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AN EVALUATION OF THE ROLE OF DIURNAL TEMPERATURES IN THE HARDENING OF BARE ROOT WHITE SPRUCE SEEDLINGS

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DALY E.J. and HODDINOTT J. *An evaluation of the role of diurnal temperatures in the hardening of bare root white spruce seedlings.* BIOTRONICS **19**, 61–70, 1990. Bare root seedlings are often lifted and placed in cold storage for overwintering. It is critical that lifting occur when the seedlings are best able to survive the stresses of storage and spring outplanting. To predict the optimal lifting time for white spruce (*Picea glauca* (Voss) Moench) seedlings, field grown seedlings were placed in growth chambers and exposed to a range of diurnal thermoperiods in short day illumination conditions. Seedlings were then cold stressed at a range of temperatures down to −35°C and survival was estimated on the basis of TTC reduction tests and visual observation of regrowth. Pre-stress exposure to a range of thermoperiods showed that seedlings survived well when stressed to −15°C. The best survival occurred when they had been subjected to day temperatures of 6 and night temperatures of −5°C which enhanced their ability to withstand cold stress down to −25°C.

Key words: *Picea glauca*; hardening; inductive temperatures; stress resistance.

INTRODUCTION

In preparation for spring planting it is an accepted practice to lift nursery grown conifer stock in the autumn for winter storage by cold storage or other means (*13*, *14*, *16*, *18–20*, *24*, *27*), and questions concerning the optimal lifting season to ensure maximum survival have been raised (*15–17*). This study attempted to define an optimum autumn lifting time for bare root white spruce seedlings based on a knowledge of photoperiod and air temperatures, and it was carried out in controlled environment chambers set to simulate autumn conditions in Northern Alberta. Optimal lifting time was evaluated from data on the influence of a range of thermal hardening conditions on the survival of seedlings following exposure to freezing stress and subsequent rewarming.

A provisional guide to lifting time is available for several tree species based upon the accumulation of a critical number of Degree-Hardening-Days (D-H-D) (*14*). This method is based on the assumption that the hardening of roots, influenced by the cumulative effect of cold soil temperatures, has a greater effect on

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successful frozen storage than above ground air temperatures, but it requires additional environmental monitoring to obtain the soil temperature data. Electrical impedance measurements (*15*), square wave oscilloscope techniques (*8*), and chlorophyll fluorescence analysis (*23*, Vidaver, personal communication) have also been used to predict lifting times but they require even more sophisticated instrumentation. This study attempted to rely on air temperature measurements to provide the data used to predict lifting times.

Evidence indicates that the induction of cold hardiness in many tree species is strongly influenced by environmental factors (*1*, *2*, *7*, *9*, *12*, *25*), and it is known that short day illumination and variations in diurnal temperature regimes are involved (*3–6*, *10*). In the present study the cold hardiness of seedlings following cold stress during storage was evaluated by observing growth following rewarming and, to obtain a more empirical estimate of the level of hardiness induced by the thermal hardening treatments, the Triphenyl Tetrazolium Chloride assay (*21*).

Seedlings best withstood cold stress during storage when they were lifted following exposure to thermoperiods providing low day air temperatures and freezing nights.

MATERIALS AND METHODS

White Spruce seedlings (3+0), *Picea glauca* (Voss) Moench, without resting buds, were obtained from the Provincial Tree Nursery at Smoky Lake, Alberta, in late August 1985 (Year 1) and 1986 (Year 2). Seedlings were transferred to 15 cm plastic pots containing a potting mix of peatmoss (0.165 m^3), Vermiculite (0.165 m^3), limestone ($CaCO_3$) (3.0 kg), Nutricote (Type 100, 14•14•14) (3.2 kg), Frittered trace elements (15 g), a soil wetting agent (industrial grade surfactant, 20 ml) and tap water (81.0 l). Five seedlings were planted per pot with optimal spacing between the seedlings, and pots were watered to field capacity with tap water.

Dormancy was induced in both batches after transferring all plants to a controlled environment chamber. Over a five week equilibration period the photoperiod and the day:night (D:N) thermoperiod were sequentially reduced at regular intervals from a photoperiod of 13.5 h and a thermoperiod of 13:7°C D:N (values similar to those found in Northern Alberta during the last two weeks of August in 1985/86) to a maintenance condition with a 10 h photoperiod and a 10:5°C thermoperiod, at about 50%RH. Light intensity was 394 ± 75 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by cool white fluorescent tubes supplemented by incandescent lamps.

In the dormancy induction chamber, the pots of seedlings were arranged according to a split-plot sampling design. All plants were watered regularly with tap water. Dead or damaged trees were replaced during the five week equilibration period from a stock of spare trees kept in the dormancy chamber. Replacement was discontinued after equilibration to prevent disturbance of established rooting systems. No sampling was done on damaged trees found during the experiment.

Plants were exposed to one of a series of randomly selected thermoperiod

Table 1. Hardening day: night thermoperiods (°C) over a 10 h photoperiod used to induce frost hardening in Year 1 and 2 white spruce seedlings

15 : 10*	12 : 10		
15 : 5	12 : 5	9 : 5	
15 : 0*	12 : 0	9 : 0	6 : 0
15 : –5*	12 : –5	9 : –5*	6 : –5*

Asterisks indicate thermoperiods used for Year 2.

hardening regimes chosen to simulate common autumn temperatures at seedling lifting time in Northern Alberta. A smaller selection of thermoperiods were used in Year 2 (Table 1). For each hardening regime, groups of 12 (Year 1) or 15 (Year 2) pots were used. Pots were placed for five days in a reach-in chamber preset for a specific hardening thermoperiod. The chamber provided cool white fluorescent light at 220 ± 10 μmol m^{-2} s^{-1} PAR, over a 10 h photoperiod. At the end of the hardening period well watered pots were transferred to a storage chamber and maintained in the dark at -2°C for 60 h to provide an equivalent of commercial seedling storage conditions. Control plants were then removed to a warm recovery chamber maintained at 23°C and 18 h photoperiod under cool white fluorescent light providing 138 ± 8 μmol m^{-2} s^{-1} PAR. Concurrently, treatment plants were transferred to a dark test chamber where they were rapidly cooled (5°C h^{-1}) using one of two sub-zero stress regimes. Year 1 hardened plants were cooled to -5°C and were held at that temperature for 2 h at which time a subsample of three pots was removed to the recovery chamber. The remaining plants were then cooled to -10°C and held for 2 h when a further subsample of three pots was removed to the recovery chamber. This procedure was repeated at -15°C for the remaining three pots. For Year 2 the stress conditions consisted of -15, -25, -30 and -35°C using the same protocol.

Plants were sampled, qualitatively and quantitatively, 1 day, 1 week and 1 month after transfer to the recovery chamber. For Year 1 plants a visual assessment of signs of frost damage was made based on needle color, the presence or absence of needle browning, and bud growth. Bud swelling, the separation of bud scales and the emergence of green foliage beneath the scales, and the expansion of terminal and lateral buds were recorded. For Year 2 hardened plants, seedling survival was determined 2.5 months after the stress treatments when a visual estimate was made of the percentage of shoot regeneration indicated by the presence of new growth.

A quantitative determination of tissue damage in both batches of hardened plants was made by the 2,3,5-Triphenyl Tetrazolium Chloride (TTC) reduction assay modified from Steponkus and Lanphear (*21*). Three replicates of 100 ± 10 mg of fresh one year old needle tissue were randomly selected from branches of treated and control plants from each hardening regime and stress treatment. The weighed samples were lightly scored with a razor blade and placed into 3 ml of 0.6% (W/V) TTC in 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.4) containing 0.05% (V/V) Triton

X-100 as a wetting agent. Samples were vacuum infiltrated for 1 h (3×20 min intervals) and placed into a dark incubation chamber at 30°C for 17 h. The TTC solution was then decanted off and the tissue was washed twice with 20 ml aliquots of distilled water. The formazan derivative was extracted from the leaf tissue with a 95% (V/V) ethanol solution (7 ml) in a hot water bath (80°C, 5 min). Extracts were cooled for 10 min then made up to 10 ml with 95% ethanol. Absorbance values were recorded at 530 nm using a PYE UNICAM SP6-550-UV/VIS Spectrophotometer. The absorbance values of treated tissues were also recorded as a percentage of the values of their controls. One-way ANOVA was performed on the absorbance values, and two-way ANOVA on the arcsin transformed percentage values.

RESULTS

Visual surveys of bud and shoot regeneration

Year 1 plants usually showed browning of the needles after stress treatments when they had been hardened in thermoperiods with night temperatures above zero (Table 2). More severe browning occurred at 10°C than at 5°C night temperatures. Plants hardened with night temperature below zero showed no signs of leaf browning except in the case of the 12:−5 (1 month) regime. All unstressed control plants remained green and supple, with no browning.

After four weeks of recovery from applied cold stress, Year 1 hardened plants showed bud growth activity in the form of bud swelling, meristematic growth and the subsequent expansion of the green foliage revealing itself between the bud scales. Bud growth activity resumed faster in plants exposed to mild hardening

Table 2. Presence (+) and absence (−) of needle browning and bud growth activity (in parenthesis) in Year 1 hardened white spruce seedlings exposed to −5, −10 or −15°C cold stress, visually assessed one day, one week and one month after transfer to recovery conditions. Estimates are based on observations of 15 plants per treatment

Night temperature (°C)		Day temperature (°C)			
		15	12	9	6
10	Day	+ (−)	+ (−)		
	Week	+ (+)	+ (−)		
	Month	+ (+)	+ (+)		
5	Day	+ (−)	− (−)	− (−)	
	Week	+ (−)	+ (+)	− (−)	
	Month	+ (+)	+ (+)	+ (+)	
0	Day	− (−)	− (−)	− (−)	+ (−)
	Week	− (−)	− (−)	− (−)	+ (−)
	Month	− (+)	− (+)	+ (+)	+ (+)
−5	Day	− (−)	− (−)	− (−)	− (−)
	Week	− (−)	− (−)	− (−)	− (−)
	Month	− (+)	+ (+)	− (+)	− (+)

Table 3. A survey of shoot survival based on a visual assessment of shoot regeneration in Year 2 hardened white spruce seedlings 2.5 months after exposure to −15, −25, −30 or −35°C stress treatments

Hardening treatment (°C)	Stress temperature (°C)			
	−15	−25	−30	−35
15 : 10	(A)*	(D)	(D)†	(D)†
15 : 0	(B)	(C)	(D)	(D)†
15 : −5	(A)	(C)	(D)†	(D)†
9 : −5	(B)	(D)	(D)†	(D)†
6 : −5	(A)	(B)	(D)	(D)†

* Each letter is representative of a percentage range for shoot regeneration (i.e. presence of new growth) in three pots of five plants per pot, based on a visual comparison to an unstressed control. Unstressed control plants showed 100% regeneration. A: 100–75% of control, B: 74–50% of control, C: 49–25% of control, D: 24–0% of control.

† All shoots dead at the time of observation.

thermoperiods (15 : 10 and 12 : 5) where it was observed one week after the stress treatments (Table 2). The 15 : 10 treatment showed some growth activity prior to the application of stress conditions. After night temperatures at or below zero, the resumption of growth activity was delayed.

Year 2 plants were subjected to a wider range of stress levels (−15°C to −35°C) as all plants had survived in Year 1. After 2.5 months of recovery the highest survival occurred after the mildest stress (−15°C) regardless of the hardening treatment (Table 3). The visual assessments were made later in Year 2 to include the possibility of more long term damage becoming apparent. As the plants were subjected to harsher stress fewer plants survived. A critical temperature value, in terms of long term survival, occurred between −15 and −25°C. In most cases, plants stressed between those levels showed at least a 25% reduction in survival. Almost all seedlings exposed to −30 and −35°C died. The LT50, the temperature at which 50% of the treated plants died, showed that the lowest survival rates after stresses below −15°C followed the 15 : 10 and 9 : −5 hardening thermoperiods, with 15 : 0 and 15 : −5°C also showing less than 50% survival. The 6 : −5°C thermoperiod was associated with the best survival percentage at −25°C.

Triphenyl Tetrazolium Chloride test

Year 1 plants all gave TTC values greater than 61% of control values. Steponkus and Lanphear (*21*) considered the value of 50% as lethal, therefore the data indicated that the trees were not severely damaged by stresses between −5 and −15°C. In 1986, hardened plants of Year 2 were subjected to more extreme stress which resulted in lower TTC values. TTC absorbance value for stressed plants of Year 2 are given in Table 4. One-way ANOVAs for each hardening period showed a significant trend to lower TTC values with increasing stress at all sampling times after stress. The absorbance data could only be compared across a row as the data was relative to the control value on that row. Row controls could not be compared as they were obtained at different times with fresh batches of reagent.

Table 4. Reduced Tetrazolium Chloride (TTC) absorbance values for Year 2 white spruce seedlings sampled 1 day, 1 week and 1 month after cold stress application

Hardening condition (D : N)	Control	Stress condition (°C)				*F* value
		−15	−25	−30	−35	
1 day						
15 : 10	0.272±0.046	0.301±0.053†	0.016±0.026	0.176±0.064	0.131±0.036	29.9*
15 : 0	0.313±0.026	0.114±0.037	0.120±0.027	0.108±0.026	0.100±0.032	52.3*
15 : −5	0.362±0.054	0.303±0.049	0.176±0.035	0.159±0.038	0.117±0.025	70.6*
9 : −5	0.323±0.065	0.239±0.044	0.212±0.068	0.208±0.045	0.162±0.016	11.2*
6 : −5	0.364±0.034	0.229±0.056	0.254±0.053	0.163±0.038	0.219±0.045	18.2*
1 week						
15 : 10	0.265±0.047	0.267±0.063†	0.158±0.045	0.152±0.076	0.110±0.031	19.4*
15 : 0	0.303±0.036	0.260±0.060†	0.211±0.045	0.153±0.027	0.144±0.043	23.9*
15 : −5	0.216±0.026	0.205±0.057†	0.118±0.023	0.116±0.033	0.108±0.030	22.1*
9 : −5	0.297±0.055	0.188±0.031	0.174±0.044	0.147±0.025	0.116±0.029	28.7*
6 : −5	0.294±0.062	0.207±0.039	0.216±0.042	0.158±0.024	0.192±0.030	13.4*
1 month						
15 : 10	0.283±0.025	0.179±0.031	0.125±0.035	0.114±0.024	0.093±0.017	57.6*
15 : 0	0.308±0.047	0.180±0.031	0.192±0.023	0.162±0.030	0.156±0.018	31.0*
15 : −5	0.345±0.026	0.327±0.062†	0.237±0.043	0.221±0.019	0.186±0.036	30.2*
9 : −5	0.343±0.036	0.202±0.023	0.181±0.027	0.162±0.033	0.161±0.025	46.7*
6 : −5	0.237±0.049	0.283±0.047†	0.226±0.031†	0.174±0.033	0.207±0.025†	17.4*

TTC absorbance values±SD (n=15). Asterisks indicate significance in an F test at the * 1% level. In a Student's t test treatments are significantly different from the control values at the 5% level except for those marked † (no significant difference).

When the Year 2 TTC absorption data was expressed in the usual way as a percentage of the control values two-way ANOVAs could be performed. Significant effects were seen due to the applied stress treatment, the hardening thermoperiod and their interaction at all three sampling times after the stress treatments (Table 5). At lower applied stress temperatures there was a trend to lower TTC% values (row effect). With more severe hardening regimes there was a trend to increased TTC% values (column effect). The highest TTC% values were seen following severe hardening regimes and mild stress temperatures, and the lowest values after mild hardening and very low stress temperatures (interaction effect).

DISCUSSION

Bare root seedlings must be adaptable to field conditions to ensure maximum survival. In North America, cold hardiness is an important aspect of that adaptability. It is generally accepted that both short days and low temperatures are important for the induction of cold hardiness in many plant species (*3*, *4*, *9*), and a model describing the influence of biological and physical factors on the development of cold hardiness was proposed by Weiser (*25*) where plants were said to pass through a series of acclimation steps possibly involving a dormancy require-

Table 5. TTC percentage values for Year 2 hardened white spruce seedlings sampled at 1 day, 1 week and 1 month after stress treatments

	Hardening conditions (D : N)	Stress temperature (°C)			
		−15	−25	−30	−35
A) Day	15 : 10	110.5±19.6	64.8± 9.6	64.7±23.7	48.2±13.0
	15 : 0	36.2±11.7	38.2± 8.6	34.5± 8.3	31.9±10.2
	15 : −5	83.8±13.5	48.7± 9.6	44.0±10.5	32.3± 6.8
	9 : −5	73.8±13.7	65.7±21.0	64.2±13.9	50.2± 5.0
	6 : −5	62.4±15.5	70.0±14.5	44.9±10.6	60.3±12.4
		F row=55.1*, F column=59.6*, F interaction=12.5*			
B) Week	15 : 10	100.7±24.0	59.5±17.0	57.5±28.6	41.6±11.7
	15 : 0	85.7±19.8	69.6±14.9	50.4± 8.8	47.3±14.1
	15 : −5	94.9±26.6	54.4±10.4	53.9±15.1	50.1±13.7
	9 : −5	63.3±11.0	58.5±14.6	49.4± 8.4	39.0± 9.7
	6 : −5	70.4±13.4	73.4±14.2	53.8± 8.1	66.2± 9.5
		F row=5.1*, F column=65.2*, F interaction=3.9*			
C) Month	15 : 10	63.2±10.8	44.0±12.5	40.0± 8.5	32.7± 5.8
	15 : 0	58.5±10.2	62.4± 7.4	52.6± 9.8	50.6± 5.7
	9 : −5	58.9± 6.6	52.6± 7.7	47.2± 9.7	46.9± 7.4
	6 : −5	119.1±19.7	95.4±12.9	73.1±13.9	87.2±10.6
		F row=61.1*, F column=49.5*, F interaction=6.4*			

Values were derived from $n=15$ samples. For percent values greater than 100 an arcsin value of 90.0 was used. Asterisks indicate significance in an F test at the * 0.1% level in the transformed data.

ment. Levitt (*9*) has reviewed these acclimation steps and the influences of photoperiod, temperature and water stress and their relation to tissue damage by cold temperatures, and some workers have suggested that a short day induced increase in hardiness is a phytochrome mediated response essential to the initial stages of acclimation (*11*, *22*, *26*). As a result, it might be asked whether or not these factors could be used to predict a period in which nursery stock could be lifted, to ensure maximum regeneration after cold storage and also maintain the condition of maximum cold hardiness required for successful outplanting.

The present data suggest that specific temperature criteria, with short photoperiods, are required for the maximum survival of outplanted, cold stressed, conifer stock. Values for percent seedling survival (Table 2) and for TTC reduction as a percentage of the controls (Table 5), show that maximum survival occurs at lower stress temperatures following cool daytime temperatures (6°C) and mild sub-zero night temperatures (−5°C). In Year 1 hardened plants at all thermoperiods tested, maximum survival occurred when plants were stressed down to −15°C. This was interpreted as being an influence of photoperiod on early stages of the acclimation process rather than a temperature induced hardiness factor. This corresponds to a condition in early autumn when growth has stopped and short days induce a phytochrome dependent sequence of events leading to Weiser's first stage of cold acclimation. Year 1 plants were hardened such that a −15°C stress may not have been cold enough to adversely affect their survival.

Increasing cold stress caused a decrease in survival in hardened plants (Table 5). With increased cold stress, plants treated with warm day and night-time temperatures, especially 15 : 10, showed a decline in survival. Under those conditions it seems likely that the temperature response needed for the induction of cold hardiness was not triggered, and in Year 1 the 15 : 10 thermoperiod even allowed some bud growth prior to the application of stress. This was qualitatively observed in the considerable reduction in percent survival of Year 2 after 2.5 months of recovery (Table 3). Year 2 survival data suggest that the greatest survival occurred when plants were stressed to no lower than −15°C (Table 3) and indicates that white spruce were sufficiently hardened to that level by short photoperiods alone. It seems that a critical stress temperature value existed between −15 and −25°C at which point a further hardening process was required so that white spruce could withstand temperatures colder than −15°C, and that role was played by the 6 : −5 thermoperiod in enhancing survival at −25°C. The virtual lack of survival of plants at temperatures below −30°C suggests that short days and the thermoperiods tested were insufficient to fully harden off the plants as defined by Weiser's third stage of cold acclimation (*25*). According to his hypothesis complete hardening in many species occurs only after a prolonged period of intense subzero temperatures.

The TTC% values in Table 5 were a measure of the damage done to the previous years photosynthetic tissue as a result of the stress treatments, while the survival data in Table 3 was a measure of the damage to the regrowth potential of overwintering buds. It was anticipated that the trends observed in both tables would be similar as both would have had an influence on plant survival. Steponkus and Lanphear (*21*) suggested that TTC% values above 50% indicated plant survival after cold stress, and using that criterion the TTC% data obtained from plants one month after stress showed a good agreement with the regrowth data. The agreement between the one week TTC% values and regrowth was less clear, but if a 60% of the control criterion was used there was a better agreement. One day after stress the TTC% data showed even less agreement with the regrowth data, and it is possible that the plants were still dormant when sampled.

In establishing a TTC% criterion to predict survival, care must be taken to ensure that the plants have had time to become fully released from dormancy. The limitation of the TTC% method was also seen when the one month TTC% data was compared to regrowth activity after 2.5 months. The 6 : −5 thermoperiod plants after a −35°C stress showed very active TTC reduction but the plants were brown and dead after 2.5 months. The needles had been capable of reducing TTC when tested but they later died of other causes, possibly frost damage to the cambium and the disruption of transport systems.

To enhance the survival of bare root seedlings following cold stress and subsequent spring planting, lifting should be delayed until the seedlings have been exposed to several days of cold air temperatures (at least −5°C) accompanied by sub-zero night air temperatures.

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