MEASURING THE EFFECTS OF FREEZING INJURY ON RED SPRUCE (*Picea rubens* Sarg.) NEEDLES

By
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An Abstract of the Thesis Presented in Partial
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Red spruce (*Picea rubens* Sarg.) decline has been observed in northeastern North America for the last 30 years. A major inciting stress involved in this decline is freezing injury of foliage (DeHayes 1992). The objectives of this study were to: (i) relate gas exchange and chlorophyll loss to visual symptoms and electrolyte leakage, (ii) determine if thawing rate can influence the amount of freezing injury, and (iii) measure how *Rhizosphaera kalkhoffii* Bubák interacts with freeze-injured needles. Two trials, 60 and 80 seedlings respectively, were conducted in which measurements of photosynthesis (4 branches per seedling), needle electrolyte leakage (1 sample per seedling), chlorophyll loss (1 sample per seedling), needle reddening and loss (4 branches per seedling) and bud break (1 sample per seedling) were performed to measure injury after a single freezing event. In addition, for each seedling, two of the branches used for photosynthesis measurements were inoculated with *R. kalkhoffii* after freezing. Seedlings were frozen to test temperatures of -5, -20, -25, -30, -35 and -45°C in trial 1 and -5, -25, -35 and -45°C in trial 2. In the second trial, half the seedlings were covered with plastic bags, which doubled the thawing time.

Measurements of freezing injury showed that photosynthesis was consistently the most sensitive measure, detecting non-visible injury on uncovered seedlings ($p<0.05$ at -25 to -30°C for trial 1 and -25 to -35°C for trial 2). All other measures were less sensitive to freezing injury and did not detect injury ($p<0.05$) until -30°C in trial 1 and -35°C in trial 2. For the covered seedlings, chlorophyll loss and % bud break were the only measures to detect damage at -35 and -45°C, respectively. Faster thawing rates increased the amount of damage ca. 2-3 fold after a single freezing event ($p<0.05$) at -35 and -45°C for all measures. Infection by *R. kalkhoffii* increased significantly after colder freezing treatments, with an 83% increase in infection rate at -40°C in trial 1, and an 81% and 40% increase at -45°C in trial 2 for uncovered and covered seedlings, respectively ($p<0.05$). Fungal inoculations caused a ca. 60% reduction in photosynthesis after treatment at -40°C in trial 1 and ca. 40% and 60% reductions after treatment at -45°C in trial 2 for covered and uncovered seedlings, respectively ($p<0.05$). This study suggests two new factors which influence freezing injury; thawing rate and fungal (*R. kalkhoffii*) infections. The results of this study are consistent with the growing knowledge that freezing injury is a complex phenomenon in red spruce.

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"Are the trees really sick?"

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF TABLES iv

INTRODUCTION 1

METHODS 6
- Seedling Material and Freezing Tests 6
- Electrical Conductivity 7
- Gas Exchange 8
- Chlorophyll Loss 9
- *Rhizosphaera kalkhoffii* Inoculations 9
- Visual Observations 10
- Thawing rate 11
- Analysis 11

RESULTS 13
- ANOVA Tests 13
- Detecting Freezing Injury 13
- Thawing rate 14
- *Rhizosphaera kalkhoffii* Inoculations 15

DISCUSSION 16

LITERATURE CITED 22

APPENDIX A 34

APPENDIX B 37

BIOGRAPHY 41

LIST OF TABLES

Table 1. Percent of needles infected by *Rhizosphaera kalkhoffii* over time for a stand of trees at Isle au Haut, Maine. 26

Table 2. Thawing rates of covered vs. uncovered seedlings. 27

Table 3. Trial 1 means and standard errors for damage measurements after freezing uninoculated and *Rhizosphaera kalkhoffii* inoculated branches. 28

Table 4. Trial 2 means and standard errors for damage measurements after freezing uncovered seedlings having uninoculated and *Rhizosphaera kalkhoffii* inoculated branches. 29

Table 5. Trial 2 means and standard errors for damage measurements after freezing covered seedlings having uninoculated and *Rhizosphaera kalkhoffii* inoculated branches. 30

Table 6. Changes in damage measurements resulting from covering seedlings during freezing. 31

Table 7. Pearson correlations (r) of *Rhizosphaera kalkhoffii* infections with all other measures of freezing injury. 32

Table 8. Changes in damage measurements resulting from *Rhizosphaera kalkhoffii* inoculation on uncovered and covered seedlings after freezing. 33

INTRODUCTION

An abnormal decline in growth and an increased dieback in red spruce (*Picea rubens* Sarg.) populations have been attributed to weather patterns (McLaughlin et al. 1987, Conkey 1988, Johnson et al. 1988, Cook and Johnson 1989). Furthermore, a number of scientists have suggested that these weather patterns have reduced growth, specifically at high elevations, via winter injury (Pomerleau 1962, Friedland et al. 1984, DeHayes et al. 1990, Adams et al. 1991, Perkins et al. 1991, DeHayes 1992, and Johnson et al. 1992). The common symptom of winter injury is reddening and mortality of the younger foliage (Friedland et al. 1984, Evans 1986, Wilkinson 1990, Strimbeck et al. 1991). Studies concerning the mechanism and causes of winter injury in red spruce are important for understanding red spruce decline and dieback.

Red spruce is unique in exhibiting winter injury within its normal range; sympatric species exhibit little or no winter injury (DeHayes et al. 1990, DeHayes 1992). Several hypotheses have been proposed for the inherent dysfunction of this species to respond to environmental changes, and these relate to freezing injury. Explanations for inadequate freezing tolerance in red spruce include: genetic variability is lacking, maximum freezing tolerance is insufficient, timing and rate of increasing freezing tolerance in autumn is too slow, and the timing and rate of reducing freezing tolerance in late winter is too fast (DeHayes 1992).

Investigating how freezing tolerance influences winter injury requires reliable laboratory measurements of freezing injury. Freezing injury is commonly measured by subjecting shoots or needles to specified sub-zero temperatures, followed by measuring

the electrical conductivity (EC) of a bathing solution over a range of temperatures (Flint et al. 1967, DeHayes and Williams 1989). The increased EC due to freezing damage is caused by either a loss in cell membrane permeability or by rupturing of the cell membranes allowing increased electrolyte leakage from the damaged cells (Calkins and Swanson 1990). Although this technique may be relatively quick and precise, it has certain limitations reducing its usefulness to represent freezing damage in red spruce. Electrolyte leakage should be measured immediately after freezing, i.e. prior to natural leaching of the electrolytes. Therefore, EC cannot be repeated over time after freezing to detect injury and/or recovery levels (Palta et al. 1978). Also, the EC method does not always clearly show at what temperature or injury level tissues are killed (Fullerton 1979). A third concern is that EC cannot demonstrate or quantify changes in physiological processes or morphology, such as carbon fixation or membrane damage, that would occur in the plant after a freezing event. Methods that quantify the relationship between freezing injury and needle physiology would be useful in predicting future growth and survival of freeze-injured tissues.

Measuring the rate of photosynthesis via gas exchange has been used to study the effects of stress on needle physiology (e.g. Hadley et al. 1993). Since photosynthesis is a fundamental process responsible for the growth and survival of living tissue, its measurement after freezing should be correlated with long term freezing injury effects on needle physiology, growth and survival. Photosynthesis is a dynamic process which can be influenced by both physiological and environmental factors including: age, temperature, water, nutrition content, CO_2 concentration, and others. However, if these

factors are standardized before and during measurement, gas exchange can be a valuable index in evaluating freezing injury in plant species (Calkins and Swanson 1990).

Freezing injury could also be measured with another physiological index, chlorophyll content. Chlorophyll is a key compound involved in the photosynthetic processes of a plant, and its concentration should therefore be an indirect measure of a plant's photosynthetic capacity. Changes in chlorophyll content can also be measured over time in conifer needles. Therefore, chlorophyll loss could represent the effect freezing injury has on long term needle physiology, survival and growth.

Most freezing tolerance experiments simply reference plant damage to absolute minimum temperature. However, winter injury is likely influenced by co-factors such as acidic mist (Amundson et al. 1990, DeHayes et al. 1991, Vann et al. 1992, Sheppard 1994) and solar irradiation (Hadley et al. 1991, Hadley and Amundson 1992). Strimbeck et al. (1993) discussed some of the current hypotheses concerning how sun-exposed foliage suffers damage due to rapid freezing. Gross et al. (1991) suggest that sun exposure will increase the number of freeze/thaw cycles causing more damage. However, none of these hypotheses consider if rapid thawing of tissues can also cause freezing injury. Based on a field trial on March 6, 1993, we found that needle temperatures may rise from -6 to 0 °C in 20 minutes when frozen needles are first exposed to sunlight (unpubl. data). It is not known if such rapid thawing could cause increased damage in freeze-injured tissue.

Freezing injury alone may not fully account for the damage to conifer needles; needle fungi may take advantage of the weakened tissue and cause additional damage.

Rehfuess (Rehfuess and Rodenkirchen 1984, Rehfuess 1987) has proposed that freezing shocks in 1982 and 1983 caused damage and shedding of needles to older Norway spruce stands in southern Germany. In addition to suffering freezing injury, weakened needles were subsequently infected by needlecast fungi. *Rhizosphaera kalkhoffii* Bubák, *Lophodermium piceae*, and *L. macrosporum* were the most common fungi found in these freeze-injured needles, causing premature needle shedding (Kowalski and Lang 1984). Most likely, freezing injury caused damage to mesophyll cells, guard cells and the cuticle (Adams et al. 1991) allowing the fungi to invade foliage otherwise resistant.

Rhizosphaera kalkhoffii has been found in a red spruce stand on Isle Au Haut, Maine, where trees frequently show symptoms of winter injury (unpubl. data). Survey work at this site included observation of 15 trees over a period of 11 months. Infected needles were detected by incubating needles for two weeks after which pycnidia of the fungus were visible. While both symptomatic and symptomless needles harbored *R. kalkhoffii*, the fungus was detected more frequently in symptomatic needles. Furthermore, the proportion of infected needles in these trees increased in the colder months (Table 1). The role of secondary needle pathogens in connection with freezing injury of red spruce needs to be studied.

To better understand how freezing injury impacts needle functions of red spruce, the following objectives and hypotheses were tested in this study:

Objective 1. Compare freezing injury as measured by visual symptoms and electrolyte leakage (EC) to physiological measurements (gas exchange, chlorophyll loss).

Hypothesis: The physiological measures will be as or more sensitive in detecting freezing injury as visual symptoms or EC.

Objective 2. Determine if thawing rate can influence freezing injury.

Hypothesis: An increased freezing rate will cause more freezing injury.

Objective 3. Determine the influence of freezing damage on *Rhizosphaera kalkhoffii* infection and if *R. kalkhoffii* has any effect on the host vigor.

Hypothesis: Freezing injury will allow the fungus to infect damaged needles at a higher rate than undamaged needles causing increased needle loss and decreased net photosynthesis.

METHODS

Seedling Material and Freezing Tests

Red spruce seedlings, three years old in 6" pots, were obtained from the Tree Breeding Center, Department of Natural Resources, Debert, Nova Scotia, in December 1992. Seedlings were kept in an outdoor holding area on the University of Maine, Orono campus until testing. Two trials were conducted with the live seedlings. The first trial included 60 seedlings exposed to -5, -20, -25, -30, -35, and -40 °C beginning in February 1994. In the second trial 80 seedlings were frozen to -5, -25, -35, and -45 °C in March 1994. Half of the seedlings in the second trial were covered with plastic bags which provided approximately a two-fold decrease in thawing rate (Table 2). Due to space limitations in the freezers, the seedlings were frozen in blocks of 16. Temperatures were decreased at 3°C per hour and held at each test temperature for 1 hour after which selected seedlings were removed. Once removed, seedlings were placed in a +3°C incubator to thaw. The process was then repeated five consecutive days per trial, ten replicates per test temperature. Seedlings remained intact and potted while freezing, with insulation and heating tape around the pots to ensure that roots were not frozen. Above and below-ground temperatures were recorded at 10 minute intervals using Type T thermocouples attached to a data logger (5000 series, Model 5100 Logger Lab, Electronics Controls Design, Milwaukie, OR). After freezing, seedlings were placed in the greenhouse under a 16 hour photoperiod until needed for further treatments (day temperatures ca. 22 °C, night temperatures ca. 19 °C). All needles used in additional treatments were confined to the terminal, first-year segments from lateral branches. To

ensure adequate number of branches, sampling was conducted on the bottom two-thirds of the seedling.

Electrical Conductivity

For each seedling three terminal branches were removed pre- ($EC_{control}$) and post-freezing (EC_t). Electrical conductivity measurements were obtained by stripping needles from 2 cm tips and placing them in a test tube with 10 ml of .01% tween 20 (0.1 ml tween 20 in 1 L H_2O). After 24 hours, electrical conductivity ($EC_{control}$ and EC_t) was measured for each test-tube with a 1481-00 Conductivity Meter with a gold dip cell (Cole-Parmer, Chicago, IL). Needles were then autoclaved for 30 minutes. After 24 hours electrical conductivity (EC_k) was remeasured. Relative conductivity (RC)was then calculated [EC/EC_k]. This same technique was also performed on branch tips whose needles had been killed by quick immersion in liquid nitrogen. This was done to measure the maximum amount of leakage possible due to freezing injury. Finally, index of injury (Flint et al. 1967) was modified such that an adjusted index of injury (AI_t) was calculated for each seedling where $AI_t = (RC_t\text{-}RC_{control})/(RC_{\text{liquid nitrogen}}\text{-}RC_{control})$. The resultant estimate of electrolyte leakage is adjusted for differences in leakage rates. Therefore, AI_t can be used to compare differences in freezing damage between samples. Without adjustment, differences in I_t could be due to different rates of electrolyte leakage and/or differences in amounts of freezing damage.

Gas Exchange

Terminal current-year segments from four intact branches on the freeze-treated seedlings were used. Two branches were inoculated with *R. kalkhoffii* (see methods below) and the other two were uninoculated. Gas exchange measurements were made using a Li-Cor 6200 Portable Photosynthesis System with a 0.25 L chamber (Lincoln, NE). The methods employed in the use of the Li-Cor were similar to those outlined by Sulzer et al. (1993). The chamber was placed around a 2.5 cm segment of the branch and after one minute of equilibration, four consecutive 15 sec measurements were made. In order to ensure that there were no leaks in the chamber, needles around the 2.5 cm segment were removed and Silly Putty® was placed around the stem segment. The shoot was illuminated with 1200-1500 $\mu mol\ m^{-2}\ s^{-1}$. Light saturation for red spruce trees occurs at about 1000 $\mu mol\ m^{-2}\ s^{-1}$ (Hadley et al. 1993). Photosynthesis was expressed in the following units: (i) CO_2 per 2.5 cm segment of branch or NPS=$\mu mol\ CO_2 * 2.5\ cm^{-1} * s^{-1}$, (ii) CO_2 per unit projected surface area or NPS/SA=$\mu mol\ CO_2 * m^{-2} * s^{-1}$, and (iii) CO_2 per unit dry weight or NPS/DW=$mg\ CO_2 * g\ dry\ weight^{-1} * h^{-1}$.

Determination of total dry weight utilized all of the needles from the gas exchange segments. Needles were dried at 65 °C for 72 hours prior to weighing. Leaf area of these needles was also calculated using the projected leaf area emulator found in Agimage Ver. 1.08 (Agvision, Decagon Devices, Inc. Pullman, WA 99163). Once leaf area and dry weight were determined, photosynthesis was expressed as NPS, NPS/SA and NPS/DW.

In the some cases, observations were not possible due to needle loss or death. For these cases, in order to maintain a balanced design, all photosynthesis measures were set to zero instead of being treated as missing observations.

Chlorophyll Loss

Needles for measuring chlorophyll loss came from three current-year segments, selected at random, at three different times; pre-freezing, 10 and 21 days after freezing. Chlorophyll was extracted from 0.1g of needles using dimethyl sulfoxide technique (Hiscox and Israelstam 1979) with modifications by Greenwood et al. (1989). Chlorophyll content was measured by using a Spectronic 1201 spectrophotometer (Milton Roy Company, Rochester, NY 14625) and extinction coefficients previously determined by Greenwood et al. (1989). Another 0.1g of needles per sample were dried at 65°C for 72 hr. Results were expressed as mg chlorophyll (chl) * mg dry weight^{-1}. For analysis, chlorophyll loss was the percent reduction in chlorophyll content in a tree based on samples collected before and after freezing. Therefore, chlorophyll loss was measured at 10 and 21 days after freezing.

***Rhizosphaera kalkhoffii* Inoculations**

Five isolates of *R. kalkhoffii* were obtained from red spruce needles on Isle Au Haut, ME, in order to represent possible variation in virulence of *R. kalkhoffii*. Pure cultures were made by single spore techniques onto 2% malt extract. Approximately 40 autoclaved red spruce needles were then placed on a pure fungal culture. After ca. 14 days, needles were transferred to sterile filter paper in petri dishes. Pycnidia were present on needles after ca. 7 days of incubation. Needles from five cultures, one per

fungal isolate, were then placed in 50 ml sterile water (ca. 10^6 conidia per ml) for inoculations (Dotzler 1991). The two current-year segments used for the NPS measurements plus one other current-year segment per seedling were inoculated by dipping each branch segment into the spore solution for ca. 3 seconds. After inoculation, the latter segment was placed in a moist chamber for two weeks, after which the percentage of infected needles was measured as the percent of needles producing pycnidia.

Visual Observations

After freezing, all seedlings were placed in a heated greenhouse (average day temperature of 22°C, average night temperature of 19°C), watered daily and exposed to a 16 hour photoperiod. After 21 days, all visual observations were measured. For each seedling, the total number of buds and the number of flushed buds were recorded on three lateral branches selected at random. This data was then expressed as the percentage of flushed buds.

The other two visual observations, needle loss and needle symptoms (reddening), were obtained from the segments used in the gas exchange measurements. Percent needle loss was obtained by counting the number of needles present at the time of gas exchange(21 days after freezing) and the potential number of needles on that segment by counting the number of needle pegs.

Needle symptoms were based on the number of needles possessing visible patches of red symptoms, and were divided into the following classification scheme.

Symptom Category	Percent of Needles with Symptoms
0	0%
1	1-10%
2	11-30%
3	31-60%
4	61-90%
5	91-99%
6	100%

Thawing rate

In a preliminary experiment, it was determined that covered (plastic bags) tissues warmed from -5 to 0 °C approximately two times slower than uncovered tissue (ca. 1904-1996 sec. vs. ca. 782-1006 sec.), independent of the starting temperatures of -25, -35 and -45 °C (Table 2). Therefore, covered and uncovered trees exposed to a single freezing event were used to compare how thawing rate influences the amount of injury. This treatment was only used in the second trial, conducted in March 1994.

Analysis

All analyses were conducted using the SAS GLM procedure (SAS Institute, Inc. Cary, NC 27511-8000). In the following models, "block" refers to a group of seedlings frozen on the same day, "temp" is the test temperature, "cover" refers to whether a seedling was covered or not, "inoc" is whether a branch was inoculated or not, and the dependent variable is the injury measurement. The design employed was a randomized complete block for the temperature and cover treatments. For inoculation treatments, a split-plot design was used where main plot factors were block, temp and cover and the sub-plot factor was inoculation. The ANOVA model used was:

Dependent variable = constant + block + temp +cover + temp*cover + error A + inoc + inoc*temp + inoc*cover + inoc*temp*cover + error B.

error A = block*temp + block*cover + block*temp*cover + tree(block*temp*cover)

error B = inoc*tree(block*temp*cover) + inoc*block(temp*cover) + branch(block*temp*cover*tree*inoc)

For trial 1, the cover effects were removed from the model. Also, for both trials the inoc effect was used for photosynthesis (NPS, NPS/SA and NPS/DW), needle loss and needle symptom measurements only.

For all ANOVAs, equality of the variances was evaluated by Levene's test. Post-test mean comparisons were made according to the suggested analysis of a balanced split-plot design by Milliken and Johnson (1992). In this study, pre-planned comparisons for the temp effect were test temperature (-20 to -45°C) versus -5 °C, the control. This would indicate at which test temperature a measurement first detected freezing damage. These mean comparisons were completed within each level of inoc*cover so that actual damage levels, not just significant trends, could be reported. For the same reason, comparison of means for inoc were completed by temp*cover and comparisons for cover were completed by temp*inoc. Pearson correlations were calculated between *R. kalkhoffii* infections and damage measurements to examine if a particular form of damage was responsible for *R. kalkhoffii* infection.

RESULTS

ANOVA Tests

All variances tested equal by Levene's test ($p>0.01$). All ANOVA tables are presented in the Appendices A and B. F-tests for the treatment effect of the model (e.g. temp+cover+temp*cover) were significant ($p<.05$) for all freeze-damage measurements. Therefore, LSD was used for comparing means (Milliken and Johnson 1992).

Detecting Freezing Injury

For inoculated branches in trial 1 (Table 3), NPS/DW detected significant damage levels ($p<0.05$) at -25°C. NPS and NPS/SA detected significant non-visible damage ($p<0.05$) at -30°C. Needle symptoms and % needle loss detected damage ($p<0.05$) at -35°C. Infections of *R. kalkhoffii* increased significantly ($p<0.05$) at -35°C. For the uninoculated branches (Table 3), NPS/SA and NPS/DW were the most sensitive detecting damage at -30°C ($p<0.05$). NPS detected significant damage at -35°C. Chlorophyll loss (21 days) and % bud break were the second most sensitive measurements by detecting damage at -30°C ($p<0.05$ and $p<0.01$, respectively). The remaining measurements detected damage at -35°C ($p<0.05$). In trial 2, for the inoculated branches of the uncovered seedlings (Table 4), all photosynthesis measurements detected damage ($p<0.05$) at -25°C. Needle symptoms and % needle loss were significant at -35°C. Infections by *R. kalkhoffii* increased significantly ($p<0.05$) also at -35°C. For the uninoculated branches of the uncovered seedlings (Table 4), all measurements detected significant damage ($p<0.05$) at -35 °C. When seedlings were covered and branches inoculated (Table 5), only photosynthesis detected significant damage ($p<0.05$) at -35 or

-45°C. Under this treatment, *R. kalkhoffii* infections did not significantly increase with temperature. For the uninoculated branches of the covered seedlings (Table 5), only % bud break and % chlorophyll loss (10 or 21 days) detected damage ($p<0.05$) at -35 or -45°C.

For all measurements, freezing damage increased with the decreasing test temperatures. However, no measurement reached a maximum damage level; damage at -40 or -45°C increased from 43.7% to 93.1% compared to the -5°C control (Table 3-5). Based on the visual symptoms alone, maximum damage or complete mortality (100% needle loss and/or 0% bud break) was not reached for a group of seedlings at the minimum temperature in either trial.

Thawing rate

Covering of seedlings during the freezing treatment was responsible for a highly significant reduction in freezing damage (Appendix B; Table 4-6). The cover effect was detected by all measures and, in general, did not vary between the inoculated and uninoculated branches (Table 6). Covering decreased the amount of damage ca. 40-75% at the -35°C treatment and ca. 40-80% at -45°C.

Rhizosphaera kalkhoffii Inoculations

Rhizosphaera kalkhoffii infection rates increased significantly due to the freezing temperature treatment ($p<0.05$) at -35 °C in trial 1 and for the uncovered seedlings in trial 2 (Tables 3 and 4). For the covered seedlings of trial 2, the temperature effect caused no significant differences in the amount of *R. kalkhoffii* infected needles. The fungus was able to infect 4.3% of the needles on the control seedlings (-5 °C) in trial 1; 7.38% & 13.10% in trial 2 for covered and uncovered seedlings, respectively. Needle infection by *R. kalkhoffii* was correlated with injury, but correlations showed that infection was not strongly related to any one measure (Table 7). For example, needle symptoms had the best correlation in trial 1 ($r=0.533$), but AI_t correlated the best ($r=0.704$) in trial 2.

R. kalkhoffii infection was not only influenced by freezing injury but also caused significant increases in needle damage as well (Appendices A and B; Table 8). This phenomenon occurred primarily below -35 °C, where infection rates were significantly higher (Table 8). In trial 1, all measures except NPS/DW detected this change at the -35°C test temperature ($p<0.05$). In trial 2 with covered seedlings, all measurements except for % needle loss were affected by *R. kalkhoffii* inoculation primarily at -35 to -45°C. For the uncovered seedlings in trial 2, only the photosynthesis measurements could detect significant increases in damage due to *R. kalkhoffii* infections ($p<0.05$ for NPS/DW and NPS/SA at -35°C; $p<0.05$°C for NPS at -45°C). Other measures (e.g. chlorophyll loss, AI_t, and % bud break) were not conducted on the inoculated needles.

DISCUSSION

In this study, decreases in photosynthesis (NPS, NPS/SA and NPS/DW) were the most sensitive indicators of freezing injury. These measures consistently detected damage at -25 or -35°C for uncovered seedlings (Table 3 and 4). They were closely followed by % bud break and chlorophyll loss measured 21 days after freezing, which detected freezing damage at -30 and -35°C for uncovered seedlings (Table 3 and 4) and were the only measures to detect damage in uninoculated, covered seedlings (Table 5). Because of the design employed in this experiment, some of the measures were based on 10 observations (% *R. kalkhoffii* infections, AI_t, % bud break, and chlorophyll loss) and others were based on a split-plot design with 20 observations (photosynthesis, needle symptoms and % needle loss). Therefore, increased sensitivity may occur for the photosynthesis, needle symptoms and needle loss measures due to larger sample sizes.

Photosynthesis measurements indicated freezing injury prior to noticeable visible symptoms. The occurrence of non-visible injury is consistent with previous observations of sub-lethal or non-visible injury due to freezing (Palta et al. 1977, 1978; Adams et al. 1991). Declining photosynthesis due to freezing injury in red spruce also has been reported by Hadley et al. (1993).

Photosynthesis rates and chlorophyll loss have advantages for measuring freezing injury in experiments. First, they are more sensitive than visible symptoms, indicating the presence of sub-lethal injury. Second, they satisfy criteria for a good measure of freezing injury (Steponkus and Lanphear 1967) in that they are quantitative, objective, and relate to visual symptoms (lethal damage) and future growth. The primary

disadvantage of using photosynthesis is the time required to process a large number of samples. Also, it is sensitive to changing environmental conditions such as temperature, humidity and light. Chlorophyll loss, on the other hand, uses small amounts of tissue that can be processed in large numbers under uniform conditions. Unfortunately, this process requires several days for the chlorophyll to degrade. This poses a problem for those who are looking for a quick method of detecting freezing injury.

In comparison to photosynthesis and chlorophyll loss, electrolyte leakage (AI_t in this study) is a quick measure. It also has been used successfully when freezing detached needles (DeHayes et al. 1990) which was not evaluated in this study. However, electrolyte leakage has disadvantages because it does not directly measure needle functions, is an arbitrary measure of irreversible damage (Adams and Perkins 1993), and cannot be used to evaluate long term tissue responses to freezing injury (Palta et al. 1977, 1978).

Therefore, in applications using intact tissue where quick results are not necessary, chlorophyll loss has the potential to be a more useful measure of freezing injury than photosynthesis rates or electrolyte leakage because (i) chlorophyll loss can be measured easily on many samples, (ii) changes in chlorophyll loss can be measured over time, and (iii) chlorophyll loss has an influence on future tree growth. As with photosynthesis rates and electrolyte leakage, chlorophyll loss can not be used as a measure of freezing injury in studies, such as surveys, where nonfrozen tissue is not available.

Percent bud break was the most sensitive visible response to freezing tests. Christersson (1978), using *Pinus silvestris* and *Picea abies* seedlings, also found that the number of flushing terminal buds was an accurate measure of freezing injury. However, he noted that freezing injury to foliage did not always correlate with bud survival. Sakai and Larcher (1987) also noticed this phenomenon and suggest that buds and foliage respond differently to the various factors influencing freezing injury. Most importantly buds are more sensitive to the rate of freezing. Therefore, even though we are starting to understand which factors influence freezing injury, more work needs to be conducted on how different tissues react to factors influencing freezing injury.

In this study, slower thawing rates decreased the amount of freezing injury in red spruce foliage by as much as 82.1% (Table 6). This decrease in damage was evident only at the lower freezing temperatures. Other studies have not evaluated this relationship, but faster thawing rates influencing red spruce winter injury is consistent with reports of increased injury on needles exposed to the sun (Hadley et al. 1991, Hadley and Amundson 1992), where increased thawing rates should occur. Steponkus (1984) suggests freezing and thawing influences lethal injury in cells by (i) causing cell lysis due to osmotic expansion of the protoplast after freezing, or (ii) altering semipermeable properties of the plasma membrane such that cells do not expand to their normal state after thawing. It seems plausible that when tissues thaw faster, the large amounts of extracellular ice quickly changing into water increases cell damage by: (i) causing a rapid influx of water into the high solute, cold acclimated cells resulting in cell lysis, or

(ii) evaporating prior to being reabsorbed by the freeze-damaged cells such that cells suffer increased desiccation stress.

In this study of current-year needles, infection rates by *R. kalkhoffii* increased significantly with colder temperature treatments. However, for covered seedlings in trial 2, this trend for increasing infection rates with colder temperatures was not statistically significant. For these seedlings the covering treatment apparently decreased damage levels so that the needles were not significantly more susceptible to *R. kalkhoffii* infection than the control needles. No single damage measurement correlated the best with fungal infections (Table 7). Therefore, there was no clear explanation for increased fungal infection on needles following seedling exposure to subfreezing temperatures.

Needles in trial 2, which was conducted later in the dormant season, generally had higher infection rates for a given test temperature when compared to trial 1. Bernstein and Carroll (1977) also observed that fungal infections increased during the dormant season. Sieber-Canavesi and Sieber (1987) found that *Abies alba* needles were infected mainly during the winter by fungi from the *Xylariaceae* family. They explained this phenomenon by (i) lowered physiological activity of trees during that season resulting in slower defense responses to penetrating fungal hyphae, and (ii) frost damage to the cuticle which enhanced penetration. The first hypothesis is not likely in this study, because the seedlings from both trials should have been at similar physiological states. The second seems more plausible due to the increased infection in response to both the freezing treatment and the longer time trees were exposed to ambient winter conditions in the second trial.

Freezing injury may be responsible for increased *R. kalkhoffii* infection, but fungal inoculation of red spruce needles was also responsible for increased freezing damage by up to 68.9% (Table 8) at the coldest test temperatures. Less freezing damage at warmer temperatures did not increase fungal infections, but fungal inoculation did cause detectable increases in freezing damage at the warmer temperatures (Tables 3-5). Even though *R. kalkhoffii* could not successfully colonize needles exposed to warmer test temperatures, the fungus could have damaged tissue in its unsuccessful attempt to infect the needle.

Increases in needle damage due to fungal inoculations on freeze-stressed seedlings could be due to a higher number of fungal infections and/or an increase in activity of hyphae in the needle. The former is proposed by Tanaka and Chiba (1971) who concluded that availability of entry sites most severely limits the success of *R. kalkhoffii* within a given host. Freezing injury causes cuticle damage on red spruce needles (Adams et al. 1991) and could increase the entry sites for *R. kalkhoffii*.

Results of this study support the observation of Rehfuess (Rehfuess and Rodenkirchen 1984, Rehfuess 1987) that *R. kalkhoffii* was an important secondary stress in the Norway spruce decline of the 1980's. He suggested that the high rates of needle loss were due to several freezing shocks followed by fungal infection, and that both stresses were responsible for the premature loss of the older needles in Norway spruce. A test of his hypothesis has not yet been reported. Results of our study suggest that a similar mechanism could be possible in current-year red spruce needles.

In conclusion, freezing injury as a cause of winter injury is a complex phenomenon, where the amount of damage is influenced by several factors. Previous work has shown that these include absolute temperature (DeHayes 1992), rapid freezing (Strimbeck et al. 1993), the number freeze/thaw cycles (Gross et al. 1991), solar irradiation (Hadley et al. 1991, Hadley and Amundson 1992), and acidic mist (Amundson et al. 1990, DeHayes et al. 1991, Vann et al. 1992, and Sheppard 1994). This study also shows that thawing rate and fungal infection also should be considered. In addition, ability to detect freezing damage and to study its effect on needle physiology will depend on the type of measurement or index employed. Based on the results of this study, photosynthesis and/or chlorophyll loss are the best measures to detect lethal and sub-lethal freezing injury on intact, red spruce needles.

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Table 1. Percent of needles infected by *Rhizosphaera kalkhoffii* over time for a stand of trees at Isle au Haut, Maine[a].

Date	Percent Infected	SE
June '93	8.00	2.29
July '93	7.33	1.78
August '93	8.00	1.94
September '93	12.4	2.71
October '93	8.00	1.91
November '93	20.6	5.68

February '94	15.5	5.16
March '94	16.8	5.08
April '94	15.8	4.10

[a] Needles from the 1993 age class were surveyed from 15 trees at each date for the presence of *Rhizosphaera kalkhoffii*. Twenty needles from five branches were randomly selected and removed from each tree and placed in a moist chamber for approximately two weeks until pycnidia formed. If one or more pycnidia was observed then the needle was considered infected. The above values presented are the average percent of needles infected per tree for the stand at the given sampling date.

Table 2. Thawing rates of needles on covered vs. uncovered seedlings[a].

	Average Thawing Times (seconds) at Three Starting Temperatures					
	Covered			Uncovered		
Interval	-25 °C	-35 °C	-45 °C	-25 °C	-35 °C	-45 °C
0 to 2 °C	885	718	724	941	1287	1154
-5 to 0 °C	1948	1904	1996	918	1006	782
-15 to -5 °C	351	340	318	100	73	87
-25 to -15 °C	66	61	67	17	13	18

[a] Eight whole seedlings were frozen to -45°C and then placed in a 3°C incubator. Surface thawing rates were recorded on four needles per seedling using thermocouples. A CR10 datalogger with an AM416 multiplexer measured temperatures every 3 seconds. Data was recorded if temperatures changed more than 3 °C and every 10 minutes. The procedure was repeated on the same seedlings for test temperatures of -35°C and -25°C. Analysis was performed as follows: (i) time intervals (TI) for each temperature interval were calculated, and (ii) ANOVA of TI was used with the following model:

TI(-45) TI(-35) TI(-25)=constant + Rep + Cover + Rep*Cover + Error

There were no differences in time intervals due to starting temperature. Covered seedlings thawed slower than uncovered at all temperature intervals ($p<0.05$).

Table 3. Trial 1 means and standard errors for damage measurements after freezing uninoculated and *Rhizosphaera kalkhoffii* inoculated branches.

Inoculated									
Measurement[a]	n	SE[b]	-5[c]	-20	-25	-30	-35	-40	% change -5 to -40°C
NPS	20	1.51	18.8	18.0	16.7	**11.9***	**6.41****	**2.26****	-88.0
NPS/SA	20	0.36	5.03	4.33	4.14	**2.60****	**2.09****	**1.23****	-75.5
NPS/DW	20	0.84	4.02	3.35	**2.93***	**1.72****	**1.52****	**0.55****	-86.3
Needle symptoms	20	0.26	1.00	1.70	0.65	1.80	**3.20****	**3.90****	-
% Needle loss	20	4.24	24.2	22.0	16.4	37.8	**58.3****	**63.7****	62.0
% Rhizosphaera Infections	10	6.32	4.30	6.90	7.80	7.70	**22.0***	**25.4****	83.1

Uninoculated									
Measurement[a]	n	SE[b]	-5[c]	-20	-25	-30	-35	-40	% change -5 to -40°C
NPS	20	1.51	19.3	18.4	16.8	13.0	**11.0***	**6.89****	-64.3
NPS/SA	20	0.36	5.22	4.85	4.15	**3.16****	**3.21****	**2.94****	-43.7
NPS/DW	20	0.84	3.82	3.44	2.97	**2.21****	**2.14****	**1.71****	-55.2
Needle symptoms	20	0.26	0.80	1.10	0.60	1.55	**2.10****	**3.05****	-
% Needle loss	20	4.24	22.3	21.6	12.5	26.5	**42.1***	**46.5****	48.0
Adjusted I_t (%)	10	7.46	10.6	27.5	23.0	26.8	**42.3****	**57.7****	81.6
% Bud Break	10	7.01	80.1	76.7	83.4	**15.9****	**19.0****	**14.8****	-81.5
% Chlorophyll loss (10 days)	10	4.32	16.0	25.8	26.0	25.8	**37.3****	**36.3****	55.9
% Chlorophyll loss (21 days)	10	6.31	14.8	21.7	21.5	**33.5***	**43.2****	**46.6****	68.2

[a] NPS=µmol CO_2*2.5 cm*s^{-1}, NPS/SA=µmol CO_2*m^{-2}*s^{-1} and NPS/DW=mg CO_2*g dry $weight^{-1}$*h^{-1}

[b] SE = standard error used in LSD when comparing main-plot effects within sub-plot effects (inoc) (Milliken and Johnson 1992).

[c] Mean comparisons were made between -5°C with -20, -25, -30, -35 or -40°C within each measurement. $^{*}0.01 < p < 0.05$ $^{**}p \leq 0.01$

Table 4. Trial 2 means and standard errors for damage measurements after freezing uncovered seedlings having uninoculated and *Rhizosphaera kalkhoffii* inoculated branches.

Inoculated							
Measurement[a]	n	SE[b]	-5[c]	-25	-35	-45	% change -5 to -40°C
NPS	20	1.31	22.0	**15.7***	**4.49****	**2.51****	-88.6
NPS/SA	20	0.33	5.30	**3.38****	**1.14****	**0.57****	-89.2
NPS/DW	20	1.09	4.41	**3.08***	**0.92****	**0.45****	-89.8
Needle symptoms	20	0.32	0.30	1.15	**3.30****	**3.90****	-
% Needle loss	20	5.29	21.7	35.9	**60.3****	**61.5****	64.7
% *Rhizosphaera* Infections	10	6.32	13.1	15.9	**48.9****	**68.0****	80.7

Uninoculated							
Measurement[a]	n	SE[b]	-5[c]	-25	-35	-45	% change -5 to -40°C
NPS	20	1.31	21.5	15.8	**7.30****	**7.88****	-63.3
NPS/SA	20	0.33	4.75	3.83	**2.00****	**1.47****	-69.1
NPS/DW	20	1.09	4.03	3.26	**1.64****	**1.15****	-71.5
Needle symptoms	20	0.32	0.20	0.80	**3.20****	**3.60****	-
% Needle loss	20	5.29	17.3	30.6	**55.3****	**60.5****	71.4
Adjusted I_t (%)	10	2.22	6.93	5.25	**25.9****	**34.2****	79.7
% Bud Break	10	3.97	90.0	81.5	**37.4****	**6.20****	-93.1
% Chlorophyll loss (10 days)	10	2.35	9.20	13.2	**30.8****	**45.5****	79.8
% Chlorophyll loss (21 days)	10	2.39	7.61	14.9	**34.7****	**49.4****	84.2

[a] NPS=µmol CO_2 * 2.5 cm * s^{-1}, NPS/SA=µmol CO_2 * m^{-2} * s^{-1} and NPS/DW=mg CO_2 * g dry weight^{-1} * h^{-1}.

[b] SE = standard error used in LSD when comparing main-plot effects within sub-plot effects (inoc) (Milliken and Johnson 1992).

[c] Mean comparisons were made between -5°C with -25, -35 or -45°C within each measurement. *$0.01 < p < 0.05$ **$p \leq 0.01$

Table 5. Trial 2 means and standard errors for damage measurements after freezing covered seedlings having uninoculated and *Rhizosphaera kalkhoffii* inoculated branches.

Inoculated							
Measurement[a]	n	SE[b]	-5[c]	-25	-35	-45	% change -5 to -40°C
NPS	20	1.31	18.2	18.4	12.7	7.91**	-56.5
NPS/SA	20	0.33	4.22	3.85	**2.95***	**2.09****	-50.5
NPS/DW	20	0.77	3.39	2.99	**2.29***	**1.50****	-55.8
Needle symptoms	20	0.32	0.10	0.50	1.10	1.15	-
% Needle loss	20	5.29	20.8	29.4	37.0	38.3	45.7
% *Rhizosphaera* Infections	10	6.32	7.38	18.9	16.2	12.2	39.5

Uninoculated							
Measurement[a]	n	SE[b]	-5[c]	-25	-35	-45	% change -5 to -40°C
NPS	20	1.31	19.8	21.6	14.8	14.1	-28.8
NPS/SA	20	0.33	4.43	4.37	3.78	3.61	-18.5
NPS/DW	20	0.77	3.41	3.42	2.72	2.74	-19.6
Needle symptoms	20	0.32	0.10	0.45	0.00	0.20	-
% Needle loss	20	5.29	21.9	30.8	27.6	27.2	19.5
Adjusted I_t (%)	10	2.22	5.53	4.65	6.16	11.2	50.6
% Bud Break	10	3.97	92.5	87.8	**66.8****	**34.2****	-63.0
% Chlorophyll loss (10 days)	10	2.35	5.96	9.62	10.95	**19.2****	69.0
% Chlorophyll loss (21 days)	10	2.39	5.63	6.96	9.65	**22.4****	74.9

[a] NPS=µmol CO_2 * 2.5 cm * s^{-1}, NPS/SA=µmol CO_2 * m^{-2} * s^{-1} and NPS/DW=mg CO_2 * g dry weight^{-1} * h^{-1}.

[b] SE = standard error used in LSD when comparing main-plot effects within sub-plot effects (inoc) (Milliken and Johnson 1992).

[c] Mean comparisons were made between -5°C with -25, -35 or -45°C within each measurement. *$0.01 < p < 0.05$ **$p \leq 0.01$

Table 6. Changes in damage measurements resulting from covering seedlings during freezing. For needle symptoms, the values are the differences between means; other measures are the percent difference between means.

		Change due to cover					
		Inoculated			Uninoculated		
Measurement[a]	SE[b]	-25[c]	-35	-45	-25	-35	-45
NPS	3.05	14.7	**64.8****	68.3	26.9	**50.7***	**44.1***
NPS/SA	0.63	12.2	**61.4****	**72.7****	12.4	**47.1****	**59.3****
NPS/DW	0.52	2.92	**59.8****	**70.0***	4.68	**39.7***	**58.0****
Needle symptoms	0.56	0.65	**2.20****	**2.75**	0.35	**3.20****	**3.40****
% Needle loss	10.3	18.1	**38.6***	**37.7***	0.65	**50.2****	**55.0****
% *Rhizosphaera* Infections	11.4	15.9	**66.9****	**82.1****	-	-	-
Adjusted I_t (%)	3.60	-	-	-	11.4	**76.2****	**67.3****
% Bud Break	8.07	-	-	-	7.18	**44.0****	**81.9****
% Chlorophyll loss (10 days)	3.99	-	-	-	26.9	**64.4****	**57.8****
% Chlorophyll loss (21 days)	4.44	-	-	-	53.2	**72.1****	**54.7****

[a] NPS=µmol CO_2*2.5 cm*s^{-1}, NPS/SA=µmol CO_2*m^{-2}*s^{-1} and NPS/DW=mg CO_2*g dry $weight^{-1}$*h^{-1}.

[b] SE = standard error used in LSD when comparing main-plot effects within sub-plot effects (inoc) for original vales (Table 4 & 5) (Milliken and Johnson 1992).

[c] Mean comparisons of original values (Tables 4 & 5) were made between cover treatments within the sub-plot effects (temp*inoc). $^{*}0.01 < p < 0.05$ $^{**}p \leq 0.01$

Table 7. Pearson correlations (r) of *Rhizosphaera kalkhoffii* infections with all other measures of freezing injury.

Measure[a]	Trial 1	Trial 2
NPS	-0.416	-0.403
NPS/SA	-0.400	-0.390
NPS/DW	-0.296	-0.362
Needle symptoms	0.533	0.647
% Needle loss	0.490	0.558
Adjusted I_t (%)	0.488	0.704
% Bud Break	-0.345	-0.342
% Chlorophyll loss (10 days)	0.499	0.505
% Chlorophyll loss (21 days)	0.500	0.494

[a] NPS=μmol CO_2*2.5 cm*s^{-1}, NPS/SA=μmol CO_2*m^{-2}*s^{-1} and NPS/DW=mg CO_2*g dry $weight^{-1}$*h^{-1}. Values for covered and uncovered seedlings were pooled for trial 2. All correlations are significant ($p \leq 0.01$).

Table 8. Changes in damage measurements resulting from *Rhizosphaera kalkhoffii* inoculation on uncovered and covered seedlings after freezing. For needle symptoms, the values are the differences between means; other measures are the percent difference between means.

	Percent difference due to inoculation								
	Trial 1			Trial 2					
				Covered			Uncovered		
Measurement[a]	SE[b]	-35[c]	-40	-25	-35	-45	-35	-45	SE
NPS	1.91	**41.5***	**67.1***	**14.8***	14.2	**44.0****	38.4	**68.4****	1.56
NPS/SA	0.44	**35.0***	**58.3****	11.9	**23.7***	**43.2****	**45.0***	**60.0***	0.40
NPS/DW	0.34	29.3	**68.1****	12.6	15.8	**45.2****	**43.7***	**61.0***	0.32
Needle symptoms	0.36	**1.10****	**0.85***	0.05	**1.10****	**0.95***	0.10	0.30	0.38
% Needle loss	5.92	**27.8****	**27.0****	4.54	25.4	29.0	8.13	1.63	6.58

[a] NPS=µmol CO_2∗2.5 cm∗s^{-1}, NPS/SA=µmol CO_2∗m^{-2}∗s^{-1} and NPS/DW=mg CO_2∗g dry $weight^{-1}$∗h^{-1}

[b] SE = standard error used in LSD when comparing sub-plot effects within whole-plot effects (temp∗cover) for original values (Table 3-5) (Milliken and Johnson 1992).

[c] Mean comparisons of original values (Table 3-5) were made between inoculation treatments within the main effects (temp∗cover). Means did not differ at temperatures above -35°C except as shown. *$0.01 < p < 0.05$ **$p \leq 0.01$

Appendix A. Trial 1: Analyses of variance for all measures of injury

Table 1

Dependent Variable: ADJUSTED INDEX OF INJURY (%)
Number of observations in data set = 60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	5195.1	1298.8		
TEMP	5	13511.1	2702.2	6.517	.000
ERROR A	50	23232.4	414.6		

Table 2

Dependent Variable: *% Rhizosphaera kalkhoffii* INFECTIONS
Number of observations in data set = 60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	2395.1	598.8		
TEMP	5	3970.7	794.1	2.553	.039
ERROR A	50	15552.4	311.0		

Table 3

Dependent Variable: % BUD BREAK
Number of observations in data set = 60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	1353.0	338.3		
TEMP	5	60729.7	12145.9	20.779	.000
ERROR A	50	29226.9	584.5		

Table 4

Dependent Variable: % CHLOROPHYLL LOSS (10 DAYS)
Number of observations in data set = 60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	316.6	79.2		
TEMP	5	3118.2	623.6	4.181	.003
ERROR A	50	7457.8	149.2		

Appendix A (cont.)

Table 5

Dependent Variable: % CHLOROPHYLL LOSS (21 DAYS)
Number of observations in data set = 60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	2173.2	543.3		
TEMP	5	8226.7	1645.3	4.693	.001
ERROR A	50	17529.0	350.6		

Table 6

Dependent Variable: PHOTOSYNTHESIS (NPS)
Number of observations in data set = 240

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	1217.2	304.3		
TEMP	5	6669.7	1333.4	6.647	.000
ERROR A	50	10030.3	200.6		
INOC	1	227.2	227.2	6.208	.014
TEMP*INOC	5	221.1	44.2	1.208	.307
ERROR B	174	6367.6	36.6		

Table 7

Dependent Variable: PHOTOSYNTHESIS (NPS/SA)
Number of observations in data set = 240

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	98.5	24.6		
TEMP	5	293.3	58.7	5.411	.000
ERROR A	50	542.0	10.8		
INOC	1	28.3	28.3	14.268	.000
TEMP*INOC	5	19.9	4.0	2.010	.079
ERROR B	174	344.8	2.0		

Appendix A (cont.)

Table 8

Dependent Variable: PHOTOSYNTHESIS (NPS/DW)
Number of observations in data set = 240

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	49.7	12.4		
TEMP	5	216.8	43.4	10.614	.000
ERROR A	50	204.3	4.1		
INOC	1	10.2	10.2	9.061	.003
TEMP*INOC	5	11.3	2.3	2.015	.079
ERROR B	174	195.7	1.1		

Table 9

Dependent Variable: NEEDLE SYMPTOMS
Number of observations in data set = 240

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	63.1	15.8		
TEMP	5	233.9	46.8	6.749	.000
ERROR A	50	346.6	6.9		
INOC	1	15.5	15.5	12.295	.001
TEMP*INOC	5	9.4	1.9	1.486	.197
ERROR B	174	219.4	1.3		

Table 10

Dependent Variable: % NEEDLE LOSS
Number of observations in data set = 240

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	7228.4	1807.1		
TEMP	5	53950.9	10790.2	8.914	.000
ERROR A	50	60525.5	1210.5		
INOC	1	4318.4	4318.4	12.332	.001
TEMP*INOC	5	2732.1	546.4	1.560	.174
ERROR B	174	60928.8	350.2		

Appendix B. Trial 2: Analyses of variance for all measures of injury.

Table 1

Dependent Variable: ADJUSTED INDEX OF INJURY (%)
Number of observations in data set = 80

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	151.1	37.8		
TEMP	3	918.2	306.1	4.730	.005
COVER	1	179.4	179.4	2.772	.101
TEMP*COVER	3	46.8	15.6	0.241	.867
ERROR A	68	4399.7	64.7		

Table 2

Dependent Variable: *% Rhizosphaera kalkhoffii* INFECTIONS
Number of observations in data set = 80

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	1830.9	457.7		
TEMP	3	6792.0	2264.0	3.486	.020
COVER	1	465.7	465.7	0.717	.400
TEMP*COVER	3	2555.9	852.0	1.312	.278
ERROR A	68	44164.3	649.5		

Table 3

Dependent Variable: % BUD BREAK
Number of observations in data set = 80

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	428.5	107.1		
TEMP	3	8746.9	2915.6	8.964	.000
COVER	1	552.0	552.0	1.697	.197
TEMP*COVER	3	1002.1	534.0	1.027	.386
ERROR A	68	22118.3	325.3		

Appendix B (cont.)

Table 4

Dependent Variable: % CHLOROPHYLL LOSS (10 DAYS)
Number of observations in data set = 80

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	70.2	17.6		
TEMP	3	499.3	166.4	1.823	.151
COVER	1	201.6	201.6	3.351	.072
TEMP*COVER	3	1023.0	341.0	4.093	.010
ERROR A	68	5405.6	79.5		

Table 5

Dependent Variable: % CHLOROPHYLL LOSS (21 DAYS)
Number of observations in data set = 80

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	312.0	78.0		
TEMP	3	888.0	296.0	2.596	.059
COVER	1	136.3	136.3	1.807	.183
TEMP*COVER	3	482.6	160.9	1.901	.138
ERROR A	68	6692.6	98.4		

Table 6

Dependent Variable: PHOTOSYNTHESIS (NPS)
Number of observations in data set = 320

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	3482.3	870.6		
TEMP	3	8642.7	2880.9	17.847	.000
COVER	1	1151.3	1151.3	7.132	.009
TEMP*COVER	3	1277.4	425.8	2.638	.057
ERROR A	68	10976.8	161.4		
INOC	1	545.3	545.2	22.553	.000
TEMP*INOC	3	297.6	99.2	4.103	.007
INOC*COVER	1	36.9	36.9	1.522	.219
TEMP*INOC*COVER	3	44.9	15.0	0.619	.603
ERROR B	232	5608.9	24.2		

Appendix B (cont.)

Table 7

Dependent Variable: PHOTOSYNTHESIS (NPS/SA)
Number of observations in data set = 320

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	67.1	16.8		
TEMP	3	374.2	124.7	19.279	.000
COVER	1	60.7	60.7	9.387	.003
TEMP*COVER	3	89.0	29.7	4.585	.006
ERROR A	68	439.9	6.5		
INOC	1	29.4	29.4	18.740	.000
TEMP*INOC	3	22.2	7.4	4.703	.003
INOC*COVER	1	2.9	2.9	1.840	.176
TEMP*INOC*COVER	3	2.6	0.9	0.547	.651
ERROR B	232	364.4	1.6		

Table 8

Dependent Variable: PHOTOSYNTHESIS (NPS/DW)
Number of observations in data set = 320

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	59.9	15.0		
TEMP	3	288.7	96.2	22.285	.000
COVER	1	15.6	15.6	3.610	.062
TEMP*COVER	3	62.7	20.9	4.840	.004
ERROR A	68	293.7	4.3		
INOC	1	13.9	13.9	14.051	.000
TEMP*INOC	3	13.9	4.6	4.684	.003
INOC*COVER	1	1.1	1.1	1.059	.305
TEMP*INOC*COVER	3	2.0	0.7	0.658	.579
ERROR B	232	230.0	1.0		

Appendix B (cont.)

Table 9

Dependent Variable: NEEDLE SYMPTOMS
Number of observations in data set = 320

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	52.8	13.2		
TEMP	3	222.4	74.1	15.549	.000
COVER	1	206.4	206.4	43.289	.000
TEMP*COVER	3	134.0	44.7	9.365	.000
ERROR A	68	324.2	4.8		
INOC	1	10.9	10.9	7.528	.007
TEMP*INOC	3	5.0	1.7	1.149	.330
2INOC*COVER	1	2.0	2.0	1.352	.246
TEMP*INOC*COVER	3	5.7	1.9	1.305	.274
ERROR B	232	335.3	1.4		

Table 10

Dependent Variable: % NEEDLE LOSS
Number of observations in data set = 320

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BLOCK	4	8888.8	2222.2		
TEMP	3	36064.6	12021.5	7.074	.000
COVER	1	16813.5	16813.5	9.894	.002
TEMP*COVER	3	12964.6	4321.5	2.543	.063
ERROR A	68	115557.1	1699.4		
INOC	1	1269.2	1269.2	2.934	.088
TEMP*INOC	3	584.2	194.7	0.450	.718
INOC*COVER	1	23.3	23.3	0.054	.816
TEMP*INOC*COVER	3	840.2	280.1	1.647	.586
ERROR B	232	100364.9	432.6		

BIOGRAPHY

Daniel Manter was born and raised in New Hampshire where he survived high school. He graduated with a Bachelor of Science in Biology from Bates College in 1991. After spending some time with the U.S. Forest Service in Hamden, CT, he moved to Vermont to ski and climb. In September of 1992 he enrolled for graduate study at the University of Maine where he is now a candidate for the degree of Master of Science (in Forestry). His degree will be conferred in December, 1994.