ELECTROLYTE LEAKAGE FROM STEM TISSUE AS AN INDICATOR OF HARDWOOD SEEDLING PHYSIOLOGICAL STATUS AND HARDINESS

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ABSTRACT.—It is important to identify rapid and accurate methods for assessing hardwood seedling quality and physiological status. Evaluation of electrolyte leakage (EL) from plant tissues is promising for this purpose. It has successfully predicted the physiological status of conifer seedlings and has been used experimentally on European hardwood species. Three species of hardwoods, northern red oak (*Quercus rubra* L.), black walnut (*Juglans nigra* L.), and black cherry (*Prunus serotina* Ehrh.), were evaluated for cold hardiness after being subjected to three storage methods (freezer, cooler, ambient) of varying duration. Higher EL values at longer durations represent a loss of dormancy and increase in cell damage over time. For all species, an increase in EL over time corresponded to a decrease in the number of days required for budbreak under greenhouse conditions. While trends were similar for all species and storage methods, DBB and EL levels did not appear to be related to greenhouse height growth. Further study is needed to assess the viability of EL as a predictor of seedling hardiness and quality for commonly produced hardwoods.

The assessment of seedling quality is an important component of reforestation and afforestation programs. Use of low quality seedlings may result in low growth rates and plantation establishment failure (Sampson et al. 1996). It is important to identify high quality stock that has the potential for vigorous root and stem growth. New roots are more efficient in supplying the newly planted seedling with water, which is essential for withstanding transplant stress (Stone 1955, Nambiar et al. 1979, Rietveld 1989, Larcher 1995). Vigorous seedlings grow at increasingly faster rates compared to seedlings of low vigor (Vyse 1982; Burdett 1990) and growth differences evident shortly after transplanting may be maintained over the life of a planting (Paterson and Fayle 1984). Plantings established with high quality seedlings may ultimately require less maintenance (Paterson and Fayle 1984, Burdett 1990) and are more resistant to insect and disease stress (Schoeneweiss 1981, Cordell et al. 1989, Landis et al. 1989; Sutherland et al. 1989). Lifting procedures, post-lifting cold storage, and choice of planting site are important considerations in maintaining a high level of seedling quality. Transporting seedlings from the nursery to storage and then to the planting site subjects them to numerous physical and environmental stresses that lead to a reduction in vigor needed for establishment success (McKay 1996, Maki and Colombo 2001). Seedling vigor is associated with physiological activity of healthy tissue (Sampson et al. 1996). Therefore, the need for practical and efficient methods to monitor changes in seedling physiological status is apparent.

The primary focus of most quality assessment research has been on conifer species, as these are produced in the greatest quantities (Moulton and Hernandez 2000). However, hardwood seedling demand is increasing, primarily as a result of ecological restoration and conservation practices (King and Keeland 1999, Stanturf et al. 2000). Consequently, research seeking to improve quality assessment methods for hardwood seedlings is increasing in significance.

Cold hardiness assessment is one method for evaluating quality. It provides a measure of dormancy status (Ritchie 1984); predicts the ability of seedlings to withstand stresses associated with lifting, storing, and planting (O'Reilly et al. 1999); and provides an indication of field performance potential

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(Pardos et al. 2003). Physiological methods of testing cold hardiness are also rapid (McKay 1992), allowing for timely management decisions in nursery operations.

Many methods have been employed for evaluating cold hardiness of plants. Visual methods such as whole plant freeze tests and shoot tissue browning tests have been effective (Timmis 1976; Liu et al. 1998), but are more time consuming. Water relations (Ameglio et al. 2001), bud mitotic activity (Calme et al. 1994), abscisic acid concentration (Li et al. 2003), soluble sugar concentration (Tinus et al. 2000), and chlorophyll fluorescence (Rose and Haase 2002) are among the many physiological indicators used for conifers. Measurement of these indicators, while more rapid, has seen limited application to hardwood forestry. The electrolyte leakage (EL) method was chosen for this study. It has been used extensively in conifer research, where it was shown to be a reliable approach to determining hardiness (Colombo et al. 1995, Bigras 1997, Tinus and Burr 1997). It is expected that similar results can be achieved with hardwood seedlings.

Electrolytes are contained within the membranes of plant cells. These membranes are sensitive to environmental stresses such as chilling and freezing conditions. Cold temperatures reduce enzymatic activity, alter metabolism, and decrease the photosynthetic capacity of plant tissues (Dubey 1997). In plant membranes, these changes are often associated with increases in permeability and loss of integrity (Campos et al. 2003). Unstressed, undamaged plant cells maintain electrolytes within the membrane. As the cells are subjected to stress, electrolytes leak into surrounding tissues. An estimation of cell damage and hardiness can be made by comparing the conductivity of the leaked contents from injured and uninjured tissues in water (Mattsson 1996, McNabb and Takahashi 2000).

For conifers, needles (Burr 1990) are the most commonly sampled tissues for EL. Sampling of leaves is particularly applicable to evergreens, which can be collected and tested throughout the dormant period. Roots (McKay 1992) and stem tissues (Colombo et al. 1995) have also been used. The efficacy of EL in predicting hardiness has resulted in its use in operational practice at some nurseries, particularly for determining lifting windows and storability (Tinus 1996). For hardwoods, relatively little information is available. Most hardwood EL research in the past was performed using roots of European species (Edwards 1998; McKay et al. 1999; O'Reilly et al. 2001). Important hardwood species of eastern North America have not been extensively studied. In addition, the impacts of different storage regimes on hardwood seedling EL and physiological status have not been widely published. Therefore, the objectives of this study were to 1) evaluate EL from stem tissues as a method for estimating hardiness and physiological status in eastern hardwoods and 2) observe changes in physiological status that occur in response to different methods and duration of post-lifting storage.

Methods

1-year-old bare-root northern red oak (*Quercus rubra* L.), black walnut (*Juglans nigra* L.), and black cherry (*Prunus serotina* Ehrh.) seedlings of bulk seed origin were hand-lifted from Vallonia Nursery (Vallonia, IN, Indiana DNR Division of Forestry) on 14 November 2002 and divided among two storage treatments: cold storage (2°C) and freezer storage (-2°C). A third treatment consisted of non-lifted seedlings of each species remaining in one of three respective nursery beds to receive ambient environmental conditions during the storage period. Seedlings were not specifically selected for uniform size, with initial heights ranging from 23-90 cm for northern red oak, 13-75 cm for black cherry, and 24-105 cm for black walnut. All seedlings were grown under standard nursery practices and selected from a small section of one of three beds (one bed per species). For each species, seedlings were bundled with moistened peat moss into two kraft paper rolls, with one bundle assigned to the cold storage treatment and one to the freezer storage treatment. The bundles were then immediately transported to Purdue University, West Lafayette, IN for storage. Cold storage was in a thermostatically controlled walk-in cooler and freezer storage was in a Conviron PGR-15 (Controlled Environments Ltd., Winnipeg, MB) growth chamber. Beginning January 2003 and ending April 2003, seedlings of each species were removed from each of the storage regimes at monthly intervals (28 January, 25 February, 27 March, 26 April). Removal dates corresponded to four storage durations. Weather data for the Vallonia Nursery area during the storage period are shown in Table 1.

Table 1.—Weather conditions for Seymour, IN (24 km from Vallonia State Nursery) during the period from 15 November 2002 to 26 April 2003.

Month	Temperature (°C)[1]	Precipitation (cm)	Snow cover (days)
November[2]	2.86	1.04	0
December	0.49	6.53	6
January	-4.75	4.09	6
February	-2.93	13.28	20
March	6.59	3.35	3
April[3]	11.77	7.90	0

[1] *Daily average*
[2] *15 November – 30 November*
[3] *1 April– 26 April*

For each species on each removal date, five trees per storage method were potted into 4-gallon Treepots™ containers (Stuewe and Sons, Inc., Corvallis, OR) using Scotts Metro-Mix 366-P (Scotts Co., Marysville, OH) and placed in a greenhouse at Purdue University's Horticulture and Landscape Architecture Plant Growth Facility. The exception was ambient trees in January, which could not be lifted because of frozen soil. Greenhouse environmental conditions were maintained at 23.9°C day and 17.8°C night, with a photoperiod determined by natural day-length. Water containing a complete fertilizer solution was applied as needed. This solution contained (in mg/liter) 200 N, 29 P, 167 K, 67 Ca, 30 Mg, and micronutrients. Nutrients were supplied from a 1000 mg/liter 15-5-15 commercial fertilizer (Miracle Gro® Excel® Cal-Mag; Scotts Co., Marysville, OH). The pH was adjusted to range from 5.7 - 6.0, with alkalinity reduction achieved via 93% sulfuric acid (Ulrich Chemical, Indianapolis, IN) at 0.08 ml/liter. The number of days to first budbreak (DBB) was recorded for each seedling as an indicator of dormancy status and physiological activity (Englert et al 1993). Measurements of height were recorded at potting and at 30, 60, and 90 days after potting (DAP).

For every storage method-duration combination, a 1 cm long section of stem, cut at both ends, was removed from the top one-third of sixteen seedlings of each species. The sixteen stem samples were individually placed into 20 ml copolymer polypropylene vials (RPI Corp., Mt. Prospect, IL) containing 15 ml of deionized water. Four sample vials were randomly assigned to one of four freeze test treatments: 3° C (control), -10° C, -20° C, and -40° C. Sample vials were capped and the control treatment placed into a 3 C refrigerator where it was not exposed to freezing temperatures. The remaining treatments were placed into a Cryomed 1010 programmable freezing unit (Thermo Forma, Marietta, OH) cooled by liquid nitrogen. Before placement of samples into the freezing chamber, the unit was first cooled to a temperature of 0°C. Once the samples were in the chamber, the rate of temperature decrease was set at –0.25°C/minute. Upon reaching each test treatment, the temperature was held for 20 minutes before decreasing again. After the 20 minute hold time, the respective vials were removed and placed in a refrigerator to thaw overnight. Sample vials of all treatments were then removed from refrigeration to complete thawing at room temperature. After thawing, stem EL (total dissolved solids, ppm) was measured with a HI 9813 portable conductivity meter (Hanna Instruments, Inc., Woonsocket, RI). A measure of maximum conductivity was obtained by placing the vials in a Getinge/Castle autoclave (Getinge USA, Inc., Rochester, NY) for steam sterilization at 110 C for 20 minutes. EL values were expressed as a percentage of maximum conductivity.

Experimental Design and Data Analysis

Descriptions here are for each species. For brevity, -20°C was selected being representative of trends observed during the freeze tests, and only its results are presented and discussed. With the -20°C test, the general experimental design is a split-plot with storage method as the whole-plot factor and duration as the sub-plot factor. Since there was only one replication of each storage method, a storage method main effect or storage method × duration interaction cannot be tested. However, within storage method there is a completely randomized design of 4 durations with 4 seedling replicates per duration for the -20° C test and 5 seedling replicates per duration for the greenhouse growth portion of

Table 2.—Mean (± SE) percent electrolyte leakage of stem samples frozen to -20° C (EL) and number of days to first budbreak (DBB) of selected hardwood species. Seedlings were stored by one of three methods and removed after durations ranging from 2 to 5 months.

			Response/	Storage method	
	Species	Duration	Ambient[1]	Cooler	Freezer
Northern	EL	2	27.81 (1.28) ab	20.79 (1.14) b	24.59 (0.48) b
red oak	EL	3	19.87 (1.88) b	24.53 (2.61) ab	24.85 (1.97) b
EL	4	31.25 (5.86) ab	30.67 (4.91) ab	42.03 (2.11) a	
EL	5	40.48 (0.77) a	39.15 (5.97) a	40.83 (1.82) a	
DBB	2	—	42.20 (2.35) a	45.60 (2.56) a	
	DBB	3	32.20 (1.32) a	28.40 (1.12) b	34.20 (3.69) b
	DBB	4	26.60 (4.41) a	29.00 (5.62) b	26.40 (2.38) bc
	DBB	5	0 (0.00) b	12.80 (1.83) c	19.25 (0.75) c
Black	EL	2	30.54 (3.70) a	27.29 (2.59) b	25.18 (1.35) c
cherry	EL	3	40.18 (4.40) a	42.94 (4.26) ab	46.01 (5.23) b
	EL	4	43.25 (3.19) a	62.51 (10.30) a	82.78 (0.96) b
	EL	5	49.02 (3.52) a	47.89 (0.79) ab	56.12 (5.96) a
DBB	2	—	16.40 (0.87) a	18.60 (6.60) a	
DBB	3	8.00 (1.90) a	10.20 (1.50) b	17.25 (7.42) a	
DBB	4	0 (0.00) b	4.60 (1.25) c	9.60 (1.21) a	
DBB	5	0 (0.00) b	1.20 (1.20) c	9.00 (1.68) a	
Black	EL	2	28.98 (2.55) b	19.81 (1.52) b	32.94 (1.64) b
walnut	EL	3	20.44 (2.24) b	24.75 (3.19) b	29.51 (1.12) b
	EL	4	43.07 (8.88) ab	24.90 (0.42) b	44.90 (2.27) ab
	EL	5	62.71 (9.86) a	52.79 (8.18) a	50.11 (6.90) a
	DBB	2	—	31.20 (1.56) a	29.20 (5.66) a
	DBB	3	16.00 (0.63) a	17.00 (1.97) b	22.40 (1.47) a
	DBB	4	5.60 (0.51) b	12.80 (1.43) b	25.00 (5.93) a
	DBB	5	0 (0.00) c	5.00 (1.05) c	17.40 (0.75) a

[1]For measured responses within each storage method, means followed by the same letter in a column did not differ significantly ($P \leq 0.05$, Tukey method).

the study. Therefore, EL and DBB differences within each storage method that result from changes in duration can be tested. Analysis of variance was performed using SAS statistical software version 8.2 (SAS Institute, Cary, NC). Results are summarized in table format (Table 2). Regression analyses were also performed to assess the relationship of EL to DBB for each species (Table 3).

Results And Discussion

Electrolyte Leakage

All species showed a similar trend when exposed to progressively longer storage durations. This was evident for all three storage methods (Table 2). For northern red oak, stem EL of ambient seedlings was 27.81% after 2 months storage and rose to 40.48% after 5 months. During the same period, stem EL of cooler-stored and freezer-stored seedlings rose from 20.79% to 39.15% and 24.59% to 40.83%, respectively. Regardless of storage method, EL levels increased in response to extended storage duration. Stem EL from ambient black cherry seedlings increased from 30.54% after 2 months storage to 49.02% after 5 months. Cooler-stored black cherry exhibited EL levels ranging from 27.29% (2 months) to 47.89% (5 months). Freezer-stored black cherry showed the greatest increase in stem EL from 2 to 5 months, rising from 25.18% to 56.12%. Again, duration impacted EL in all storage methods. Stem EL of black walnut seedlings left in the nursery beds began at 28.98% after 2 months

Table 3.—Simple linear regression of electrolyte leakage (EL) values at -20°C on number of days to first budbreak (DBB) for northern red oak, black cherry, and black walnut seedlings across all storage methods. Paired values from all durations were used.

Species	R^2	P-value	Regression equation
Northern red oak	0.60	0.0049	DBB = 63.17 – 1.17(EL)
Black cherry	0.14	0.2457	*no observed relationship*
Black Walnut	0.40	0.0359	DBB = 32.78 – 0.44(EL)

and ended at 62.71% after 5 months. Stem EL of cooler-stored seedlings rose from 19.81% to 52.79%, while that of freezer-stored seedlings rose from 32.94% to 50.11%. The change of EL over the course of storage was smallest for freezer-stored seedlings.

Increasing levels of stem EL with extended storage indicates the seedlings' chilling requirements had been met, resulting in a loss of dormancy and cold hardiness over time. Individual species responded differently to the different storage methods. The response of northern red oak differed little among storage methods. This might be attributed to a greater chilling requirement or higher level of dormancy compared to the other species. Stem EL of freezer-stored black cherry seedlings appeared to be somewhat higher than the other storage methods. This may be a result of a low chilling requirement and an increased sensitivity and susceptibility to freezing temperatures in a storage environment. EL of ambient-stored black walnut seedlings at the end of the 5-month period was 10-12 percentage points greater than that of cooler and freezer-stored seedlings. It may be that some form of cold storage is better able to maintain a level of hardiness for prolonged durations compared to the other two species.

Budbreak

The number of days to budbreak (DBB) was used as an indicator of seedling dormancy status. DBB followed a trend that was opposite that of stem EL (Table 1). DBB decreased substantially with longer storage durations, while stem EL generally increased. For northern red oak, ambient-stored seedlings required the fewest DBB, decreasing from 32.20 to 0 during the storage period. DBB of cooler-stored seedlings was 42.20 after 2 months and 12.80 after 5 months, while that of freezer-stored seedlings ranged from 42.60 to 19.25 during that period.

Black cherry seedlings required few DBB in all storage methods. Seedlings left in the nursery needed only 8 DBB after 3 months and had already leafed out after 4 months. DBB of cooler-stored seedlings decreased from 16.40 after 2 months to 1.20 after 5 months. Freezer-stored seedlings needed 18.60 DBB after 2 months and 9 DBB after 5 months. Although it could not be tested, it appeared that method of storage influenced DBB to some extent. Seedlings stored in the freezer for 4 and 5 months required more DBB than those left in the nursery during the same period. From this data, it would appear that overwinter storage is essential for late planting of black cherry.

DBB of ambient-stored black walnut seedlings decreased from 16 after 3 months to 0 after 5 months. Cooler-stored seedlings needed 31.20 DBB after 2 months and 5 DBB after 5 months, while DBB of freezer-stored seedlings only decreased from 29.20 to 17.40. There were clear differences due to storage duration, with seedlings stored for 2-3 months generally requiring more DBB that those stored for 4-5 months. However, when looking at this effect within each storage method, only ambient and cooler-stored seedlings followed this trend. Freezer-stored seedlings tended to require more DBB than the other storage methods and did not show a great decrease in DBB with increased storage duration.

Overall, DBB decreased with longer storage durations, while stem EL levels decreased. This is consistent with the results of Calme et al. (1994). Fewer DBB and high EL levels both point to an increase in seedling physiological activity and loss of hardiness. A significant negative relationship ($p \leq 0.05$) between EL and DBB was shown for northern red oak and black walnut, but not black cherry

(Table 3). The lack of a relationship in black cherry might be attributed to its behavior in freezer storage. High levels of shoot desiccation, although not quantified, were observed in the freezer. Packaging materials did not completely enclose the seedlings, thereby exposing much of the stem tissue to freezing conditions. Consequently, there was a higher occurrence of terminal bud mortality compared to cold-stored and ambient seedlings. This mortality may have altered seedling budbreak patterns and also resulted in the high EL levels. This could explain seedlings having high EL and high DBB at the same time compared to the other storage methods.

Height Growth

Height growth of all species did not seem to be extensively affected by the different storage regimes (data not shown). Height growth of northern red oak in each storage method appeared to increase over time to a point and then decline. Black cherry seedlings lifted from the nursery at the end of 5 months were already growing and did not survive transplanting to greenhouse conditions. Growth of ambient and cooler-stored black walnut seedlings was similar, while freezer-stored seedlings generally had the smallest height increase. It is difficult to discern relationships between height growth and EL or DBB. It is possible that a seedling's growth response is more closely associated with its inherent genetic capability than either EL or DBB.

Future Considerations

Future hardwood EL studies should be expanded to compare different plant tissues such as roots and buds. Investigating lateral vs. terminal buds or fine vs. coarse roots would also be useful to increasing our understanding of EL in hardwoods. Seedling packaging methods should also be considered, particularly when dealing with sub-freezing conditions. In addition, because the seedlings for this project were germinated from seeds collected over a large geographic range in Indiana, it is likely that genetic variability is responsible for some of the observed differences or lack thereof. Therefore, it may be helpful to reduce this variation by using plant material of known parentage. Rapid methods for measuring cold hardiness and dormancy status will benefit foresters by allowing prompt assessment of a seedling's ability to withstand transportation to field planting sites without excessive desiccation or loss of stored reserves. This knowledge is useful in planning when certain batches of seedlings should be shipped and matching a seedling's physiological status with appropriate planting sites. Information gathered during the course of this preliminary study will be valuable in planning and implementing related projects in the future.

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