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Original article

Cold hardiness and transplant response of *Juglans nigra* seedlings subjected to alternative storage regimes

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Abstract –
• Effects of overwinter storage regimes on seedling cold hardiness and physiological vigor are relatively unexplored, particularly for temperate deciduous forest tree species.
• We evaluated influence of storage duration (0, 66, 119, or 175 d) on electrolyte leakage of stem and root collar tissues following exposure to a series of freeze-test temperatures in black walnut (*Juglans nigra* L.) seedlings sampled from cold (3 °C) or freezer (–2 °C) storage. Seedlings were subsequently transplanted into a controlled growth chamber environment for two months.
• Regardless of storage temperature, mean LT_{50} was lowest for seedlings stored for 66 d (≤ –34 °C) and increased dramatically after 119 d (≥ –13 °C).
• Root collar tissue had lower LT_{50} than stem tissue after 119 d for cold-stored seedlings, reflecting importance of evaluative tissue type. Days to bud break shortened with increasing storage duration up to 119 d and stabilized thereafter for both storage regimes. Root growth potential was maximized after 119 d of storage, and subsequently declined for cold-stored seedlings. Height growth increased following storage, regardless of duration.
• To promote stress resistance and transplant growth response, we recommend that black walnut seedlings from this genetic source be outplanted after approximately 66–119 d of storage.

black walnut / bud break / cold hardiness / cold storage / electrolyte leakage / freezer storage / root growth potential

Résumé – Endurcissement au froid et réponse des semis de *Juglans nigra* transplantés après exposition à différentes modalités de stockage.
• Les effets de différentes modalités de stockage hivernal sur la résistance au froid des semis et sur leur vigueur physiologique ont été relativement inexplorés, en particulier pour les arbres forestiers décidus tempérés.
• Nous avons évalué l'influence de la durée de stockage (0, 66, 119 ou 175 jours) sur la perte d'électrolyte de la tige et des tissus du collet racinaire exposés à une série de tests (témoin 4 %C, –10 °C, –20 °C, –40 °C) de température de congélation de semis de noyer noir (*Juglans nigra* L.), après stockage au froid (3 °C) ou au gel (–2 °C). Les semis étaient ensuite transplantés dans une chambre climatisée pour une durée de deux mois. Indépendamment de la température de stockage, la moyenne de LT50 (température létale correspondant à un endommagement de 50 % des plants) a été plus basse pour les semis stockés pendant 66 jours (≤ –34 °C) et s'est accrue de façon spectaculaire après 119 jours (≥ –13 °C).
• Les tissus du collet racinaire avaient un plus bas LT50 que les tissus de la tige, après 119 jours pour les semis stockés au froid, reflétant l'importance du type de tissu pour l'évaluation. Le nombre de jours jusqu'au débourrement a été raccourci avec l'accroissement de la durée de stockage jusqu'à 119 jours et s'est stabilisé par la suite pour les deux modalités de stockage. Le potentiel de croissance racinaire a été maximisé après 119 jours de stockage et a décliné par la suite, pour les semis stockés au froid. La croissance en hauteur s'est accrue à la suite du stockage, indépendamment de sa durée.
• Pour promouvoir une résistance élevée au stress et une forte reprise de croissance des semis transplantés, nous recommandons que les semis de noyer noir de cette source génétique soient plantés après approximativement 66 à 119 jours de stockage.

noyer noir / débourrement / endurcissement au froid / stockage au froid / perte d'électrolyte / stockage en congélation / potentiel de croissance racinaire

1. INTRODUCTION

Black walnut (*Juglans nigra* L.) occurs throughout the deciduous forests in the central and eastern parts of the United States north into southern Ontario, Canada (Williams, 1990). It is considered one of the most ecologically important (Goheen and Swihart, 2003) and economically valuable native hardwoods (McGuire et al., 1999; Shifley, 2004). For these reasons, much research has focused on production of high quality black walnut seedlings and subsequent performance upon outplanting (Beineke, 1989; Jacobs et al., 2005; Rink and Van Sambeek, 1985; Seifert et al., 2006).

Quality seedlings that meet expectations or standards of performance on a particular planting site (Duryea, 1984) are necessary for reforestation success. Most research on seedling quality assessment has focused on conifer species. Increasing demand for hardwood species as a result of ecological restoration and conservation practices (King and Keeland, 1999; Stanturf et al., 2000), however, has led to increased efforts to develop seedling quality assessment methods specific to

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hardwoods (Wilson and Jacobs, 2006). One method for evaluating seedling physiological quality, cold hardiness assessment, provides a measure of dormancy status (Ritchie, 1984), predicts ability of seedlings to withstand stresses associated with lifting, storing, and planting (O'Reilly et al., 1999), and serves as an indicator of field performance potential (Pardos et al., 2003). Physiological methods of testing cold hardiness are also rapid (McKay, 1992), allowing for timely management decisions in nursery operations.

Many techniques have been employed to assess cold hardiness. Visual methods such as whole plant freeze tests and shoot tissue browning tests are effective (Liu et al., 1998; Timmis, 1976), yet 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. Though these measurements are rapid, they have had limited application in hardwood regeneration programs (Cabral and O'Reilly, 2005; Généré et al., 2004; O'Reilly et al., 2003; Radoglou and Raftoyannis, 2001).

The electrolyte leakage (EL) method, used extensively as a reliable approach to determine cold hardiness in conifer research (Bigras, 1997; Colombo et al., 1995; Jacobs et al., 2008; Tinus and Burr, 1997), was chosen for this study as it has recently shown success with hardwood seedlings (Cabral and O'Reilly, 2005; Garriou et al., 2000; Généré et al., 2004; Mortazavi et al., 2004; O'Reilly et al., 2003; Radoglou and Raftoyannis, 2001). Cold temperatures reduce enzymatic activity, alter metabolism, and decrease photosynthetic capacity of plant tissues (Dubey, 1997). These changes are often associated with increased membrane permeability and degradation of membrane integrity (Campos et al., 2003), causing electrolytes from stressed cells to leak into the surrounding apoplast. EL estimates cell damage and hardiness by comparing conductivity of leaked contents from injured and uninjured tissues (Mattsson, 1996; McKay, 1992).

For conifers, needles (Burr, 1990) are the most commonly sampled tissues for EL. Sampling of foliage is particularly applicable to evergreens, which can be collected and tested throughout the dormant period. Roots (McKay et al., 1999) and stem tissues (Colombo et al., 1995) have also been used. Efficacy of EL in predicting hardiness has resulted in its operational use 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., 2003). Many important hardwood species of eastern North America, including black walnut, have not been extensively studied. Furthermore, impacts of different storage regimes on tissue EL and physiological status of hardwood seedlings have not been widely published. Thus, the objectives of this research were to examine effects of storage duration on (i) cold hardiness (assessed via EL following subjection to various freeze test temperatures) and (ii) dormancy status and plant vitality (assessed via root growth potential and days to terminal bud break) in black walnut under cold (3 °C) and freezer (–2 °C) storage regimes varying in duration.

Table I. Mean (± SE) temperature (°C) and total precipitation (mm) in October, November, and December 2003 (data from Purdue Agronomy Research Center, West Lafayette, Indiana).

Month	Temperature (°C)	Precipitation (mm)
October	11.89 (0.82)	30.95
November	7.50 (1.22)	86.03
December	0.37 (1.08)	13.42

2. MATERIALS AND METHODS

2.1. Plant material

Seeds were collected from various black walnut parent trees located in close proximity to West Lafayette, IN, USA (40 ° 25´ N, 86 ° 54´ W) in November 2002. Seeds were pooled from five parent trees and processed by physically removing the periderm from the nut and cleaning the remaining residue with a power sprayer. Seeds were stored in perforated plastic bins filled with moist, finely milled peat moss at 2 °C until March 2003. Seeds were sprouted by placing them in flats lined with fine vermiculite under a programmed misting cycle (8 s every 6 min) to keep them moist (Davis et al., 2004) at 24 °C air temperature. Seeds were removed from flats as they sprouted and placed in refrigerated storage (2 °C) until a sufficient number had been collected.

Sprouted seeds were sown into Styroblock™ 623A containers (28 (500 mL) cells per container, Beaver Plastics, Edmonton, Alberta, Canada) with Scotts Metro-Mix 560 soil mix (O.M. Scotts, Marysville, OH, USA) and grown in a greenhouse under 24 °C day/17 °C night environmental conditions until late-October 2003, when they were placed in an outdoor lathe house under ambient environmental conditions (Tab. I) to induce hardening.

2.2. Cold storage treatments

In mid-December 2003, 400 seedlings were removed from styroblocks and divided into two groups of 200 seedlings. One group was placed into four 3-ply storage bags with polyethylene inner sheets (Portco Packaging, Vancouver, WA, USA) fully enclosed and placed into cold storage (3 °C); another group was similarly processed and placed into freezer storage (–2 °C). Seedling transfer to over-winter storage at this time frame is consistent with operational practice in this region (Jacobs, 2003). A group of 15 seedlings (23.7 (mean) ±1.11 (SE) cm height and 5.7 ± 0.13 mm RCD) was immediately removed from each storage regime for initial (0 d storage) assessment of morphology and cold hardiness. Subsequent seedling removals occurred after 66 d (29.5 ± 1.79 cm height and 7.1 ± 0.19 mm RCD), 119 d (28.6 ± 1.82 cm height and 7.5 ± 0.18 mm RCD), and 175 d (28.2 ± 2.12 cm height and 6.5 ± 0.25 mm RCD) of storage.

2.3. Cold hardiness evaluation

Cold hardiness was estimated using the electrolyte leakage (EL) method (Burr, 1990; Colombo et al., 1995; McKay, 1992). Twenty

seedlings from each storage temperature were randomly selected and removed at end of each storage duration treatment for destructive sampling. Five seedlings were randomly assigned to each of four freeze test temperatures: control (4 °C), –10, –20, and –40 °C. From each seedling, two plant parts were used for testing: a 1-cm section of stem tissue two nodes (i.e., two points of leaf attachment) below the terminal bud and a 1-cm section of stem tissue at the root collar.

Individual samples were placed into 20-mL copolymer polypropylene vials (RPI Corp., Mt. Prospect, IL, USA) containing 15 mL of deionized water. Sample vials were capped and control vials placed into a 4 °C refrigerator. The remaining treatments were placed into a Cryomed 1010 programmable freezing unit (Thermo Forma, Marietta, OH, USA) cooled by liquid nitrogen. The freezing chamber was cooled to 0 °C before samples were placed into the unit. Air temperature was lowered by 0.25 °C min^{-1} and held for 20 min at each test treatment before removing respective vials. Once removed, vials were placed in a refrigerator to thaw overnight. Sample vials of all treatments were then removed from refrigeration to complete thawing and leaching at 22 °C. Freeze-induced EL (total dissolved solids; ppm) was measured with a portable conductivity meter (HI 9813, Hanna Instruments, Inc., Woonsocket, RI, USA). Measure of maximum EL (after total cell death) was obtained by placing vials in a Getinge/Castle autoclave (Getinge, Inc., Rochester, NY, USA) for steam sterilization at 120 °C for 20 min. For each sample, an Index of Damage (I_d) was calculated using the following formula:

$$I_d(\%) = \left(\frac{Freeze\text{-}induced EL}{Maximum EL} \right) \times 100. \quad (1)$$

2.4. Transplant performance

After removal from cold or freezer storage, 15 seedlings from each storage regime and duration (with each seedling representing an experimental unit) were measured for root volume using the water displacement method (Pinkas et al., 1964), potted into Tall One Treepot^{TM} containers (2.83 L, Stuewe & Sons, Corvallis, OR, USA) with Scotts Metro-Mix 560 media, and randomly distributed into two Conviron E8 growth chambers (Controlled Environments, Ltd., Winnipeg, Manitoba, Canada) for two months. Environmental conditions in the chambers were maintained at temperatures of 24 °C day/17 °C night, 16-h photoperiod, and 60% RH. A second measurement of root volume was made at the end of the 2-month period. Root growth potential was determined by assessing absolute change in root volume over the 2-month period. Seedlings in growth chambers were monitored daily for terminal bud break (defined as time when bud scales began to separate and reveal leaves).

2.5. Data analysis

The experiment was limited to one storage chamber per storage regime so storage temperature could not be replicated. The EL component of the experiment within each storage temperature was a completely randomized factorial design with three independent variables: storage duration, freeze test temperature, and plant part. The dependent variable was I_d. The post-storage growth chamber portion of the study was a completely randomized design with storage duration as the independent variable and root growth potential (RGP), root collar diameter (RCD), height, and days to terminal bud break (DTB) as dependent variables. Each seedling randomly selected from storage chambers on each date in each part of the study was considered as one experimental unit.

All data were analyzed using SAS Software (SAS, Inc., Cary, NC, USA). ANOVA was performed on DTB, and changes in root volume, RCD, and height using PROC GLM with Tukey's separation of storage duration means. Because all two-way interactions in the EL portion of the study were significant, analyses of simple main effects were performed using the LSMEANS/ SLICE feature in the PROC MIXED statement. Interaction between plant tissue and freeze test temperature was sliced by temperature to compare plant parts separately at each freeze test temperature. Similarly, interaction between freeze test temperature and storage duration was sliced by temperature and interaction between plant part tissue and storage duration was sliced by duration. Significant differences at each slice level were further analyzed using LSMEANS/ DIFF of the PROC MIXED procedure. Temperature and storage duration were between-subject variables and plant part was the within-subjects variable.

In addition to I_d, cold hardiness data were expressed as LT_{50} (i.e., lethal temperature at which 50% of plant material is damaged; Burr, 1990). Each LT_{50} value was calculated by fitting simple quadratic regressions for each treatment based on EL values at each test temperature. Differences in mean LT_{50} values across storage duration treatments and plant tissues were identified using ANOVA at a significance level of $\alpha = 0.05$ and ranked using Tukey's Least Squares Means with adjustment for multiple comparisons.

3. RESULTS AND DISCUSSION

3.1. Cold hardiness development

For freezer-stored seedlings, I_d did not differ between plant parts, but did differ among freeze test temperatures and storage duration treatments (Tab. II). For cold-stored seedlings, I_d differed between plant parts and among freeze test temperatures and storage duration treatments (Tab. II). All two-way interactions were statistically significant for both cold- and freezer-stored seedlings (Tab. II). I_d differed little among storage types, durations, or tissue types for seedlings subjected to freeze tests temperatures of 4 °C (control) and –10 °C (mean $I_d < 50\%$). Following exposure to –20 °C, however, higher I_d was observed among seedlings stored for either 119 d or 175 d (mean I_d ranged from 51% to 81%) compared to those not stored or stored for only 66 d (mean I_d ranged from 17% to 56%). This trend was more pronounced for cold-stored seedlings (mean I_d separation of 55%) compared to freezer-stored seedlings (mean I_d separation of 25%) and for stem tissue (mean I_d separation of 45%) compared to root collar tissue (mean I_d separation of 35%). High I_d (mean $I_d > 50\%$) resulted after exposure to –40 °C, regardless of storage type, duration, or tissue type.

Fitting simple quadratic regression equations for replicate measures of I_d (dependent variable) on freeze test temperatures (independent variable) for each combination of plant part, storage temperature, and storage duration yielded R^2 values ranging from 0.86 to 0.95 for freezer-stored seedlings and from 0.92 to 0.97 for cold-stored seedlings. Similar to results for I_d, the temperature at which 50% of tissue was damaged (LT_{50}) differed among storage durations for both cold- and freezer-stored black walnut seedlings (Figs. 1 and 2).

Table II. Summary of ANOVA for index of damage (I_d) resulting from electrolyte leakage testing of freezer- (–2 °C) and cold- (3 °C) stored black walnut seedlings. Plant part (part), freeze test temperature (temp), and storage duration (dur) are independent variables; I_d is the dependent variable.

Storage temperature	Source of variation	DF	Sum of squares	Mean square	*F* value	*P* value
–2 °C	part	1	1.57	1.57	0.01	0.9091
	temp	3	68939.2	22979.7	191.24	< 0.0001
	part × temp	3	1575.51	525.17	4.37	0.0058
	dur	3	12013.1	4004.36	33.32	< 0.0001
	part × dur	3	1234.19	411.4	3.42	0.0193
	temp × dur	9	6686.43	742.94	6.18	< 0.0001
	part × temp × dur	9	939.28	104.36	0.87	0.5552
	Error	127	15260.5	120.16		
3 °C	part	1	580.99	580.99	9.97	0.002
	temp	3	63533.8	21177.9	363.29	< 0.0001
	part × temp	3	546.42	182.14	3.12	0.0282
	dur	3	25057.9	8352.64	143.28	< 0.0001
	part × dur	3	2020.27	673.42	11.55	< 0.0001
	temp × dur	9	16513.4	1834.82	31.47	< 0.0001
	part × temp × dur	9	295.31	32.81	0.56	0.8253
	Error	128	7461.76	58.30		

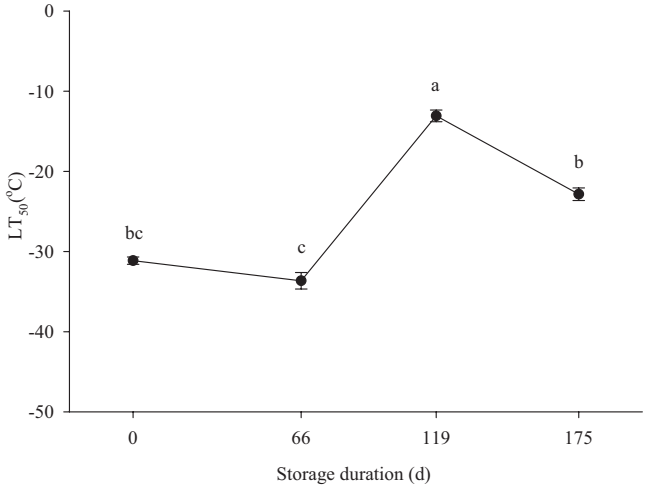

Figure 1. Mean (± SE) freezer-stored black walnut cold hardiness expressed as LT_{50} across storage duration treatment (0, 66, 119, and 175 d) averaged across plant tissue types (stem and root collar). Each LT_{50} was calculated by fitting simple quadratic regressions based on electrolyte leakage values at 4, –10, –20, and –40 °C. Different letters indicate significant differences (α = 0.05).

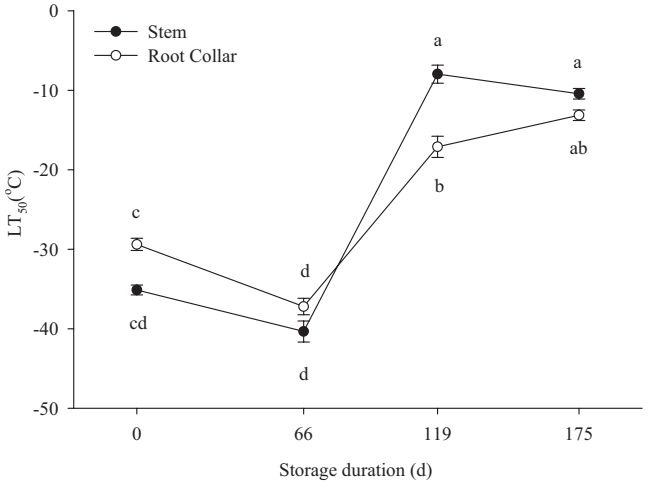

Figure 2. Mean (± SE) cold-stored black walnut cold hardiness expressed as LT_{50} across storage duration treatments (0, 66, 119, and 175 d) for each plant tissue type (stem and root collar). Each LT_{50} was calculated by fitting simple quadratic regressions based on electrolyte leakage values at 4 °C, –10 °C, –20 °C, and –40 °C. Different letters indicate significant differences (α = 0.05).

For freezer-stored seedlings, only the main effect of storage duration was significant ($p < 0.001$). Mean LT_{50} (averaged across plant tissues) was lowest for seedlings taken from 66 d of freezer storage and increased significantly from 66 to 119 d of storage (Fig. 1), indicating that seedlings had increased physiological activity and were emerging from dormancy. LT_{50} declined after 175 d of storage (Fig. 1), suggesting that additional freezer storage (i.e., beyond 119 d) maintained or slightly increased cold hardiness.

For cold-stored seedlings, LT_{50} results showed similar patterns to that of freezer-stored seedlings. However, interaction between storage duration and plant tissue type was significant ($p < 0.001$). Mean LT_{50} decreased slightly from 0 to 66 d, which was significant only for root collar tissue (Fig. 2), indicating that seedlings were most cold hardy and dormant during this time. A large increase in LT_{50} then occurred from 66 to 119 d for both tissue types, with stem tissue having significantly greater LT_{50} than root collar tissue (Fig. 2). Unlike freezer-stored seedlings, values for both tissue types stabilized between 119 and 175 d (Fig. 2), indicating that additional storage did not significantly affect cold hardiness. Observed variation in cold hardiness of the different tissue

types (i.e., greater cold hardiness of root collar vs. stem tissue after 119 d) suggests that tissues higher on the stem (i.e., closer to the terminal bud), deharden more rapidly than basal tissues in freezer-stored seedlings. Increased cold hardiness of basal tissues may be advantageous to survival of planted seedlings as these tissues contain dormant buds that facilitate resprouting following cold damage to shoots associated with late spring frosts, which is common for black walnut in this region (Jacobs et al. 2004). Interestingly, these same two tissues from freezer-stored seedlings showed no such differentiation, indicating that the lower temperature (–2 °C) may have been cold enough to prolong dormancy of the upper stem tissues.

Lack of foliage during dormancy in temperate deciduous forest tree species has prompted investigation of use of other plants parts for EL sampling with these species, most commonly involving root tissues (Wilson and Jacobs, 2006). For example, Mortazavi et al. 2004 assessed taproots of ash (*Fraxinus excelsior* L.), maple (*Acer pseudoplatanus* L.), and sessile oak (*Quercus petraea* (Mattuschka) Liebl.) to examine seasonal variation in stress resistance in response to heat treatments applied in vitro. Past studies have identified relationships between fine root EL and seedling physiological quality or field performance potential in various hardwood species (e.g., McKay et al., 1999; Garriou et al., 2000), though O'Reilly et al. (2002) reported no correlation with height growth in freshly lifted or cold stored ash and maple. As in our study, sampling EL of stem (Deans et al., 1995; Sarvaš, 2001) and root collar tissues (Schute and Sarvaš, 1999) has also proved useful in evaluating seedling physiological activity of hardwood species. The potentially non-destructive nature of stem tissue sampling may offer an advantage over using roots or root collar tissue in evaluating physiological status of hardwoods (Wilson and Jacobs, 2006).

3.2. Transplant performance

DTB differed significantly across storage duration treatments for both cold- and freezer-stored seedlings such that DTB generally shortened with increasing storage duration up to 119 d and stabilized thereafter (Fig. 3A). For both cold- and freezer-stored seedlings, DTB was greatest for non-stored seedlings (0 d; Fig. 3A), indicating that seedlings were highly dormant and exposure to ambient environmental conditions during late-October through mid-December (Tab. I) did not fully satisfy chilling requirements. DTB was substantially and significantly lower in seedlings stored for 66 d (Fig. 3A), indicating that a minimal chilling requirement may have been met. Specifically, both cold- and freezer-stored seedlings required fewer than half the number of days to break bud after 66 d of storage compared to non-stored seedlings, reducing bud dormancy from approximately 43 d without storage to 18 d or fewer depending on storage temperature. This confirms that heat requirements for bud break in black walnut are reduced given chilling accumulation, as demonstrated for other hardwood species (O'Reilly et al., 2003). Mean DTB declined further between 66 and 119 d (significant in freezer-stored seedlings; Fig. 3A). DTB was not significantly different between seedlings stored for 119 or 175 d for either storage treatment (Fig. 3A), indicating that additional chilling (i.e., beyond 119 d) was not necessary. Our results correspond to findings of Rietveld and Williams (1978), which showed that physiological dormancy of black walnut seedlings ended following approximately 129 d of storage at 3 °C.

Root growth potential (RGP), measured via absolute change in root volume over the 2-month growth period, differed significantly across storage duration treatments for both freezer- and cold-stored seedlings (Fig. 3B). For both storage treatments, root volume growth was lowest for seedlings not stored (significant in cold-stored seedlings), greater in seedlings taken from storage after 66 d, greatest in seedlings taken from storage after 119 d, and declined after 175 d in storage (Fig. 3B). RGP fluctuated widely in relation to storage duration for cold-stored seedlings; root growth was significantly greatest in seedlings stored for 119 d and statistically equal between 66 and 175 d (Fig. 3B). RGP fluctuated less among freezer stored seedlings; RGP was significantly lower among seedlings stored for 0 and 66 d than those stored for 119 and 175 d (Fig. 3B). RGP is the most common physiological quality testing protocol for conifers (Simpson and Ritchie, 1997), and positive relationships between RGP and survival or field performance have consistently been reported for a variety of hardwood species (Garriou et al., 2000; Lindqvist, 1998; O'Reilly et al., 2002; Pardos et al., 2003). Additionally, RGP may provide a good indicator of stress tolerance and storability as RGP immediately after lifting was negatively correlated with survival for silver birch (*Betula pendula* Roth.) and pedunculate oak (*Quercus robur* L.) (Lindqvist, 1998). RGP is known to fluctuate seasonally under ambient conditions and in overwinter storage (Simpson and Ritchie, 1997), generally rising with accumulation of cold hardiness according to the degree growth stage model (Fuchigami and Nee, 1987; Fuchigami et al., 1982). This relationship is supported by our study results, which showed high RGP for seedlings stored for 119 d or more (Fig. 3B) under both storage regimes.

RCD growth did not differ significantly across storage duration treatments for either freezer- or cold-stored seedlings (Fig. 3C), which may have been associated with relatively high inherent variation among seedlings. Height did not differ among storage duration treatments (66, 119, or 175 d), but non-stored seedlings (0 d) had significantly less height growth at end of the 2-month growth period compared to seedlings that had been either freezer- or cold-stored (Fig. 3D), indicating that at least 66 d of storage under either temperature increases early height growth.

4. CONCLUSIONS

Despite prevalence of overwinter storage of nursery seedlings, little is known about physiological effects of varying types and durations of storage, particularly for temperate deciduous forest tree species. Our research begins to shed light on these effects by documenting changes in black walnut seedling cold hardiness and transplant performance as a result of varying durations of cold or freezer storage. Our

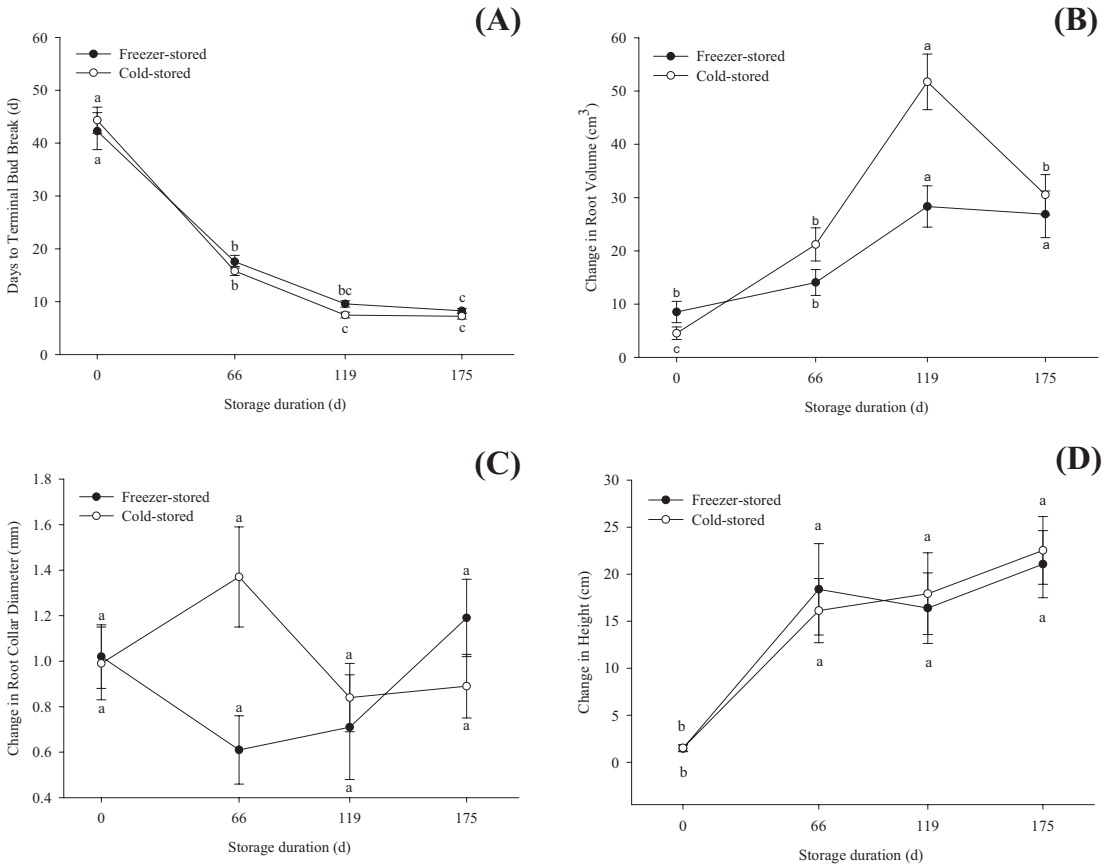

Figure 3. Mean (± SE) (A) days to terminal bud break (DTB), and change in (B) root volume, (C) root collar diameter (RCD), and (D) height (H) of freezer- (–2 °C) and cold- (3 °C) stored black walnut seedlings after 2 months in a growth chamber. Different letters indicate significant differences ($\alpha = 0.05$) across storage duration treatments.

data indicate that regardless of storage temperature, maximum cold hardiness was attained after 66 d of storage and physiological dormancy ended after 119 d. Highest RGP occurred after 119 d of storage, and RGP declined thereafter for cold-stored seedlings. Despite high RGP with ≥ 119 d of storage, we recommend that seedlings of this genetic source be outplanted after approximately 66–119 d of storage to prevent gradual loss of plant vigor (e.g., stress resistance).

Additional research should be designed to more precisely identify the time period when black walnut seedling chilling requirements are fulfilled, and if this time differs between cold- and freezer-stored seedlings, to better understand specific durations that seedlings of this species can be stored without considerable loss of cold hardiness and stress resistance.

Future studies using EL as a means to assess cold hardiness should consider the tissue for which these measurements are made as significant differences were observed between stem and root collar tissues taken from the same individual seedlings. In the future, it will be useful to identify plant tissues that are strongly reflective of changes associated with stress or dormancy, well correlated with field performance, and simultaneously produce low variation in EL among individual samples, as these would have the most promise in consistent prediction of hardiness and physiological status. This variation, which has been documented for cold hardiness assessments of various black spruce (*Picea mariana* (P. Mill.) B.S.P.) tissues (Colombo et al., 1995), must be accounted for in determining whether this is a suitable means for non-destructive testing (stem tissue) or must be destructive (root tissue) to achieve the desired level of information. Finally, variability among different species and genetic sources will also need to be evaluated to further refine applicability of target EL thresholds for lifting, storing, and planting.

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