400 h/1700h).

Enriched pathways	**NOM p-value**	**Enriched pathways**	**NOM p-value**
Enriched pathways for 450 h chilling	**NOM p-value**	**Enriched pathways for 750 h chilling**	**NOM p-value**
VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.037	VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.002
VV10904DITERPENOID_BIOSYNTHESIS	0.022	VV50101CHANNELS_AND_PORES	0.035
VV11040BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS	0.047	VV60003AP2_EREBP	0.033
VV60011BHLH	0.016	VV60032GRAS	0.010
VV60034HB	0.004	NA	NA
Enriched pathways for 750 h chilling	**NOM p-value**	**Enriched pathways for 1000 h chilling**	**NOM p-value**
VV10220UREA_CYCLE_AND_METABOLISM_OF_AMINO_GROUPS	0.042	VV10195PHOTOSYNTHESIS	0.020
VV10564GLYCEROPHOSPHOLIPID_METABOLISM	0.015	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.000
VV10565ETHER_LIPID_METABOLISM	0.046	VV10710CARBON_FIXATION	0.044
VV10906CAROTENOID_BIOSYNTHESIS	0.029	VV10942ANTHOCYANIN_BIOSYNTHESIS	0.049
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.005	VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.015
VV34020CALCIUM_SIGNALING_PATHWAY	0.021	VV23010RIBOSOME	0.000
VV34626PLANT-PATHOGEN_INTERACTION	0.027	VV23018RNA_DEGRADATION	0.005
VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.000	VV23050PROTEASOME	0.031
VV44146PEROXISOME	0.046	VV30003AUXIN_SIGNALING	0.003
VV50101CHANNELS_AND_PORES	0.029	VV40006CELL_WALL	0.006
VV50111TETHERING_FACTORS	0.045	VV50105TRANSPORT_ELECTRON_CARRIERS	0.000

VV50121PORTERS_CAT_1_TO_6	0.019	VV50113THYLAKOID_TARGETING_PATHWAY	0.041
NA	NA	VV60011BHLH	0.016
NA	NA	VV60016C2C2-GATA	0.043
NA	NA	VV60034HB	0.000
Enriched pathways for 1000 h chilling	**NOM p-value**	**Enriched pathways for 1400 h chilling**	**NOM p-value**
VV10010GLYCOLYSIS	0.033	VV10220UREA_CYCLE_AND_METABOLISM_OF_AMINO_GROUPS	0.006
VV10051FRUCTOSE_AND_MANNOSE_METABOLISM	0.045	VV10330ARGININE_AND_PROLINE_METABOLISM	0.009
VV10480GLUTATHIONE_METABOLISM	0.007	VV23010RIBOSOME	0.000
VV10760NICOTINATE_AND_NICOTINAMIDE_METABOLISM	0.017	VV23050PROTEASOME	0.006
VV10900TERPENOID_BIOSYNTHESIS	0.036	VV30003AUXIN_SIGNALING	0.021
VV10910NITROGEN_METABOLISM	0.049	VV30005BRASSINOSTEROIDS_SIGNALING	0.038
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV40006CELL_WALL	0.000
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.016	VV44810REGULATION_OF_ACTIN_CYTOSKELETON	0.005
VV34626PLANT-PATHOGEN_INTERACTION	0.045	VV50004AUXIN_TRANSPORT	0.004
VV50109INCOMPLETELY_CHARACTERIZED_TRANSPORT_SYSTEMS	0.012	VV60007AS2	0.033
VV50135PRIMARY_ACTIVE_TRANSPORTER_CAT_D3_TO_E2	0.018	VV60011BHLH	0.000
VV52010ABC_TRANSPORTERS	0.044	NA	NA
VV60037HSF	0.029	NA	NA
Enriched pathways for 1400 h chilling	**NOM p-value**	**Enriched pathways for 1700 h chilling**	**NOM p-value**
VV10195PHOTOSYNTHESIS	0.045	VV10061FATTY_ACID_BIOSYNTHESIS	0.041
VV10480GLUTATHIONE_METABOLISM	0.000	VV10062FATTY_ACID_ELONGATION_IN_MITOCHONDRIA	0.030
VV10860PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	0.046	VV10230PURINE_METABOLISM	0.045
VV10940PHENYLPROPANOI	0.002	VV10240PYRIMIDINE_METAB	0.030

D_BIOSYNTHESIS		OLISM	
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.000	VV10300LYSINE_BIOSYNTHESIS	0.040
VV60003AP2_EREBP	0.023	VV10460CYANOAMINO_ACID_METABOLISM	0.008
VV60037HSF	0.034	VV10900TERPENOID_BIOSYNTHESIS	0.035
VV60073ORPHANS_ZF-B_BOX	0.043	VV11000SINGLE_REACTIONS	0.019
NA	NA	VV20970AMINOACYL-TRNA_BIOSYNTHESIS	0.004
NA	NA	VV23010RIBOSOME	0.000
NA	NA	VV40006CELL_WALL	0.004
NA	NA	VV50131PRIMARY_ACTIVE_TRANSPORTER_CAT_A2_TO_A4	0.016
NA	NA	VV60011BHLH	0.020
NA	NA	NA	NA

The number of enriched pathways increased with increased chilling hours in Marquette. Plant pathogen interaction and phenylpropanoid biosynthesis pathways were enriched in 1000 chilling hours. Cell wall and auxin signaling pathways were enriched in the 1400 chilling hours.

Table 4-3. Enriched pathways in Marquette during increased natural chilling hours. Pairwise comparisons of increased chilling (450 h/650 h, 650 h/750 h, 750 h/950h, 950 h/1000h).

Enriched pathways	NOM p-value	Enriched pathways	NOM p-value
Enriched pathways for 450 h chilling	**NOM p-value**	**Enriched pathways for 650 h chilling**	**NOM p-value**
VV10071FATTY_ACID_METABOLISM	0.031	VV10030PENTOSE_PHOSPHATE	0.011
VV10100BIOSYNTHESIS_OF_STEROIDS	0.045	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.026
VV10350TYROSINE_METABOLISM	0.018	VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0
VV10360PHENYLALANINE_METABOLISM	0.000	VV23010RIBOSOME	0.002
VV10400PHENYLALANINE_TYROSINE_AND_TRYPTOPHAN_BIOSYNTHESIS	0.049	VV23013RNA_TRANSPORT	0.005
VV10410BETA-ALANINE_METABOLISM	0.002	VV23040SPLICEOSOME	0.002
VV10902MONOTERPENOID_BIOSYNTHESIS	0.010	VV34070PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM	0.031
VV10910NITROGEN_METABOLISM	0.014	VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.011
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV50113THYLAKOID_TARGETING_PATHWAY	0.031
VV10941FLAVONOID_BIOSYNTHESIS	0.003	VV50132PRIMARY_ACTIVE_TRANSPORTER_CAT_A5_TO_A8	0.002
VV11000SINGLE_REACTIONS	0.015	VV60038JUMONJI	0.034
VV30008ETHYLENE_SIGNALING	0.033	VV60085MTERF	0.041
VV30010GIBBERELLIN_SIGNALING	0.000	NA	NA
VV50104GROUP_TRANSLOCATORS	0.046	NA	NA
VV60003AP2_EREBP	0.028	NA	NA
VV60037HSF	0.034	NA	NA

VV60044MYB	0.008	NA	NA
Enriched pathways for 650 h chilling	**NOM p-value**	**Enriched pathways for 750 h chilling**	**NOM p-value**
VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.046	VV10230PURINE_METABOLISM	0.027
VV10564GLYCEROPHOSPHOLIPID_METABOLISM	0.029	VV10910NITROGEN_METABOLISM	0.037
VV10565ETHER_LIPID_METABOLISM	0.016	VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000
VV10943ISOFLAVONOID_BIOSYNTHESIS	0.015	VV30005BRASSINOSTEROIDS_SIGNALING	0.027
VV10966GLUCOSINOLATE_BIOSYNTHESIS	0.004	VV30008ETHYLENE_SIGNALING	0.019
VV11000SINGLE_REACTIONS	0.029	VV50122PORTERS_CAT_7_TO_17	0.014
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.000	VV50123PORTERS_CAT_18_TO_29	0.009
VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.043	VV50125PORTERS_CAT_66_TO_94	0.032
VV60085MTERF	0.035	VV60003AP2_EREBP	0.046
Enriched pathways for 750 h chilling	**NOM p-value**	**Enriched pathways for 950 h chilling**	**NOM p-value**
VV10100BIOSYNTHESIS_OF_STEROIDS	0.028	VV10360PHENYLALANINE_METABOLISM	0
VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.042	VV10562INOSITOL_PHOSPHATE_METABOLISM	0.016
VV23010RIBOSOME	0.000	VV10592ALPHA-LINOLENIC_ACID_METABOLISM	0.000
VV23013RNA_TRANSPORT	0.002	VV10680METHANE_METABOLISM	0.009
VV23050PROTEASOME	0.006	VV10910NITROGEN_METABOLISM	0.013
VV23060PROTEIN_EXPORT	0.014	VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000
VV44145PHAGOSOME	0.011	VV10941FLAVONOID_BIOSYNTHESIS	0.005
VV44146PEROXISOME	0.028	VV10942ANTHOCYANIN_BIOSYNTHESIS	0.013

VV50112NUCLEAR_PORE_COMPLEX	0.009	VV10950ALKALOID_BIOSYNTHESIS_I	0.024
VV50132PRIMARY_ACTIVE_TRANSPORTER_CAT_A5_TO_A8	0.000	VV30008ETHYLENE_SIGNALING	0.000
VV60042MADS	0.043	VV30011JASMONATE_SIGNALING	0.000
VV60085MTERF	0.023	VV34020CALCIUM_SIGNALING_PATHWAY	0.000
NA	NA	VV34626PLANT-PATHOGEN_INTERACTION	0.000
NA	NA	VV60003AP2_EREBP	0.000
NA	NA	VV60011BHLH	0.043
NA	NA	VV60034HB	0.016
NA	NA	VV60044MYB	0.043
NA	NA	VV60046NAC	0.000
NA	NA	VV60058SNF2	0.007
NA	NA	VV60066WRKY	0.000
Enriched pathways for 950 h chilling	**NOM p-value**	**Enriched pathways for 1000 h chilling**	**NOM p-value**
VV10360PHENYLALANINE_METABOLISM	0.000	VV10051FRUCTOSE_AND_MANNOSE_METABOLISM	0.036
VV10592ALPHA-LINOLENIC_ACID_METABOLISM	0.000	VV10195PHOTOSYNTHESIS	0.035
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.007
VV10942ANTHOCYANIN_BIOSYNTHESIS	0.000	VV23050PROTEASOME	0.048
VV30008ETHYLENE_SIGNALING	0.000	VV24130SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	0.047
VV30011JASMONATE_SIGNALING	0.000	VV50135PRIMARY_ACTIVE_TRANSPORTER_CAT_D3_TO_E2	0.024
VV34020CALCIUM_SIGNALING_PATHWAY	0.006	NA	NA
VV34626PLANT-PATHOGEN_INTERACTION	0.008	NA	NA
VV60003AP2_EREBP	0.000	NA	NA
VV60066WRKY	0.000	NA	NA

A greater number of pathways were enriched in 750 and 950 chilling hours in natural conditions than higher chilling hours in Marquette. Phenylpropanoid biosynthesis and flavonoid biosynthesis pathways were enriched in all chilling hours. Transcription regulation pathways were enriched at 950 chilling hours.

Table 4-4. Enriched pathways in Brianna during increased controlled chilling hours. Pairwise comparisons of increased chilling (450 h/750 h, 750 h/1000 h, 1000 h/1400h, 1400 h/1700h).

Enriched pathways	**NOM p-value**	**Enriched pathways**	**NOM p-value**
Enriched pathways for 450 h chilling	**NOM p-value**	**Enriched pathways for 750 h chilling**	**NOM p-value**
VV10195PHOTOSYNTHESIS	0.000	VV10030PENTOSE_PHOSPHATE	0.029
VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.000	VV10195PHOTOSYNTHESIS	0.000
VV10511N-GLYCAN_DEGRADATION	0.000	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.000
VV10520NUCLEOTIDE_SUGARS_METABOLISM	0.037	VV10400PHENYLALANINE_TYROSINE_AND_TRYPTOPHAN_BIOSYNTHESIS	0.010
VV10600SPHINGOLIPID_METABOLISM	0.015	VV10511N-GLYCAN_DEGRADATION	0.000
NA	NA	VV10600SPHINGOLIPID_METABOLISM	0.015
NA	NA	VV10640PROPANOATE_METABOLISM	0.036
NA	NA	VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.014
NA	NA	VV23050PROTEASOME	0.004
NA	NA	VV40006CELL_WALL	0.000
NA	NA	VV50105TRANSPORT_ELECTRON_CARRIERS	0.000
Enriched pathways for 750 h chilling	**NOM p-value**	**Enriched pathways for 1000 h chilling**	**NOM p-value**
VV10010GLYCOLYSIS	0.004	VV10942ANTHOCYANIN_BIOSYNTHESIS	0.020
VV10360PHENYLALANINE_METABOLISM	0.026	VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.000
VV10511N-GLYCAN_DEGRADATION	0.020	VV23010RIBOSOME	0.007
VV10530AMINOSUGARS_METABOLISM	0.017	VV23030DNA_REPLICATION	0.041
VV10564GLYCEROPHOSPHOLIPID_METABOLISM	0.022	VV30003AUXIN_SIGNALING	0.011
VV10900TERPENOID_BIOSYNTHESIS	0.004	VV30005BRASSINOSTEROIDS_SIGNALING	0.048
VV10902MONOTERPENOID_BIOSYNTHESIS	0.002	VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.002
VV10940PHENYLPROPANOI	0.000	VV50125PORTERS_CAT_66_	0.039

D_BIOSYNTHESIS		TO_94	
VV10941FLAVONOID_BIOSYNTHESIS	0.009	VV60011BHLH	0.005
VV23050PROTEASOME	0.037	VV60015C2C2-DOF	0.047
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.000	VV60034HB	0.000
VV30011JASMONATE_SIGNALING	0.015	VV60058SNF2	0.007
VV50135PRIMARY_ACTIVE_TRANSPORTER_CAT_D3_TO_E2	0.027	NA	NA
Enriched pathways for 1000 h chilling	**NOM p-value**	**Enriched pathways for 1400 h chilling**	**NOM p-value**
VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.017	VV10300LYSINE_BIOSYNTHESIS	0.035
VV10530AMINOSUGARS_METABOLISM	0.000	VV10941FLAVONOID_BIOSYNTHESIS	0.037
VV10564GLYCEROPHOSPHOLIPID_METABOLISM	0.029	VV20970AMINOACYL-TRNA_BIOSYNTHESIS	0.008
VV10900TERPENOID_BIOSYNTHESIS	0.000	VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.000
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV23010RIBOSOME	0.000
VV10943ISOFLAVONOID_BIOSYNTHESIS	0.010	VV23013RNA_TRANSPORT	0.001
VV11000SINGLE_REACTIONS	0.006	VV23018RNA_DEGRADATION	0.017
VV34710CIRCADIAN_RHYTHM	0.047	VV23040SPLICEOSOME	0.006
VV44140REGULATION_OF_AUTOPHAGY	0.043	VV23050PROTEASOME	0.000
VV44146PEROXISOME	0.000	VV30003AUXIN_SIGNALING	0.014
VV60037HSF	0.007	VV40006CELL_WALL	0.017
VV60046NAC	0.049	VV44810REGULATION_OF_ACTIN_CYTOSKELETON	0.007
VV60066WRKY	0.008	VV60007AS2	0.000
VV60078OTHER_ZF-C3HC4	0.000	VV60011BHLH	0.002
NA	NA	VV60034HB	0.016
Enriched pathways for 1400 h chilling	**NOM p-value**	**Enriched pathways for 1700 h chilling**	**NOM p-value**
VV10271METHIONINE_METABOLISM	0.000	VV10052GALACTOSE_METABOLISM	0.019
VV10480GLUTATHIONE_METABOLISM	0.000	VV10195PHOTOSYNTHESIS	0.000
VV10750VITAMIN_B6_METABOLISM	0.037	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.000

VV10920SULFUR_METABOLISM	0.017	VV10460CYANOAMINO_ACID_METABOLISM	0.000
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV10511N-GLYCAN_DEGRADATION	0.006
VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.000	VV10900TERPENOID_BIOSYNTHESIS	0.004
VV23010RIBOSOME	0.002	VV10902MONOTERPENOID_BIOSYNTHESIS	0.025
VV23013RNA_TRANSPORT	0.033	VV23020RNA_POLYMERASE	0.027
VV23040SPLICEOSOME	0.027	VV40006CELL_WALL	0.006
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.006	VV44810REGULATION_OF_ACTIN_CYTOSKELETON	0.022
VV60042MADS	0.033	VV50105TRANSPORT_ELECTRON_CARRIERS	0
NA	NA	VV60001ABI3VP1	0.032
NA	NA	VV60058SNF2	0.025
NA	NA	VV60093TRAF	0.008
NA	NA	NA	NA

The number of enriched pathways increased with increased chilling hours in controlled conditions for Brianna. The greatest number of enriched pathways were found in the 1000/14000 comparison in controlled chilling for Brianna. Plant hormone signaling and transcription factor pathways were enriched at 1000 chilling hours. Like Marquette, phenylpropanoid biosynthesis and flavonoid biosynthesis pathways were enriched in 1000 and 1400 chilling hours condition in Brianna.

Table 4-5. Enriched pathways in Brianna during increased natural chilling hours. Pairwise comparisons of increased chilling (450 h/650 h, 650 h/750 h, 750 h/950h, 950 h/1000h.

Enriched pathways	**NOM p-value**	**Enriched pathways**	**NOM p-value**
Enriched pathways for 450 h chilling	**NOM p-value**	**Enriched pathways for 650 h chilling**	**NOM p-value**
VV10100BIOSYNTHESIS_OF_STEROIDS	0.000	VV10196PHOTOSYNTHESIS_ANTENNA_PROTEINS	0.000
VV10460CYANOAMINO_ACID_METABOLISM	0.036	VV10760NICOTINATE_AND_NICOTINAMIDE_METABOLISM	0.046
VV10511N-GLYCAN_DEGRADATION	0.002	VV23008RIBOSOME_BIOGENESIS_IN_EUKARYOTES	0.000
VV10600SPHINGOLIPID_METABOLISM	0.010	VV23013RNA_TRANSPORT	0.000
VV10640PROPANOATE_METABOLISM	0.042	VV23040SPLICEOSOME	0.002
VV10900TERPENOID_BIOSYNTHESIS	0.006	VV50123PORTERS_CAT_18_TO_29	0.042
VV10902MONOTERPENOID_BIOSYNTHESIS	0.006	VV50132PRIMARY_ACTIVE_TRANSPORTER_CAT_A5_TO_A8	0.046
VV10908ZEATIN_BIOSYNTHESIS	0.027	VV50133PRIMARY_ACTIVE_TRANSPORTER_CAT_A9_TO_A18	0.020
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV60007AS2	0.015
VV10941FLAVONOID_BIOSYNTHESIS	0.006	VV60032GRAS	0.012
VV10942ANTHOCYANIN_BIOSYNTHESIS	0.000	NA	NA
VV30005BRASSINOSTEROIDS_SIGNALING	0.002	NA	NA
VV34626PLANT-PATHOGEN_INTERACTION		NA	NA
VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.018	NA	NA
VV50121PORTERS_CAT_1_TO_6	0.025	NA	NA
VV50125PORTERS_CAT_66_TO_94	0.008	NA	NA

VV60011BHLH	0.013	NA	NA
VV60034HB	0.000	NA	NA
VV60044MYB	0.019	NA	NA
Enriched pathways for 650 h chilling	**NOM p-value**	**Enriched pathways for 750 h chilling**	**NOM p-value**
VV10564GLYCEROPHOSPHOLIPID_METABOLISM	0.023	VV10230PURINE_METABOLISM	0.032
VV10565ETHER_LIPID_METABOLISM	0.026	VV10940PHENYLPROPANOID_BIOSYNTHESIS	0
VV10943ISOFLAVONOID_BIOSYNTHESIS	0.010	VV30005BRASSINOSTEROIDS_SIGNALING	0.016
VV10966GLUCOSINOLATE_BIOSYNTHESIS	0.007	VV30008ETHYLENE_SIGNALING	0.022
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.000	VV50122PORTERS_CAT_7_TO_17	0.003
VV60085MTERF	0.033	VV50123PORTERS_CAT_18_TO_29	0.012
NA	NA	VV50125PORTERS_CAT_66_TO_94	0.032
NA	NA	VV10230PURINE_METABOLISM	0.032
Enriched pathways for 750 h chilling	**NOM p-value**	**Enriched pathways for 950 h chilling**	**NOM p-value**
VV23010RIBOSOME	0.000	VV10360PHENYLALANINE_METABOLISM	0.000
VV23013RNA_TRANSPORT	0.000	VV10562INOSITOL_PHOSPHATE_METABOLISM	0.011
VV23050PROTEASOME	0.002	VV10592ALPHA-LINOLENIC_ACID_METABOLISM	0.000
VV23060PROTEIN_EXPORT	0.021	VV10680METHANE_METABOLISM	0.009
VV24141PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM	0.018	VV10910NITROGEN_METABOLISM	0.020
VV44145PHAGOSOME	0.000	VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000
VV44146PEROXISOME	0.019	VV10941FLAVONOID_BIOSYNTHESIS	0.002
VV50112NUCLEAR_PORE_COMPLEX	0.009	VV10942ANTHOCYANIN_BIOSYNTHESIS	0.009
VV50132PRIMARY_ACTIVE_TRANSPORTER_CAT_A5_TO_A8	0.000	VV10950ALKALOID_BIOSYNTHESIS_I	0.018
VV60042MADS	0.022	VV30008ETHYLENE_SIGNALING	0.000

VV60085MTERF	0.029	VV30011JASMONATE_SIGNALING	0.000
NA	NA	VV34020CALCIUM_SIGNALING	0.000
NA	NA	VV34626PLANT-PATHOGEN_INTERACTION	0.003
NA	NA	VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.046
NA	NA	VV60003AP2_EREBP	0.000
NA	NA	VV60011BHLH	0.041
NA	NA	VV60034HB	0.019
NA	NA	VV60046NAC	0.000
NA	NA	VV60058SNF2	0.002
Enriched pathways for 950 h chilling	**NOM p-value**	**Enriched pathways for 1000 h chilling**	**NOM p-value**
VV10350TYROSINE_METABOLISM	0.014	VV10251GLUTAMATE_METABOLISM	0.021
VV10360PHENYLALANINE_METABOLISM	0.000	VV10562INOSITOL_PHOSPHATE_METABOLISM	0.010
VV10400PHENYLALANINE_TYROSINE_AND_TRYPTOPHAN_BIOSYNTHESIS	0.000	VV10906CAROTENOID_BIOSYNTHESIS	0.043
VV10530AMINOSUGARS_METABOLISM	0.002	VV11013ABA_BIOSYNTHESIS	0.036
VV10910NITROGEN_METABOLISM	0.042	VV23040SPLICEOSOME	0.004
VV10940PHENYLPROPANOID_BIOSYNTHESIS	0.000	VV34627R_PROTEINS_FROM_PLANT-PATHOGEN_INTERACTION	0.009
VV10941FLAVONOID_BIOSYNTHESIS	0.037	VV50133PRIMARY_ACTIVE_TRANSPORTER_CAT_A9_TO_A18	0.000
VV23010RIBOSOME	0.002	VV60017C2H2	0.002
VV23060PROTEIN_EXPORT	0.029	VV60032GRAS	0.038
NA	NA	VV60058SNF2	0.022

A greater number of enriched pathways were found in the comparison between 750 and 950 natural chilling condition for Brianna. Marquette transcription factor pathways were enriched in 950 and 1000 chilling hours. Plant pathogen interaction, jasmonate signaling, and flavonoid biosynthesis pathways were enriched in 950 chilling

hours. Like Marquette transcription factors were enriched in the 950 and 1000 chilling hours.

4.5 Discussion

Changes in expression levels were coordinated with the increasing accumulation of chilling hours. In a study of increased chilling in *V. riparia* many differentially expressed genes were involved in metabolism, cell defense/stress response, and genetic information processing (Mathiason et al., 2008). Increased chilling showed bud break for Brianna was at 701 – 901 chilling hours while Marquette required more than 901 chilling hours. Chilling fulfillment measured across all cultivars showed that there was no difference in response to chilling in natural and controlled conditions; however, there was cultivar by condition interactions. Here were gene expression differences between controlled and natural conditions in Marquette and Brianna. Increased chilling resulted in an increased rate of bud break in both chilling conditions (Yilmaz et al., 2021). In this study, we found that differential gene expression was greater with increased chilling hours in either chilling condition. The greatest number of DEG in natural conditions occurred at lower chilling hours than in controlled chilling conditions. In a related study with kiwifruit, free radical scavenging activity was increased from early chilling to end in both controlled and field conditions (Gheshlaghi et al., 2018). In black currant, fewer DEG were found in early dormancy stages and maximum DEGs were found at bud break (Hedley et al., 2010; Shangguan et al., 2020). Differential gene expression increased from 450 to 1000 chilling hours in controlled and field conditions. Secondary metabolism, cellular metabolism (cell wall metabolism, cell cycle, and cell division), and starch-sucrose metabolism pathways were enriched at 1000 chilling hours. Proteomic analysis indicates that cell wall and secondary metabolism have significant roles in grape bud dormancy (George et al., 2018). Cell wall metabolism and phenylpropanoid-related gene increased

in abundance when grapevine buds became dormant (George et al., 2018; Victor et al., 2010). In this study, the cell cycle pathway was enriched in field conditions while the cell wall metabolism pathway was enriched in both controlled and field chilling. Carbohydrate pathways are shown to have a role in bud dormancy in grapes (George et al., 2018). Cold stress in grapevine caused starch reserves to be hydrolyzed to soluble sugars via starch degrading enzymes (Mohamed et al., 2010) and up-regulation of α-amylases (Xin et al., 2013). Starch catalysis was up-regulated in January relative to November, and β- amylase coding genes were highly expressed during December and March (Shangguan et al., 2020). In our study, starch and sucrose metabolism, and sugar metabolism was enriched in field conditions in contrast to controlled chilling which may be partly attributable to the freezing temperatures in the field. Kiwi vines had a similar pattern of DEG in controlled and natural chilling with the controlled chilling having fewer DEG than found in natural chilling. A greater number of enriched pathways were found in the field than in controlled chilling conditions in Marquette and Brianna.

4.6 Conclusion

In this study, we reported that the number of DEG increased from 450 to 1000 chilling hours in controlled and field conditions across cultivars. There were more DEG genes up and downregulated at 1000 chilling hours and as bud transitioned to ecodormancy (1400 and 1700 chilling hours) when rapid bud break can occur with favorable temperature conditions. A greater number of enriched gene pathways were found in the field than in controlled chilling conditions. There was an increasing number of DEG with increased chilling in the controlled and field chilling from 450 to 1000. We found from gene set enrichment analyses, there are enrichment pathways in controlled and fields such as phenylpropanoid biosynthesis, photosynthesis antenna proteins, and plant-pathogen interaction, in addition, although cell wall and auxin signaling were significantly enriched in controlled, jasmonate and ethylene signaling were significant in the field.

4.7 References

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