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Cold hardiness of white spruce, black spruce, jack pine, and lodgepole pine needles during
dehardening

Rongzhou Man¹, Pengxin Lu¹, and Qing-Lai Dang²

¹Ontario Ministry of Natural Resources and Forestry, Ontario Forest Research Institute, 1235
Queen Street East, Sault Ste. Marie, ON, P6A 2E5 Canada rongzhou.man@ontario.ca;
pengxin.lu@ontario.ca

²Faculty of Natural Resources Management, Lakehead University, Thunder Bay, ON P7B 5E1
qdang@lakeheadu.ca

Abstract Conifer winter damage results primarily from loss of cold hardiness during unseasonably warm days in late winter and early spring and such damage may increase in frequency and severity under a warming climate. In this study, the dehardening dynamics of lodgepole pine (*Pinus contorta* Dougl. ex. Loud), jack pine (*Pinus banksiana* Lamb.), white spruce (*Picea glauca* (Moench) Voss), and black spruce (*Picea mariana* (Mill.) B.S.P.) were examined in relation to thermal accumulation during artificial dehardening in winter (December) and spring (March) using relative electrolyte leakage and visual assessment of pine needles and spruce shoots. Results indicated that all 4 species dehardened at a similar rate and to a similar extent, despite considerably different thermal accumulation requirements. Spring dehardening was comparatively faster, with black spruce slightly harder than the other conifers at the late stage of spring dehardening. The difference, however, was relatively small and did not afford black spruce significant protection during seedling freezing tests prior to budbreak in late March and early May. The dehardening curves and models developed in this study may serve as a tool to predict cold hardiness by temperature and to understand the potential risks of conifer cold injury during warming/freezing events prior to budbreak.

Keywords: conifer winter injury, dehardening curves, budbreak, relative electrolyte leakage, visual assessment

Introduction

The ability of boreal conifers to survive winter cold depends not only on the level of cold hardiness they can potentially achieve, but also on their responses to temperature fluctuations, i.e., the ability to remain cold hardy during periods of warm days in late winter. In general, changes in cold hardiness follow seasonal changes in photoperiod and temperature, increasing with shorter photoperiod and lower temperatures in fall, reaching a maximum in mid-winter, and decreasing with higher temperatures and longer photoperiod in spring (Bigras et al. 2001; Pletsers et al. 2015). The level of maximum cold hardiness is generally well below minimum temperatures that occur within a species' natural distribution range (Halfpenny and Ozanne 1989; Bannister and Neuner 2001; Strimbeck et al. 2015); therefore, well-hardened boreal conifers are unlikely to suffer freezing injury from cold temperatures (Bannister and Neuner 2001). However, cold hardiness can be rapidly lost during natural dehardening (Bigras et al. 2001; Man et al. 2016), which typically occurs late winter to early spring (Sakai and Larcher 1987; Jönsson et al. 2004; Kalberer et al. 2006). During this period, episodes of unseasonably warm days can be followed by seasonally cold days, resulting in winter freezing; such damage has occurred on mature trees of a single species, such as lodgepole pine (*Pinus contorta* Dougl. ex. Loud.; Henson 1952; Halfpenny and Ozanne 1989) and red spruce (*Picea rubens* Sarg.; Strimbeck et al. 1995; Lund and Livingston 1999), and on saplings and mature trees of multiple species over extensive areas (Cayford et al. 1959; Man et al. 2009, 2013).

Relative to level of maximum cold hardiness, the dehardening process is less well documented, especially in relation to temperature changes, the primary factor influencing spring dehardening (Bigras et al. 2001). Observations about the annual cold hardiness of several boreal conifers indicate that spring dehardening occurs more rapidly than fall hardening and differs not

only among species groups (e.g., between pine and spruce) (Glerum 1973; Repo 1992; Silim and Lavender 1994), but also within species (Nilsson 2001; Ögren 2001) due to variations in chilling and thermal accumulations for dormancy release and budbreak (Howe et al. 2003). Although seasonal changes in cold hardiness are generally known (Bannister and Neuner 2001; Bigras et al. 2001), quantitative links between these changes and cumulative spring temperature changes are rarely established and, therefore, results are difficult to apply in modelling cold hardiness or understanding the risks of conifer winter injury. As shown by Repo and Pelkonen (1986) and Man et al. (2014, 2016), the rate of dehardening also varies with season in relation to progress towards fulfilling chilling requirements and dormancy release.

The objectives of this study were to determine the change of cold hardiness during dehardening in winter months in 4 Canadian conifer species: lodgepole pine, jack pine (*Pinus banksiana* Lamb.), white spruce (*Picea glauca* (Moench) Voss), and black spruce (*Picea mariana* (Mill.) B.S.P.), and to examine variation among species in dehardening dynamics before and after chilling requirements for dormancy release are met. Our hypothesis was that since black and white spruce flush bud later than lodgepole pine and jack pine (Man et al. 2016) they would be slower to lose cold hardiness during spring dehardening, which is when winter freezing damage generally occurs (Cayford et al. 1959; Man et al. 2009, 2013).

Methods

Seedling materials

Lodgepole pine seedlings were initially grown in containers in a greenhouse at Tree Time Services Inc./Coast to Coast Reforestation in Smoky Lake, Alberta, Canada, using seeds of open-pollinated wild trees from southwest of Whitecourt, Alberta (54° 04'N, 116 ° 41'W). Black

spruce, white spruce and jack pine seedlings were initially grown in containers at the Millson Forestry Service Inc. in Timmins, Ontario, using seeds from orchard trees established for the nearby Martel Forest area (47°50'- 48°28'N, 82°15'- 83°25'W). After cold storage in early June 2013, 1-year-old container seedlings were shipped to Sault Ste. Marie, Ontario, for use in this study. Upon arrival, seedlings were transplanted into 4" square (10 cm side x 15 cm deep) pots filled with 2:1 peat moss/vermiculite (v/v) mixture and grown in the greenhouse at the Ontario Forest Research Institute. The seedlings received natural photoperiods with temperatures 2 to 5 °C above ambient were watered as required using an automated spray walker, supplemented with hand watering, and fertilized weekly with 20-8-20 (N-P-K) (Plant Products Co Ltd, Brampton, ON) at 100 ppm N for a month before being moved outdoors in early July. They were kept outdoors for three summers with weekly watering and biweekly fertilization following standard nursery practices (20-8-20 at 100 ppm N before mid-August and 20-20-20 at 50 ppm N from late August to mid-October), and received winter snow cover from late December to late March and natural hardening and dehardening under ambient photoperiod and temperatures (Fig. 1). Average height and basal diameter of the seedlings before the dehardening processes were studied were 61.5 cm and 9.5 mm for white spruce, 76.4 cm and 9.9 mm for black spruce, 75.3 cm and 11.5 mm for jack pine, and 66.2 cm and 11.1 mm for lodgepole pine, respectively.

Dehardening conditions

Artificial dehardening was applied to 8 seedlings of each species within each of the 3 replications (different growth chambers). Growth chambers were set at 15°C/5°C (day/night) and 12 h photoperiod to represent average conditions from early April to early May in the study area, which is when spring dehardening generally occurs (Fig. 1). The dehardening experiment was

carried out at two stages of winter development, one in early December when chilling requirement was about 90% complete on spruce and 50% on pine, and the other in early March when chilling requirement was about 100% complete on spruce and 90% on pine (Man, R., Lu, P., and Dang, Q.L., unpublished data). Seedlings were moved directly from the outdoor holding area into growth chambers in early December when ambient temperatures were generally above 0 °C. In March dehardening, however, seedlings were conditioned at -2 °C for a week, followed by a week of slow thawing at 2 °C to minimize temperature shock due to below freezing night temperatures (Fig. 1).

Cold hardiness and budbreak assessments

During the artificial dehardening period, seedling cold hardiness was determined by a series of freezing tests using tissue samples collected from seedlings from each of the experimental replications. Relative electrolyte leakage (REL) (Burr et al. 2001) was measured and needle discoloration was assessed visually. Intervals between freezing tests were 2 days at the early stage of dehardening and 4 or more days when changes in cold hardiness became more gradual. For each freezing test, current pine needle fascicles and spruce shoots (buds removed) were collected from top laterals of 6 seedlings within each replication. Samples were placed into 7 test tubes, with each test tube containing 6 pine needle fascicles or 6 1-cm spruce shoot segments from 6 different seedlings. Three freezing test programs were used with testing temperatures of (1) +4, -10, -20, -30, -40, -50, and -196 °C when complete needle death occurred below -40 °C; (2) +4, -5, -10, -15, -20, -30, and -50 °C when complete needle death occurred between -20 °C and -40 °C; and (3) +4, -3, -6, -9, -12, -15, and -20 °C when complete needle death occurred between -10 °C and -20 °C. The freezing test programs were adjusted based on results of

previous cold hardness assessments to ensure that rapid changes in REL prior to needle death were captured. After being rinsed with deionized water, the control (non-freezing) samples were placed in a refrigerator at +4 °C during freezing tests, with remaining samples subjected to the testing temperatures described above in a programmable freezer (Thermotron[®] SM-32-C, Holland, MI). Temperature inside the freezer was lowered at a rate of 5 °C h⁻¹ for freezing test programs (1) and (2) and 3 °C h⁻¹ for program (3) for better differentiation of cold hardness changes at the late stage of spring dehardening. Once a target temperature was reached, it was held constant for 30 minutes. For samples targeted for -196 °C, the temperature in the freezer was lowered to -68 °C at 5 °C h⁻¹ before samples were immersed in 30 ml liquid nitrogen. To reduce post-freezing temperature change, frozen samples were stored at -2 °C for 2 hours when the testing temperatures were ≥ -10 °C or at -10 °C for 1h followed by at -2 °C for 1h when testing temperatures were < -10 °C, before thawing in a refrigerator at +4 °C overnight.

After thawing, 30 ml of deionized water was added to each test tube, which was sufficient to submerge needles or shoot segments. Tubes were then covered with plastic caps and incubated at room temperature (20 °C) for 4 days to enhance the release of diffusible electrolytes from damaged tissues (Deans et al. 1995) and promote the development of observable damage (Burr et al. 2001; Man et al. 2016). Initial post-freezing conductivity of the solution was measured at 20 °C with a Cole-Parmer Traceable Portable Conductivity Meter (Cole-Parmer Instrument Company). The solutions and samples were then placed in a hot water bath at 90 °C for 2 hours, cooled to room temperature, and measured for final post-killing conductivity after 24 hours. The REL was expressed as the ratio of initial to final conductivity.

The level of tissue damage caused by freezing temperatures was assessed at the time of initial post-freezing conductivity measurements. The three damage classes were: *undamaged* for

healthy green needles and clear solution, *damaged* for partially discoloured (yellowish green for pine and grey green for spruce) needles and somewhat cloudy solution, and *dead* for completely discoloured (completely yellow for pine and totally pale for spruce) needles and cloudy solution. Assessment consistency was validated by comparing needle and solution colour among freezing temperatures and replications.

Seedlings subjected to dehardening conditions were monitored daily for terminal budbreak and shoot elongation after first notice of bud swelling for each species. Buds were considered flushed when bud scales were open (new needles clearly visible on spruce and shoot elongation started on pine). Thermal accumulation was calculated as cumulative growing degree hours (CGDH) above the threshold of 0 °C from start of dehardening conditions (Snyder *et al.* 1999; Man and Lu 2010). Cold hardiness assessments ended when buds were completely open and black spruce shoots were elongating as it was the last of the 4 species to break bud.

Seedling freezing tests

Whole seedling freezing tests were conducted with outdoor-grown seedlings during natural spring dehardening to confirm the results of cold hardiness assessments of needles and shoot samples during artificial dehardening. The first freezing test was carried out March 19 to 21, 2016, at an early stage of spring dehardening when thermal accumulation (CGDH > 0 °C since January 1) was about 1500 and there was no sign of budbreak in the 4 species. The second freezing test was conducted May 2 to 4, 2016 at a late stage of spring dehardening when thermal accumulation was about 5800 and jack pine and lodgepole pine had flushed and white spruce buds had started to swell.

At the time of the first seedling freezing test, outdoor night temperatures were about -5 °C. Seedlings were bagged and conditioned at 0 °C overnight before they were exposed to test temperatures of -15 °C, -25 °C, and -35 °C in a programmable freezer (Thermotron[®] SM-32-C, Holland, MI). At the time of the second freezing test, outdoor night temperatures were close to 0 °C and seedlings were directly exposed to testing temperatures of -5 °C, -10 °C, and -15 °C. The freezer temperature was reduced at 5 °C h⁻¹ for the first seedling freezing test and 3 °C h⁻¹ for the second test; the later allowed for better differentiation of cold hardiness change at the late stage of spring dehardening. After 30 minutes at the target temperature, seedlings were conditioned for 2 hours at -10 °C or 0 °C overnight before being returned outdoors. Each freezing treatment was replicated 3 times (days), with 4 seedlings from each of the 4 species per replication. For both freezing tests, unfrozen outdoor stored seedlings were used as controls.

Percentage needle death was visually assessed 1 month after the first seedling freezing tests and about 8 days after the second, which was when needle browning and mortality were clearly visible.

Data analysis

Cold hardiness assessed using REL method was determined at LT50 (50% tissue mortality) through nonlinear regression of REL versus temperature for each replication and the formula by Anderson et al. (1988):

$$Y_T = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + e^{k(T_m - T)}}$$

where Y_T is the REL at temperature T , $Y_{\min}$ is the minimum value of the response variable for non-stressed sample, $Y_{\max}$ is the maximum value of freeze-stressed samples, k represents the steepness of the response curve, and T_m is the midpoint of the symmetrical curve (an estimate of

LT50). In black spruce and white spruce, the short exposure to liquid nitrogen did not kill all the needles at the beginning of the dehardening process; as a result, the mean maximum values in subsequent cold hardiness assessments of the same replications when needles were completely dead were used.

The cold hardiness determined by visual assessment was the lowest *damaged* temperature prior to complete needle death or midpoints between the lowest *undamaged* temperature and the highest *death* temperature when damaged temperature was not captured. REL values were interpolated to assist cold hardiness determination when lowest ‘damaged’ temperature was between -60 °C and -196 °C.

Cold hardiness was compared among the conifer species tested using a randomized complete block design with 4 treatments (species) and 3 replications (growth chambers). For each cold hardiness assessment, multiple contrasts were conducted to examine differences in cold hardiness among species when the species effect was significant ($P < 0.05$). The randomized complete block design with 16 treatments (4 species and 4 freezing temperatures) and 3 replications was also applied to each of the two whole seedling freezing tests (March and May) to compare needle damage among species. Analyses were conducted using the statistical software SAS (SAS 9.4, SAS Institute Inc. 2012).

Results

Needle cold hardiness during dehardening

Dehardening trends in needles were similar among the 4 species tested, with generally faster and more advanced dehardening in March than December, although seedlings were more cold hardy in March prior to artificial dehardening (Table 1; Figs. 2 and 3). Maximum cold

hardiness prior to the March dehardening was below -60°C , while the minimum cold hardiness at the end of artificial dehardening was -7°C to -10°C . Differences in cold hardiness among the 4 species were significant at the early stage of December dehardening ($\text{CGDH} < 4000$), with black spruce and white spruce 10°C hardier than jack pine and 20°C hardier than lodgepole pine. At the late stage of March dehardening ($\text{CGDH} > 4000$), black spruce was 2 to 3°C hardier than the other conifers (mostly lodgepole pine and occasionally white spruce). Jack pine showed a greater gain in cold hardiness than other conifers in the December dehardening when the seedlings were stored for 10 days at 0°C , covered in large plastic bags, between freezing tests.

Fitting an exponential growth curve model ($a + b \cdot (1 - \exp(-c \cdot x))$) to the mean cold hardiness of March dehardening data indicated a dehardening rate (c) of 0.0006, 0.0007, 0.0007, and 0.0006, and minimum cold hardiness ($a+b$) of -7.1 , -8.2 , -8.0 , and -10.4 for lodgepole pine, jack pine, white spruce, and black spruce, respectively ($R^2 > 0.95$ for all). Corresponding values for (a) were -69.6 , 80.0 , -85.2 , and -69.9 , and (b) were 62.6 , 71.7 , 77.2 , and 59.5 . Fitting of the December dehardening data produced an average dehardening rate (c) generally less than 0.0001 and $R^2 < 0.83$.

Cold hardiness determined using REL and visual assessments was generally consistent, with differences within 1 to 2°C for the species tested, except for the beginning of March dehardening when cold hardiness was below -60°C and not well captured by the testing temperatures (Figs. 2, 3, 4 and 5).

Seedling freezing tests

In early spring (late March, Fig. 1), visible needle death was evident for all species after they were exposed to -25°C and -35°C for 30 minutes ($P < 0.001$ for temperature effect) (Fig.

6a). At the time of the freezing test, seedling cold hardiness determined by ambient thermal accumulation (CGDH above 0 °C since January 1 was about 1500) on March dehardening curves was -25 °C for lodgepole pine, -28 °C for jack pine, and -31 °C for black and white spruce (Figs. 3 and 5). Lodgepole pine was the only species with a substantial increase in needle death, from 5% to over 40%, with a decrease in testing temperature from -25 °C to -35 °C, although the effects of tree species ($P = 0.368$) and species by temperature interaction ($P = 0.083$) were not statistically significant. In the late spring freezing test (early May, Fig. 1), needle death exceeded 50% at -10 °C and -15 °C for all 4 species ($P < 0.001$ for temperature effect) (Fig. 6b) when the cold hardiness determined by ambient thermal accumulation (CGDH was about 5800) on March dehardening curves was -9.8 °C for lodgepole pine, -11.5 °C for jack pine, -10.7 °C for white spruce, and -13.4 °C for black spruce, respectively. The point at which needle death occurred differed among the 4 conifer species ($P = 0.028$), with the highest differences between white spruce and jack pine.

Discussion

Our hypothesis that black and white spruce are slower to lose their cold hardiness than the pine species during spring dehardening was only partially true. The 4 conifer species dehardened at similar rates and to a similar extent as shown by the dehardening curves and fitted exponential growth models, despite the fact that they differed considerably in timing of budbreak (Table 1; Figs. 2, 3, 4, and 5). The relatively greater cold hardiness shown by black spruce at the late stage of March dehardening is possibly due to its delayed budbreak. However, compared to the level of cold hardiness prior to the artificial dehardening, differences were small and did not give black spruce significant protection against late spring freezing temperatures at -10 °C and -15 °C

in early May (Fig. 6b) when bud swelling had not yet occurred. Comparatively, white spruce did not show any advantage over the pine species in cold hardiness resulting from its late budbreak.

Our assessments of cold hardiness prior to spring (March) dehardening were consistent with those observed by Glerum (1973) who indicated that the maximum cold hardiness of spruce and pine trees was well below -40°C , while the minimum cold hardiness differed by several degrees, likely reflective of the differences between mature needles used in this study and the newly flushed needles in his study. The more gradual change of cold hardiness during winter (December) dehardening could have resulted from incomplete fulfilment of chilling requirements, as insufficient chilling is associated with a greater need for thermal accumulation for budbreak in all 4 conifer species (Man, R., Lu, P., and Dang, Q.L., unpublished data).

Our findings suggest that dehardening of mature conifer needles continues during budbreak (Glerum 1973; Bigras and Hébert 1996). For example, both jack and lodgepole pine broke bud earlier than spruce and continued to lose cold hardiness during and after budbreak (Fig. 3). Comparatively, changes in the cold hardiness of white and black spruce during budbreak were small and may not be significantly sufficient to be captured by some of the previous studies, which suggested that minimum cold hardiness in spruce is generally reached at the time of budbreak (Peace 1962; Holzer 1969; Nienstaedt and King 1969).

Lodgepole pine was relatively less cold hardy than the other conifer species as indicated by cold hardiness levels at the beginning of winter dehardening and the end of spring dehardening and the amount of needle death at -35°C in early spring freezing tests. Halfpenny and Ozanne (1989) reported considerable damage to lodgepole pine in Colorado during a false spring when a warm spell (6-day mean maximum temperature of 6.4°C) in January was followed by severe cold in February (minimum temperature of -34°C). Lodgepole pine also

shows higher sensitivity to warming/temperature fluctuations and greater level of dehardening than other conifers in controlled conditions (Nilsson 2001; Ögren 2001; Man et al. 2016). The red belt damage, likely a result of winter freezing (Robin and Susut 1974; Man et al. 2013), occurs on lodgepole pine more so than other boreal conifers (Robin and Susut 1974).

Our results show that all 4 conifer species were vulnerable to freezing temperatures following quick loss of cold hardiness during spring dehardening, as shown by the thermal accumulations and night temperatures associated with 3 historical conifer winter browning events (Fig. 5). Slower dehardening would give black spruce mature needles some level of protection as they were less damaged than other conifers in reported winter browning events (Cayford et al. 1959; Man et al. 2009), but more protection for this species may come from its delayed new growth (Glerum 1973; Bigras et al. 2001). Compared to spruce, pine trees were notably less hardy in early winter (Figs. 2 and 4), possibly due to their different capacities for hardening (lodgepole pine) and dehardening (jack pine), and therefore have an additional vulnerability to winter cold. The substantial increase in cold hardiness of jack pine following 10 days of cold storage at 0 °C indicated a greater capacity to re-harden and potentially reduce vulnerability to freezing temperatures if the return to cold temperatures after warming is more gradual (Pukacki and Kamińska-Rózek 2013; Man et al. 2016).

Caution is required when applying the findings of this study to natural forests due to possible differences between artificial freezing and natural freezing events — the latter have more gradual changes of temperatures and possibly less damage (Sakai and Larcher 1987), which may be offset by longer exposure to cold and stronger post-freezing radiation from sunlight and therefore more damage (Dang et al. 1992) — and between seedlings and mature trees in frost resistance (Sakai and Larcher 1987; Vitasse 2013). Nevertheless, the spring

dehardening curves from REL and visual assessments were comparable and supported by the results of whole seedling freezing tests (Fig. 6) and the observations from the reported conifer winter browning events that occurred in spring prior to black spruce budbreak (Fig. 5) (Cayford et al. 1959; Man et al. 2009, 2013). These dehardening curves can serve as tools to predict the level of cold hardiness from natural thermal accumulations and inform understanding of the potential effects of winter warming/freezing events that may increase in frequency and scale under a warming climate (Schär et al. 2004; Rigby and Porporato 2008; Man et al. 2009; Augspurger 2013; Strimbeck et al. 2015). Based on the findings of this study, the effects of winter freezing may be reduced with northward migration of tree species, as southern populations require more chilling and thermal accumulations for dormancy release and budbreak (Howe et al. 2003).

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Table 1. Variance analysis and thermal accumulations (cumulative growing degree hours $>0^{\circ}\text{C}$) required for budbreak in winter (December) and spring (March) dehardening experiments.

Variance analysis	Treatment effect	Species	Time	Species*Time
	Probability (<i>P</i>)	<0.001	<0.001	0.227
Mean values	Lodgepole pine	Jack pine	White spruce	Black spruce
Winter	4381	3332	7880	9851
Spring	2899	1841	6030	8700

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Fig. 1. Outdoor air temperatures (daily high and low) at the study location in Sault Ste. Marie, Ontario, during winter 2015-16.

Fig. 2. Cold hardness of 4 boreal conifers assessed based on changes in relative conductivity during artificial dehardening in winter (December). Asterisks above points indicate significant differences among species.

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Fig. 3. Cold hardiness of 4 boreal conifers assessed based on changes in relative conductivity during artificial dehardening in spring (March). Asterisks above points indicate significant differences among species.

Fig. 4. Level of needle damage assessed visually after freezing tests during artificial dehardening in winter (December).

Fig. 5. Level of needle damage assessed visually after freezing tests during artificial dehardening in spring (March). White circles indicate the thermal accumulation (cumulative growing degree hours (CGDH) > 0 °C) and night temperatures from three historical conifer winter browning events, 1958 in central Canada (CGDH = 6400 on April 29), 2007 in northeastern Ontario (CGDH = 7600 on May 19), and 2012 in northwestern Ontario (CGDH = 3800 for the Geraldton area and 4400 for Thunder Bay area on April 17).

Fig. 6. Percent needle death of seedlings grown outdoors by species (PI-lodgepole pine, Pj-jack pine, Sw-white spruce, and Sb-black spruce), season, and testing temperature for seedling freezing tests conducted in late March (19 - 21) and early May (2 - 4).