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Seasonal Variation in Freezing Tolerance and Dehydrin Protein Expression in Canes of Frontenac and Seyval blanc Grapevine Cultivars

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Abstract

The cold hardiness of primary buds of grapevine cultivars (*Vitis* sp.) is typically used as a principal indicator of grapevine cold hardiness; however, freezing tolerance of cane tissues has received little attention. Our investigation characterized freezing tolerance and protein profiles (including dehydrins) of grapevine cultivars that are very hardy (Frontenac) and moderately hardy (Seyval blanc) during the autumnal cold acclimation, mid-winter, and vernal deacclimation period (20 Aug to 22 Apr) in a research vineyard. Cane samples collected in the field (a total of 15 sampling dates with ~2 samples/month) were subjected to controlled laboratory freezing tests to assess the freezing tolerance of bark + vascular cambium and xylem + pith tissues. SDS-PAGE profiles and immunoblots were used to determine qualitative and quantitative changes in proteins, including dehydrins. During early autumn (20 Aug to 28 Oct), an increase in bark + vascular cambium and xylem + pith freezing tolerance of the two cultivars was similar (by about 12 °C relative to the starting date). 'Frontenac' bark + vascular cambium appears more responsive to colder/freezing temperatures for cold acclimation during mid-winter (28 Oct to 5 Jan) than 'Seyval blanc', and at the peak hardiness, it was about 15 °C more freeze tolerant. For xylem + pith tissues of both cultivars, the kinetics of cold acclimation and differences in cultivars' peak freezing tolerance were similar to those for bark + vascular cambium tissues. The bark + vascular cambium and xylem + pith of both cultivars deacclimated between 15 Jan and 23 Mar, with 'Seyval blanc' losing ~3 to 6 °C more in hardiness than 'Frontenac'. Both cultivars exhibited reacclimation following deacclimation in response to freeze events of late March to early April, whereby 'Seyval blanc' showed a greater reacclimation capacity. Polypeptides in bark + vascular cambium and xylem + pith of 'Seyval blanc' (23kDa and 17 kDa) and 'Frontenac' (23 kDa, 19kDa, and 17 kDa) accumulated during cold acclimation, followed by reduced abundance with deacclimation. A ~13 kDa dehydrin protein in the bark + vascular cambium of both cultivars exhibited a similar accumulation pattern and was not detected in xylem + pith tissues.

Introduction

Increased interest in grape and wine production in northern climates has necessitated the development of cultivars derived from *Vitis riparia* Michaux that are adapted to cold climates. Severe cold temperatures and extreme temperature fluctuation in the winter months pose a risk of freeze damage to grapevines grown in cold-climate regions. The ability of a plant to efficiently acclimate in the fall and reacclimate upon the return of cold after a transient warm spell is an adaptive attribute for plant survival. Grapevine cultivars are selected for their ability to withstand freezing temperatures, a critical trait for overwinter survival that ensures both crop productivity and vineyard profitability.

Screening for freezing tolerance of new cultivars is typically conducted by examining primary bud survival after exposure to the lowest temperature

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expected in a region (Cragin et al. 2017; Hébert-Haché et al. 2023; Londo and Kovaleski 2017; Slater et al. 1991). Freezing tolerance of the rest of the vine has received relatively little attention, although many interspecific cultivars will produce a crop from secondary buds if primary buds are injured (Zabadal et al. 2007). Functionality of stem/cane tissues, i.e., phloem, cambium, and xylem, is crucial for the restart of the vegetative cycle; healthy and functional phloem is necessary for the transport of carbohydrate reserves for budbreak and similarly xylem for hydraulic conductivity. Cane tissues are also essential to support secondary buds (Slater et al. 1991) in case primary buds are irreversibly injured, and such production from surviving secondary buds could be limited if vascular tissues are injured. However, reports on freeze-damage of stem/cane damage in grapes are scarce (Gonzalez Antivilo et al. 2020).

Freezing injury typically results from severe dehydration stress caused by the formation of extracellular ice within tissues (Arora 2018). Dehydrins are proteins found in many woody plants, including *Vitis* sp. (Hébert-Haché et al. 2023; Salzman et al. 1996; Xiao and Nassuth 2006), and are associated with some protection from cellular desiccation (Campbell and Close 1997; Hughes and Graether 2011; Peng et al. 2008). Differences in dehydrin gene/protein expression across cultivars and tissue types in grapevine have been reported, along with their association with freezing tolerance (Hébert-Haché et al. 2023; Rubio et al. 2016; Xiao and Nassuth 2006).

This study aimed to characterize the freezing tolerance of two grapevine cultivars, Frontenac (very hardy) and Seyval blanc (moderately hardy), by examining bark with vascular cambium and xylem with pith tissues during autumnal acclimation, midwinter, and vernal deacclimation. It also investigated dehydrin protein expression as potential biochemical markers of freezing tolerance to support grapevine breeding programs.

Materials and Methods

Plant materials. The study used six-year-old grapevines of ‘Seyval blanc’ (syn. Seyve-Villard 5-276) and ‘Frontenac’ established in a vineyard at the Iowa State University Horticulture Research Station (HRS) near Ames, IA (lat. 42º06’41”N, long. 93°35’19”W), which has a continental climate. The research vineyard encompassing 0.69 ha was characterized predominantly by Clarion loam soil, classified as mixed, superactive, mesic Typic Hapludolls, occurring on slopes ranging from 0 to 9% (NRCS, 2009). Vines were spaced 2.6 × 3.3 m apart and trained to a bilateral high cordon system that accommodated the procumbent growth of ‘Frontenac’ vines and a mid-wire cordon system with catch wires to accommodate upright growth of ‘Seyval blanc’ vines. Cultivars were randomly arranged in the vineyard as three plants per panel in each of 15 rows planted in a north-south orientation.

Sampling. Ten to 12 canes per cultivar were harvested from ten vines that were randomly selected on each of 15 sampling dates between August 2008 and April 2009. Specific sampling dates were 20 Aug, 9 Sept, 30 Sept, 16 Oct, 28 Oct, 11 Nov, 5 Dec, 15 Dec, and 23 Dec in 2008, and 5 Jan, 15 Jan, 18 Feb, 23 Mar, 9 Apr, and 22 Apr in 2009. By August 20, the season’s shoots began changing from green to a brown color as a protective periderm developed. Sampling dates were determined based on the variation between the daily high (of the date prior) and low air temperatures. Variation in temperature of ± 5 °C determined the sampling dates, and sampling occurred monthly. Samples were 30-cm basal sections of canes from the current growing season, averaging 0.76 ± 0.3 cm in diameter. Plant samples were collected between 8:00 and 9:00 AM each day of sampling, transported to the laboratory in insulated chests with ambient field air, and held in the laboratory at the same air temperature ± 2 °C as occurred in the vineyard at the time of sampling until processed. Daily high and low air temperatures were recorded from a remote weather monitoring station (HMP45, Vaisala, Minneapolis, MN, USA) located at HRS.

Freezing. Two chambers were used to freeze the 2.5 cm long internodal segments of canes (divided at 4 °C) as per the requirement of coldest target treatment temperature. An Isotemp 3028 refrigerated glycol bath (Fisher Scientific, Pittsburgh, PA, USA) was used when the coldest treatment temperature did not exceed -20 °C, the operational limit of this equipment. Scientemp programmable chest-freezer (Scientemp, Adrian, MI, USA) can achieve temperatures to -80 °C and was used for freezing canes to -60 °C. For freezing in a glycol bath, randomly selected segments were individually placed in 75-mL test tubes containing 150 µL of deionized water to initiate ice nucleation, which was confirmed by an exothermic event detected by thermocouples attached to the reference samples. Shoots or canes used for freezing in the Scientemp programmable chest freezer were prepared according to Kalberer et al. (2007), where paper tissues wetted with deionized water were wrapped at the base of shoot or cane segments to facilitate ice nucleation and then sealed in polyethylene bags (7.5 × 12.5 cm).

Each segment was cooled to a set minimum temperature, ranging between -1 and -60 °C. Freezing tests were carried out in a glycol bath until October and in the Scientemp freezer between November through the following April, when the Isotemp glycol bath could no longer reach the required temperature for 100% tissue mortality. Freezing protocol was similar to that described by Kalberer et al. (2007) and Lim et al. (1998), ensuring relatively slow cooling and thawing. Four replications for each treatment temperature were tested. Temperatures were lowered at a rate of 1 ºC h^{-1} for the first 6 h, 2 ºC h^{-1} for the next 2 h, 3 ºC h^{-1} for the following 4 h, and 4 ºC h^{-1} for the remainder of the test, such that the cooling rate was gradually increased as the treatment temperatures became progressively colder. Test tubes were removed after exposure to their respective treatment temperature and thawed on ice overnight. The following morning, test tubes were removed from ice and held at 4 ºC for 2 h to complete the thawing of tissues. Finally, tissues were incubated at 22 ± 2 °C for 3 d to develop any injury symptoms.

Injury evaluation. Cane segments were individually sectioned transversally and longitudinally to examine the percentage of browning of tissues visually. Bark with vascular cambium tissues (bark + vascular cambium) included all layers of periderm, phloem, cork cambium, and the vascular cambium layer. Xylem with pith tissues (xylem + pith) included all xylem and pith of the cane segment. A dissecting microscope was used to rate injury on a five-point scale with increments at 0, 25, 50, 75, and 100% (Ashworth et al. 1983; Lim et al. 1998; Warmund et al. 1986). Injury percentages at each treatment temperature were averaged over four replications. The resultant averages were fitted with a sigmoidal Gompertz function (Lim et al., 1998) using R 2.9.2 (R Development Core Team, 2009). The freezing tolerance, defined as LT_{50}, the temperature resulting in 50% injury for bark + vascular cambium and xylem + pith tissues for both cultivars at each sampling date, was obtained from the corresponding Gompertz function. Differences in LT_{50} values were evaluated between tissue types and cultivars at the same sampling date by overlapping confidence intervals ($\alpha \leq 0.05$) adjusted using Bonferroni's correction for multiple comparisons (R Development Core Team 2009).

Protein extraction and separation. Subsamples of tissues were randomly obtained for protein extraction from the same pool of cane segments as used for freezing tests. Bark + vascular cambium and xylem + pith tissue samples were separated from cane segments with a razor blade, ground in liquid nitrogen, and stored at -80 ºC immediately. Proteins were extracted using a sodium borate extraction method (Wetzel et al. 1989), modified by Arora et al. (1992). Protein concentrations were estimated as stated by Esen (1980).

Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Arora et al. (1992) on 4% stacking and 12.5% resolving gel. Ten µg or 6 µg of bark + vascular cambium or xylem proteins, respectively, were loaded in each lane. The gels were stained with Coomassie Brilliant Blue G-250 (Sigma, St. Louis, MO) in methanol and destained in 25% methanol. Molecular weights of protein bands were determined in reference to the distance traveled by molecular weight standards (BioRad, Hercules, CA).

Dehydrin immunoblotting. Proteins (2.0 or 2.5 µg) were transferred from SDS-PAGE gels to a 0.45 µm nitrocellulose membrane, according to a method of Arora and Wisniewski (1994). Membranes were then probed with a 1:1500 dilution of the primary dehydrin antibody (AbCam, Cambridge, MA, USA) and immunoreactive bands were detected using an anti-rabbit alkaline phosphatase conjugate with a BCIP/NBT color development kit (Promega, Madison, WI, USA). Dehydrin protein detection was confirmed by comparison to parallel blots of preimmune serum (AbCam, Cambridge, MA, USA).

Results

Freezing tolerance. Daily mean maximum and minimum temperatures between August and the following April are reported in Fig. 1. Freezing tolerance (LT_{50} values) of bark + vascular cambium and xylem + pith tissues for both cultivars at each of the 15 sampling dates are shown in Figs. 2 A–D. Cold acclimation (seasonal induction of freezing tolerance) in both cultivars commenced soon after the 20 Sept sampling and progressively continued until 5 Dec sampling. A slight reduction in freezing tolerance for both tissues of both cultivars was noticeable on 15 Dec, after which further cold acclimation resumed until 15 Jan. From February sampling onward, a progressive reduction in freezing tolerance, i.e., deacclimation, was observed for both cultivars until 23 Mar, whereafter a transient increase in freezing tolerance (reacclimation) was observed for 9 Apr samples. Deacclimation resumed, thereafter, until the final sampling on 22 Apr. Maximum freezing tolerance for each cultivar and tissue type was observed after vines were exposed to temperatures as low as -25 °C on 23 Dec in the vineyard (Fig. 1). Between 18 Feb and 22 Apr, the frequency of air temperatures above 0 °C increased except for freezing events between 23 Mar and 9 Apr during which temperatures as low as -5 °C were recorded (Fig. 1).

Bark + Vascular Cambium. During the early phase of cold acclimation (20 Aug to 28 Oct), freezing tolerance increased by ~12 °C for both 'Frontenac' and 'Seyval blanc', i.e., from about -3 °C to -15 °C, respectively. Periderm development on canes was 0.52% and 0.2% for 'Frontenac' and 'Seyval blanc', respectively, on 20 Aug, followed by a progressive increase, reaching 40.5% and 84.9%, respectively, by Oct 28. However, after the first severe freeze in the vineyard on 27 Oct when the temperature dropped to -5.6 °C, 'Frontenac' began to gain more freezing tolerance than 'Seyval blanc', reaching approximately -26 °C by 11 Nov compared to -14°C for 'Seyval blanc' (Figs. 2A and C). During the later cold acclimation period, the bark + vascular cambium of 'Frontenac' consistently showed greater freezing tolerance than for 'Seyval blanc'. The largest difference occurred on 5 Jan when both cultivars reached their maximum freezing tolerances: -49 °C for Frontenac and -34 °C for Seyval blanc (Figs. 2A and C). As temperatures warmed, both cultivars began to deacclimate. By March 23, freezing tolerance

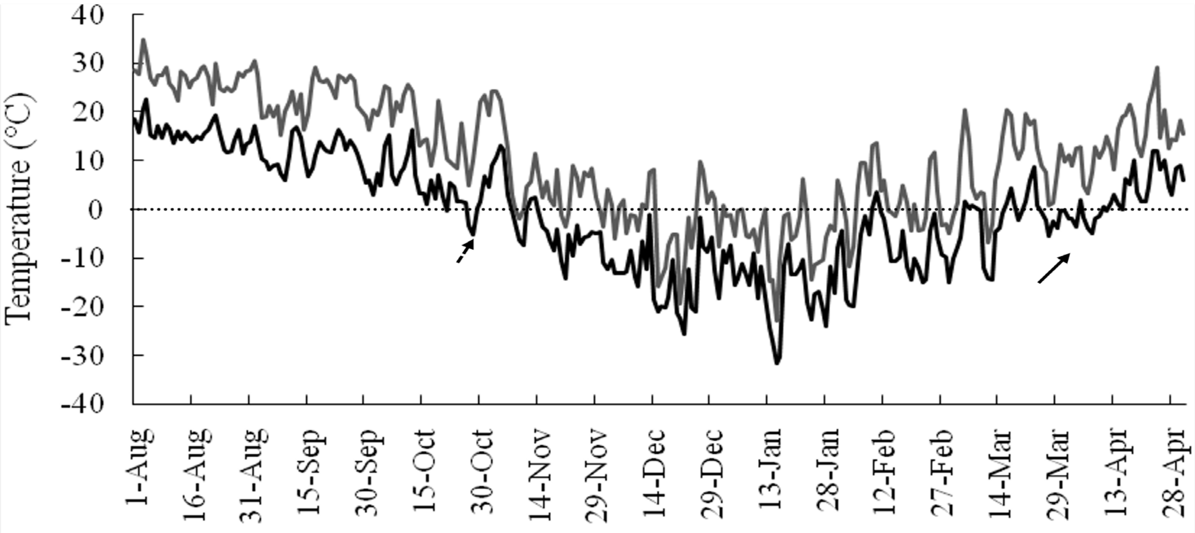

Fig. 1. Daily mean of high and low air temperatures recorded between Aug 2008 and Apr 2009 in a research grape vineyard, Ames, Iowa. A dashed line represents 0 °C.

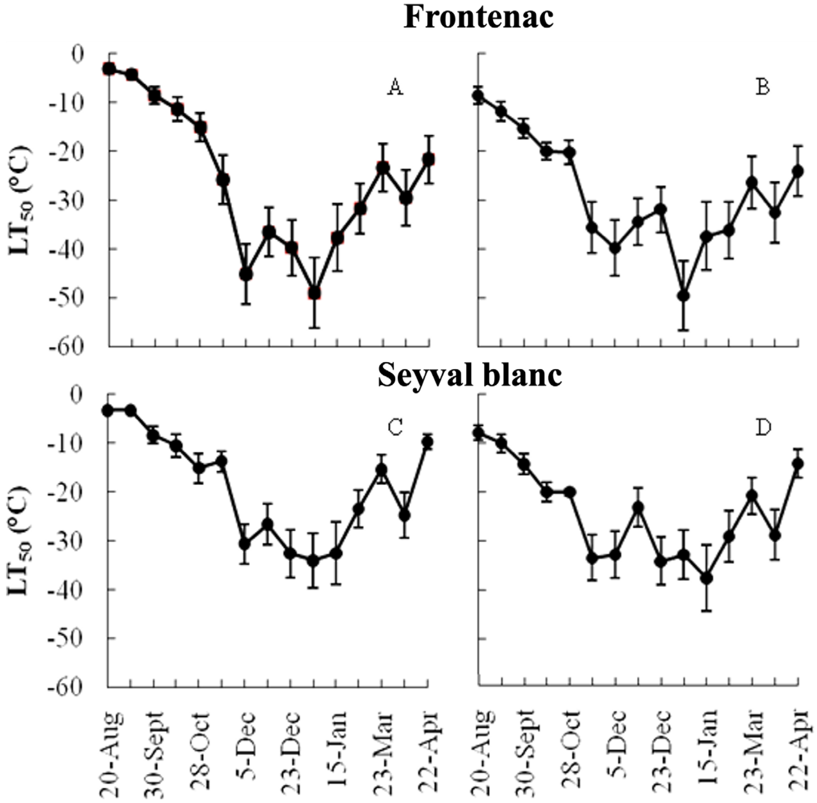

Fig. 2. LT$_{50}$ values (means ± standard error) for grapevine cane tissues of 'Frontenac' bark + vascular cambium (A) and xylem + pith (B), and 'Seyval blanc' bark + vascular cambium (C) and xylem + pith (D). Fifteen sampling dates were 20 Aug, 9 Sept, 30 Sept, 16 Oct, 28 Oct, 11 Nov, 5 Dec, 15 Dec, 23 Dec, in 2008, and 5 Jan, 15 Jan, 18 Feb, 23 Mar, 9 Apr, 22 Apr, in 2009.

had declined to about -23°C for 'Frontenac' and -15 °C for 'Seyval blanc'. Relative to their peak freezing tolerance, 'Seyval blanc' bark + vascular cambium tissues deacclimated about 1.4 times more than 'Frontenac' (Figs. 2A and C). Following the freezing events between 23 Mar to 9 Apr. (Fig. 1), both cultivars showed reacclimation. 'Seyval blanc' bark + vascular cambium regained about 9 °C (-24 °C compared to -15 °C), and 'Frontenac' gained about 6 °C -29 °C compared to -23 °C (Figs. 2A and C).

Xylem + Pith. Between 20 Aug to 28 Oct, freezing tolerance of xylem+ pith increased in both 'Frontenac' and 'Seyval blanc', from about -8 °C to -20 °C. By 5 Jan, maximum freezing tolerances were reached at about -49 °C for 'Frontenac' and -33 °C for 'Seyval blanc' (Figs. 2B and D). From 18 Feb onward, both cultivars began to deacclimate, and by 22 Apr, their tolerances had declined to approximately -24 °C for 'Frontenac' and -14 °C for 'Seyval blanc' (Figs. 2B and D). During the deacclimation period, both cultivars showed a brief reacclimation following freezing events of 23 Mar to 9 Apr (Fig. 1). Freezing tolerance in 'Frontenac' increased from about -26 °C on 23 Mar to -32 °C on 9 Apr (a 6 °C gain), while 'Seyval blanc' increased from about -21 °C to -29°C (an 8 °C gain; Figs. 2B and D).

SDS Page: Bark + Vascular Cambium. Protein profiles for 'Frontenac' and 'Seyval blanc' are presented in Figs. 3A and B, respectively. Both cultivars exhibited an incremental abundance of 23 kDa polypeptide during cold acclimation from October onwards, reaching its apparent maximum by February in 'Frontenac' (Fig. 3A) and by January in 'Seyval blanc' (Fig. 3B). Its abundance decreased thereafter by March sampling. A similar accumulation and reduction pattern was observed in 'Frontenac' for a 19 kDa polypeptide, which was not detected in 'Seyval blanc'. A 17-kDa band was detected in both 'Frontenac' and 'Seyval blanc' bark + vascular cambium,

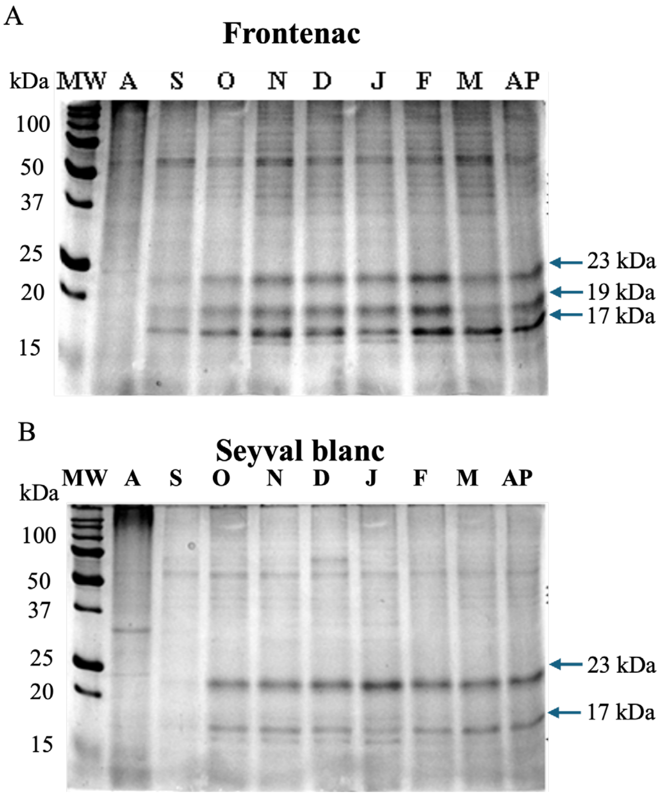

Fig. 3. SDS-PAGE profiles of proteins extracted from bark + vascular cambium tissues of cane samples from 'Frontenac' (A) and 'Seyval blanc' (B) grapevines. MW = molecular weight, A = August, S = September, O = October, N = November, D = December, J = January, F = February, M = March, AP = April.

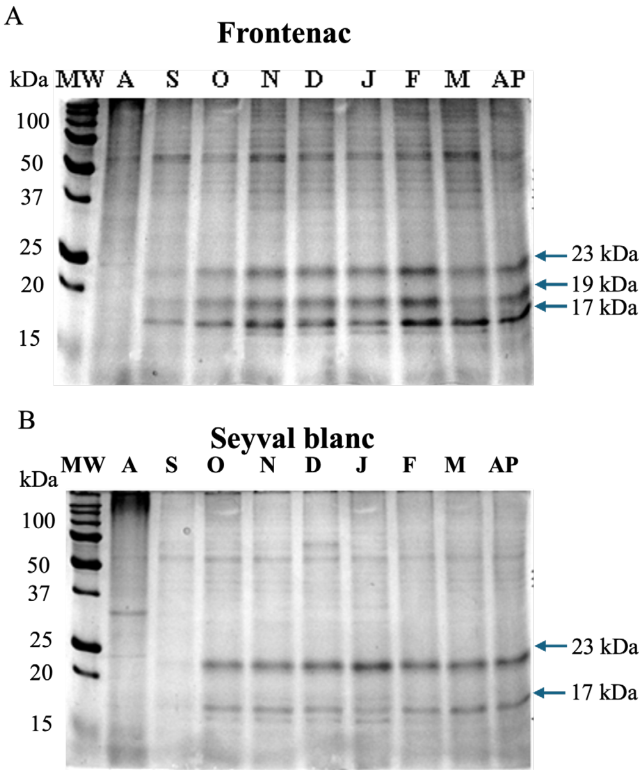

Fig. 4. SDS-PAGE profiles of proteins extracted from xylem + pith tissues of cane samples from 'Frontenac' (A) and 'Seyval blanc' (B) grapevines. MW = molecular weight, A = August, S = September, O = October, N = November, D = December, J = January, F = February, M = March, AP = April.

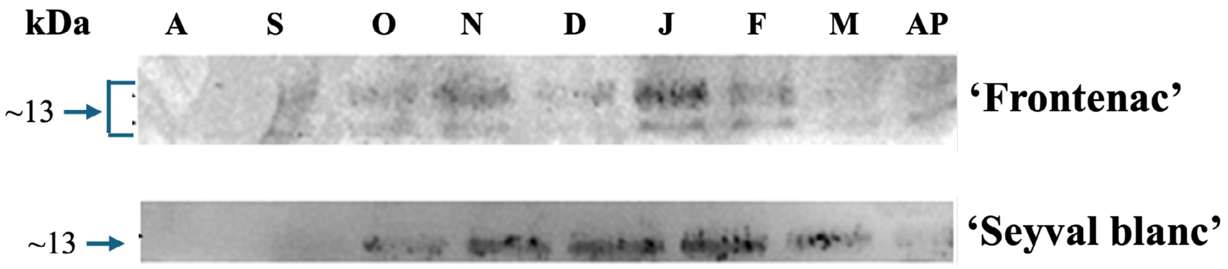

Fig. 5. Anti-dehydrin immunoblots for 'Frontenac' and 'Seyval blanc' bark + vascular cambium tissues. A = August, S = September, O = October, N = November, D = December, J = January, F = February, M = March, AP = April.

displaying a pattern similar as the other two polypeptides. It was more abundant in 'Frontenac' profiles.

SDS Page: Xylem + Pith. Protein profiles from 'Frontenac' and 'Seyval blanc' are presented in Figs. 4A and B, respectively. Overall, these profiles were qualitatively and pattern-wise similar to those of bark + vascular cambium tissues, but, visually, the band intensities of these polypeptides appear more intense than in bark + vascular cambium samples.

Immunoblots. Anti-dehydrin immunoblots are shown for corresponding SDS-PAGE profiles in Fig. 5. Dehydrin-like protein bands of ~ 13kDa were observed in bark + vascular cambium tissues of both 'Frontenac' (twin bands) and 'Seyval blanc'; no such immunoreactive band was detected in xylem + pith tissues. Accumulation pattern (appearance, high abundance, and then disappearance) of this dehydrin paralleled seasonal cold acclimation and deacclimation. None of these dehydrin-like proteins was visible in parallel immunoblots with preimmune serum (data not shown).

Discussion

Our study investigated indicators of grapevine freezing tolerance beyond primary bud cold hardiness. Specifically, we compared the freezing tolerance of bark + vascular cambium tissues to that of xylem + pith tissues, both within and between two cultivars: Frontenac, a highly cold-hardy cultivar, and Seyval blanc, a moderately hardy cultivar. These comparisons of tissues were conducted across seasonal phases of cold acclimation and deacclimation.

Seasonal cold acclimation. Between 20 Aug and 28 Oct, shoot and cane tissues of both cultivars exhibited a small but significant cold acclimation. During this period, mean daily maximum temperatures ranged from 13.7 to 27 °C, while mean minimum temperatures were 3.7 to 15 °C. This period was also devoid of any significant freeze event; the first hard freeze was not detected until the night of 28 Oct (Fig. 1). By 28 Oct, freezing tolerance of bark + vascular cambium and xylem, + pith was almost identical in both cultivars, at about -15 °C and -20 °C, for 'Frontenac' and 'Seyval blanc', respectively (Fig. 2). This early acclimation occurring without significant cold exposure is noteworthy because *V. riparia*, a parent species of 'Frontenac', is valued by grapevine breeders for its excellent cold hardiness (Hemstad and Luby 2000). The native range of *V. riparia* extends into Canada and has been recorded as cold hardy to about -40 ºC (Pierquet and Stushnoff 1980). It was demonstrated that *V. riparia* buds exhibited some degree of cold acclimation under short photoperiods even before the onset of cold/freezing temperatures in the fall (Fennell and Hoover, 1991; Fennell and Mathiason, 2002). This finding suggests that *V. riparia* undergoes the "first stage of cold acclimation", a process characteristic of woody perennials in temperate zones (Arora et al. 1992). Our data substantiates this observation in that the LT_{50} of bark + vascular cambium and xylem + pith changed from about -3 °C and -8 °C on 20 Aug to about -15 °C and -20 °C by 28 Oct, respectively. The day length on 20 Aug was 13.7 h, and on 28 Oct, it was 10.5 h. This "first-stage of cold acclimation" during early fall is typically a gradual process. On the other hand, shoots or canes of 'Seyval blanc', which has a parentage that includes *V. vinifera* L., *V. rupestris* Scheele, and *V. lincecumii* Buckley (University of California, Davis 2025), are not as cold hardy as *V. riparia*, but had similar freezing tolerance as 'Frontenac' during 20 Aug to 28 Oct. Unlike *V. riparia, V. vinifera*, one of the parent species of 'Seyval blanc' used in our study, showed no increase in freezing tolerance under short days (Schnabel and Wample 1987). However, both short days and cooler temperatures, working in synergy, induced cold acclimation in *V. vinifera* 'White Riesling' (Schnabel and Wample 1987). To our knowledge, no reports exist on cold acclimation induced by short days in either *V. rupestris* or *V. lincecumii*, the other two parent species of 'Seyval blanc'. Based on the above discussion, we had expected 'Frontenac' to have acquired greater freezing tolerance than 'Seyval blanc' during 20 Aug to 28 Oct. But they both acclimated to a similar extent during this period. The precise nature of the environmental cue inducing the observed early phase of cold acclimation, as observed during 20 Aug and 28 Oct in 'Seyval blanc', is unclear at present and warrants further investigation.

'Frontenac' appears to be more responsive to colder/freezing temperatures for the 'second stage of cold acclimation' than 'Seyval blanc' as indicated by an overall greater freezing tolerance of 'Frontenac' from 28 Oct to 5 Jan, a period when freezing events in the field became more regular; at the peak cold acclimation, 'Frontenac' bark + vascular cambium and xylem + pith cane tissues were about 15 °C more freeze-tolerant than 'Seyval blanc' counterparts (Fig. 2). This "second stage of cold acclimation" in late fall/early winter appears more accelerated than that in early fall.

While freezing tolerance of 'Frontenac' xylem + pith on 20 Aug, 9 Sept, 30 Sept, 16 Oct, 28 Oct, and 11 Nov was about 5 to 7 °C greater than the bark + vascular cambium tissues, this comparison was reversed for 5 Dec, 15 Dec, and 23 Dec samples when bark + vascular cambium tissues were hardier than the xylem + pith; essentially no difference in freezing tolerance of bark + vascular cambium and xylem + pith tissues was detected for 5 Jan and 15 Jan. In contrast, xylem + pith tissues of 'Seyval blanc' canes, in general, had greater freezing tolerance than bark + vascular cambium tissues throughout the cold acclimation period (Fig. 2). Our observations are not entirely consistent with a report by Slater et al. (1991), who noted bark to be, typically, less freezing tolerant than xylem. However, our data on greater freezing tolerance of the bark + vascular cambium over xylem + pith during the mid-winter period (Dec samples) were similar to results found in deciduous peach (*Prunus persica* [(L.) Batsch]; Arora et al. 1992) and other woody perennials (Ashworth and Wisniewski 1991; Malone and Ashworth 1991; Mathers 2004). We are not aware of any previous study on the comparison of seasonal cold acclimation in *Vitis* bark, vascular cambium, xylem, and pith tissues. Differential cold acclimation as noted above and the deacclimation behavior (discussed below) by the two tissues may be associated with strategies of bark and xylem to survive freezing temperatures. Living xylem tissues of several woody species, including grapes, survive cold winters by deep supercooling, a freezing-avoidance process in which cellular water is isolated from the dehydrative and nucleating effects of extracellular ice; in contrast, bark tissues survive freezing by tolerating freeze-desiccation (Wisniewski and Arora, 1992).

Seasonal Deacclimation. The chilling requirement must be met to allow the grapevine to resume growth, provided the temperatures are appropriate. Occasionally, temperatures may rise above 4 °C in January in some climates, only to decline again. These "winter thaws" can cause severe injury if chilling requirements have been met (buds are no longer endodormant) and the grapevine begins to deacclimate (Londo and Kovaleski, 2017). Chilling requirements for many interspecific cultivars are largely unknown and were not determined for the two cultivars in the present study.

'Frontenac' bark + vascular cambium and xylem + pith tissues deacclimated by ~14 °C and 11°C (loss of ~37% and 29%), respectively, between 15 Jan and 23 Mar 2009 while both bark + vascular cambium and xylem + pith of 'Seyval blanc' deacclimated by about 17 °C (loss of ~52% and 45%), respectively, indicating a relatively greater deacclimation by 'Seyval blanc'. Deacclimation rates (loss of freezing tolerance per day) further indicated 'Seyval blanc' to have deacclimated slightly faster (0.25 °C / day for both bark + vascular cambium and xylem + pith) than 'Frontenac', which deacclimated at 0.16 °C / day (xylem + pith) and 0.2 °C /day (bark + vascular cambium). The parentage of *V. riparia* in 'Frontenac' lends to a higher likelihood of greater chilling requirement and later exit from endodormancy compared with 'Seyval blanc', which possesses a lineage of *V. vinifera* and other American *Vitis* species that are generally less freezing tolerant than *V. riparia*.

Our data showed that both cultivars reacclimated to some extent in response to freezing events during late March and mid-April (Fig. 1). 'Seyval blanc' bark + vascular cambium reacclimated by ~ 9 °C (58%) and xylem + pith reacclimated by 8 °C (39%), while bark + vascular cambium and xylem + pith of 'Frontenac' reacclimated by ~ 6 °C (26% and 22%, respectively; Fig. 2). Kalberer et al. (2006) found that deciduous azalea buds were unable to reharden until sufficient deacclimation had occurred and suggested that the capacity for rehardening in azalea was influenced by a threshold level of dehardening, for which the cellular/molecular explanation is unknown. 'Seyval blanc' deacclimated more within the same time span compared with 'Frontenac' in our study. We suggest that, as in the mentioned azalea study, 'Seyval blanc' may have attained the requisite "threshold" seasonal deacclimation (higher loss than 'Frontenac'), thus allowing for a greater reacclimation capacity than 'Frontenac' during the same time.

Protein Profiles and Dehydrins. Seasonal protein profiles of bark + vascular cambium and xylem + pith of 'Frontenac' and 'Seyval blanc' grapevines during cold acclimation and deacclimation are presented in figures 3 and 4. The SDS-PAGE indicates a substantial accumulation of three major polypeptides, ~23, 19, and 17 kDa during cold acclimation (August to January/February) followed by relatively lower band intensities coincident with deacclimation (March/April) in 'Frontenac' bark + vascular cambium tissues (Fig. 3A). 'Seyval blanc' bark + vascular cambium proteins exhibited similar seasonality except that the 19 kDa protein was not detected (Fig. 3B). Similar patterns were also noted for the xylem + pith tissues, except that the protein bands were apparent at relatively higher intensities (Figs. 4A and B). Numerous electrophoretic studies have investigated the qualitative and quantitative changes in proteins during seasonal cold acclimation and deacclimation in both herbaceous and woody species (Arora et al. 1992; Guy 1990; Kang and Titus 1987). In general, most studies have reported similar quantitative seasonality as in the present study, while qualitative differences are diverse. Such protein changes are believed to be associated with the adjustment of metabolism to cold temperatures and/or for inducing actual freezing tolerance. In woody perennials, including fruit trees, bark + vascular cambium storage proteins that accumulate during fall and winter have been ascribed functional roles (Arora et al. 1996; O'Kennedy and Titus 1979; Wetzel et al. 1989). Some of these proteins may be associated with dormancy transitions in woody perennials, which overlap with seasonal cold acclimation and deacclimation cycling (Arora et al., 1997, 2003). However, the characterization and functional roles of the three grapevine proteins observed in the present study were not examined.

Immunoblotting was performed to identify a specific protein class, dehydrins, and their seasonality in bark + vascular cambium and xylem + pith of two cultivars. Our data indicate an accumulation of ~13 kDa dehydrin in the bark + vascular cambium tissues of both cultivars during cold acclimation, followed by its reduced intensity during deacclimation (Fig. 5). Unlike 'Seyval blanc', two, not one, closely placed dehydrin bands at ~ 13 kDa were evident in 'Frontenac' bark + vascular cambium tissues. No dehydrin could be detected in the xylem + pith tissues of these cultivars. Dehydrins are highly hydrophilic proteins that accumulate in plants when exposed to stresses that induce cellular dehydration (Campbell and Close, 1997). Winter freezes typically involve the formation of ice in between cells, resulting in cellular desiccation due to exosmosis to extracellular ice via a vapor pressure gradient (Arora 2018). Therefore, seasonal cold acclimation, a multifactorial process, is expected to involve dehydration tolerance as one of the components. Dehydrin accumulation may be a strategy to protect cell membranes and macromolecules from acute desiccation due to their hydrophilicity. Many reports have previously linked dehydrin abundance to freezing tolerance (Hughes and Graether 2011; Kosová et al. 2007 and references therein). Several groups have reported the presence of dehydrins in grapevine buds, including their association with cold acclimation, cold exposure, or in vitro cryoprotection of proteins (Hughes and Graether 2011; Rubio et al., 2016; Salzmann et al., 1996; Xia and Nassuth, 2006; Yang et al., 2012). Hébert-Haché et al. (2023) reported that the relative band intensities of five dehydrins, ranging from 26 to 90 kDa, were positively correlated (quantitatively) with the freezing tolerance of Sauvignon blanc grapevine buds, a *V. vinifera* cultivar. However, their study did not provide data for stem tissues. Similarly, Xian and Nassuth (2006) identified five dehydrin genes within the *V. vinifera* genome. The predicted masses for these putative dehydrins are 13.2, 13.9, 18.9, 20.1, and 23.5 kDa, with the first two, *VvDHN1a* and *VvDHN1b*, being highly homologous to each other. No comparable data are currently available for *V. riparia*, *V. rupestris*, or *V. lincecumii*. Nevertheless, assuming a high homology among dehydrins across grape species, it is reasonable to infer that the 13 kDa dehydrin identified in the present study may be encoded by a gene similar to *VvDHN1*.

Conclusions

The purpose of this study was to characterize the freezing tolerance of bark + vascular cambium and xylem + pith tissues of two grape cultivars with different genetic pedigrees. In early autumn, 'Frontenac' (very hardy) and 'Seyval blanc' (moderately hardy) exhibited similar increases in the freezing tolerance of bark + vascular cambium and xylem + pith. By midwinter, 'Frontenac' bark + vascular cambium demonstrated enhanced cold acclimation relative to 'Seyval blanc', achieving approximately 15 °C higher freeze tolerance at peak hardiness. Xylem + pith tissues in both cultivars had a comparable cold acclimation pattern to bark + vascular cambium in terms of cold acclimation. 'Seyval blanc' exhibited a greater degree and rate of deacclimation in both bark + vascular cambium and xylem + pith compared to 'Frontenac'. Both cultivars showed evidence of reacclimation in response to spring freeze events. Relatively faster cold acclimation, slower deacclimation under sudden warm spells, and efficient reacclimation upon the return of freezing temperatures are desirable traits for breeding programs aimed at developing climate-resilient grapevines. Protein analysis showed the accumulation of 23 kDa and 17 kDa polypeptides in 'Seyval blanc', and 23 kDa, 17 kDa, and 19 kDa polypeptides in 'Frontenac' bark + vascular cambium during cold acclimation, with levels declining during deacclimation. An approximate13 kDa dehydrin protein, a potential biochemical indicator of cold hardiness, was detected in the bark + vascular cambium of both cultivars, exhibiting a similar accumulation pattern, but was undetected in the xylem + pith tissues.

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