

Cold Hardiness of *Vitis vinifera* Roots

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Abstract: Grapevine buds have identifiable patterns of cold acclimation and deacclimation, but it is not known whether roots also follow these patterns. A better understanding of root cold hardiness thresholds and/or acclimation patterns would enable mitigation strategies to be developed to reduce the likelihood of root damage. This study had two major objectives: 1) to improve protocols for electrolyte leakage and then quantitatively compare the results to differential thermal analysis; and 2) to quantify whether *Vitis vinifera* roots acclimate to preconditioning temperatures. Existing protocols were optimized and root cold hardiness was evaluated on own-rooted *Vitis vinifera* Merlot and Chardonnay. To determine whether grapevine roots acclimate to their environment, three preconditioning regimes were applied: ambient air temperature during active vine growth; 12°C for one week during dormancy; and 0°C for one week during dormancy. Following preconditioning, root samples were collected and exposed to fixed temperatures of -2.0, -4.0, -6.0, or -8.0°C, and electrolyte leakage, the standard indicator of root cold damage, was measured. The incidence of low temperature exotherms from differential thermal analysis was used as a potential alternative to identify temperatures that cause tissue damage. Overall, Chardonnay and Merlot roots did not have dynamic cold acclimation patterns like grapevine buds. There was little variation in maximum cold hardiness (<1.2°C) regardless of preconditioning. Max root cold hardiness was experimentally derived for both varieties (median = -5.9°C for Chardonnay and -5.7°C for Merlot). Differential thermal analysis was an effective alternative method to electrolyte leakage for estimating damage thresholds for these varieties. The lethal temperature at which 10, 50, and 90% of roots were killed was calculated for grapevine roots, with values of -4.0, -5.8, and -7.0°C, respectively.

Key words: cold hardiness, differential thermal analysis, electrolyte leakage, root

Chilling and freezing injury to plants causes millions of dollars of annual crop loss worldwide (Snyder and De Melo-Abreu 2005). Because of this climatic impact on sustainability and profitability, there has been much research on cold hardiness and cold acclimation/deacclimation in aerial portions of plants, including grapevine (Richards 1983, Mills et al. 2006, Keller and Mills 2007, Ferguson et al. 2011, 2014). In contrast, studies on root hardiness of perennial plants are typically limited to forest species of economic importance such as Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) (Smit-Spinkins et al. 1985, Lindstrom 1986). This

work indicated differences between genera in absolute cold hardiness: -16°C (*P. sylvestris*) and -20°C (*Pc. abies*). Further exploration of seasonal patterns of root cold hardiness in Scots pine, Norway spruce, lodgepole pine (*P. contorta*), and other conifers suggests some form of cold acclimation in roots (Timmis and Worrall 1975, Lindstrom and Nystrom 1987).

Currently, there are few techniques by which vineyard managers can determine if winter soil temperatures have fallen to damaging levels prior to subsequent mid-season vine collapse. At that point, little can be done to salvage the vines, and production during that and the next several growing seasons may be reduced or lost. If information on cold damage were available earlier in the season, cultural practices could be adopted to improve the possibility of vine survival from this damage. If damage requires replant, that decision can be made earlier, and in the case of Washington, limit production losses to one year.

Literature on cold hardiness of grapevine roots is sparse, as frequently noted in studies of the grape root system (Richards 1983, Comas et al. 2005). To our knowledge, no research has examined the ability of grapevine roots to acclimate to seasonal variations in soil temperature. Following a severely cold winter in Washington, Ahmedullah and Kawakami (1986) suggested an absolute lethal temperature for Concord (*Vitis labruscana*) roots at ~ -5°C. A similar study in Japan demonstrated a cold-hardiness threshold of -4.0°C for *V. vinifera* Kyoho roots (Okamoto et al. 2000). Guo et al. (1987) demonstrated maximum root cold hardiness for 19 varieties and species including *V. vinifera* Muscat Hamburg (-5.2°C), *V. riparia* (-11.4°C), and *V. amurensis* (-15.5°C). These studies indicate that max cold hardiness differs among *Vitis* species,

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but there is no literature on within-season variation in acclimation. These three studies, the most recent of which was performed 17 years ago, make up the entire body of literature on grapevine root hardiness. Current technological advances in assessing root cold hardiness merit a reexamination of these results, with additional exploration into cold hardiness acclimation.

Electrolyte leakage (EL) analysis is the gold standard for root cold hardiness estimation. This analysis, first described by Dexter et al. (1930, 1932), involves measuring the conductivity of a bathing solution in which the tissues in question are soaked. In the event of damage, more electrolytes are leaked from tissue, leading to measurable increases in bathing solution conductivity. The general protocol framework described herein was developed from techniques described by Wilner (1955, 1959), Wilner et al. (1960), and McKay (1992), with consideration of technical concerns presented by Whitlow et al. (1992) and Deans et al. (1995). These protocols have been used in all known published work on grapevine root hardiness (Ahmedullah and Kawakami 1986, Guo et al. 1987, Okamoto et al. 2000). Past studies of EL were criticized for using short tissue soak times, which do not allow enough time for completion of EL from damaged tissue, nor does it allow sufficient time for electrolyte reuptake (Deans et al. 1995). Whitlow et al. (1992) also expressed concern about the use of EL, and in particular, relative conductivity, as a means to quantify damage, suggesting that membrane permeability would not be captured. These authors suggested the addition of a tissue ionic conductance value (g_{Ti}) to account for the chemical driving force and tissue surface area; this is understandable when using tissues that vary in size and structure (e.g., leaves, shoots), which may be difficult to normalize prior to evaluation. While we recognize the possibility of increased accuracy by using the (g_{Ti}) in calculations, the protocols of Whitlow et al. (1992) were optimized for leaf tissue and also had the limitation of a short soak duration.

Differential thermal analysis (DTA) is a rapid technique (requiring <12 hr to complete) that is commonly used for determining grapevine bud and cane cold hardiness (Pierquet and Stushnoff 1980, Wample et al. 1990, Mills et al. 2006, Ferguson et al. 2014). Unfortunately, in some past studies (e.g., Burr et al. 1986), a lack of distinct high- and low-temperature exotherms for tissue (*Pinus spp.* buds) prevented researchers from quantifying absolute cold-hardiness temperature thresholds. No published work indicates whether root tissues have the ability to supercool or acclimate to sub-freezing temperatures like grapevine buds; such a scenario seems unlikely given the different tissue organization and connectivity of these two organs (Ashworth 1990). Because of the lack of published information, DTA was included in this study to determine whether root exotherm patterns and EL were correlated, as the adoption of a more rapid evaluation method is more amenable for the development of grower-related advisory systems.

The objectives of this study were twofold: 1) to improve EL protocols and quantitatively compare EL to DTA to determine a more rapid method for root cold hardiness assess-

ments; and 2) to quantify whether *V. vinifera* roots acclimate to preconditioning temperatures.

Materials and Methods

Data were generated simultaneously to evaluate and optimize EL protocols against DTA, and to determine root cold acclimation and max hardiness. A diagram of the process is presented in Supplemental Figure 1.

Plant materials. For these studies, *V. vinifera* Chardonnay and Merlot were selected based on differences seen in their acclimation and deacclimation patterns as measured in dormant buds (Ferguson et al. 2011). Dormant cuttings of both varieties were rooted in March 2014. Source material was obtained from the grapevine foundation block of the Clean Plant Center-Northwest (Prosser, WA), a repository of plant material that has undergone virus elimination. Cuttings were callused and rooted in a plywood callusing box filled with perlite (Therm-o-Rock West, Inc.) and covered with a heating mat (Hydrofarm) set to 25°C and damp burlap bags to retain moisture. Rooted plants were potted on 17 May 2014 in 13-L blow-molded plastic nursery pots (#3, McConkey Grower Supplies). Potting media consisted of 1:1:1 potting mix (Sunshine Mix #3 Professional Growing Mix, Sun-Gro Horticulture); peat moss (Nature's, Sun-Gro Horticulture); and perlite (Therm-o-Rock West, Inc.). Plants were moved to the greenhouse, grown for five weeks, then relocated to outdoor aboveground plant beds. Irrigation was delivered via drip two times daily for 10-min intervals at a rate of 2 L/hr (~0.66 L/plant/day). During times of extreme heat, the duration of drip delivery was increased to 1 L/plant/day. Plants were staked and tied to 1.5-m bamboo stakes and “hedged” if growth exceeded 0.9 m past the top of the stake; two shoots per plant were retained in year 1. In fall, plants were allowed to go dormant naturally (indicated by the formation of periderm), were pruned to two 2-bud spurs, and were placed into climate-controlled storage (7 to 15°C, avg. = 12°C) on 10 Nov 2014. Storage conditions were under continuous darkness, with the exception of light used when plant materials were removed. In 2015, the same vines were moved out of cold storage on 5 June and given 20 g of nitrogen urea (46-0-0). Irrigation was delivered as in 2014, and four shoots developed. Vines were moved back into cold storage after periderm formation in the fall, pruned to two 2-bud spurs, and placed into climate-controlled storage on 15 Oct 2015. Dormant vines were used for various root cold hardiness assays in both study years (2014 to 2015 and 2015 to 2016).

EL protocol optimization. Recent literature has debated whether the duration of the soak time needed to capture complete EL and potential partial electrolyte reuptake should be improved (Deans et al. 1995). Healthy tissue, which releases small amounts of electrolytes when soaked, needs enough time to complete leakage to subsequently be used to “zero” the conductivity of the bathing solution. To determine the duration of an adequate soak time for healthy grapevine roots, 20 replications of Chardonnay root tissue (1.0 to 2.0 mm diam, 1 cm long) were collected and placed individually in 15-mL conical tubes with 8 mL of deionized water at 4.0°C and allowed to soak for seven days. Solution conductivity

was measured daily for each replication (S230 SevenCompact Conductivity meter, Mettler-Toldeo). The minimum number of days required for max EL was determined and used in all subsequent assays.

To determine the duration of an adequate posttreatment (post-freeze) soak time for grapevine roots, 20 replications of Chardonnay root tissue (1.0 to 2.0 mm diam, 1 cm long) were collected and placed individually in 15-mL conical tubes with 8 mL of deionized water, held at -80°C for 12 hr (to ensure complete kill of the tissue), thawed at room temperature, and allowed to soak in the deionized water for 10 days at 4°C . Conductivity was measured daily for each replication. The min number of days required for max EL was determined and that post-damage soak duration was used in all future assays.

To begin optimizing protocols for grapevine roots, it was necessary to determine the temperature range needed in order to evaluate potential cold damage to root tissue. The original freezing program for grapevine buds (Mills et al. 2006) was applied to 20 dormant Chardonnay root replications during this preliminary testing phase. The program slowly reduces temperature from 4°C to -40°C by $4^{\circ}\text{C}/\text{hr}$, and was chosen as the test program during protocol development because of its general accepted use in grape cold-hardiness experiments. The programmable freezer setup was as described by Wample et al. (1990). Following the initial tests, a freeze protocol was designed specifically for roots, which attempted to mimic the slower temperature variations of the soil compared to those of the air. Slower chilling also minimizes inaccurate exotherm interpretation resulting from the development of unrealistic ice formation sites in the tissues (Burke et al. 1976). The programmed freeze temperature protocols are described in Table 1; the final selected temperature minimums were -2 , -4 , -6 , and -8°C .

Determining cold hardiness following preconditioning.

Vines were subjected to three preconditioning temperature regimes prior to assessing root cold hardiness in 2014 to 2015 and 2015 to 2016 (experiment conducted twice). Twenty vines per variety were used for each preconditioning regime in each year. The first preconditioning regime occurred during active vine growth: vines were subjected to ambient temperature.

Table 1 Programmed freezing protocols used to determine the temperature at which damage occurs in grapevine root tissue. Total duration for Steps 4 and 6 depended on the minimum temperature selected.

Step	Duration (min)	Action
1	15	Decrease sample temperature to 4°C from ambient temperature
2	60	Hold at 4°C
3	180	Decrease sample temperature by $2^{\circ}\text{C}/\text{hr}$ to -2°C
4	0-360	Decrease temperature by $1^{\circ}\text{C}/\text{hr}$ to desired freeze temperature
5	30	Hold at minimum freeze temperature (-2 , -4 , -6 , or -8°C)
6	90-180	Increase sample temperature by $4^{\circ}\text{C}/\text{hr}$ to 4°C
7	Variable	Hold at 4°C until samples are removed from freezer

Typical daytime high temperatures during sampling were between 28°C and 32°C , and nighttime low temperatures ranged from 14°C to 21°C (AgWeatherNet 2016). The second and third preconditioning regimes were imposed when vines were fully dormant after three weeks in cold storage. In the second, vines were brought out of cold storage and held at a consistent temperature of 12°C in a dedicated cold storage unit for a min of one week; in the third regime, vines were brought out of cold storage and held at a consistent temperature of 0°C in a dedicated cold storage unit for a min of one week. To ensure that root temperature equilibrated with air temperature, temperature sensors (HOBO, U-Series, Onset Computer Corporation) were placed in the pots and temperature was monitored over the first two days of placement into cold storage. Root temperature stabilized to air temperature within 12 hr (data not shown). At the end of preconditioning, root tissue was sampled and subjected to programmed freezing as described below.

Two root/soil samples per plant were collected using a 2.54-cm diam soil corer inserted to the floor of the pot equidistant between the vine main trunk and the pot boundary (~ 17 cm in length). Soil cores were rinsed through a 2.0-mm pore mesh sieve (U. S. Standard Sieve Series, The W. S. Tyler Company) to separate root debris from the soil matrix. Roots were then washed with tap water to remove excess soil and rinsed with deionized water to remove any surface electrolytes. Following this rinsing procedure, roots were sorted, and roots between 1.0 and 2.0 mm diam were retained. Frayed root ends were cut with a razor blade and discarded. Ten within-treatment replications weighing 0.040 to 0.055 g (Model XS64, Mettler-Toledo) were retained. Samples were soaked for 24 hr at 4°C in 15 mL conical tubes containing 8 mL deionized water (Falcon Conical Centrifuge tubes, Sigma-Aldrich Co. LLC) to collect baseline EL prior to treatment.

Following the collection and initial processing of root materials subjected to each of the preconditioning treatments (described above under “Plant Materials”), conductivity measurements of the bathing solution were recorded following the 24-hr initial soak. As previously mentioned, this value was used to zero the conductivity of the bathing solution. Roots were then removed from tubes (with care to remove as little bathing solution as possible), dried with a task wipe (Kimberly-Clark Professional Kimtech Science Kimwipes), and individually wrapped in a 6-cm \times 6-cm piece of aluminum foil to create a small packet.

These 10 packets per variety and preconditioning regime were then placed into individual thermoelectric modules in the programmable freezer as described by Mills et al. (2006). After the programmed freezing protocol, root samples were removed from the foil packets and returned to their original 15-mL tubes containing the original bathing solution. Samples were allowed to soak for seven days, and solution conductivity was measured on day 7. The sample tubes were then held at -80°C for a min of 10 hr to kill the tissue, after which the samples were thawed and held at 4°C for seven days. On day 7, a final conductivity measurement was recorded. Relative conductivity was calculated to estimate tissue damage

($R = L_t / L_k$), where L_t = conductance of bathing solution from a sample frozen at temperature (t) in the programmable freezer, and L_k = conductance of bathing solution from sample frozen at temperature (t), soaked for seven days, and then killed following -80°C freeze (Green and Warrington 1978). Relative conductivity values >0.5 are associated with dead tissue,

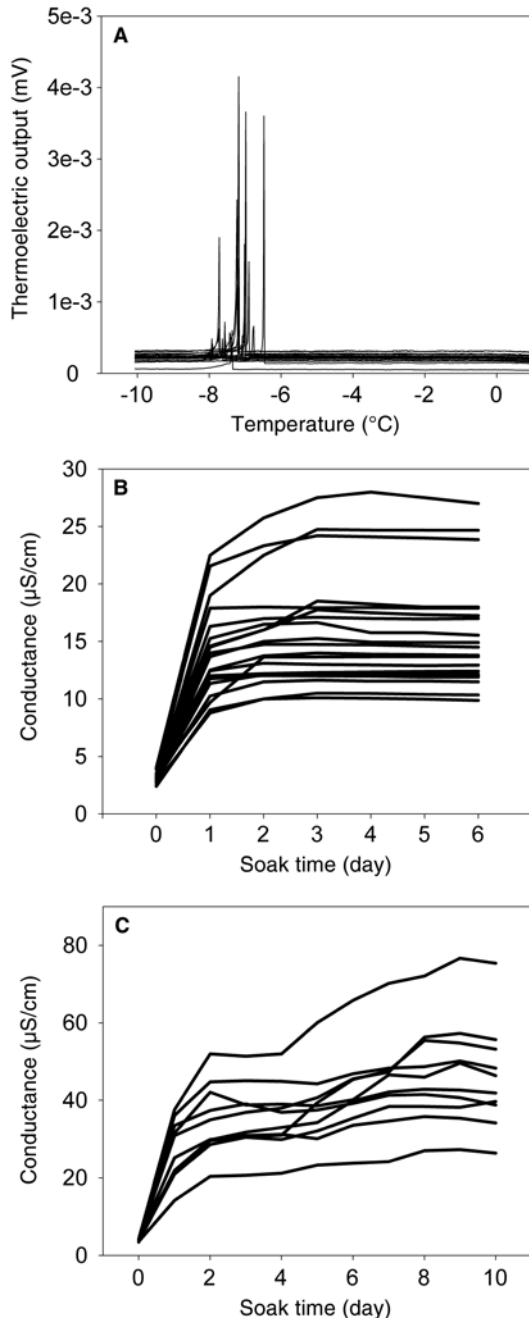


Figure 1 Optimization of cold-hardiness evaluation protocols. **(A)** Differential thermal analysis trace after subjecting root samples to freeze protocols for grapevine buds (Mills et al. 2006). Graph was truncated at -10°C due to lack of peaks below -8°C . **(B)** Conductivity of bathing solution of uninjured grapevine roots. Samples were collected by length rather than mass, explaining the variance in absolute conductivity values for the 20 replicates. **(C)** Conductivity of bathing solution containing damaged grapevine roots after soaking for 10 days. Samples were collected by length rather than mass, explaining the variance in absolute conductivity values for the 10 replicates.

and values <0.5 indicate live tissue or tissue that can fully recover (Green and Warrington 1978). Relative conductivity is an alternative means for interpreting electrical conductivity data; historically, electrical conductivity was compared to visual ratings of damage to quantify treatment effects. Relative conductivity is faster and yields similar results (Deans et al. 1995). The lethal temperatures at which 10 (LT_{10}), 50 (LT_{50}), and 90% (LT_{90}) of tissues were killed were also calculated.

The programmable freezer used to reproduce controlled freezing events was also equipped to capture low- and high-temperature exotherms (LTE and HTE) of freezing tissue. The incidence of exotherm occurrence (indicating tissue damage; HTE = apoplastic freezing; LTE = symplastic freezing) was compared to relative conductivity values for each sample to determine whether DTA could be used as a rapid substitute for the EL assay.

Statistical analysis was performed using Minitab 17 (Minitab 17 Statistical Software). Analysis of variance (ANOVA) and Tukey's HSD were used to determine differences among treatments for all analyses except EL year-to-year varietal comparisons, which were carried out using two-sample t -tests. These results were confirmed using an ANOVA mixed-model with year, variety, and preconditioning as fixed effects, and the relative conductivity values following each programmed freeze (-2 , -4 , -6 , -8°C) as the response variable. Departure from normality was not great enough to warrant transformation of the data (McDonald 2014). Statistical differences were assigned at $p < 0.05$. The efficacy of DTA for predicting tissue death was calculated by comparing outcomes to EL evaluations and calculating true positives (where positives were determined by the presence of an exotherm peak, i.e., tissue death), true negatives, false positives, and false negatives. The sensitivity (true-positive rate), specificity (true-negative rate), positive predictive value (probability of predicting a positive event and it actually occurring), and negative predictive value (probability of predicting a negative event and it actually occurring) (Ott and Longnecker 2001) of DTA as an assay was then calculated.

Results

To narrow the range of temperatures evaluated for cold-damage thresholds, a preliminary experiment using the grape bud freeze program (Mills et al. 2006) on roots was conducted. In that experiment, 100% of exotherm peaks from root tissue occurred between -4.0°C and -7.9°C , with a mean of -5.9°C and a standard deviation of 1.1°C (Figure 1A). The median temperature at which exotherms were observed was -6.0°C . No exotherms occurred below 7.9°C . Only one exotherm was apparent for each root tissue sample (rather than one HTE and one LTE). There did not appear to be LTEs and HTEs that could be differentiated. From this, temperatures between -2°C and -4°C were selected to represent limited tissue damage, -6°C represented variable tissue damage, and -8°C represented complete tissue damage for subsequent experiments. Given the small differences between these temperatures, an alternative freezing program was selected for the actual experiments, as presented in Table 1 and described

in the Materials and Methods. Freeze data from test runs with Merlot root tissue were found to be encompassed by the parameters set by Chardonnay, so a full evaluation and separate protocol development for Merlot was deemed unnecessary.

EL protocol optimization. Of the total potential electrolytes that could be leaked from healthy (undamaged) excised root tissue, an average of $84 \pm 3.8\%$ of the total electrolytes that were going to be released were released into the bathing solution within 24 hrs of the pretreatment soak (Figure 1B). Because leakage from healthy tissue is of much smaller magnitude (3 to 5 times less) than that from damaged tissue, and because of the relative shape of the leakage curve, the 24-hr presoak was deemed sufficient to calibrate the bathing solution for potential pretreatment differences, and was incorporated into all EL protocols.

After complete tissue death from exposure to -80°C , 10% of samples showed complete leakage by day 6 postthaw (Figure 1C). By day 7, 90% of samples had reached max EL, and at day 8, 100% of samples had reached max EL. Seven days

Table 2 Mixed-model analysis of variance results with year, variety, and preconditioning as fixed effects, and the relative conductivity values after each programmed freeze as the response variable. Asterisks indicate significant differences ($p < 0.05$).

	Programmed freeze temperature			
	-2.0°C	-4.0°C	-6.0°C	-8.0°C
Year	0.125	0.490	0.038*	0.025*
Variety	0.053	0.995	0.827	0.843
Preconditioning	0.000*	0.000*	0.283	0.013*
Year*Variety	0.485	0.038*	0.637	0.195
Year*Preconditioning	0.000*	0.786	0.000*	0.012*
Variety*Preconditioning	0.000*	0.000*	0.000*	0.000*

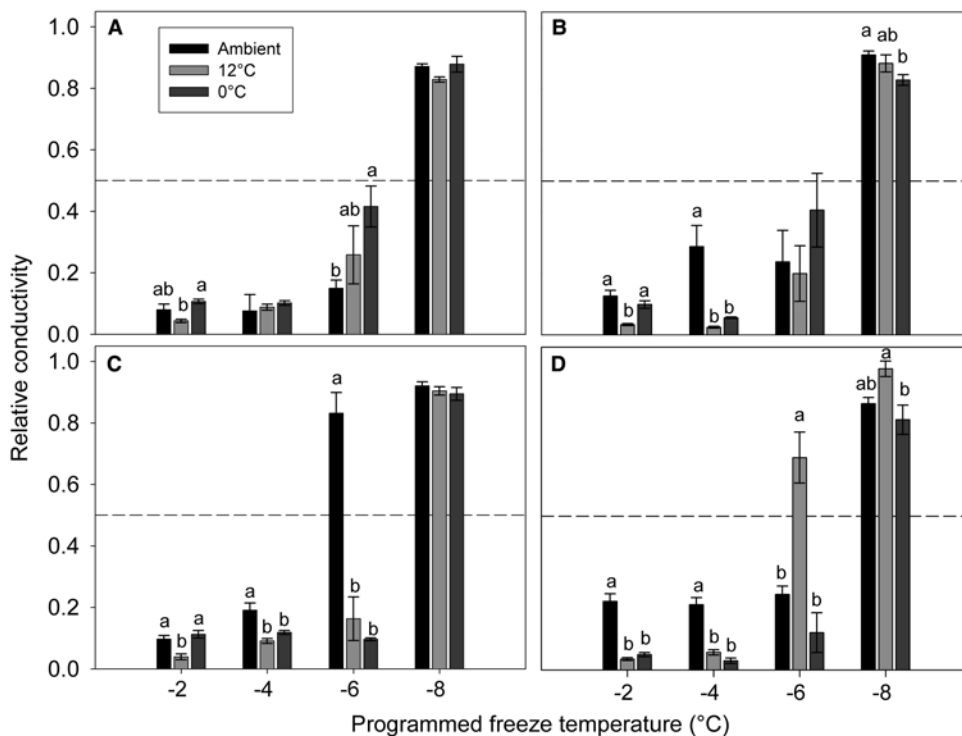


Figure 2 Mean relative conductivity of bathing solution for *Vitis vinifera* Chardonnay and Merlot roots after preconditioning, followed by controlled decreases in temperature below freezing. (A) Chardonnay, 2014; (B) Merlot, 2014; (C) Chardonnay, 2015; and (D) Merlot, 2015. Relative conductivity values above 0.5 indicate dead tissue (dashed line). Error bars represent $\pm\text{SE}$. Different letters indicate significant differences among treatments within a low temperature exposure using Tukey's HSD, $\alpha = 0.05$. Ambient = preconditioned to ambient temperature during active growth; 12°C = preconditioned during dormancy at 12°C for a min of one week; 0°C = preconditioned during dormancy at 0°C for a min of one week.

of leakage time was deemed adequate to elicit leakage and minimize protocol time, and this soak time was incorporated into all subsequent EL protocols.

Determining cold hardiness following preconditioning.

Regardless of year or variety, there was no evidence that temperature preconditioning influenced max root cold hardiness. Results from mixed model ANOVA are shown in Table 2. The variety-preconditioning interaction was significant for all programmed freeze temperatures. However, interpretation of statistical significance should not be confused here with practical significance, especially when data has a threshold response, such as with the interpretation of relative conductivity values. A graphical representation of these values and differences are also presented to highlight where these differences occur, and if they are practically important. In both 2014 (Figure 2A and 2B, Figure 3A and 3B) and 2015 (Figure 2C and 2D, Figure 3C and 3D), there was no consistent pattern in preconditioning that was associated with significantly higher or lower relative conductivity or incidence of exotherms to -2 , -4 , -6 , or -8°C temperature exposure. The only consistent pattern in the data was between the relative conductivity values and exotherm incidence within the cold temperature exposures, regardless of variety, year, or preconditioning. Exposure to -8.0°C was associated with leakage values that surpassed the relative conductivity threshold (0.5) 100% of the time in all cases, and had 100% incidence of exotherms. Conversely, exposure to -2.0°C and -4.0°C never surpassed the threshold and only exhibited exotherms 1.7% of the time. Exposure to -6.0°C was variable; 31.7% of the time, relative conductivity values exceeded 0.5, and in 30.8% of samples, the exposure resulted in an exotherm (Figures 2 and 3). Relative conductivity values are shown in Table 3.

While individual preconditioning regimes often resulted in a relative conductivity that was different from others at a specific temperature exposure, in almost all cases, the average relative conductivity for all preconditioning regimes at a specific temperature exposure was either well below, or well above, the 0.5 threshold. For example, in Chardonnay in 2014, exposure to -2.0°C resulted in higher relative conductivity values in the dormant preconditioning at 0°C than in the dormant 12°C preconditioning (Figure 2A), yet all preconditioning values at this low temperature exposure remained <0.5. Similarly, in 2015 (Figure 2C), exposure to -2.0°C resulted in lower relative conductivity values in dormant 12°C preconditioning than the other two regimes, but relative conductivity for all pretreatments at this low temperature exposure remained below 0.5. In Merlot in 2014 (Figure 2B), exposure to -2.0°C resulted in lower relative conductivity (less damage) for the dormant 12°C preconditioning than the other preconditioning regimes, but all values were <0.5. Following the -8.0°C exposure, ambient outdoor preconditioning showed significantly higher relative conductivity than the 0°C preconditioning, but all preconditioning relative conductivity values remained above 0.5. In 2015 (Figure 2D), following a -8.0°C low temperature exposure, higher relative conductivity occurred in the 12°C preconditioning than in the 0°C preconditioning, but all relative conductivity values among preconditioning regimes were >0.5. Preconditioning was less a factor in tissue damage than was degree of low temperature exposure.

Similar results, and a lack of consistent patterns between root preconditioning and cold hardiness, were also seen across years and varieties in DTA (Figure 3). In 2014 in Chardonnay (Figure 3A), the 6.0°C low temperature exposure resulted in higher exotherm incidence in the dormant 0°C precondition-

ing than in the actively growing, ambient outdoor temperature preconditioning. At the same exposure temperature in 2015 (Figure 3C), the actively growing preconditioning had a higher exotherm incidence than the other preconditioning regimes. In 2014 in Merlot (Figure 3B), no significant differences in exotherm incidence were found following any of the exposures, and in 2015 (Figure 3D), following a 6.0°C exposure, there was a higher exotherm incidence in the 12°C preconditioning than in the other preconditioning regimes. As seen in EL, preconditioning was not a good predictor of max cold hardiness.

Few differences were observed between varieties in response to preconditioning and freeze protocols (Figure 4). In most cases, Merlot and Chardonnay responses mirrored each other. There were a few instances where significant differences were biologically meaningful; for example, in 2015 ambient outdoor preconditioning (Figure 4B) and the 12°C preconditioning (Figure 4D) following the -6°C freeze protocol. Other significant differences were found between

Table 3 Mean values of relative conductivity of bathing solution for all preconditioning regimes combined across temperature exposure. Standard deviation presented in parentheses.

Variety/ vintage	Temperature exposure			
	-2.0°C	-4.0°C	-6.0°C	-8.0°C
Chardonnay				
2014	0.077 (0.045)	0.089 (0.096)	0.278 (0.229)	0.866 (0.054)
2015	0.083 (0.046)	0.133 (0.062)	0.364 (0.373)	0.906 (0.050)
Merlot				
2014	0.086 (0.055)	0.122 (0.168)	0.281 (0.327)	0.874 (0.070)
2015	0.103 (0.097)	0.100 (0.093)	0.353 (0.308)	0.887 (0.121)

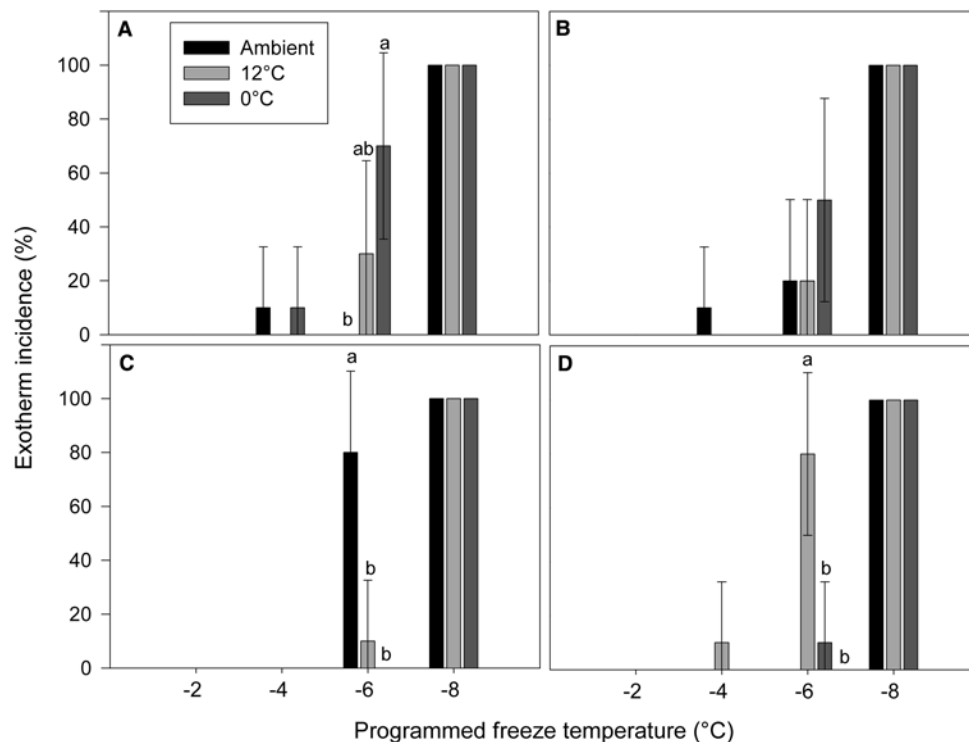


Figure 3 Mean incidence of low-temperature exotherms for *Vitis vinifera* Chardonnay and Merlot roots after preconditioning, followed by controlled decreases in temperature below freezing. (A) Chardonnay, 2014; (B) Merlot, 2014; (C) Chardonnay, 2015; and (D) Merlot, 2015. Error bars represent standard error. Different letters indicate significant differences between treatment means within a temperature exposure using Tukey's HSD, $\alpha = 0.05$. Ambient = preconditioned to ambient temperature during active growth; 12°C = preconditioned during dormancy at 12°C for a min of one week; 0°C = preconditioned during dormancy at 0°C for a min of one week.

varieties, but as was observed in relative conductivity, all of these differences occurred between tissues that both fell well below or above the 0.5 damage threshold.

Comparing assays. When comparing samples that were first evaluated with DTA (observed for exotherms) and followed by EL (comparing their relative conductivity after a subzero temperature exposure; Figure 5), samples producing an exotherm also had a relative conductivity rating above the 0.5 threshold 97.9% of the time. There was a high level of similarity between the incidence of DTA HTE exotherm events and the EL relative conductivity ratings that indicate damage; DTA was a relatively robust assay with high sensitivity (98.1%), specificity (96.2%), positive predictive value (98.4%), and negative predictive value (95.6%).

Discussion

Grapevine roots did not acclimate to preconditioning temperatures, and max cold hardiness showed little variation regardless of preconditioning. We expected that more damage

would occur after preconditioning at the actively growing, ambient outdoor temperature than after dormant preconditioning at 0°C, but this was not the case. There were no consistent differences in tissue damage among preconditioning regimes at any of the low-temperature exposures (-2.0, -4.0, -6.0, or -8.0°C). Regardless of preconditioning, the median freezing temperatures for Chardonnay and Merlot root tissues were -5.9°C and -5.7°C, respectively. The LT₁₀, LT₅₀, and LT₉₀ were calculated, with values of -4.0, -5.8, and -7.0°C, respectively. EL accuracy was found to be improved when adequate tissue soak times were used. Differential thermal analysis was found to have high levels of agreement with EL when the presence of an exotherm was used to indicate tissue vitality. Further examination into the possibility of root order affecting hardiness, and further evaluation of the technique when comparing between different species of *Vitis* is necessary prior to using DTA as a replacement for EL.

EL was chosen as the standard to which DTA was compared. The EL protocols presented here used a calculated

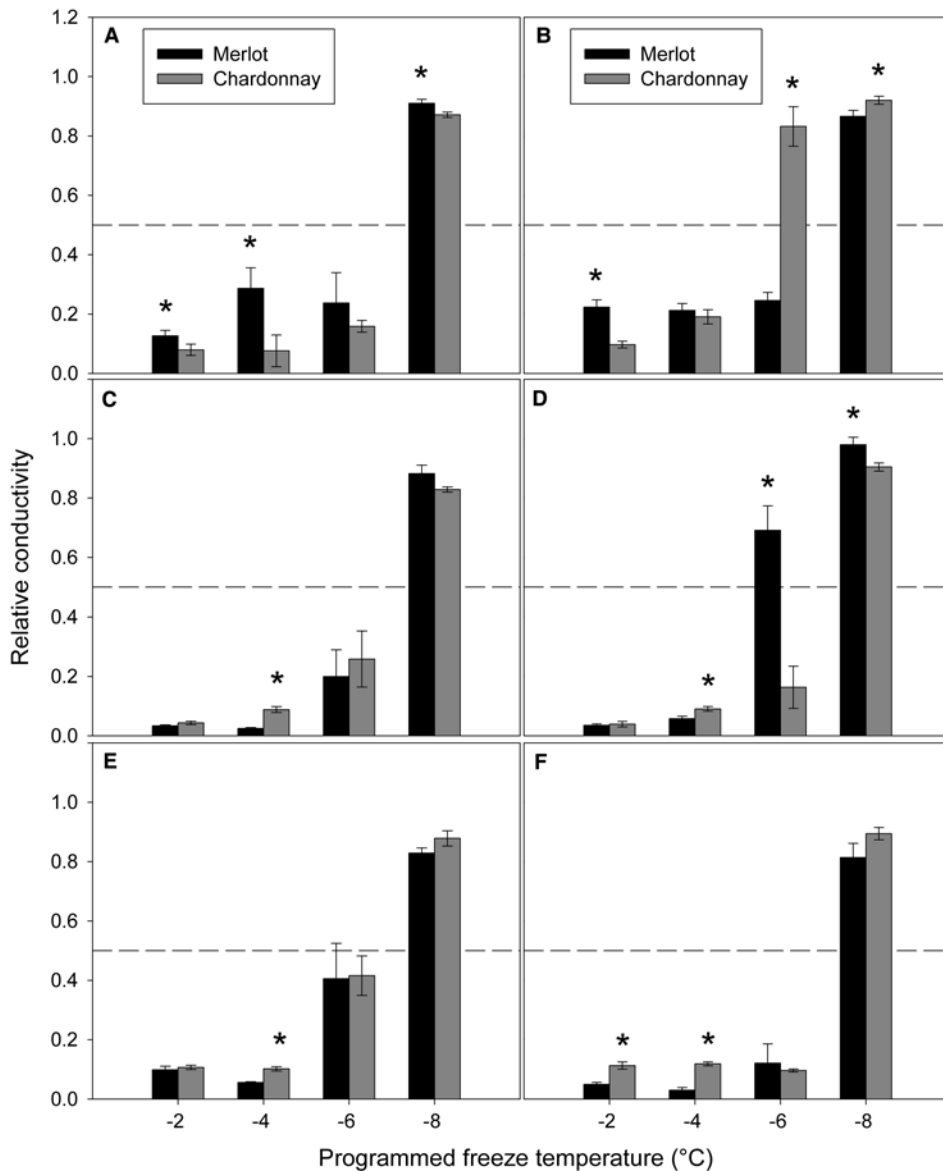


Figure 4 Mean relative conductivity following preconditioning for Merlot and Chardonnay in 2014 (A, C, E) and 2015 (B, D, F). Preconditioning regimes: (A, B) active growth at ambient outdoor temperatures; (C, D) dormant vines at 12°C for a min of one week; and (E, F) dormant vines at 0°C for a min of one week. Relative conductivity values above 0.5 indicate dead tissue (dashed line). Error bars are standard error. Significant differences ($p < 0.5$) between varieties at each controlled temperature are denoted with an asterisk.

variable, relative conductivity (comparing leakage before and after a low-temperature event), to rate samples as alive or dead (Wilner 1960, Deans et al. 1995), where a threshold of 0.5 or higher was delineated as the point where the tissue was no longer viable (Green and Warrington 1978, Hallam and Tibbits 1988). During the protocol optimization process for EL, we found that extending soak times to 24 hr prior to tissue treatment and allowing tissue to soak for seven days following treatment was optimal for assessment.

In the literature, the accuracy of DTA to determine cold hardiness thresholds is questioned if the procedure fails to produce clearly recognizable HTE and LTE peaks (Burr et al. 1986). However, other authors who have used DTA, particularly on conifers, have reported a lack of multiple exotherms (Coleman et al. 1992), but still viewed the procedure as a viable evaluation option. During this study, only a single, individual exotherm per sample was observed when root tissue was subjected to subfreezing temperatures. This suggests that grapevine roots cannot supercool, and the HTE coincides with cellular freezing and tissue damage/death. The strong agreement between EL and DTA here suggests that DTA may be used as a fast and reliable method for evaluating grapevine root cold hardiness in *V. vinifera*. Further evaluation of different *Vitis* species (e.g., comparison between *V. vinifera* and *V. amurensis* [-15.5°C; Okamoto et al. 2000]), comparing DTA with EL, is necessary before completely validating the use of DTA as a general substitute for EL, particularly for the development of management-based temperature thresholds.

The relative conductivity threshold of 0.5 used to distinguish live from dead tissue assumes that tissue will not recover if more than 50% of available electrolytes are leaked. Following comparison between EL and DTA, the 0.5 thresh-

old failed to capture a small number of samples that produced exotherms but subsequently were rated as alive by EL. We suggest that the relative conductivity threshold for live-dead determination in grapevine roots should be lowered to 0.4. This value better captures damage in grapevine root tissues after exposure to subfreezing temperatures and provides a more conservative estimate by reducing false positives (results indicating live tissue, when in fact it is dead). Following the proposed lowering of the live-dead threshold to 0.4, the sensitivity, specificity, positive predictive value, and negative predictive value improved to 98.9, 96.3, 98.4, and 97.5%, respectively. This finding corroborates the suggestion by Deans et al. (1995) that decreasing the relative conductivity threshold from 0.5 to 0.23 for *Quercus patraea* increased accuracy in assessing tissue death. Of course, this threshold is specific for the size of roots used in this study; additional work will be needed to establish thresholds for larger roots or for conclusions based on root order.

Additional improvements in protocols could come from evaluating the type and size of roots used for cold-hardiness assays. Roots smaller than 2.0-mm diam comprise the overall majority of root mass (~80%; Bassoi et al. 2003), so their testing is the logical place to start when examining tissues most affected by cold damage. Small-diameter roots (<2 mm) are the standard sample diameter for testing root hardiness in conifers (Coleman et al. 1992) and apples (Wilner et al. 1960). Okamoto et al. (2000) found that, in *Vitis* spp., larger diameter roots exhibited less cold hardiness than fine roots. Reexamination of this finding using optimized EL and DTA would shed light on the validity of DTA and further the ability to pinpoint lethal soil temperature thresholds for grapevine roots.

It does not appear that grapevine roots can acclimate to cold temperatures to the same extent as grapevine buds. In

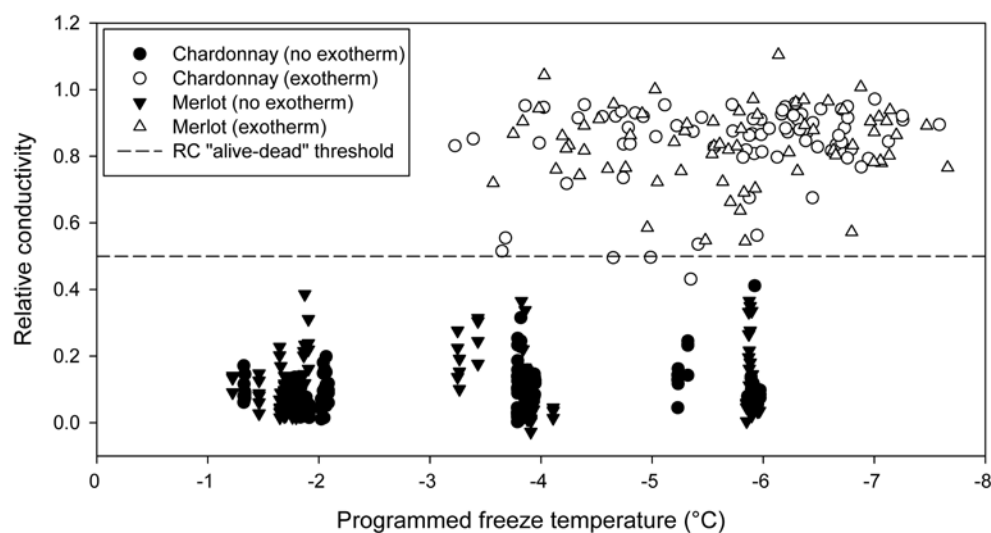


Figure 5 Relationship between relative conductivity (RC) of root tissue and the incidence of low temperature exotherms (LTE). RC values from electrolyte leakage were compared to whether or not differential thermal analysis indicated samples were undamaged (closed symbols; no LTE) or lethally damaged (open symbols; LTE) during programmed freezes. While programmed freeze events were to set temperatures, individual thermocouples for each root sample recorded the actual temperature achieved at that root during the programmed freeze; that actual temperature is presented here. RC values above 0.5 are rated as dead (dashed line), while those below are rated as alive. All samples above the 0.5 threshold corresponded to damaged tissue (open symbols). The majority of samples below the 0.5 threshold corresponded to undamaged tissue (closed symbols), with only one exception.

both Merlot and Chardonnay, across both years, there were instances where roots exposed to warmer temperatures prior to freezing were more cold hardy than roots exposed to colder temperatures, based on absolute EL values. This is not what would be expected if roots acclimated in the same way as buds (Ferguson et al. 2014). However, despite differences in cold hardiness, the absolute level of relative conductivity was still below the damage threshold of 0.5. Differences across preconditioning regimes should be interpreted with caution. While significantly different from each other, if values are below the “kill” threshold for relative conductivity, they may not be biologically different. Interestingly, there were very few instances in which relative conductivity values fell near the dead/alive threshold of 0.5. The only time this occurred was after exposure to -6.0°C ; while the average relative conductivity may have approached the threshold, the distribution of individual data points were either well below or well above the threshold. In other words, root tissue response at this exposure temperature was either substantially above or below the threshold, as this was a transition temperature for potential damage.

Lack of cold acclimation likely reflects an evolutionary response to temperature variation in the rhizosphere and the conditions under which the plant evolved. For grapevine buds, which are surrounded by air, cold temperatures and large temperature swings are a constant threat during dormancy. In contrast, grapevine roots are surrounded by soil, which has an infinitely larger thermal mass than air, and subsequently changes temperature slowly. Because of temperature buffering in the soil, grapevine roots likely did not evolve the complex mechanisms of acclimation essential to survival above the earth’s surface like grapevine buds, although the absolute difference between grapevine species in their root cold hardiness does likely differ, given the wide geographical distribution of different *Vitis* species (Okamoto et al. 2000).

This study, limited to Chardonnay and Merlot, found little difference in max root cold hardiness. Max hardiness of grapevine roots for several *Vitis* spp. have been described previously (Ahmedullah and Kawakami 1986, Guo et al. 1987, Okamoto et al. 2000) and correspond with the levels found here, where median values were -5.7°C to -5.9°C . We suspect that the EL protocols used in this study parsed out roots that were ‘damaged but will recover’ more accurately than past studies in which there were shorter intervals between the damaging events and ultimate data collection (Deans et al. 1995).

Soil temperatures in Washington State rarely fall below the max hardiness temperatures calculated in this study, but potentially damaging soil temperatures do occur during particularly cold and dry winters, especially on sites with shallow soil and low water holding capacity. Desiccation injury could be a major factor in what typically is diagnosed as root freeze damage in situ. Drought-tolerant rootstocks with deeper rooting patterns offer potential to decrease winter root damage because soil temperature variation is dampened with depth. Smart et al. (2006) suggested that overall rooting patterns are more heavily influenced by soil properties than by genotype. Irrigation is also important. High soil water content

in fall damps temperature fluctuations because of the higher heat capacity of water than dry soil. In-season irrigation can also play a role in mitigating potential root damage. For example, frequent, short-duration irrigation sets encourage root development near the soil surface, exposing the root system to greater temperature fluctuations than if the roots were established deeper (Anderson et al. 2003).

Washington State’s grape industry predominantly uses own-rooted grapevines. Consequently, we evaluated the cold hardiness of *V. vinifera* roots. While more-common *Vitis* rootstocks were not included, we hope that the information and techniques described here will provide a foundation for further study.

Conclusion

When examining viability assessment techniques for grapevine roots, EL and DTA were comparable for two *V. vinifera* varieties. A modified EL protocol was developed that optimized soak periods for max ion release while minimizing assay duration. DTA, once questioned in tissues that failed to produce two distinct exotherms (HTE and LTE), was faster and had similar accuracy as EL in determining the freezing point of excised grapevine root tissues. This study found little evidence of grapevine root acclimation to cold temperatures in the *V. vinifera* varieties evaluated. Max root hardiness for Chardonnay and Merlot had median values of -5.9°C and -5.7°C , respectively. LT_{10} , LT_{50} , and LT_{90} values were calculated for grapevine roots, with values of -4.0 , -5.8 , and -7.0°C , respectively.

Literature Cited

- AgWeatherNet. 2016. Historical Weather Data - WSU Hamilton Station. Accessed: 1 Nov 2016. <http://weather.wsu.edu/?p=93050>.
- Ahmedullah M and Kawakami A. 1986. Evaluation of laboratory tests for determining the lethal temperature of *Vitis labruscana* BAILEY Concord roots exposed to subzero temperatures. *Vitis* 25:142-150.
- Anderson LJ, Comas LH, Lakso AN and Eissenstat DM. 2003. Multiple risk factors in root survivorship: A 4-year study in Concord grape. *New Phytol* 158:489-501.
- Ashworth EN. 1990. The formation and distribution of ice within Forsythia flower buds. *Plant Physiol.* 92:718-725.
- Basso LH, Hopmans JW, Jorge LAC, Alencar CM and Silva JAM. 2003. Grapevine root distribution in drip and microsprinkler irrigation. *Sci Agric* 60:377-387.
- Burke MJ, Gusta LV, Quamme HA, Weiser CJ and Li PH. 1976. Freezing and injury in plants. *Ann Rev Plant Physiol* 27:507-528.
- Burr KE, Tinus RW, Wallner SJ and King RM. 1986. Comparison of four cold hardiness tests on three western conifers. *In Proceedings of the Combined Western Forest Nursery Council and Intermountain Nursery Association Meeting.* pp. 87-95. Tumwater, WA.
- Coleman MD, Hinckley TM, McNaughton G and Smit BA. 1992. Root cold hardiness and native distribution of subalpine conifers. *Can J Forest Res* 22:932-938.
- Comas LH, Anderson LJ, Dunst RM, Lakso AN and Eissenstat DM. 2005. Canopy and environmental control of root dynamics in a long-term study of Concord grape. *New Phytol* 167:829-840.
- Deans JD, Billington HL and Harvey FJ. 1995. Assessment of frost damage to leafless stem tissues of *Quercus patraea*: A reappraisal of the method of relative conductivity. *Forestry* 68:25-34.

- Dexter ST, Tottingham WE and Graber LF. 1930. Preliminary results in measuring the hardiness of plants. *Plant Physiol* 5:215-223.
- Dexter ST, Tottingham WE and Graber LF. 1932. Investigations of the hardiness of plants by measurement of electrical conductivity. *Plant Physiol* 7:63-78.
- Ferguson JC, Tarara JM, Mills LJ, Grove GG and Keller M. 2011. Dynamic thermal time model of cold hardiness for dormant grapevine buds. *Ann Bot* 107:389-396.
- Ferguson JC, Moyer MM, Mills LJ, Hoogenboom G and Keller M. 2014. Modeling dormant bud cold hardiness and budbreak in twenty-three *Vitis* genotypes reveals variation by region of origin. *Am J Enol Vitic* 65:59-71.
- Green LM and Warrington IJ. 1978. Assessment of frost damage in radiata pine seedlings using the diffusate conductivity technique. *NZ J For Sci* 8:344-350.
- Guo XW, Fu WH and Wang GJ. 1987. Studies on cold hardiness of grapevine roots. *Vitis* 26:161-171.
- Hallam PM and Tibbitts WN. 1988. Determination of frost hardiness of *Eucalyptus* using the electrical conductivity of diffusate in conjunction with a freezing chamber. *Can J Forest Res* 18:595-600.
- Keller M and Mills LJ. 2007. Effect of pruning on recovery and productivity of cold-injured Merlot grapevines. *Am J Enol Vitic* 58:351-357.
- Lindström A. 1986. Freezing temperatures in the root zone - effects on growth of containerized *Pinus sylvestris* and *Picea abies* seedlings. *Scand J Forest Res* 1:371-377.
- Lindström A and Nyström C. 1987. Seasonal variation in root hardiness of container-grown Scots pine, Norway spruce, and lodgepole pine seedlings. *Can J Forest Res* 17:787-793.
- McDonald JH. 2014. One-way ANOVA. *In Handbook of Biological Statistics*. pp. 145-156. Sparky House Publishing, Baltimore, Maryland.
- McKay HM. 1992. Electrolyte leakage from fine roots of conifer seedlings: A rapid index of plant vitality following cold storage. *Can J Forest Res* 22:1371-1377.
- Mills LJ, Ferguson JC and Keller M. 2006. Cold-hardiness evaluation of grapevine buds and cane tissues. *Am J Enol Vitic* 57:194-200.
- Okamoto G, Wang S and Hirano K. 2000. Cold resistance in root and cane of own-root 'Kyoho' grapevines. *In Scientific Reports of the Faculty of Agriculture, Okayama University*. 89:23-29.
- Ott RL and Longnecker M. 2001. Bayes' Formula. *In An Introduction to Statistical Methods and Data Analysis*. Crockett C (ed.), pp. 136-140. Thomson Learning, Pacific Grove, CA.
- Pierquet P and Stushnoff C. 1980. Relationship of low temperature exotherms to cold injury in *Vitis riparia* Michx. *Am J Enol Vitic* 31:1-6.
- Richards D. 1983. The grape root system. *In Horticultural Reviews*. pp. 5:127-168. John Wiley & Sons, Inc., Hoboken, New Jersey.
- Smart DR, Schwass E, Lakso A and Morano L. 2006. Grapevine rooting patterns: A comprehensive analysis and review. *Am J Enol Vitic* 57:89-104.
- Smit-Spinks B, Swanson BT and Markhart III AH. 1985. The effect of photoperiod and thermoperiod on cold acclimation and growth of *Pinus sylvestris*. *Can J Forest Res* 15:453-460.
- Snyder RL and de Melo-Abreu JP. 2005. Frost protection: Fundamentals, practice, and economics. Environmental and Natural Resources Series #10. Food and Agriculture Organization of the United Nations. Rome, Italy. 1:1-126.
- Timmis R and Worrall J. 1975. Environmental control of cold acclimation in Douglas fir during germination, active growth, and rest. *Can J Forest Res* 5:464-477.
- Wample RL, Reisenauer G, Bary A and Schuetze F. 1990. Microcomputer-controlled freezing, data acquisition and analysis system for cold hardiness evaluation. *HortScience* 25:973-976.
- Whitlow TH, Bassuk NL, Ranney TG and Reichert DL. 1992. An improved method for using electrolyte leakage to assess membrane competence in plant tissues. *Plant Physiol* 98:198-205.
- Wilner J. 1955. Results of laboratory tests for winter hardiness of woody plants by electrolytic methods. *Proc Amer Soc Hort Sci* 66:93-99.
- Wilner J. 1959. Note on an electrolytic procedure for differentiating between frost injury of roots and shoots in woody plants. *Can J Plant Sci* 39:512-513.
- Wilner J. 1960. Relative and absolute electrolytic conductance tests for frost hardiness of apple varieties. *Can J Plant Sci* 40:630-637.
- Wilner J, Kalbfleisch W and Mason WJ. 1960. Note on two electrolytic methods for determining frost hardiness of fruit trees. *Can J Plant Sci* 40:563-565.