

# Physiological Responses of Planting Frozen and Thawed Douglas-Fir Seedlings

M Anisul Islam  
Kent G Apostol  
Douglass F Jacobs  
R Kasten Dumroese

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## M ANISUL ISLAM

Post-doctoral Research Scientist  
Hardwood Tree Improvement and  
Regeneration Center (HTIRC)  
Department of Forestry and Natural Resources  
Purdue University  
West Lafayette, IN 47907-2061  
Tel: 765.494.3587  
E-mail: mislam@purdue.edu

## KENT G APOSTOL

Assistant Professor  
Department of Biological Sciences  
Bethel University  
3900 Bethel Drive  
St Paul, MN 55112  
E-mail: k-apostol@bethel.edu

## DOUGLASS F JACOBS

Associate Professor  
Hardwood Tree Improvement and  
Regeneration Center (HTIRC)  
Department of Forestry and Natural Resources  
Purdue University  
West Lafayette, IN 47907-2061  
E-mail: djacobs@purdue.edu

## R KASTEN DUMROESE

Research Plant Physiologist  
National Nursery Specialist  
USDA Forest Service  
Southern Research Station  
1221 South Main Street  
Moscow, ID 83843-4211  
E-mail: kdumroese@fs.fed.us

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## ABSTRACT

We studied the short-term (7-day) physiological responses of planting thawed and frozen root plugs of Douglas-fir (*Pseudotsuga menziesii*) seedlings in 2 separate experiments under cool-moist and warm-dry growing conditions, respectively. Our results showed that shoot water potential, root hydraulic conductance, net photosynthesis (*A*), and transpiration (*E*) were significantly lower in frozen seedlings compared with thawed seedlings under both growing conditions. Chlorophyll fluorescence values in frozen and thawed seedlings were similar throughout the measurement

period at both growing conditions except at 0 hours. We detected no significant differences in electrolyte leakage and chlorophyll content between frozen and thawed seedlings under both environmental regimes. When planted under warm-dry conditions, thawed seedlings had more buds that began to elongate and had more new roots than frozen-planted seedlings at the end of the experiment. When planted in cool-moist conditions, however, neither frozen nor thawed seedlings had buds that resumed growth. Comparatively higher photosynthesis rates observed in thawed seedlings planted under warm-dry conditions might have contributed toward the production of more new roots, and could be advantageous for survival and early growth after outplanting.

#### KEYWORDS

chlorophyll fluorescence, gas exchange, *Pseudotsuga menziesii*, root hydraulic conductance, transplant stress, water relations

#### Introduction

Dormant conifer seedlings are often stored in freezers prior to spring outplanting in temperate climates (McKay 1997; Paterson and others 2001). Freezer storage mainly provides flexibility in scheduling seedling delivery to outplanting sites in spring (Rose and Haase 1997). Frozen seedlings, however, require thawing prior to outplanting to facilitate seedling separation because root plugs freeze together during storage (Kooistra 2004). Proper thawing of root plugs demands additional nursery resources, and can have potential negative impacts on seedling health and physiology (Hocking 1971; Puttonen 1986). These factors suggest that direct outplanting of frozen root plugs after removal from storage would streamline the seedling production process and improve logistics of seedling delivery (Kooistra and Bakker 2002).

Several studies have reported that outplanting frozen seedlings had no damaging effects on seedling performance compared with thawed seedlings (Silim and Guy 1998; Kooistra and Bakker 2002, 2005). When seedlings are outplanted into warm soil, (18 to 32 °C [64 to 90 °F]), thawing of frozen root plugs is unnecessary (Camm and

others 1995). However, Helenius (2005) reported higher mortality and reduced growth in Norway spruce (*Picea abies* L. Karst) seedlings outplanted frozen compared with thawed cohorts.

Spring outplanting of trees usually starts when average ambient temperature is above freezing, and could continue at difficult-to-access sites until the average ambient temperature rises to 30 °C (86 °F). These conditions are likely to occur under certain circumstances, such as when snow limits access to high elevation sites until early summer. For example, frozen root plugs of Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) outplanted in Colorado, and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) and western white pine (*Pinus monticola* Dougl. ex D. Don) outplanted in Idaho, under hot, sunny weather showed nearly 100% mortality (Jacobs, personal observation; Dum-roese, personal observation). Heikurinen (1981) reported similar plantation failure using frozen root plugs. Therefore, it is possible that the physiological dysfunctions may occur immediately, and be expressed in a short time interval after outplanting.

Although some studies have focused on the effects of thawing regime and long-term response of frozen-planted root plugs (Camm and others 1995; Kooistra and Bakker 2002; Helenius and others 2004; Helenius 2005), little is known about the short-term physiological changes occurring in seedlings planted with frozen root plugs under relatively high or low ambient air temperatures. In the present study, we examined short-term (7-day) responses of planting frozen and thawed Douglas-fir (*Pseudotsuga menziesii*) seedlings into 2 growing conditions—cool-moist at 10 °C (50 °F) and relative humidity (RH) of 75%; hot-dry at 30 °C (86 °F) and RH 50%—to better understand the response mechanisms that take place immediately after transplanting.

#### Study Procedure

##### Plant Material

Douglas-fir seeds were collected from the Flat-head National Forest in western Montana (Hun-

gry Horse Ranger District; elevation 1675 m [5495 ft]) and were grown in 3 x 15 cm (1.2 x 6 in) containers (315B [160/90] Styroblock™, Beaver Plastics, Ltd, Edmonton, Alberta, Canada) filled with 1:1 (v:v) peat:vermiculite medium at the USDA Forest Service research facility in Moscow, Idaho (46.7°N, 117°W) for one season using standard operational methods. A total of 125 seedlings were placed in groups of 20, sealed in plastic bags, placed in boxes, and shipped to Purdue University in West Lafayette, Indiana in December 2006. Upon arrival at Purdue University, the root plug of each seedling was wrapped with Saran™ premium wrap and the group of 20 seedlings were placed into sealed plastic bags and stored in a freezer at approximately -2 °C (28 °F) until the experiment started.

#### Root Plug Treatments, Growing Conditions, and Experimental Design

At the beginning of the experiment, a sub-sample ( $n = 5$ ) of seedlings had the following characteristics (mean  $\pm$  SE): height ( $19.0 \pm 0.9$  cm [ $7.5 \pm 0.35$  in]); root collar diameter ( $1.90 \pm 0.08$  mm); shoot ( $0.66 \pm 0.01$  g) and root ( $0.62 \pm 0.04$  g) dry mass. For frozen root (FR) planting, seedlings remained in freezer storage until the time of planting for both experiments. For thawed root (TR) planting, seedlings were taken out of freezer storage and kept at room temperature for 24 hours to ensure proper thawing prior to the start of the experiment. Thawing was done in dark conditions. Seedlings were planted into Treepot™-Tall One (36 x 10 cm [14 x 4 in]; 2.83 L [0.75 gal]) pots (Stuewe and Sons, Inc, Corvallis, Oregon, USA) filled with 2:1 (v:v) peat:vermiculite and immediately transferred to the growth chamber.

#### Experiment 1— Cool-Moist Conditions

Seedlings were transferred to a controlled environment chamber with a day temperature of 10 °C (50 °F) and night temperature of 6 °C (43 °F), relative humidity of  $75\% \pm 2.5\%$ , and an 18-hour photoperiod with photosynthetic photon flux density, measured at seedling top height, of

300  $\text{mmol/m}^2/\text{s}$ , provided by fluorescent lamps and incandescent bulbs.

Two groups of root plug treatments (a total of 60 seedlings), frozen roots and thawed roots, were randomly distributed within the growth chamber. Chlorophyll fluorescence, gas exchange, and shoot water potential measurements were taken at 0, 6, 12, and 24 hours, and 3 and 7 days, while root hydraulic conductance, electrolyte leakage, chlorophyll content, and root respiration measurements were taken only at 0 hours, and 1, 3, and 7 days. At each measurement time, measurements were taken on 5 randomly selected seedlings from each treatment. The experimental design was completely randomized.

#### Experiment 2— Warm-Dry Conditions

For the second experiment, the conditions, experimental design, and sampling were the same as Experiment 1, except that the controlled environment chamber was set at a day temperature of 30 °C (86 °F) and a night temperature of 20 °C (68 °F), with a relative humidity of  $50\% \pm 2.5\%$ .

#### Chlorophyll Fluorescence and Gas Exchange Measurements

Leaf photochemical efficiency was expressed as leaf chlorophyll fluorescence ( $F_v/F_m$ ). Chlorophyll fluorescence (CF) was measured on the upper 3 cm (1.2 in) portion of the shoot using an integrated fluorescence chamber head, LI-6400-40 leaf chamber fluorometer (LI-COR, Inc, Nebraska), on 5 different seedlings from each treatment at 0, 6, 12, and 24 hours, and 3 and 7 days after planting.

The terminal shoots were allowed to dark-adapt by covering the shoots with Ultra-black film for 20 minutes before CF measurements. Maximum fluorescence ( $F_m$ ) was determined following a red light saturating pulse ( $> 7000 \mu\text{mol photons/m}^2/\text{s}$ ) and centered at wavelength 630 nm. The  $F_v/F_m$  ratio estimates maximal quantum yield of PS II photochemistry in dark-adapted needles.

Gas exchange measurements were performed after chlorophyll fluorescence measurements using a LI-6400 portable photosynthesis system and 6400-05 conifer chamber on the same 5 different seedlings from each treatment at 0, 6, 12, and 24 hours, and 3 and 7 days after planting. Shoot water potential was determined using a pressure chamber immediately following gas exchange measurements.

#### Root Hydraulic Conductance

Roots hydraulic conductance was measured in intact roots of the same seedlings used for the gas exchange measurements with a high pressure flow meter (HPFM) as described by Tyree and others (1995). The use of the HPFM allows for measurement of intact roots, because water is applied under increasing pressure through an excised stem (around root collar level) into the whole root system (Tyree and others 1995). Stems of both frozen and thawed seedlings were cut 2 cm (0.8 in) above the root collar, and flow rates of all seedlings were measured over a range of 0 to 2.75 MPa (0 to 27.5 bars) to obtain a linear pressure-flow relationship (Tyree and others 1995). Root hydraulic conductance of 5 root systems was measured for each treatment on each measurement period and expressed as kg/MPa/s.

#### Needle Electrolyte Leakage

Following the measurement of root hydraulic conductance, needle electrolyte leakage (a measure of cell integrity and cell membrane leakiness) was measured on the same seedlings with a Seven-Easy Conductivity meter as described by Zwiazek and Blake (1990). Approximately 100 mg (fresh weight) of needles were taken from 5 seedlings per treatment, washed with deionized water, and placed in separate vials, each containing 15 ml of deionized water. After incubation for 6 hours on an orbital shaker, electrical conductivity of each solution (initial conductivity) was measured. Total electrolytes of the samples were obtained by autoclaving the samples at 120 °C (248 °F) for 20 minutes. The autoclaved samples were allowed to

cool, total electrolytes of the sample solutions were measured, and electrolyte leakage (EL) was calculated as initial conductivity as a percentage of the total electrolytes.

#### Chlorophyll Content

Needle chlorophyll was measured using the method of Arnon (1949) as modified to use dimethyl sulfoxide (DMSO) (Hiscox and Israelstam 1979). Needles (100 mg) were placed in test tubes with 7 ml of DMSO and transferred to an oven at 68 °C (154 °F) for 30 minutes, with a marble on top of each tube to prevent solvent evaporation. The sample was then removed from the oven and made up to a total volume of 10 ml with DMSO. A 3-ml aliquot was transferred to a cuvette to measure absorbance. A Perkin-Elmer LC-95 UV/Visible spectrophotometer was used to measure the absorbance of the solution at 645 and 663 nm. Chlorophyll *a* ( $C_a$ ), chlorophyll *b* ( $C_b$ ), and total chlorophyll ( $C_T$ ) were determined from the absorbance at 645 ( $D_{645}$ ) and 663 ( $D_{663}$ ) according to Arnon's formulae (1949).

#### Root Respiration

Root respiration, which was measured as oxygen uptake using an oxygen electrode (Model 58, Yellow Springs Instruments, Inc, Ohio), was determined at 1, 3, and 7 days after planting. The oxygen probe and the root system were placed in a 1500 cm<sup>3</sup> (91.5 in<sup>3</sup>) airtight cylinder filled with aerated distilled water that was continuously stirred with a magnetic stirrer. Root respiration measurements were made in respective growing temperatures and were monitored for 20 minutes by recording the oxygen uptake every 4 minutes. Root respiration rates were calculated as a mean of oxygen uptake over time and values were expressed in mmol O<sub>2</sub>/cm<sup>3</sup>/min.

#### Statistical Analysis

Analysis of variance (ANOVA) was performed using SAS (SAS Institute Inc, Cary, North Carolina). The means were compared using Tukey's pairwise multiple comparisons test, and were

considered significantly different at  $P \leq 0.05$ . Gas exchange,  $F_v/F_m$ , root hydraulic conductance, shoot water potential, needle electrolyte leakage, chlorophyll content, and root respiration were analyzed for each measurement period.

## Results

### Experiment 1—Cool-moist Conditions

$F_v/F_m$  values for frozen and thawed seedlings were 0.69 and 0.72, respectively, at the beginning of the experiment (0 hours), but they were not significantly different.  $F_v/F_m$  values for frozen- and thawed-planted seedlings varied from 0.60 to 0.73 for the duration of the experiment.

Negative mean values for A were recorded in frozen seedlings at 0 hours, but photosynthesis rates continued to increase as the experiment progressed. Although thawed-planted seedlings exhibited significantly higher rates of photosynthesis than frozen-planted seedlings, rates dropped on day 3, and increased on day 7. Photosynthesis rates ranged from -0.5 to 2.16 mmol CO<sub>2</sub>/m<sup>2</sup>/s in frozen seedlings, and from 0.77 to 2.7 mmol CO<sub>2</sub>/m<sup>2</sup>/s in thawed seedlings during the experiment. Thawed-planted seedlings maintained higher rates of stomatal conductance during the measurement period compared to frozen-planted seedlings. Stomatal conductance ranged from 0.01 to 89.48 mmol H<sub>2</sub>O/m<sup>2</sup>/s in frozen seedlings, and from 36.63 to 117.79 mmol H<sub>2</sub>O/m<sup>2</sup>/s during the experiment. A similar trend in transpiration was observed for both thawed- and frozen-planted seedlings, where it ranged from -0.001 to 1.08 mmol H<sub>2</sub>O/m<sup>2</sup>/s in frozen seedlings, and from 0.33 to 1.58 mmol H<sub>2</sub>O/m<sup>2</sup>/s in thawed seedlings during the experiment.

The root treatments had significant effect on shoot water potential ( $y_w$ ). Thawed-planted seedlings had significantly less negative  $y_w$  compared to frozen-planted seedlings at 12 hours, but they maintained a less negative water potential compared to frozen seedlings throughout the experiment. Although thawed-planted seedlings showed slightly higher rates of root hydraulic conductance than frozen-planted seedlings, they

were not significantly different. Overall, root hydraulic conductance was also significantly higher in thawed-planted seedlings compared to frozen-planted seedlings. There were no significant differences in needle electrolyte leakage, root respiration rates, and chlorophyll content between frozen- and thawed-planted seedlings.

No terminal or lateral buds began to elongate either in frozen- or thawed-planted seedlings during the duration of the experiment.

### Experiment 2—Warm-dry Conditions

At the beginning of the experiment (0 hours),  $F_v/F_m$  values for frozen and thawed seedlings were 0.64 and 0.74, respectively, and they were significantly different. Thereafter, the  $F_v/F_m$  values ranged from 0.72 to 0.75 in both frozen- and thawed-planted seedlings for the duration of the experiment. Although  $F_v/F_m$  values were higher for thawed than for frozen seedlings at 12 hours and 7 days, the differences were not statistically significant.

In general, thawed seedlings maintained significantly higher rates of photosynthesis than frozen-planted seedlings. Frozen-planted seedlings had a very low mean value for photosynthesis (0.110  $\mu$ mol CO<sub>2</sub>/m<sup>2</sup>/s) at 0 hours compared to a significantly higher rate of A (3.049  $\mu$ mol CO<sub>2</sub>/m<sup>2</sup>/s) in thawed seedlings. Photosynthesis increased gradually in frozen-planted seedlings from 0 hours to 3 days, but declined on day 7. Thawed seedlings had significantly higher rates of photosynthesis than frozen-planted seedlings at 12 hours, but their overall rates fluctuated throughout the measurement period. Photosynthesis rates ranged from 0.11 to 2.5  $\mu$ mol CO<sub>2</sub>/m<sup>2</sup>/s in frozen seedlings, and from 2.35 to 4.13  $\mu$ mol CO<sub>2</sub>/m<sup>2</sup>/s in thawed seedlings during the experiment. Stomatal conductance and transpiration measurements showed the same overall trend. The rates of  $g_s$  and  $E$  were higher in thawed-planted seedlings, except for day 1. Values for  $g_s$  and  $E$  were significantly lower in seedlings planted while root plugs were frozen compared with thawed root plugs at 0 hours. Thawed

seedlings maintained higher rates of stomatal conductance and transpiration after 7 days compared to frozen-planted seedlings, but differences were not significant.

The root treatment had significant effects on shoot water potential ( $\psi_w$ ). Over the course of the experiment, thawed-planted seedlings had significantly less negative  $\psi_w$  compared to frozen-planted seedlings at 0 hours and 1 day. Time zero  $\psi_w$  values were  $-1.34 \pm 0.10$  MPa ( $-13.4 \pm 1.0$  bar) and  $-1.03 \pm 0.04$  MPa ( $-10.3 \pm 0.4$  bar) for frozen and thawed seedlings, respectively. The frozen seedlings showed more negative  $\psi_w$  over the measurement period. Frozen seedlings had a gradual increase in  $\psi_w$  after 3 and 7 days. Overall, root hydraulic conductance was also significantly higher in thawed-planted seedlings compared to frozen-planted seedlings. Needle electrolyte leakage was significantly higher on day 3 in frozen-planted seedlings, although EL was not significantly different at any other measurement period. One day after planting, root respiration rates were 25% higher in frozen-planted seedlings ( $2.15 \text{ mmol O}_2/\text{cm}^3/\text{min}$ ) compared to thawed-planted seedlings ( $1.61 \text{ mmol O}_2/\text{cm}^3/\text{min}$ ). However, root respiration remained the same for frozen and thawed seedlings after 3 and 7 days.

Thawed-planted seedlings had a significantly higher number of new roots on day 7 ( $38 \pm 5$ ) compared to frozen-planted seedlings ( $20 \pm 5$ ) (Figure 1). Although thawed-planted seedlings had a higher mean number of broken terminal and lateral buds ( $3.6 \pm 0.2$ ) than frozen-planted seedlings ( $0.4 \pm 0.2$ ) at 7 days, differences were not significant.

## Discussion

Chlorophyll fluorescence ( $F_v/F_m$ ) value reflects the potential quantum efficiency of PS II and provides a sensitive indicator of plant photosynthetic performance (Björkman and Demmig 1987) and plant stress.  $F_v/F_m$  value close to 0.80 indicates a healthy seedling, while a decrease from this value indicates a stress (Fracheboud and others 1999). In our study, we observed high-



**Figure 1.** Development of new roots in frozen (left) and thawed (right) root plugs 7 days after planting.

er ranges of  $F_v/F_m$  values for frozen and thawed seedlings, even at 0 hours. The lower initial (0 hours)  $F_v/F_m$  values in frozen and thawed seedlings may suggest that physiological processes (for example, photosynthetic apparatus of PS II) were not yet metabolically activated to resume normal growth. Both frozen and thawed root plugs showed an increase in  $F_v/F_m$  values 6 hours after planting under both environmental regimes. The increase in  $F_v/F_m$  values in both treatments after 6 hours may suggest that they began to become metabolically active.

In order to meet transpirational demands, plant roots must efficiently and continuously absorb and transport water from soil to shoot. An initial higher resistance to root water uptake soon after outplanting causes seedling water stress, and this may subsequently lead to outplanting failures if frozen root plugs are outplanted. In both experiments, we have shown that  $y_w$  was significantly higher under cool-moist and warm-dry conditions, respectively, in thawed-planted seedlings than in frozen-planted seedlings. This water deficit condition in frozen was possibly because of lower root plug water content in frozen seedlings compared with thawed seedlings, suggesting that the water stored in the frozen seedlings was not available to roots. This is reflected by relatively low root hydraulic conductance in frozen roots compared to thawed roots in both growing environment experiments. Because root plugs were still frozen at 0 hours, the lower  $y_w$  observed in frozen seedlings compared with thawed seedlings could be related to a reduction in root water uptake resulting in decreased  $g_s$  and  $E$  and, in turn, resulting in much lower  $A$  rates than where roots are thawed. Previous studies revealed that both  $g_s$  and root water uptake were reduced when plants were exposed to different environmental stresses (Wan and others 1999; Kamaluddin and Zwiazek 2001), suggesting that root water flow and overall plant water status are interrelated. In our present study, it is plausible that the decline in  $E$  and  $g_s$  in frozen-planted root plugs was partly due to the reduction in root hydraulic conductance.

Although no significant new root growth was observed in either frozen or thawed seedlings under cool-moist conditions, thawed seedlings grown under warm-dry conditions had significantly more new roots than frozen-planted seedlings (Figure 1). Thawed seedlings had a greater mean number of broken buds compared to frozen-planted seedlings under warm-dry conditions. Higher net photosynthesis rates probably contributed towards emergence of more new roots in thawed seedlings. This has been confirmed in a study by van den Driessche (1987), where new root growth in Douglas-fir and Sitka spruce (*Picea sitchensis*) seedlings was associated with current photosynthesis.

In our present study, we observed reduced levels of root hydraulic conductance in frozen-planted seedlings under both environmental regimes. Disruption of root plasma membrane functions could be a factor that would interfere with water uptake (Crane and Möller 1988). Apostol and Zwiazek (2003) have shown that an increase of tissue ion leakage indicates loss of membrane integrity and, consequently, leads to failure in root functions. The comparatively higher root respiration rates in frozen seedlings compared with thawed seedlings, as an initial transient response, might reflect increased respiratory substrates from damaged cells, which is commonly observed as a wounding response (Klotz and others 2003). On the contrary, the higher oxygen uptake by thawed roots planted under cool-moist environment could possibly be due to resumption of metabolic and cell repair processes. It is quite possible that frozen-planted roots planted under cool-moist conditions had not started the same processes because the soil temperature was not very conducive for higher oxygen uptake. We hypothesize that a similar mechanism affecting survival in frozen seedlings is partly due to membrane permeability, resulting in increased membrane electrolyte leakage. This requires further investigation.

## Conclusions

In both cool and hot environments, net photosynthesis, transpiration, stomatal conductance, shoot water potential, and root hydraulic conductance (RHC) for thawed-planted seedlings were higher than frozen-planted seedlings. We conclude, however, that higher photosynthesis and water conductance rates in thawed seedlings planted at both cool-moist and warm-dry conditions would help them overcome initial outplanting stress, and the contribution of higher photosynthesis rates in thawed-planted seedlings may prove advantageous for survival and early growth. Our results, combined with those of other studies, suggest that field establishment success of frozen root plugs is dictated by environmental conditions to which they are exposed at outplanting. Under hot conditions with high vapor pressure deficit combined with cold and/or dry soils, thawing delay could lead to an imbalance between root water uptake and transpiration that can cause desiccation and may lead to mortality. Potential for frozen plugs to establish and survive is high, however, when outplanting under cloudy, cool conditions with high relative humidity and low vapor pressure deficit combined with warm and moist soils. On sites and at outplanting dates when these stressful environmental conditions are likely to prevail, it may be advisable to avoid outplanting frozen root plugs. Future studies (longer duration) are needed to find the link between more specific physiological mechanisms (for example, root hydraulic conductance, root membrane injury) that may dictate outplanting performances of frozen root plugs from a wider range of species and stocktypes.

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