AJEV Papers in Press. Published online June 22, 2017.

AJEV PAPERS IN PRESS • AJEV PAPERS IN PRESS

American Journal of Enology and Viticulture (AJEV). doi: 10.5344/ajev.2017.16109

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Research Article

Cold Hardiness of *Vitis vinifera* Roots

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Acknowledgments: Work presented here was completed in partial fulfillment of a Master of Science in Horticulture from Washington State University. Funding was provided by the Washington State University Irrigated Agriculture Research and Extension Center and the Washington State University Viticulture and Enology Program. Plant materials were provided by the Clean Plant Center Northwest. Thank you to Dr. Amit Dhingra and Dr. Markus Keller for their suggestions and insight. An additional thank you to Drs. Bill Riley and Julie Tarara for their manuscript review. This work was partially supported by the USDA National Institute of Food and Agriculture Hatch project #226789.

Manuscript submitted Nov 2016, revised Mar 2017, Apr 2017, accepted Apr 2017

Abstract: Grapevine buds have identifiable cold acclimation and deacclimation patterns. Whether roots follow these patterns is unknown. If root cold hardiness thresholds and/or acclimation patterns were better understood, mitigation strategies could be developed to reduce the likelihood of root damage. This study encompassed two major objectives: 1) To improve protocols for electrolyte leakage and then quantitatively compare 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 when vines

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were actively growing; 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: -2.0°C, -4.0°C, -6.0°C, or -8.0°C. 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. Maximum root cold hardiness was experimentally derived for both varieties (Chardonnay median -5.9°C; Merlot median -5.7°C). Differential thermal analysis was an effective alternative method to electrolyte leakage in estimating damage thresholds for these two varieties The lethal temperature at which 10%, 50%, and 90% of roots were killed were calculated for grapevine roots, with values of -4.0°C, -5.8°C, and -7.0°C, respectively.

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

Introduction

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

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1986, Smit-Spinks et al. 1985). 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 (*Pinus contorta*), and other conifers suggests some form of cold acclimation in roots (Lindstrom and Nystrom 1987, Timmis and Worrall 1975).

Currently, vineyard managers have little way of telling 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 informed 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.

There is sparse literature on the cold hardiness of grapevine roots, and this fact is regularly commented on in studies of the grape root system (Comas et al. 2005, Richards 1983). To the author's knowledge, no research has examined the ability of grapevine roots to acclimate to seasonal variations in soil temperature. Following a winter of severe cold temperatures in Washington, Ahmedullah and Kawakami (1986) suggested an absolute lethal temperature for Concord (*Vitis labruscana*) roots at around -5°C. A similar study in Japan demonstrated a threshold for cold hardiness of *V. vinifera* 'Kyoho' roots of -4.0°C (Okamoto et al. 2000). Guo et al. (1987) demonstrated maximum cold hardiness of 19 different varieties and species including *V. vinifera* 'Muscat Hamburg' roots at -5.2°C, *V. riparia* roots at -11.4°C, and that of *V. amurensis* roots at -15.5°C. Thus, there appears to be species difference(s) within *Vitis* for maximum cold hardiness, but no literature on within-season acclimation variation. These three

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studies, with the most recent study taking place 17 years ago, make up the entire body of knowledge 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 and was first described by Dexter et al. (1930, 1932). In short, EL involves the measurement of 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), Wilner (1959), Wilner et al. (1960), and McKay (1992), with technical concerns presented by Deans et al. (1995) and Whitlow et al. (1992) taken into consideration. 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). However, a criticism and limitation to the use of this technique in past studies was short tissue soak times which do not allow enough time for completion of electrolyte leakage from damaged tissue, nor does it allow sufficient time for electrolyte re-uptake (Deans et al. 1995). Whitlow et al. (1992) also expressed concerns regarding the use of electrolyte leakage, and in particular, relative conductivity as a means for quantifying damage, suggesting that it does not capture membrane permeability. They suggest that a tissue ionic conductance value (g_{Ti}) should be added to account for the chemical driving force and tissue surface area; this is understandable when using tissues of varying sizes and structure (leaves, shoots) which may be difficult to normalize prior to evaluation. While we recognize the possibility of increased accuracy through the use of the (g_{Ti})

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in calculations, their protocols were optimized for leaf tissue, and also had the limitation of a short soak duration.

Differential thermal analysis (DTA), a rapid technique commonly used for determining grapevine bud and cane cold hardiness (Ferguson et al. 2014, Mills et al. 2006, Pierquet and Stushnoff 1980, Wample et al. 1990), requires less than 12 hours to complete. Unfortunately, in some past studies (Burr et al. 1986), a lack of distinctive high- and low-temperature exotherms for tissue (*Pinus spp.* buds) prohibited researchers from quantifying absolutely cold hardiness temperature thresholds. There is no published work indicating 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: First, to improve protocols for EL and then quantitatively compare to DTA to determine a more rapid method for root cold hardiness assessments; and second, to quantify whether *Vitis vinifera* roots acclimate to preconditioning temperatures.

Materials and Methods

Data were generated simultaneously for both evaluating and optimizing EL protocols against DTA, and to determine root cold acclimation and maximum hardiness. A diagram of the process is in Supplemental Fig. 1.

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117 ***Plant materials***

118 For these studies, *V. vinifera* 'Chardonnay' and 'Merlot' were selected, based on differences seen
119 in their acclimation and deacclimation patterns as measured in dormant buds (Ferguson et al.
120 2011). Dormant cuttings of both varieties were rooted in Mar 2014. Source material was from
121 the grapevine foundation block of the Clean Plant Center-Northwest (Prosser, WA), which is a
122 repository of plant material that has undergone virus elimination. Cuttings were callused and
123 rooted in a plywood callusing box filled with perlite (Therm-o-Rock West, Inc., Chandler, AZ),
124 covered with a heating mat (Hydrofarm®, Petaluma, CA) set to 25°C and damp burlap bags to
125 retain moisture. Rooted plants were potted on 17 May 2014 in 13 L blow-molded plastic nursery
126 pots (#3, McConkey Grower Supplies, Sumner, WA). Potting media consisted of 1:1:1 potting
127 mix (Sunshine Mix #3 Professional Growing Mix, Sun-Gro Horticulture, Agawam, MA): peat
128 moss (Nature's®, Sun-Gro Horticulture, Agawam, MA): and perlite (Therm-o-Rock West, Inc.,
129 Chandler, AZ). Plants were moved to the greenhouse, grown for 5 wk, then relocated to outdoor
130 above-ground plant beds. Irrigation was delivered via drip two times daily for 10-min intervals at
131 a rate of 2 L/hr (approximately 0.66 L/plant/day). During times of extreme heat, the duration of
132 drip delivery was increased to 1 L/plant/day. Plants were staked and tied to 1.5 m bamboo stakes
133 and "hedged" if growth exceeded 0.9 m past the top of the stake; two shoots per plant were
134 retained in year 1. In fall, plants were allowed to go dormant naturally (indicated by the
135 formation of periderm), pruned to two, 2-bud spurs, and were placed into climate-controlled
136 storage on 10 Nov 2014. Temperatures in cold storage varied between 7°C and 15°C with an
137 average of 12°C. Storage conditions were under continuous darkness, with the exception of light
138 used when plant materials were being removed. In 2015, the same vines were moved out of cold

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storage on 5 Jun and given 20 g of nitrogen urea (46-0-0). Irrigation was delivered as in 2014; 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 years (2014-2015, and 2015-2016) of the study.

Electrolyte Leakage Protocol Optimization

The duration of the "soak" time needed to capture complete electrolyte leakage and potential partial electrolyte re-uptake is one area that has been highlighted as a point of change in recent literature on the subject (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 diameter, 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 7 days. Solution conductivity was measured daily for each replication (S230 SevenCompact^{TM} Conductivity meter, Mettler-Toldeo, Columbus, OH USA). The minimum number of days required for maximum electrolyte leakage was determined and used in all subsequent assays.

To determine the duration of an adequate post-treatment (post-freeze) soak time for grapevine roots, 20 replications of Chardonnay root tissue (1.0 to 2.0 mm diameter, 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 d at 4°C. Conductivity was measured daily for each

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161 replication. The minimum number of days required for maximum electrolyte leakage was
162 determined and that post-damage soak duration was used in all future assays.

163 To begin optimizing protocols for grapevine roots, it was necessary to determine the
164 temperature range needed to evaluate potential cold damage to root tissue. The original freezing
165 program for grapevine buds (Mills et al. 2006) was applied to 20 dormant Chardonnay root
166 replications during this preliminary testing phase. The program slowly reduces temperature from
167 4°C to -40°C at 4°C/hr, and was chosen as the test program during protocol development due to
168 its general accepted use in grape cold hardiness experiments. The programmable freezer setup
169 was the same as that described by Wample et al. (1990). Following these initial tests, a freeze
170 protocol was designed specifically for roots, which attempted to mimic the slower temperature
171 variations of the soil compared to those of the air. Slower chilling also minimizes inaccurate
172 exotherm interpretation resulting from the development of unrealistic ice formation sites within
173 the tissues (Burke et al. 1976). The programmed freeze temperature protocols are described in
174 Table 1, and final selected temperature minimums were -2, -4, -6, and -8°C.

175 ***Determining Cold Hardiness Following Preconditioning***

176 Vines were subjected to three preconditioning temperature regimes prior to assessment of
177 root cold hardiness in both 2014-2015 and 2015-2016 (experiment conducted twice). Twenty
178 vines of each variety were used for each preconditioning regime in each year. The first
179 preconditioning regime occurred during active vine growth: vines were subjected to ambient
180 temperature. Typical daytime high temperatures during sampling were 28°C to 32°C and
181 nighttime low temperatures ranged from 14°C to 21°C (AgWeatherNet 2016). The other
182 preconditioning regimes were imposed when vines were fully dormant after 3 wks in cold

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storage: 1) vines were brought out of cold storage and held at a consistent temperature of 12°C in a dedicated cold storage unit for a minimum of 1 wk; and 2) vines were brought out of cold storage and held at a consistent temperature of 0°C in a dedicated cold storage unit for a minimum of 1 wk. To ensure that root temperature equilibrated with air temperature, temperature sensors (HOBO, U-Series, Onset Computer Corporation, Bourne, MA, USA) 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 hours (*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 diameter soil corer inserted to the floor of the pot equidistant between the vine main trunk and the pot boundary (approximately 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, Mentor, OH) 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, keeping those 1.0 to 2.0 mm in diameter. 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©, Schwerzenbach, Switzerland) were retained. Samples were soaked for 24 hr in 8 mL of deionized water in 15 mL conical tubes at 4°C (Falcon™ Conical Centrifuge tubes, Sigma-Aldrich Co. LLC., Darmstadt, Germany) for EL analysis to collect baseline electrolyte leakage prior to treatment.

Following the collection and initial processing of root materials subjected to each of the

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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™, Dallas, TX), and were individually wrapped in a 6 cm x 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). Following the programmed freezing protocol, root samples were removed from the foil packets and returned to their original 15 mL tubes (containing original bathing solution). Samples were allowed to soak for 7 d, and solution conductivity was measured on day 7. The sample tubes were then held at -80°C a minimum of 10 hr to kill the tissue. Following this, the samples were thawed and held at 4°C for 7 d. On day 7, a final conductivity measurement was recorded. Relative conductivity was calculated to estimate tissue damage ($R_t = L_t / L_k$), 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 7 d, and then killed following -80°C freeze (Green and Warrington 1978). Relative conductivity values above 0.5 are associated with dead tissue and values below 0.5 indicate live tissue or that which is able to 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 quantity treatment effects. Relative conductivity is faster, and yields similar results (Deans et al. 1995). The lethal temperatures

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when 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 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 completed using Minitab® 17 (Minitab 17 Statistical Software, State College, PA, USA). Analysis of variance and Tukey's HSD were used to determine differences among treatments of all analyses other than that for 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°C, -4°C, -6°C, -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 $p < 0.05$. DTA efficacy at 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, or in other words, 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 negative event and it actually occurring) (Ott and Longnecker 2001) of DTA as an assay was then calculated.

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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 (Fig. 1A). The median temperature at which exotherms were observed was -6.0°C. There were no exotherms 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 low and high temperature exotherms (LTE and HTE, respectively) 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 of 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.

Electrolyte Leakage Protocol Optimization

Of the total potential electrolytes that can be leaked in healthy (undamaged) excised root tissue, an average of 84 ± 3.8% of the total electrolytes that were going to be released were released into the bathing solution within 24 hrs of a pre-treatment soak (Fig. 1B). Because the leakage of uninjured tissue is of much smaller magnitude (3x to 5x less) than that of 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 pre-treatment differences, and was incorporated into

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all EL protocols.

After complete tissue death following exposure to -80°C, 10% of samples demonstrated complete leakage by day 6 post-thaw (Fig. 1C). By day 7, 90% of samples had reached maximum electrolyte leakage, and at day 8 100% of samples had reached maximum electrolyte leakage. Seven days of leakage time was deemed adequate to elicit leakage and minimize protocol time; this soak time was incorporated into all EL protocols thereafter.

Determining Cold Hardiness Following Preconditioning

Regardless of year or variety, there was no evidence that temperature preconditioning influenced maximum root cold hardiness. Results from mixed model ANOVA are shown in Table 2. Year was a significant effect in the -6.0°C and -8.0°C freezes. Preconditioning was a significant effect in the -2.0°C, -4.0°C, and -8.0°C freezes. The year-variety interaction was significant for the -4.0°C freeze. The year-preconditioning interaction was significant in the -2.0°C, -6.0°C, and -8.0°C freezes. The variety-preconditioning interaction was significant for all programmed freeze temperatures. However, interpretation of statistical significance should not be confused here with practical significance. This is especially the case when data has a threshold response, such as the case with the interpretation of relative conductivity values. As such, 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 (Fig. 2A and 2B, Fig. 3A and 3B), and 2015 (Fig. 2C and 2D, Fig. 3C and 3D), there was no consistent pattern in preconditioning being associated with significantly higher or lower relative conductivity or incidence of exotherms to -2°C, -4°C, -6°C or -8°C temperature exposure. The only consistent pattern in the data was in the relative conductivity values and exotherm incidence within the cold

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293 temperature exposures regardless of variety, year, or preconditioning. The -8.0°C exposure was
294 associated with leakage values which surpassed the relative conductivity threshold (0.5) 100% of
295 the time in all cases, and had 100% incidence of exotherms. Conversely, the -2.0°C and -4.0°C
296 exposures never surpassed the threshold and only exhibited exotherms 1.7% of the time. The -
297 6.0°C exposure was variable; 31.7% of the time relative conductivity values exceeded 0.5 and in
298 30.8% of samples the exposure resulted in an exotherm (Fig. 2 and 3). Values of relative
299 conductivity are in Table 3.

300 While individual preconditioning regimes often resulted in a relative conductivity that
301 was different from others at a specific temperature exposure, in almost all cases the average
302 relative conductivity for all preconditioning regimes at a specific temperature exposure were
303 either well below, or well above the 0.5 threshold. For example, in Chardonnay during 2014,
304 exposure to -2.0°C resulted in higher relative conductivity values in the dormant preconditioning
305 at 0°C than in the dormant 12°C preconditioning (Fig. 2A), yet all preconditioning values at this
306 low temperature exposure remained under 0.5. Similarly, in 2015 (Fig. 2C), exposure to -2.0°C
307 resulted in lower relative conductive values in dormant 12°C preconditioning than the other two
308 regimes, but relative conductivity for all pretreatments at this low temperature exposure
309 remained below 0.5. In Merlot in 2014 (Fig. 2B), exposure to -2.0°C resulted in lower relative
310 conductivity (less damage) for the dormant 12°C preconditioning than the other preconditioning
311 regimes, but all remained under 0.5. Following the -8.0°C exposure, ambient outdoor
312 preconditioning showed significantly higher relative conductivity than the 0°C preconditioning,
313 but all preconditioning relative conductivity values remained above 0.5. In 2015 (Fig 2D),
314 following a -8.0°C low temperature exposure, higher relative conductivity occurred in the 12°C

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315 preconditioning than in the 0°C preconditioning but all relative conductivity values among
316 preconditioning regimes were above 0.5. Preconditioning was less a factor in tissue damage than
317 was degree of low temperature exposure.

318 Similar results, and a lack of consistent patterns between root preconditioning and cold
319 hardiness, were also seen across years and varieties in DTA (Fig. 3). In 2014 in Chardonnay
320 (Fig. 3A), the -6.0°C low temperature exposure resulted in higher exotherm incidence in the
321 dormant 0°C preconditioning than in the actively growing, ambient outdoor temperature
322 preconditioning. At the same exposure temperature in 2015 (Fig. 3C), the actively growing
323 preconditioning had a higher exotherm incidence than the other preconditioning regimes. In 2014
324 in Merlot (Fig. 3B), no significant differences in exotherm incidence were found following any
325 of the exposures, and in 2015 (Fig. 3D), following a 6.0°C exposure, there was a higher
326 exotherm incidence in the 12°C preconditioning than in the other preconditioning regimes. As
327 seen in EL, preconditioning was not a good predictor of maximum cold hardiness.

328 Few differences were observed between varieties in response to preconditioning
329 and freeze protocols (Fig. 4). In most cases, Merlot and Chardonnay responses mirrored
330 each other. There were a few instances where significant differences were biologically
331 meaningful; for example, in 2015 ambient outdoor preconditioning (Fig. 4B) and the 12°C
332 preconditioning (Fig. 4D) following the -6°C freeze protocol. Other significant differences
333 were found between varieties, but as was observed in relative conductivity, all of these
334 differences occurred between tissues that both fell well below or above the 0.5 damage
335 threshold.

336

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337 ***Comparing Assays***

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

345 ## Discussion

346 Grapevine roots did not acclimate to preconditioning temperatures, and maximum cold hardiness
347 showed little variation regardless of preconditioning. We expected more damage following
348 preconditioning at the actively growing, ambient outdoor temperature than following dormant
349 preconditioning at 0°C, yet this was not the case. There were no consistent differences in tissue
350 damage among preconditioning regimes at each of the low temperature exposures
351 (-2.0°C, -4.0°C, -6.0°C, or -8.0°C). Regardless of preconditioning, the median freezing
352 temperatures for Chardonnay and Merlot root tissues were -5.9°C and -5.7°C, respectively. The
353 LT_{10}, LT_{50}, and LT_{90} were calculated, with values of -4.0°C, -5.8°C, and -7.0°C, respectively.
354 Electrolyte leakage accuracy was found to be improved when adequate tissue soak times were
355 used. Differential thermal analysis was found to have high levels of agreement with EL when the
356 presence of an exotherm was used to indicate tissue vitality. Further examination into the
357 possibility of root order affecting hardiness, and further evaluation of the technique when

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358 comparing between different species of *Vitis* is necessary prior to using DTA as a replacement
359 for EL.

360 Electrolyte leakage was chosen as the standard to which DTA was compared. The EL
361 protocols presented here used a calculated variable, relative conductivity (comparing leakage
362 before and after a low temperature event), to rate samples as alive or dead (Deans et al. 1995,
363 Wilner 1960), where a threshold of 0.5 or higher was delineated as the point where the tissue was
364 no longer viable (Green and Warrington 1978, Hallam and Tibbits 1988). During the protocol
365 optimization process for EL we found that extending soak times to 24 hr prior to tissue treatment
366 and allowing tissue to soak for 7 d following treatment was optimal for assessment.

367 In the literature, the accuracy of DTA to determine cold hardiness thresholds is
368 questioned if the procedure fails to produce clearly recognizable HTE and LTE peaks (Burr et al.
369 1986). However, other authors who have used the method, particularly on conifers, have reported
370 a lack of multiple exotherms (Coleman et al. 1992), but still viewed the procedure as a viable
371 evaluation option. During this study, only a single, individual exotherm per sample was observed
372 when root tissue was subjected to sub-freezing temperatures. This suggests that grapevine roots
373 do not have the ability to supercool, and the HTE coincides with cellular freezing and tissue
374 damage/death. Strong agreement between EL and DTA here suggests that DTA may be used as a
375 fast and reliable method for evaluating grapevine root cold hardiness in *Vitis vinifera*. Further
376 evaluation of different *Vitis* species (such as a comparison between *V. vinifera and V. amurensis*
377 (-15.5°C) Okamoto et al. 2000) comparing DTA with EL is necessary before completely
378 validating using DTA as a general substitute for EL, particularly for the development of
379 management-based temperature thresholds.

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The relative conductivity threshold that is used to distinguish live from dead tissue is 0.5, which assumes a lack of tissue recovery if more than 50% of available electrolytes are leaked. Following comparison between EL and DTA, the 0.5 threshold 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 be lowered to 0.4. This value better captures damage in grapevine root tissues following sub-freezing temperature exposure 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, 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 in *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 on establishing threshold will be needed if larger roots are used, or if conclusions based on roots of a specific order are to be made.

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 diameter comprise the overall majority of root mass (approximately 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 root hardiness testing of conifers (Coleman et al. 1992) and apples (Wilner et al. 1960). Okamoto et al. (2000) found that larger diameter roots exhibited less cold hardiness than fine roots in *Vitis*. A reexamination of this finding using

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optimized EL and DTA would shed light on its validity, furthering our 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 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 one would expect if roots were acclimating in the same way as buds (Ferguson et al. 2014). However, while there were differences, the absolute level of relative conductivity was still below the damage threshold of 0.5. Differences across preconditioning regimes do need to 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 where relative conductivity values fell near the dead/alive threshold of 0.5. The only time that 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 is likely a reflection of the 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. On the other hand, grapevine roots are surrounded by soil which has an infinitely larger thermal mass than air, and subsequently changes temperature slowly. Because of

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424 temperature buffering in the soil, grapevine roots likely did not evolve the complex mechanisms
425 of acclimation essential to survival above the earth's surface like grapevine buds, although the
426 absolute difference between grapevine species in their root cold hardiness does likely differ as
427 suggested by Okamoto et al. (2000) given the wide geographical distribution of different *Vitis*
428 species.

429 This study, limited to Chardonnay and Merlot, found little difference in maximum root
430 cold hardiness. Maximum hardiness of grapevine roots for several *Vitis* spp. have been described
431 previously (Ahmedullah and Kawakami 1986, Guo et al. 1987, Okamoto et al. 2000) and
432 correspond with the levels found in the present study, where median values were -5.7°C to
433 -5.9°C. We suspect that the EL protocols used in this study parsed out roots that were 'damaged
434 but will recover' more accurately than past studies in which there were shorter intervals between
435 the damaging events and ultimate data collection (Deans et al. 1995).

436 Soil temperatures in Washington State rarely are driven below the maximum hardiness
437 temperatures calculated from this study. Potentially damaging soil temperatures do occur during
438 particularly cold and dry winters, especially on sites with shallow soil and low water holding
439 capacity. Desiccation injury could be a major factor in what typically is diagnosed as root freeze
440 damage *in situ*. Drought tolerant rootstocks with deeper rooting patterns offer potential to
441 decrease winter root damage because soil temperature variation is dampened with depth. Smart
442 et al. (2006) suggested that overall rooting patterns are more heavily influenced by soil
443 properties than by genotype. Irrigation is also important. High soil water content in fall damps
444 temperature fluctuations because of the higher heat capacity of water than dry soil. In-season
445 irrigation can also play a role in mitigating potential root damage. For example, frequent short

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duration irrigation sets encourage root development nearer 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, this study evaluated the cold hardiness of *V. vinifera* roots. While *Vitis* more commonly used as rootstocks were not included, we hope that the information and techniques described within will provide a foundation for further study.

Conclusion

When examining viability assessment techniques for grapevine roots, electrolyte leakage and differential thermal analysis were comparable for two *V. vinifera* varieties. A modified electrolyte leakage protocol was developed that optimized soak periods for maximum ion release while minimizing assay duration. Differential thermal analysis, 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. Maximum 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°C, -5.8°C, and -7.0°C, respectively.

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Table 1 Programmed freeze protocols 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 (Minutes)	Action
1	15	Decrease sample temperature to 4°C from ambient temperature
2	60	Hold at 4°C
3	180	Decrease sample temperature to -2°C at -2°C/hr
4	0-360	Decrease temperature to desired freeze temperature at 1°C/hr
5	30	Hold at minimum freeze temperature (-2°C, -4°C, -6°C, or -8°C)
6	90-180	Increase sample temperature to 4°C at 4°C/hr
7	Variable	Hold at 4°C until samples are removed from freezer

Table 2 Mixed model ANOVA results with year, variety, and preconditioning as fixed effects, and the relative conductivity values following each programmed freeze (-2°C, -4°C, -6°C, -8°C) as the response variable. Significant differences are denoted with an asterisk ($p < 0.05$).

	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*

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

		Temperature exposure			
Variety	Vintage	-2.0 °C	-4.0 °C	-6.0 °C	-8.0 °C
		Relative Conductivity			
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)

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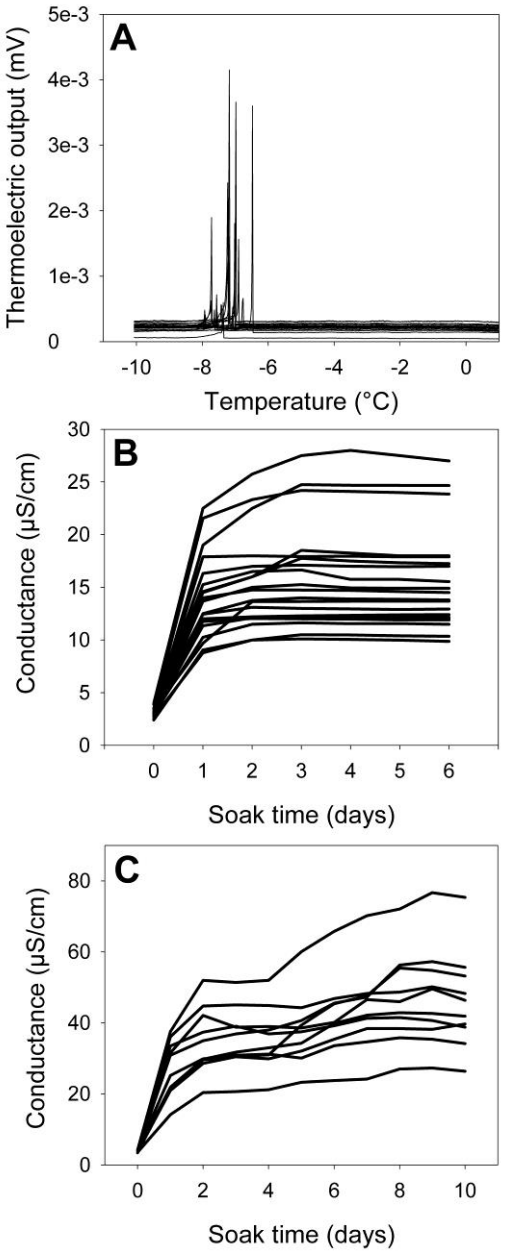

Figure 1 Optimization of cold hardiness evaluation protocols. (**A**) Differential thermal analysis trace after subjecting root samples to the freeze protocols for grapevine buds (Mills et al. 2006). Graph was truncated at -10°C due to lack of peaks below -8°C. (**B**) Conductivity of bathing solution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

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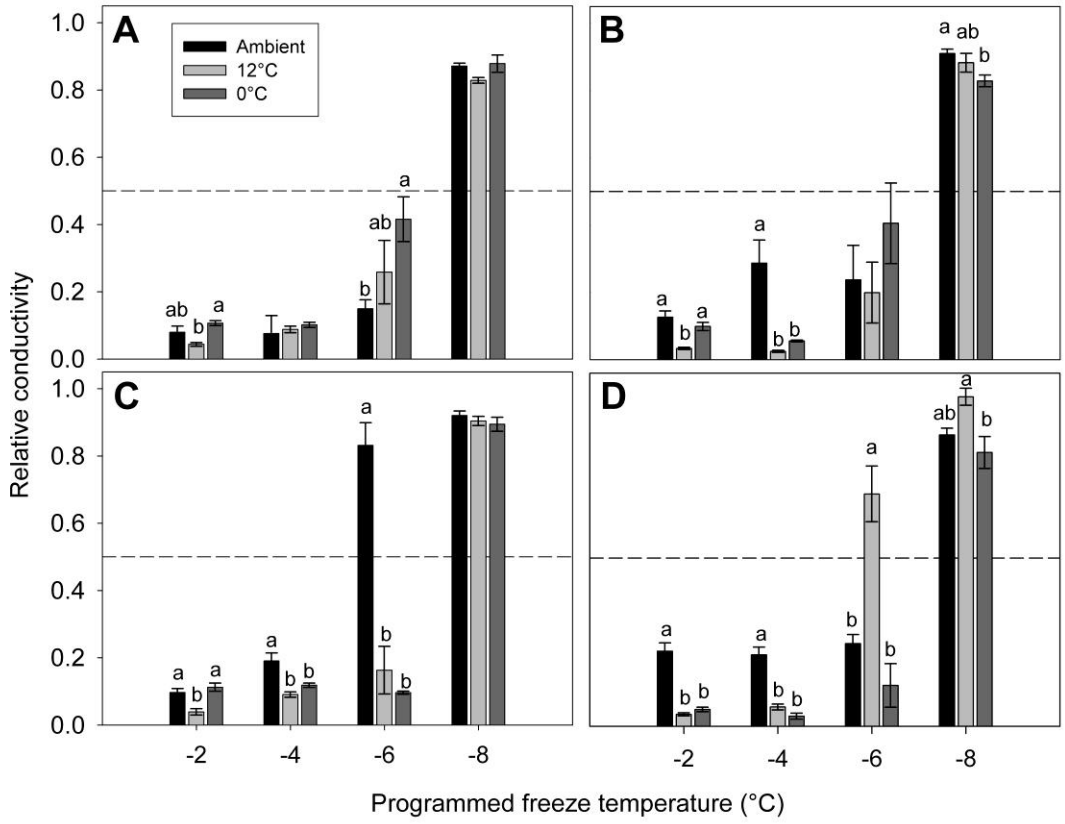

Figure 2 Mean relative conductivity of bathing solution for *Vitis vinifera* 'Chardonnay' and 'Merlot' roots after preconditioning followed by controlled decreases in temperature below freezing. (**A**) Chardonnay, 2014; (**B**) Merlot, 2014; (**C**) Chardonnay, 2015; and (**D**) Merlot, 2015. Relative conductivity values above 0.5 indicate dead tissue (dashed line). Error bars represent ±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 minimum of 1 wk; 0°C = preconditioned during dormancy at 0°C for a minimum of 1 wk.

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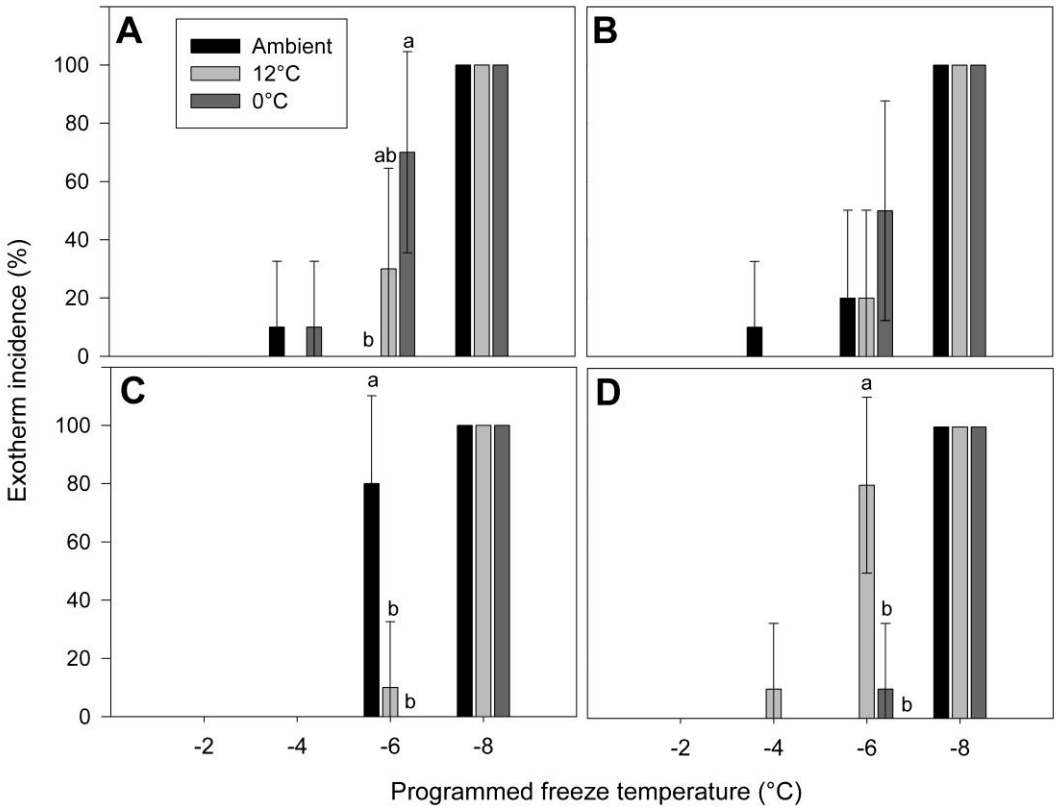

Figure 3 Mean incidence of low temperature exotherms for *Vitis vinifera* Chardonnay and Merlot roots after preconditioning followed by controlled decreases in temperature below freezing. (**A**) Chardonnay, 2014; (**B**) Merlot, 2014; (**C**) Chardonnay, 2015; and (**D**) Merlot, 2015. Error bars represent standard error. Different letters indicate significant difference between treatment means within a temperature exposure using Tukey's HSD $\alpha = 0.05$.

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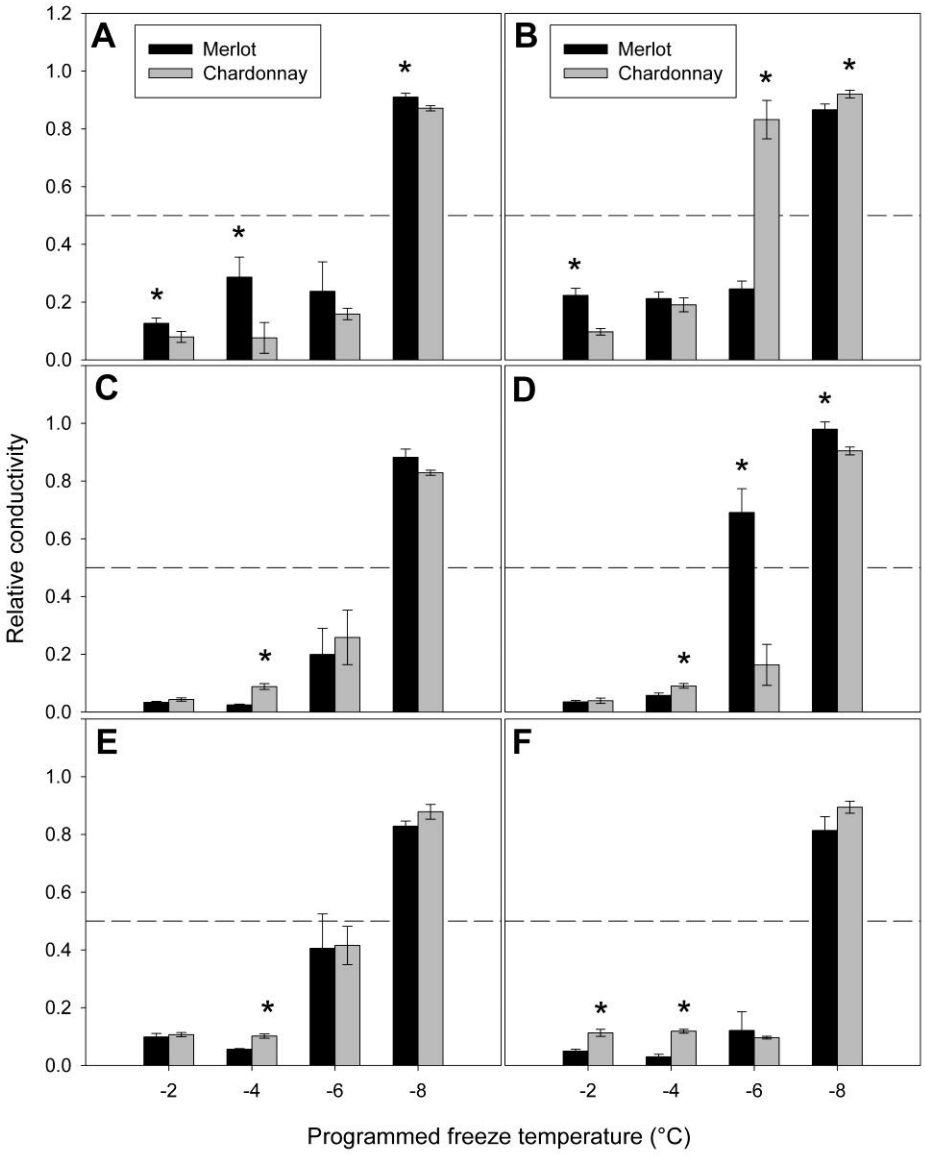

Figure 4 Mean relative conductivity following preconditioning for Merlot and Chardonnay in 2014 (**A**, **C**, **E**) and 2015 (**B**, **D**, **F**). Preconditioning regimes: (**A**, **B**) active growth at ambient outdoor temperatures; (**C**, **D**) dormant vines at 12°C for a minimum of 1 wk; and (**E**, **F**) dormant vines at 0°C for a minimum of 1 wk. 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.

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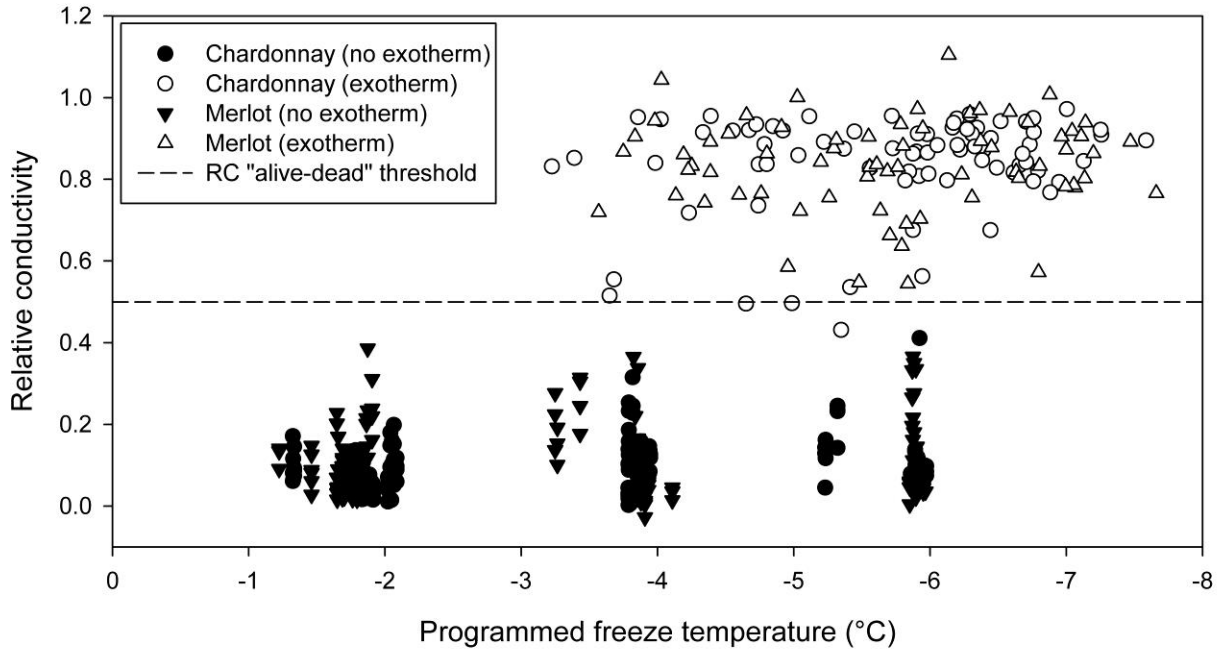

Figure 5 Relationship between relative conductivity of root tissue and the incidence of low temperature exotherms (LTE). Relative conductivity values from electrolyte leakage were compared to whether or not differential thermal analysis indicated samples were undamaged (closed symbols; no LTE) or lethally damaged (open symbols; LTE) during programed 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. Relative conductivity 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 1 exception.

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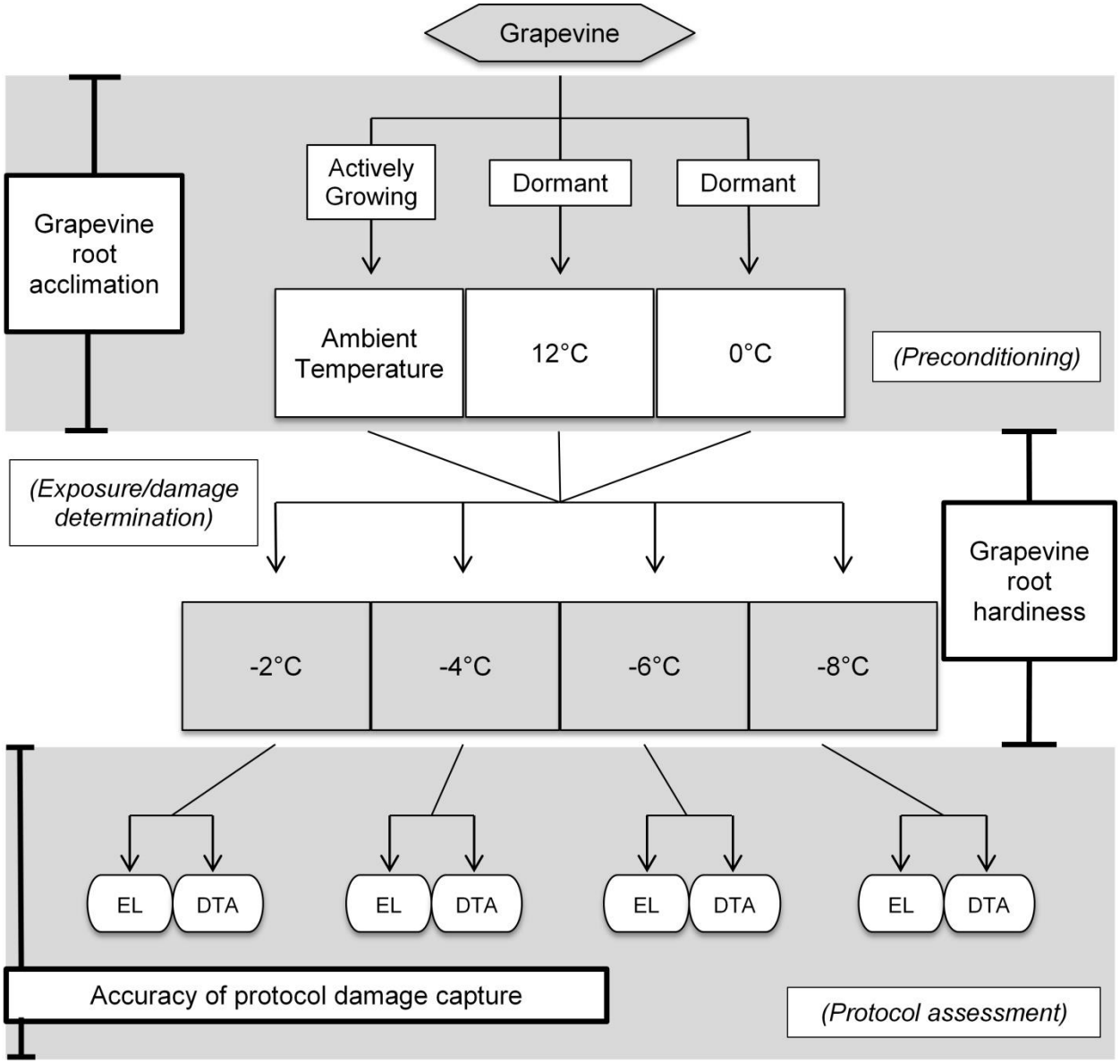

Supplemental Figure 1 Schematic of experimental protocols. Overview of the experimental process involving the pretreatments of grapevine material, freeze protocol temperatures, and the use of electrolyte leakage (EL) and differential thermal analysis (DTA) for assessing grapevine root viability. The chart is further subdivided into shaded areas that highlight which treatment addressed which unknown.