Quantifying genetic variation in needle retention and timing of bud flush in Balsam Fir Christmas Trees for improved performance under climate change in the northeast

Project

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Final Report

Submitted to:

U.S Christmas Tree Promotion Board

Submitted by:
The Wood Science and Technology Centre
University of New Brunswick
1350 Regent Street
Fredericton, NB, Canada E3C 2G6
Tel: (506) 453-4507
Fax: (506) 453-3574
E-mail: woodsci@unb.ca

Ron Smith PhD, RPF
UNB Wood Science & Technology Centre.

Joel Tremblay BScF, FIT
UNB Faculty of Forestry & Environmental Management.

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Introduction

This report summarizes the work conducted on the project "Quantifying genetic variation in needle retention and timing of bud flush in Balsam Fir Christmas Trees for improved performance under climate change in the northeast".

There were three main objectives for this project, all of which were completed.

Objective 1: Optimize needle retention testing methodologies by screening a total of sixty phenotypically superior balsam fir trees (twenty each from Quebec, New Brunswick and Nova Scotia) at each of two locations and at two collection periods over three years.

This work was completed with needle retention assessed for 64 trees over three years. The key deliverable was the following publication:

Joel D. Tremblay, Ronald F. Smith, and Loic D'Orangeville 2023. Integrating rate of moisture loss into needle retention testing for improved selection of Balsam fir (Abies balsamea) for use as Christmas trees. Forests 2023, 14, 1626. https:// doi.org/10.3390/f14081626

Objective 2: Determine the degree of correlation between the time of bud flush and time of the onset of frost hardiness.

Objective 3: Determine the effect(s) of rootstock and tree age on timing of bud flush and needle keepability.

Part of the results of Objectives 2 and 3 were reported as a chapter in the MScF thesis of Joel Tremblay:

Tremblay, J. 2024 Improving Needle Retention Selection Methods and Determining the Influence of Rootstock on Balsam Fir (*Abies balsamea*) Bud Flush and Development for Use in Christmas Trees Improvement Programs. A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Forestry in the Graduate Academic Unit of Forestry and Environmental Management.

Mr. Tremblay is scheduled to present and defend his thesis in February 2024. *A digital copy of the approved thesis will be provided to the Real Christmas Tree Board in March 2024.*

Two separate final technical reports were prepared.

1. Objective 1
2. Objectives 2 and 3

Some of the results not included in Mr. Tremblay's thesis, specifically those related to field grafting, are included in Technical Report 2.

Technical Report 1

Optimizing needle retention testing methodologies by screening a total of sixty phenotypically superior balsam fir trees (twenty each from Quebec, New Brunswick and Nova Scotia) at each of two locations and at two collection periods over three years.

Introduction

Balsam fir (*Abies balsamea*) is the primary species cultivated for the Christmas tree and greenery industry in Northeastern North America. In addition to form, colour, and fragrance, needle retention is of increasing importance as many consumers in the United States display their Christmas trees for four or more weeks during the holiday season [1]. Climate change models predict that the mean annual temperature in Atlantic Canada will increase by approximately 1.3–1.9 °C by the year 2050, and between 1.9 and 5.2 °C by the end of the century [2], resulting in multiple species ranges shifting northward [3] and a significant decline in balsam fir habitat in the Maritimes over the next 100 years [4]. Warmer temperatures and an extended growing season can create problems for Christmas trees in both the spring and fall. Growth patterns of Fraser fir (*Abies fraseri*) at different elevations in the Southern Appalachian Mountains were studied, and it was revealed that Christmas tree growers will need to adapt their management techniques to account for the warming climate [5]. Early and warm springtime temperatures can result in early bud flush and an increased risk of late spring frost damage to young expanding shoots [6]. Warmer temperatures later into the fall can delay cold acclimation. Needle retention and cold acclimation are positively correlated; thus, with warmer fall temperatures, trees will be prone to early post-harvest needle loss [7,8]. Cold acclimation in conifers is a complex process that involves numerous signaling pathways, including changes in phytohormones due to low temperatures, the circadian clock, and changes to light quality and photoperiod [9]. These processes can begin as early as July in response to photoperiod changes and its primary purpose is the protection of cell membranes from injuries during cold temperatures [10–12]. However, the most important of these factors is low temperatures [9,13]. To meet the demand for Christmas trees by early December, balsam fir trees in the Maritime provinces of Canada must be harvested in late October or early November to allow for shipping to the markets. Under warm fall temperatures, trees harvested in late October or early November often have not yet been exposed to the freezing temperatures generally required for the cold acclimation process and its associated positive effect on needle retention [1,14–16]. Testing two harvest dates, one early and one late during the normal harvest season for Christmas tree growers, can be used to identify trees that perform well under warmer conditions as well as after a period of cold acclimation. It has been suggested that trees with a natural resistance to post-harvest needle abscission will perform well regardless of harvest date, and trees that do not feature this ability will perform better when harvested later in the season after they have been exposed to cooler temperatures [17]. Early season harvesting and testing are beneficial for two reasons: (1) This mimics a season of high demand that results in growers needing to harvest sooner, and (2) the warmer temperatures that come with harvesting earlier will potentially become the new norm later in the fall because of the changing climate. So, individuals that perform well regardless of the amount of cold acclimation will be vitally important in future breeding programs. Balsam fir has a range that almost spans the width of Canada [18]. As would be expected in a species with a trans-continental range, there is significant genetic variation in the species [19,20]. Needle retention in Christmas trees varies with genotype [21], date of collection [8], and environmental

factors [22]. While correlations between needle abscission and carbohydrates such as raffinose have been reported [23], no causal relationships have yet to be identified [22,23]. Needle abscission cannot be prevented entirely; however, the selection of individuals with high needle retention abilities can lead to improving this issue [24].

Methods

Sample Site Descriptions

Three areas were selected for this study: a provincial clone bank in Kingsclear, NB, Canada (45◦57′31.5″ N 66◦48′07.2″ W); a multi-aged natural Christmas tree stand (seed production area) in New Germany, NS, Canada (44◦33′48.5″ N 64◦45′09.5″ W); and a Christmas tree plantation in Hatley, QC, Canada (45◦14′05.6″ N 71◦56′13.5″ W) (Figure 1).

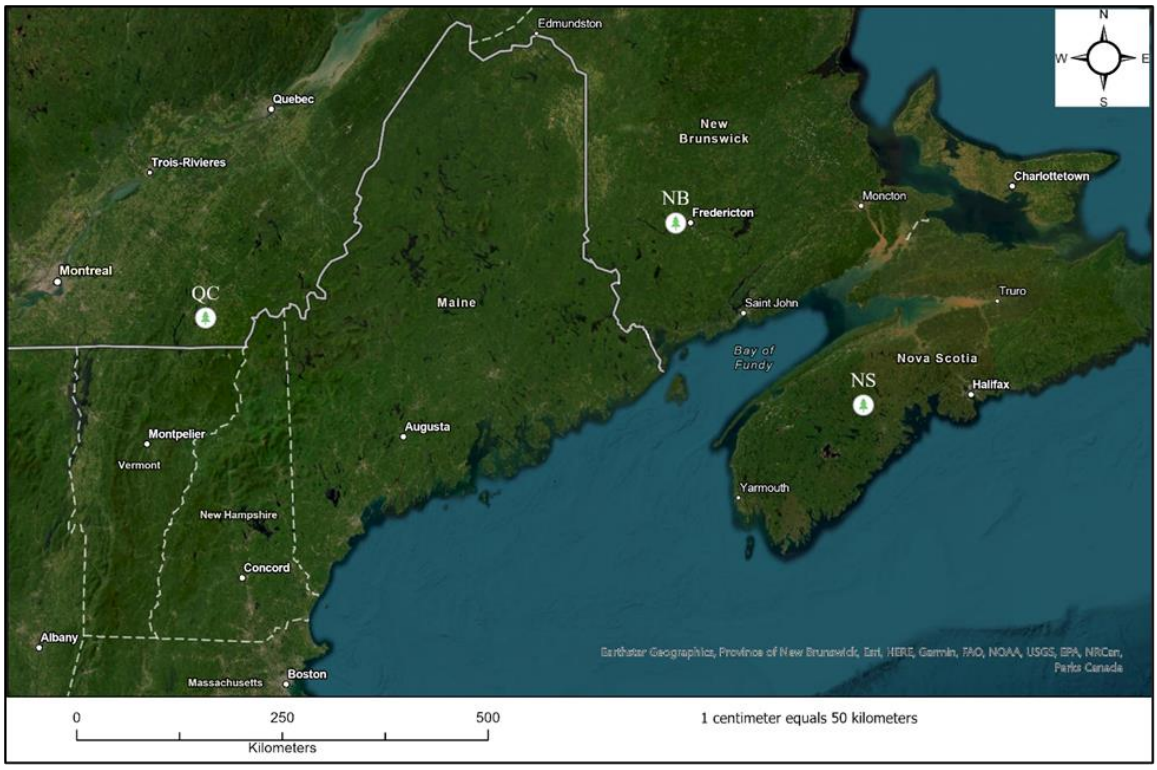

Figure 1 Map of sample origin locations: Westmost is Hatley, Quebec, Canada, denoted as QC (45°14′05.6″ N 71°56′13.5″ W). The northmost location is located just outside of Fredericton, New Brunswick, Canada, in the Kingsclear provincial tree nursery and is denoted with NB (45°57′31.5″ N 66°48′07.2″ W). The southernmost location is located in New Germany, Nova Scotia, Canada, and is denoted as NS (44°33′48.5″ N 64°45′09.5″ W).

A clonal seed orchard, comprising grafts from ‘plus tree’ selections produced by Christmas tree growers and provincial forest extension staff from around the province was established at the Kingsclear provincial tree nursery in the early 1990s [26]. A clone bank comprising regrafts of two ramets from each of the parent trees in the seed orchard was established in 2005–2006 as the trees in the original orchard had become too large to work with. All of the clones in both the original seed orchard and the clone bank were evaluated for growth and form in 2019, and

twenty-one clones were selected for this study. None of the individual selections at Kingsclear were related.

The trees from the Nova Scotia seed production area were from a six-hectare natural stand of mixed ages. The trees in this stand have undergone recurrent selection to favour late flushing. There are currently three age classes in this stand ranging from 8 to 30 years, with many being progeny of but a few trees i.e., there is a high likelihood of significant relatedness among the trees in the stand [25].

The Quebec location was originally used for agricultural purposes but developed into a Christmas tree farm after it was inherited by the current owner. The plantation from which the trees in this study originated was established using superior stock from their own open-pollinated seed orchard. The degree of relatedness among trees in this plantation is not known, and the ages of the material range from 30 to 40 years [27].

All the trees included in this study had previously been selected for good growth and form, traits desirable in Christmas trees. However, none had previously been tested for needle retention.

Tree Selection, Branch Sampling, and Handling

A total of 62 healthy, phenotypically good balsam fir trees were selected for branch sampling and testing; 21, 20, and 21 from the NB, NS, and QC sites, respectively. In the second year of the project, two trees from Nova Scotia were replaced, resulting in a total of 64 trees being tested. Branches were sampled twice annually from 2020 to 2022 (inclusive) (Table 1).

Table 1 Summary of branch sample dates (d/m/y) from the New Brunswick (NB), Nova Scotia (NS), and Quebec (QC) sites.

Year	Collection	NB	NS	QC
2020	Early	8/10/20	7/10/20	7/10/20
	Late	20/10/20	20/10/20	20/10/20
2021	Early	11/10/21	11/10/21	11/10/21
	Late	31/10/21	1/11/21	31/10/21
2022	Early	11/10/22	10/10/22	10/10/22
	Late	31/10/22	30/10/22	31/10/22

Three branches were harvested during each early and late collection period, for a total of six per tree per year. A total of 186 branches were collected twice per year, yielding a total of 1116 branches for testing at the University of New Brunswick Wood Science and Technology Centre (WSTC) in Fredericton NB, over the three-year study. At each collection time, a duplicate number of the branches were harvested and shipped to a secondary location in Hatley, QC (Downey Tree Farm & Nursery) for an additional replicate of testing. Branches in this study were randomly collected from different aspects and positions within the upper third of the tree crowns. Most samples were collected from the top five whorls, but for a few of the larger trees, a few branches were collected from as low as the eighth whorl, usually to avoid large numbers of reproductive buds. All the trees in the study were open-grown, and even the few branches collected from as low as the eighth whorl were not shade-grown. The branches collected from

the Kingsclear provincial tree nursery, nearby to Fredericton, were transported immediately to the WSTC. The branches collected from the NS and QC locations were shipped to the WSTC via ground mail on ice and in sealed coolers to minimize drying during the transportation process. The branches shipped from NS to QC followed the same methods. Upon delivery to the test sites, branches were cut approximately 1 cm below the two-year-old lateral shoots and hung for needle retention testing (Figure 2).

(a) (b)

Figure 2. Needle retention branches cut to length. Branches with a low amount of pollen buds (a) and with a high amount of pollen buds (b).

Testing Branches for Needle Retention

WSTC test site: Testing at the WSTC in Fredericton was carried out in growth chambers. The temperature of the chamber was maintained at 20 °C (+/−2 °C) and 50% (+/−5%) relative humidity throughout the tests. Fluorescent lighting was provided for 24 h during the tests. There was one difference in 2021 from 2020 and 2022 in that a different growth chamber was used. The same conditions were maintained, but in 2021, a larger chamber was used compared with the other two years. Branches were hung on racks within the growth chambers (Figure 3). Each rack supported three rows of branches vertically and horizontally. One branch from each tree was hung on the top, middle, and bottom shelf. Branches were grouped by province and were measured and returned to the same row and shelf throughout the test.

In 2020, the needle retention tests ran for a total of six weeks. There were a few branches that had retained the majority of their needles and were still pliable after six weeks. In 2021 and 2022, the tests at the WSTC were run for a total of eight weeks.

(a) (b)

Figure 2. Photos showing the racks and the growth chambers used at the WSTC for 2020 and 2022 (a) and 2021 (b).

Downey test site: In Quebec, the branches were tagged and hung in a garage/shop. There was limited temperature control and no formal humidity control at this location, but this is where their routine needle retention testing is carried out (Figure 4). Conditions in this building are generally stable at around 20 ◦C to 22 ◦C and relative humidity of approximately 60% [27]. No supplemental light was provided to the branches, and all tests ran for six weeks.

Figure 3. Photo of branches hanging at the Downey test site in 2020.

Branch 'Quality' Measurements

During sample collection, the goal was to try to avoid branches with high numbers of pollen buds, but this was often not possible, especially in 2021 (Figure 2). Therefore, to try to account for the presence of pollen buds on the branches and their potential impact on needle retention, the branches were assigned a rating of 0 to 3; 0 - no pollen, 1 and 2—low and moderate numbers

of pollen buds, respectively; and 3 - abundant pollen buds present. This quantitative assessment of the number of pollen buds was only performed for the branches tested at the WSTC.

In addition to rating the branches for the relative number of pollen buds, branches were assigned a needle configuration rating of 1 to 3, where 1 corresponds to needles with a fairly flat configuration, 2 corresponds to needles partially oriented around the shoot, and 3 corresponds to a 'bottle Brush' configuration, where needles are oriented all around the shoot.

This assessment was carried out to try to determine if there were any tendencies for needle retention to be higher for trees with a bottle-brush configuration than for the other two configurations. The initial total branch fresh weight was measured and attributed to a size class of 1 (0–26 g), 2 (>26–51 g), 3 (>51–76 g), or 4 (>76 g).

Testing for Moisture Loss and Needle Retention

Needle retention was monitored weekly at both the Downey and the WSTC sites. However, moisture loss measurements were not performed at the Downey site. Needle retention results for the two test sites are reported separately.

At the WSTC, each branch was weighed weekly. On each sample measurement day, branches were removed from the rack, weighed, and then held over an aluminum tray, and gently rubbed by hand (wearing a cotton glove) until needles stopped falling. The number of needles was counted, and the branch was classified on a 0 to 8 scale (Table 2). Branches were discarded when they either lost half of their needles or half of their weight and had mummified needles (hard and brittle).

Class	Description
0	No needles present in tray
1	1–10 needles present
2	11–20 needles present
3	21–30 needles present
4	31–40 needles present
5	41–50 needles present
6	51–100 needles present
7	>100 needles present
8	Discarded

Table 2. Needle retention classification scale: the eighth class was used for branches that had lost over 50% of their needles or 50% of their weight, with mummified needles.

At the WSTC, in 2020, the needle retention tests ran for a total of 6 weeks (41- and 43-days post-harvest) for the early and late collections, respectively. In 2021 and 2022, the tests were run for six weeks, but the branches that remained pliable were retained for eight weeks at which time a final assessment was performed. Testing at the Downey location consisted of rub tests for six weeks for all three years, and the branches were assessed for total needle loss at the end of each test period.

Some branches retained their needles even though the branch was brittle. When this occurred, class 'm' for mummified was used. A shoot was classified as mummified when needles broke during the rub test rather than falling off. For mummified branches, the lateral shoots often snapped off.

Criteria for Choosing the Select Individuals

A two-step process was used to identify and separate the 'Select' and 'Population' groups of trees for the statistical analyses and to be included in next-generation tree improvement programs in the three provinces. These steps were used to assess all the branches tested at the WSTC during each of the early and late harvests for all three years, totaling six different testing periods.

Step 1: Qualitative assessments, including pliability and colour, were carried out weekly (Table 3). These were generally subjective assessments of whether the branch was supple (not brittle) and whether needles started to turn yellow or brown. A needle loss rub test was also conducted weekly, and the needles shed during this test were counted and categorized on a 0–8 scale (Table 2). When identifying branches for each grouping, the 'Select' branches had to first satisfy these criteria of remaining green and supple and retaining over 50% of their needles by the end of the study.

Table 2. Branch quality criteria used to assess each branch in Step 1. All branches that met these criteria were then assessed using a quantitative measure of moisture loss.

Criteria	Description
Pliability	Branches were supple (not mummified = not brittle) and still had over 50% of their needles.
Colour	Branches were still green and did not have a brown or yellow designation in their latest tested week.

Step 2: Branch moisture loss was measured weekly and ranked. For each of the six testing periods, the top ten individuals were identified by first ranking all branches in descending order of moisture loss and then considering the criteria in Step 1. To be considered in the top ten, the branches that met the colour, pliability, and needle retention criteria were chosen, starting with the branches that had retained the highest amount of their initial fresh weight. In some instances, fewer than ten branches were selected as only those that met the criteria in Step 1 were included. The frequency of each tree that qualified in the top ten for each of the six periods was determined. Only trees that were present in the top ten for all six periods were chosen as 'Select' trees. This ensured that one or more branches from these 'Select' trees exhibited minimal weight

loss and needle loss, remained pliable, and had acceptable colour at the end of every test period. Individuals that qualified less than six times could still merit future study; however, for the purpose of this study, only individuals with a frequency of six were selected. Of the 64 trees that were tested at the WSTC, 7 'Select' trees were identified: 4 trees from NB, 1 tree from NS, and 2 trees from QC.

Statistical Analyses

All statistical analyses were carried out using R Studio version 4.1.0 [28]. The rate of moisture loss followed a curvilinear pattern. Different nonlinear models were examined, and the following exponential decay model, using the Dose–Response Curves package, was chosen to calculate the slope values for each individual branch for further analyses [29].

$$Y_i = c + (d - c)\exp\left(-\frac{x}{e}\right)$$

where Y_i, the response variable, is the moisture content of each branch *i*. The initial branch weight starts at d and decays to c. Time is x, and e describes the rate of moisture loss. A linear mixed effects model (lme) was used to compare the individual rates of moisture loss between the selected and unselected branch types while controlling the random effects of harvest timing and year as well as province of origin [30].

$$b_i = \beta_0 + \beta_1 Type_i + \epsilon_y + \epsilon_{py} + \epsilon_{hpy}$$

where b_i, the response variable, is the rate of moisture loss for each branch *i*; β_0 is the general intercept; and β_1 is the parameter associated with the fixed effect type. The random effects were year, province in year, and harvest in province in year. The lme was fitted in R using the nlme package with a restricted maximum likelihood approach.

The residuals from the model were positively skewed, and a log10 transformation was used to normalize them.

Initial analyses indicated that 29 branches lost moisture at a rapid rate (within one to two weeks post-harvest). Upon further analyses, an additional 80 branches were identified as having too few data points (lost moisture rapidly) to be properly tested. These branches significantly skewed the distribution of the data and were dropped from the analysis of the full dataset. The remaining 999 branches were included in the statistical analyses.

In complement to the lme described above, a series of t-tests were performed to further analyze the data and significance between the groups. A two-sample t-test assuming equal variances was used to test for differences in the rate of moisture loss (slope coefficient) among shelves at the WSTC test site. All shelf comparisons within each test series were evaluated, and no significant differences were found (data on file). Therefore, shelf was not included as a factor in subsequent analyses. The rate of moisture loss generally did not differ by branch size; thus, no further analysis of branch size was deemed necessary.

Two-sample t-tests were used to test for differences between rates of moisture loss between collection dates within the ‘Select’ and ‘Population’ groups, assuming equal variances. Comparisons between the ‘Select’ and ‘Population’ groups were performed using a Welch two-sample t-test and assuming unequal variances.

Results

Comparison of Needle Retention Test Results for the Downey and WSTC Sites

Needle retention testing at the Downey site was a less labour-intensive approach than that conducted at the WSTC. The goal was to determine how adopting an approach that could be implemented at an operational scale to evaluate a large number of trees would compare with that at the WSTC.

With two harvests each year for three years, there was a total of 6 testing periods at each location, for a total of 12 periods combined. A frequency of 12 indicates that between the two testing locations, that tree had one or more branches in the top five individuals for needle retention and quality, for every possible period when considering all quality traits. A comparison of the rankings of the trees at the two sites is provided in Table 4. Trees highlighted in green indicate the seven ‘Select’ trees that were identified through both needle loss and moisture loss testing, which in combination occurred only at the WSTC location.

The process of branch assessment slightly varied between the two test sites. The criteria of ‘Longest in the study’, ‘Most branches acceptable’, ‘Colour’, and ‘Pliability rating’ were used at both sites. At the Downey site, the total needle loss at the end of the test period was determined, whereas at the WSTC, weekly needle loss was measured. At the end of each test period at the WSTC, a final rating of ‘acceptable’ was designated for branches that exhibited good needle retention, had acceptable colour and remained pliable.

The results in Table 4 show a good overall agreement in needle loss rankings for the two sites/methods. However, it is important to note that, within a test site, the rankings provided in Table 4 are relative to the other trees from that province and collection. For the Downey test, the total needle loss was measured. In some instances, the trees such as NS 140 had a frequency of 12 for needle loss but ranked poorly for other traits and thus did not become a ‘Select’ tree.

Table 3. Summary of the frequency that branches from a tested tree were in the top five trees at each test site. A frequency of 12 indicates that between the two testing locations, that tree has had one or more branches present in the top five individuals for every possible period at both test sites (2 harvests × 3 years × 2 sites).

New Brunswick		Nova Scotia		Quebec	
Tree ID	Frequency	Tree ID	Frequency	Tree ID	Frequency
95	11	140	12	246	10
79	10	143	11	240	9
8	8	135	8	250	8
83	6	121	6	244	7
1	6	134	6	248	6
90	6	137	5	237	4
23	5	110	5	243	4
9	5	112	2	245	3
140	3	160	2	249	3
		108	1	234	2
		144	1	239	1
		136	1	236	1
				241	1
				230	1

Effects of Branch Size, Needle Configuration, and Pollen Production on Needle Retention

The branches from Nova Scotia were generally larger than those from New Brunswick and Quebec (Table 5). The rate of moisture loss generally did not differ among branches of different size classes (data on file), but one t-test comparison between the largest and smallest branch size groups was significant ($p = 0.02$). Despite this one test, it was decided to pool all branches within the ‘Select’ and ‘Population’ groups, and branch size was not included as a factor in subsequent analyses.

Table 4. Summary of mean branch weights (grams) and standard deviations (Stdev) by province and collection.

Year	Harvest	New Brunswick		Nova Scotia		Quebec	
		Mean (g)	Stdev	Mean (g)	Stdev	Mean (g)	Stdev
2020	Early	44.2	24.3	129.1	55.5	36.0	22.5
	Late	49.5	27.4	119.0	50.1	26.8	16.5
2021	Early	28.3	22.2	91.6	45.8	26.5	16.0
	Late	32.6	26.1	97.0	39.5	28.1	14.5
2022	Early	54.3	42.6	113.5	50.5	26.8	13.2
	Late	40.9	26.3	105.9	46.7	21.0	11.6

All the branches that tested well for needle retention had needle configurations of 2 and 3 (Table 6). No trees with needle configurations only in classes 1 and 2 (flat and partial bottle brush) tested well for needle retention.

Table 5. Summary of the frequency of branches by needle configuration, for the top three trees from each province; all three years combined at the WSTC. Trees are sorted in order within each province.

New Brunswick			Nova Scotia			Quebec		
Tree ID (No. Times Tree Was in Top 3 in Test)	Branch Count in Each Needle Configuration Class		Tree ID (No. Times Tree Was in Top 3 in Test)	Branch Count in Each Needle Configuration Class		Tree ID (No. Times Tree Was in Top 3 in Test)	Branch Count in Each Needle Configuration Class	
	Class 2	Class 3		Class 2	Class 3		Class 2	Class 3
9 (6)	13	5	143 (6)	5	11	240 (6)	1	15
90 (6)	5	13	140 (6)	9	9	246 (6)	9	7
95 (6)	14	4	135 (6)	16	2	249 (6)	4	14

The branches that featured a high number of pollen buds consistently dropped needles very quickly in only those areas (Figure 5). These branches retained their needles in locations that did not feature any pollen buds, and when considering only the remaining locations, the branches had a similar needle loss pattern to branches completely void of pollen buds. This pattern was present and consistent in both harvests, all three provinces, and in each of the three years.

All the branches used in this study were from the upper crown, with none having a flat needle configuration. The trees in this study had already undergone selection against a flat needle configuration, a trait that is not desirable for Christmas trees. Shaded branches originating lower in the crown, which would tend towards a flat configuration, were not used.

Figure 4. An example of a branch sample with a high level of pollen buds present, where needles have abscised predominantly only in the pollen bud areas.

Needle Retention of 'Select' versus 'Population' Trees

The pattern of needle loss differed among the three years of testing. In 2020 and 2022, the mean needle loss increased over time. In 2021, the mean needle loss did not increase in weeks four and five to the same degree as in 2020 and 2022. Within any year, the overall pattern of needle loss was generally the same for all provinces and both the early and late collection series (data on file). Needle loss for the 'Select' trees compared with the 'Population' group by year and province is shown in Figure 6.

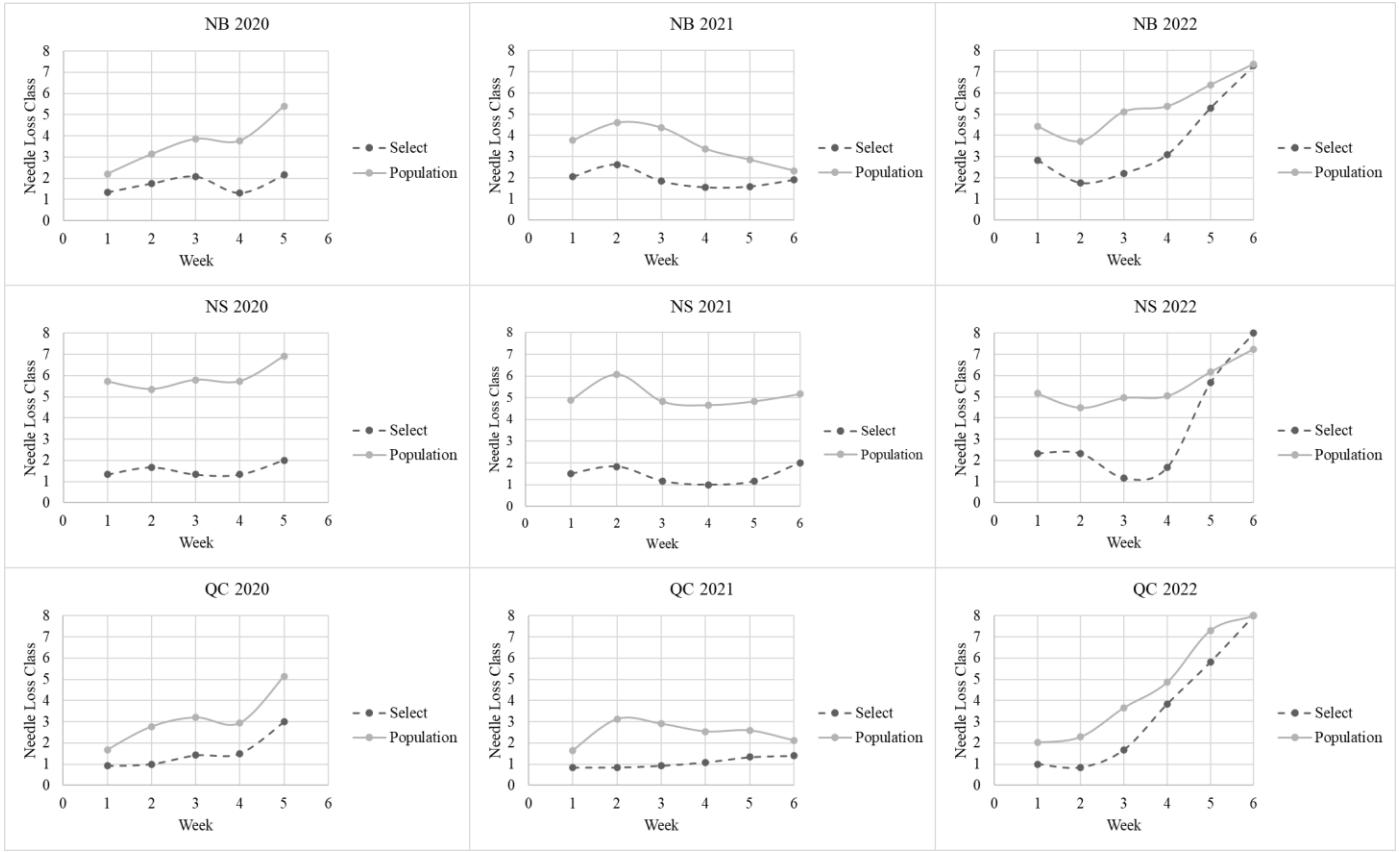

Figure 5. Mean needle loss over time, comparing ‘Select’ and ‘Population’ branch groups by province and year. Early and late collections are combined in their respective groups.

Moisture Loss Comparison between 'Select' and 'Population' Groups

All branches were weighed weekly until they were discarded. When testing moisture loss slope coefficients within the two groups of trees, comparisons between the early and late collections were statistically significant for some but not all test series (Table 7). 'Select' trees from Nova Scotia and Quebec lost moisture at a significantly slower rate than the corresponding 'Population' trees (Figure 7, Table 8). For New Brunswick trees, the differences were significant for 2021 but not in 2020 and 2022. The difference in the percent weight loss between the 'Select' and 'Population' trees was the smallest for Quebec branches but still statistically significant (Figure 7, Table 8).

Table 6. Summary of two-sample t-tests for the slope coefficient comparisons of early and late harvest periods for the 'Select' and 'Population' groups by year and province.

Year	Group Type	Comparison	*t*-Value	df	*p*-Value
New Brunswick					
2020	Select	Early vs. Late	0.253	21	0.803
	Population	Early vs. Late	1.439	93	0.154
2021	Select	Early vs. Late	1.004	22	0.326
	Population	Early vs. Late	2.249	81	0.027
2022	Select	Early vs. Late	3.415	22	0.002
	Population	Early vs. Late	0.258	88	0.797
Nova Scotia					
2020	Select	Early vs. Late	−0.909	4	0.415
	Population	Early vs. Late	−2.041	100	0.044
2021	Select	Early vs. Late	4.426	4	0.011
	Population	Early vs. Late	5.350	87	<0.001
2022	Select	Early vs. Late	3.730	4	0.020
	Population	Early vs. Late	5.577	99	<0.001
Quebec					
2020	Select	Early vs. Late	−1.738	10	0.113
	Population	Early vs. Late	−1.667	98	0.099
2021	Select	Early vs. Late	1.637	10	0.133
	Population	Early vs. Late	0.080	104	0.936
2022	Select	Early vs. Late	0.442	10	0.668
	Population	Early vs. Late	2.514	106	0.013

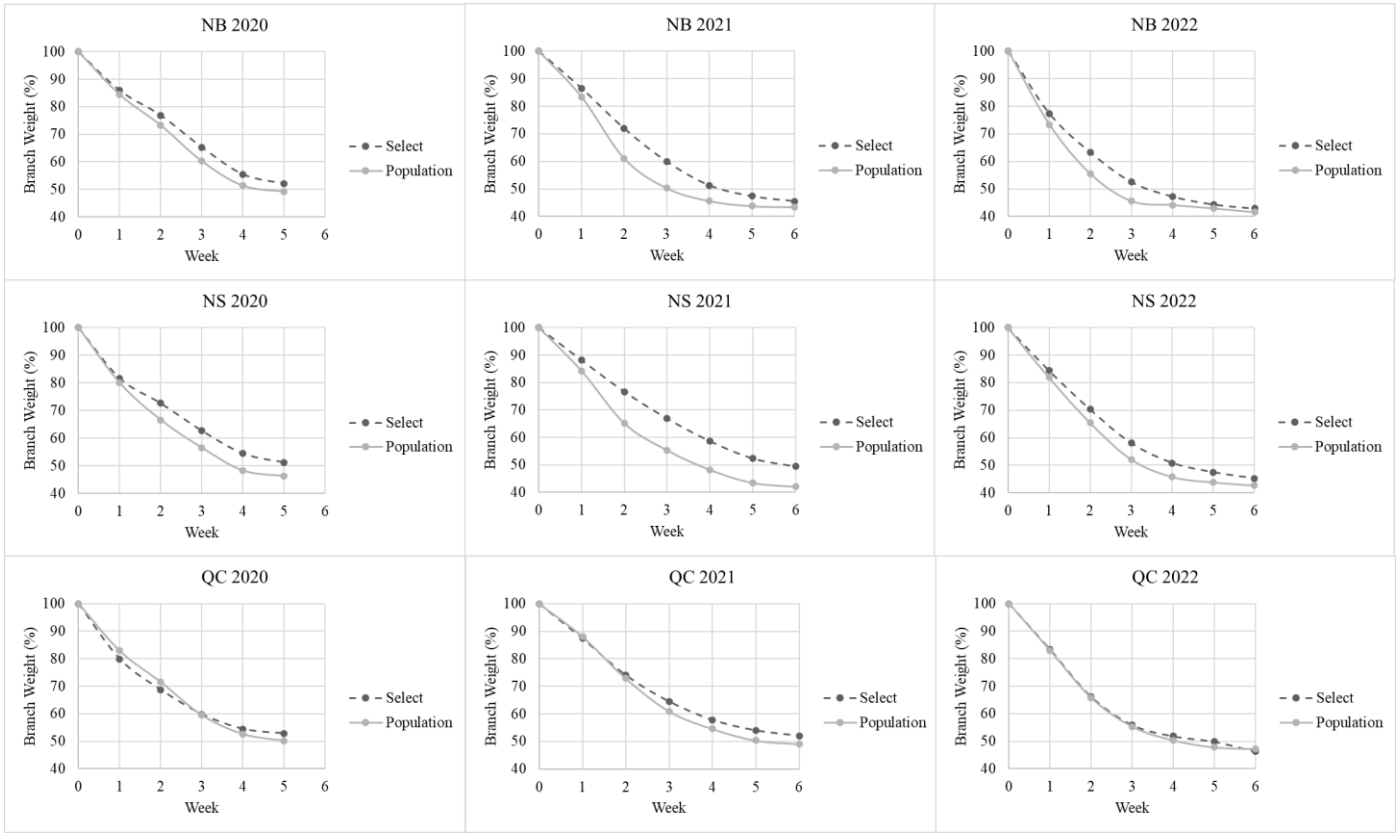

Figure 6. Comparison of percentage of branch weight over time between the 'Select' and 'Population' groups for each province and year. Early and late harvests are combined.

Table 7. Summary of Welch two-sample *t*-tests for the slope coefficient comparisons between the 'Select' and 'Population' groups by year, province, and entire datasets. Tests were conducted assuming unequal variance, thus generating degrees of freedom that are not whole numbers.

Comparison	*t*-Value	df	*p*-Value
New Brunswick			
2020 Select vs. Population	0.039	50.80	0.969
2021 Select vs. Population	2.960	104.75	0.004
2022 Select vs. Population	0.732	111.91	0.466
Nova Scotia			
2020 Select vs. Population	3.722	104.50	<0.001
2021 Select vs. Population	2.321	21.91	0.030
2022 Select vs. Population	1.750	49.26	0.086
Quebec			
2020 Select vs. Population	7.069	84.32	<0.001
2021 Select vs. Population	5.101	103.86	<0.001
2022 Select vs. Population	4.049	110.14	<0.001
All Years			
NB Select vs. Population	1.699	194.35	0.091
NS Select vs. Population	3.673	73.13	<0.001
QC Select vs. Population	8.242	247.85	<0.001
Whole Dataset			
Select vs. Population	5.588	305.76	<0.001

Discussion

The decision not to include tree age as a factor in this study was predicated on the results from a companion study in 2020 in which the needle retention of branches from fifteen 30- and 12-year-old grafts of the same clones (regrafts from the older trees) were tested at the WSTC following the same protocols as in this study [31].

Not all branches from single trees exhibited the same needle retention pattern, possibly because of the slight differences in the presence or absence of pollen buds, and the aspect and position within the crowns. All the tested branches had two years of growth and were collected from the upper crowns of the trees. This was implemented so that the age classes of needles were the same for all branches. Needle drop and replacement naturally occur in trees and increase as needles age. Balsam fir needles that are one and two years old will experience a needle drop of less than 10 percent, but this percentage increases yearly thereafter [32].

When collecting samples for testing, the branches with fewer pollen buds were prioritized but not always available. During testing, needles adjacent to pollen buds generally shed quickly

compared with the other needles on the same shoots and branches. Male and female flower buds in balsam fir differentiate on elongating shoots in July [33]. Drought and other stressors that are associated with increasing the proportion of buds that differentiate reproductively also impact needle primordia development [34]. During years with heavy flower production, overall shoot growth, including length and number of needles, as well as size and shape of needles, is severely affected as nutrients and hormones are preferentially diverted to the developing reproductive structures [33,35].

The rate of moisture loss was slower for the 'Select' group than for the 'Population' group (slopes of moisture loss over time) in all comparisons. However, these differences were only statistically significant ($p < 0.05$) for six of the nine single-year within-province comparisons, with the differences being significant for all three years for Quebec branches. The 'Select' and 'Population' groups differed significantly for Quebec and Nova Scotia branches in the pooled analyses (all three years combined) as well as in the full dataset comparison involving all three provinces and years.

The lack of statistical significance in New Brunswick tests is likely related to the population history of previously being selected for inclusion in a clonal seed orchard based on identified superior traits. This is also supported by New Brunswick having the most identified 'Select' trees, in total, four of the seven between the three provinces.

The differences in the rates of moisture loss between the early and late collections for the 'Population' trees were statistically significant for five of nine comparisons. Conversely, for the 'Select' trees, the differences were only significant for three of the nine comparisons. This observation agrees with that reported for balsam fir in which collecting branches later in the season improved needle retention more for trees with lower needle abscission resistance (NAR) than for high-NAR trees [17].

Needle retention was generally better for branches from the late than early collections, in agreement with that generally observed for balsam fir [22] and other Christmas tree species [8], but not all of the differences in this study were statistically significant.

Physiological changes that have been correlated with cold acclimation and reduced post-harvest needle loss in fir species were not measured in this study, but other studies have included the accumulation of raffinose in Fraser fir [8,23], and raffinose, galactose, and ABA in balsam fir [17]. In these and other studies, delaying harvesting improved overall post-harvest needle retention, but changes in the aforementioned compounds were not directly linked to needle abscission.

One limitation of using rub tests to evaluate needle loss is that it is a single quantitative measure that is affected by differences in branch size and the concomitant number of needles on the branches. Post-harvest moisture content has a direct influence on needle retention and branch quality. Multiple studies using various Christmas tree species have concluded that lower moisture levels lead to increased needle abscission and poor branch quality [1,8]. Because moisture loss and needle loss are directly correlated, the former was chosen as a suitable proxy

for measuring the branches' needle retention capabilities. Despite the greater control of temperature, humidity, and light at the WSTC compared with the Downey test site, both effectively identified trees that lost their needles quickly and those that kept the needles for five or more weeks.

Two different patterns of needle drop were associated with moisture loss in this study. The trees that lost needles rapidly often also exhibited rapid loss of moisture, whereas there were some that lost moisture rapidly but for which needle loss did not occur. For those trees, needles became hard and brittle but did not abscise from the shoot, i.e., they were mummified. Abscission is an energy dependent process, and if trees dehydrate too quickly, then abscission cannot occur. Needles may still crumble or break off due to brittleness, but they do not truly abscise [36].

The rate of needle loss in this study was predominantly not correlated with initial branch size (weight), contrary to that observed for Norway spruce [37]. The rates of moisture loss for the four branch size classes were compared using a Welch two-sample t-test, and of the six combinations, only the comparison between the largest and smallest classes was significant. MacDonald et al. (2014) conducted a study of 45 branches, comprising 15 each from high-, medium-, and low-NAR trees, and observed that high-NAR trees generally had smaller diameter (9.1%) and weight (25%) branches than the low-NAR genotypes [17]. Among the other physical traits they assessed, needle density (needles/cm shoot) and needle breakage strength (force required to remove a needle from a stem) were both negatively correlated with needle retention, but neither of these measurements would be practical to use operationally.

When testing branches for weight loss, a measurement was taken each week prior to that week's needle loss rub test, and a percentage value was generated for each of the eight weeks comparing branch weight to the samples' original fresh weight in week zero. This percentage was used to standardize the rate of moisture loss between the samples regardless of branch size. The standardized percentages were used to calculate the slope coefficients for each branch that was used in the primary analysis. This approach was effective in differentiating between the 'Select' and 'Population' trees.

The rate of moisture loss was inversely correlated with needle retention, similar to that previously reported for whole tree harvesting studies in balsam fir [38] and for noble and Nordmann firs (*Abies procera* and *Abies nordmanniana*) [1]. Whole tree harvesting and monitoring can be used to assess the treatment effects on needle retention that can be applied to optimize the post-harvesting handling of Christmas trees. However, it is not a tool that can be effectively used for selecting and testing trees in a tree improvement program.

Needle retention can be considered as both a quantitative and qualitative trait. Cumulative needle loss was not, by itself, the most consistent means to select superior trees. Differences in the initial number of needles present on branches of different sizes were not factored into this study. Weight measurements were an indirect measure of needle biomass. Expressing needle loss as a percentage of total needles might have accounted for this. However, moisture loss was expressed as a percentage of branch weights, and a combination of needle and moisture loss with the qualitative assessments of needle colour and branch pliability was used. Given the different

testing methods at the two test sites, slightly different criteria were used to rank the trees and a combination of the rankings was used to determine the final selections in Table 4.

Several trees from all three provinces, although ranking high in the needle retention tests, exhibited needle discolouring and some mummification, and thus were not selected. The combination of qualitative and quantitative methods resulted in four, one, and two trees being selected from NB, NS, and QC, respectively (highlighted in green in Table 4). For example, Clone no. 1 from NB ranked in the top five in four tests at the Downey site, but at the WSTC, it ranked fourth and fifth in both harvests, respectively, only in 2021. This clone is an example of a tree that generally performed well but did not meet the specific criteria for selection when the qualitative traits were included in the selection process.

One of the potential shortfalls of using needle rub tests alone for selecting trees with good needle retention is that the number of needles lost is generally proportional to branch size. Therefore, selecting branches of equal size could have improved the comparison among trees at the Downey test site.

The results from this study indicate the importance of using the quantitative assessment of moisture loss as part of the process of selecting trees with good needle retention. The branches that lost moisture rapidly often became mummified without a significant loss of needles. These branches can be identified early in the testing process and discarded early, thereby reducing the workload. The rate of moisture loss in the 'Select' trees was significantly lower than in the 'Population' group (Figure 7). Differences in the rate of moisture loss could be attributed to factors such as the rate, timing, and amount of wax deposition on the needles, but this was not examined in this study. Variability due to environmental conditions regarding stomatal response and wax deposition in conifers have been recorded [39,40].

All the trees included in this study were phenotypically good trees, having been selected for good growth and form, including crown density, acceptable crown taper, needle colour, age class retention, branch angle and needle configuration that has been associated with good needle retention [25]. As well as freedom from insects/diseases or deformities. The number of identified 'Select' trees differed for the three sample sites. No direct measures of genetic variability were analyzed as part of this study. Variation in needle retention within each site was likely influenced by the relative amount of genetic variation among the trees and thus would have influenced the number of trees exhibiting superior needle retention, i.e., selection intensity. The 'Select' trees in this study were grafted in 2021 and 2022, and future plans include further testing for the heritability of needle retention in these trees.

Conclusions

The two objectives of this study were (1) to identify balsam fir parent trees for inclusion in long-term Christmas tree improvement programs in the Northeast and (2) to determine if the rates of moisture loss from branches can be used to improve or be used alone to better identify trees with good needle retention properties than traditional needle rub tests. The hypothesis was that moisture loss will be a definitive variable to successfully select trees with good needle retention

and will be able to identify trees from the three participating provinces. Both of these objectives were met. These results further support the importance of assessing both quantitative and qualitative traits over multiple years in the process of selecting balsam fir Christmas trees that will be well adapted under future climate change conditions.

The next step in the program will be to evaluate the heritability of needle retention using open-pollinated family tests. The family tests will be established in each of the three provinces using all of the 'Select' trees plus additional selections as they are identified. These replicated family tests will be used to identify the parents that produce offspring with good growth and needle retention under different growing conditions and thus best suited for growers to use.

The trees identified in this study, and other trees as they are identified, will be used to establish seed orchards in the three partner provinces. Grafts of all seven 'Select' trees will be out planted in the fall of 2023 at each of the three respective locations. The orchard in New Brunswick has already been designed, using the Computer Organized Orchard Layout (COOL) program, and the design includes 20 ramets of 40 different clones. The COOL program maximizes the distance between the ramets of the same clone, minimizing selfing as much as practicable. The designs and number of clones for the orchards in Quebec and Nova Scotia are yet to be determined. However, these orchards will be designed using the COOL program or an alternative program [41,42].

All the trees in this study had previously been selected because they were phenotypically good trees. The testing component for needle retention was carried out to add a criterion intended to select trees that would likely perform well under climate change conditions and be suitable for early harvesting and/or harvesting in the warmer fall seasons that are predicted for the Northeast.

Neither the factors that regulate needle abscission nor the number of genes that control this natural process are fully understood. Moving forward, the intention is to graft the selected trees into seed orchards and establish family tests, the results of which will be used to confirm the level of genetic heritability for needle retention. Given the significant genetic variation in morphological traits in balsam fir provenances [20], it should be possible to select trees for traits such as the timing of cold acclimation and increased needle retention that may be associated with this process.

The second main objective of this study was to determine if combining moisture loss measurements with traditional needle rub tests could be a more reliable method for identifying trees with good needle retention properties. Our results indicate that the testing process followed here can help tests in two ways: (1) eliminating poor trees after two weeks, thereby reducing the number of trees that need to be followed for the full duration of the testing period; and (2) increasing selection intensity (i.e., selecting fewer trees), possibly increasing the likelihood of selecting genetically superior trees for a breeding program. This conclusion has to be verified with the field testing of progeny from the 'Select' trees.

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Technical Report 2

Rootstock effects on graft phenology and development

Introduction

A significant effect from the warming climate in the Northeastern Canadian region is a longer growing season, specifically earlier and warmer spring periods causing plants and trees to exit dormancy early in the spring, increasing their risk of frost damage [1]. The length of the growing season for warm season crops is projected to increase by 13.7 to 18.6 days by 2050 and 14.3 to 51.1 days by 2100 [2]. This warmer climate is expected to shift multiple species distributions and cause a significant decline in balsam fir (*Abies balsamea*) habitat in Atlantic Canada within the 21st century [3,4]. This increasing risk of late spring frost damage has created a demand from the Christmas tree industry in this region for tree improvement programs to provide climate adapted stock to mitigate this risk.

Grafting is a vegetative propagation method that is used worldwide to preserve desirable genotypes for multiple purposes including tree improvement programs [5]. A graft is composed of two pieces: a scion, which originates from the crown of the desirable tree, and the rootstock, which is the root system that will receive the scion [6]. Over time the branches of the rootstock are removed until only the scion that is being supported by the root system remains, creating a clone of the parent tree.

In the Northeastern Canadian Christmas tree industry, grafting is used to propagate desirable seed trees and a specific trait that is becoming increasingly valued is a later bud flush to limit the risk of late spring frost damage [7]. Having multiple clones of desirable genotypes also permits genetic experiments for tree improvement research, without sacrificing the original tree. Grafting can also be used to establish clone banks comprised of select trees as insurance against natural and unforeseen disturbances.

In the United States, grafting is increasingly used in the Christmas tree industry to reclaim fields contaminated by Phytophthora (*Phytophthora cinnamomi*) root rot that would otherwise be inoperable. Growers have resorted to grafting the desirable and susceptible Fraser fir (*Abies fraseri*) onto rootstock of *Abies* species such as Momi fir (*Abies firma*) that are resistant to the fungus [6]. The range of this fungus is predicted to spread northward into Canada because of increasing temperatures from the warming climate [8]. This is an example of a major problem that the Northeastern Canadian Christmas tree industry might have to eventually face. However, a known solution is the practice of grafting onto resistant rootstocks, but with the warming climate encouraging the spread of this fungus, understanding the bud flushing trait will be evermore important.

The rootstock is known to have an influence on the grafted scion in some species and it is well document in fruit where grafting is a common practice. In grapefruit (*Citrus paradisi*), rootstocks have been documented to have an effect on yield and chemical composition in fruits, physical characteristics, sodium levels as well as macro and micronutrients in the leaves [9]. In apple trees

(*Malus pumila*), a clonal grafting study reported that depending on the rootstock, the gene expression in the scion for photosynthesis, transcription/translation, cell division and stress response related genes could be influenced [10]. A peach tree (*Prunus persica*) grafting study focusing on hormone transport through xylem sap flow, suggested that this flow was regulated by the rootstock genotype and was restricted by the graft union [11]. Meaning that hormonal influence from the rootstock on the scion can largely depend on the xylem sap flow characteristics of the rootstock and graft union quality.

Less literature is available in conifers; however, it has been reported in loblolly pine (*Pinus taeda*) that the rootstock can influence graft survival, flowering, and growth [12]. This same study also suggests that the flowering vigor of the rootstock could have a direct effect on the flowering habits of the scion. Another Loblolly pine grafting study, comprised of six scion clones grafted onto 25 full-sib family (both parents are known) rootstocks, examined multiple phenological variables and found that the rootstock only had a significant influence on the diameter at breast height of the grafted scion. This study examined the effects eight years post grafting and suggested that scion clone and site have more control over scion growth and reproduction than the rootstock itself [13]. Another long-term grafting study in involving multiple pine species also found that compatibility, growth rate and needle length varied by scion and rootstock combinations. A positive correlation was found for combinations that produced the longest needles also had the highest conelet production [14].

Many of the available studies in the literature on grafting in conifers report on the different methodologies and factors that affect success, such as species, timing, and scion material [15]. An important and mostly unknown factor is the influence that the rootstock has on timing of bud flush. The heritability of the bud flush timing trait has been studied for multiple *Abies* species. In 2007, 51 full sib and six half sib (only one parent is known) families of European silver fir *(Abies alba*) were used to determine the heritability of bud flush in an effort to ameliorate late spring frost damage events. The study concluded that bud flush was under strong genetic control in this species [16]. Similar studies in Fraser fir Christmas trees and Coastal Douglas fir (*Pseudotsuga menziesii var. menziesii*), concluded that the high heritability of timing of bud flush provides an opportunity to select for late flushing trees with a concomitant reduced susceptibility to spring frost damage [17,18]. These studies however were not completed using grafted material, so it is agreed that it is a highly heritable trait, but the level of control that remains with the scion after grafting in unknown.

Christmas tree improvement programs to date primarily focus on phenotypic traits that the end consumer values, such as colour, form, fragrance, and more recently, needle retention [19]. With the increasing effects of a warming climate, these programs will need to adjust their focus to produce stock better suited for these conditions.

The objective for this study was to determine the influence of the rootstock on grafted balsam fir bud flush timing and development by grafting and monitoring a series of identified early and late flushing trees. The hypothesis for this experiment is that the rootstock material will not change the relative phenology or bud flush timing of the grafted scion.

Methods

Greenhouse Grafting

Four balsam fir trees from each of the participating provinces were identified for inclusion in this study; two early and two late flushing. The New Brunswick (NB) selections were made based on visual inspections of timing of bud flush in 2021 (Data on file). The Nova Scotia (NS) and Quebec (QC) trees were also selected using visual inspections however, they based on multiple years of historical observations by the growers. In total, 191 grafts were completed from these 12 identified 'early' and 'late' flushing trees, from each province, in 2022 (Table 1).

Rootstock Production and Grafting

Production and handling of rootstocks and grafts were completed at the Kingsclear provincial tree nursery nearby to Fredericton, NB, in March of 2022.

All rootstock material used were from Productions Resinex Inc located in QC. These rootstocks were four-years old, 215 were balsam fir and 40 were Canaan fir (*Abies balsamea var. phanerolepis*). To prepare rootstocks for grafting, they were potted early in the fall, preceding the spring grafting period.

Branches were collected in late February from the top third of each tree using pole pruners and the samples from NS and QC were shipped to Fredericton, NB, on ice and in coolers to minimize moisture loss. Following industry standards [20], the rootstocks were moved into a heated garage for approximately one week to thaw before grafting to ensure they were pliable enough to be cut. Scions were selected and cut from the terminal approximately six inches of the shoots from the harvested branches.

To prepare the scions for grafting, the needles were removed from the bottom half of the of the scion and they were stored in snow to minimize drying. A top cleft graft was used when a close match was possible between scion and rootstock diameters to ensure adequate bonding between the cambial layers. When it was not possible to match the two sides of the cambia (scion and rootstock), the cambium on one side of the scion was matched directly with one side of the cambium on the rootstock.

To prepare a scion for a top cleft graft, the bottom portion of the scion (needles removed) was sliced on both sides into a wedge shape at equal angles. The top of the leader on the rootstock was removed and a vertical slit was cut in the stem to the same length as the wedge portion of the scion. After inserting the scion into the rootstock slit, a rubber band was tied around the entire incision to hold it closed and in place and the union was sealed with grafting wax (Figure 1).

Figure 1. Steps for a Top Cleft graft: (1) matching scion and rootstock diameter; (2) slicing wedge shape into scion, approximately one third to half of the total scion length; (3) side view of scion wedge shape; (4) scion inserted into vertical incision in rootstock leader; (5) graft union tied in place with rubber band; and (6) graft union sealed with hot grafting wax.

Side veneer grafts were used when a close match was not possible between scion and rootstock diameters, these were done as per Burns et al. 1981 and followed a similar method as the top cleft (Figure 2) [20]. When slicing the base of the scion, one side was approximately half the length of the longer side. The rootstock leader then received a vertical slit off set from the center of the shoot, leaving the entire shoot intact and forming a pocket in the side of the stem. This allows for a smaller scion to still be grafted to larger rootstocks because the cambial layers are easier to place in contact with one another by cutting closer to the outside of the rootstock stem. The long side of the scion wedge was then fit in against the main stem and the outside peel of the rootstock incision was trimmed to the length of the shorter side of the scion wedge. The leader of the rootstock was initially left intact, however, after the scion began to flush, the terminal buds of the rootstock were removed.

After grafting, all samples were kept in a temperature-controlled greenhouse set at 20 ◦C and thereafter adjusted to match the exterior temperatures as they warmed. Grafts were eventually moved outside to a shade house after healing and when most of the new growth was completed. Grafts were watered regularly and fertilized as needed. Rootstock were basally pruned and upon scion flushing, the terminal new growth shoots of the lateral branches of the rootstock were also pruned. All material was overwintered in an unheated greenhouse at the Kingsclear provincial tree nursery and then moved into a shade house in the spring after snowmelt.

Figure 1 Steps for Side Veneer graft: (1) scion is too small for rootstock leader; (2) short side of scion wedge; (3) long side of scion wedge; (4) scion inserted into rootstock pocket incision, long side of wedge facing leader stem and outside peel of the incision is cut to length to match short side of wedge. Tying in place with a rubber band and sealing with grafting wax follows the same protocol as the top cleft graft (Figure 1).

Table 1. **Inventory of early and late flushing grafts completed in March of 2022.**

Province	Clone	Grafted	Successful
New Brunswick	NB-1 Early	21	14
	NB-79 Early	21	19
	NB-8 Late	22	19
	NB-95 Late	21	17
Nova Scotia	NS-IR7 Early	21	18
	NS-IR14 Early	22	22
	NS-IR1 Late	20	14
	NS-IR17 Late	19	14
Quebec	QC-14 Early	6	6
	QC-15 Early	6	6
	QC-66 Late	6	6
	QC-69 Late	6	5
Total		191	160

An additional 63 grafts were completed in 2022 for the establishment of three clonal seed orchards as an added outcome of this study. The grafting success is reported for all grafts that were completed in 2022, totalling 254 and overall success was 86.2%. This is comparable to other studies grafting *Abies* species but higher than most studies grafting other conifers [21-24]. It is generally agreed that *Abies* are easier to successfully graft than other coniferous species.

The percent success was similar for the three provinces, ranging from 82.5-95.8%. When comparing locally sourced (NB) and out-of-province scions (NS, QC), success rates varied by less than one percent (i.e., 85.7-86.5%). This indicated that province of origin, scion collection, handling and transportation did not have an adverse effect grafting success.

A total of four different graft types were used in this study, and all had relatively good success rates which ranged from 80.6-100%. The top cleft method which was used for 171 grafts a success rate of 86.5%. The top cleft one side had a slightly higher success rate at 87.2% but was only used for 39 grafts. The side veneer method was used for only 8 grafts and had a 100% success rate. The side veneer one side method had the lowest success rate which was 80.6% which was likely caused by this method having the least amount of cambial contact surface area between scion and rootstock, this method was used for 36 grafts.

Due to a shortage of balsam fir rootstock, a subset of grafts completed in 2022 used Canaan fir as rootstock material. This is a subspecies of balsam fir that originated from Virginia and West Virginia and is also used within the Christmas tree industry [25,26]. Canaan rootstocks were only used for NB and NS grafts and only for clones with a minimum number of 15 scions. When conducting inter-species grafts, it is important that they are compatible; this is known as the level of taxonomic affinity [27,28]. A total of 39 grafts were made with balsam fir scions and Canaan fir rootstocks, with a success rate for this combination of 89.7%, which is higher than the balsam fir on balsam fir grafts which was 85.6% for 215 grafts. No issue was expected because Canaan fir is so closely related to balsam fir, and it has been suggested that there is a high likelihood that all *Abies* species in North America originated from one population [29-31]. These results were like those of a study that grafted Fraser fir on rootstocks of multiple *Abies* species which included Canaan fir and had success rates above 92% [21]. Because of the comparable success rates between rootstock types, both types were combined for the analyses.

Bud Development Assessments

Weekly bud development assessments were completed using a one to ten scale of development stages adapted for balsam fir from bud development studies for other coniferous species (Figure 3) [32,33]. All grafted material were located and assessed at weekly intervals at the Kingsclear provincial tree nursery. The NB parent trees were also located at this nursery and three branches were selected from the top third of each of the four parent trees and were assessed at the same interval. The parent trees from NS and QC were assessed with a visual inspection of the top third of the crown by the participating growers and used the same one to ten scale and weekly interval. Because the NS and QC parent trees and their respective grafts were in different geographic locations, the data is not directly comparable, however the parent trees were still monitored to show their bud flush timing for the same year the grafts were monitored. The specific monitoring

of three branches per parent tree was also not feasible at the NS and QC locations, so these in-depth assessments only occurred for the NB parent trees. Graft mortality was recorded, and dead grafts were discarded to prevent the spread of any pests that could be present. Bud development was not monitored the year of grafting because of the healing shock immediately post grafting. All bud development assessments took place in 2023, the grafts which were all located at the Kingsclear provincial nursery and assessments were from April 24th to August 15th. The NB parent trees were monitored from May 2nd to August 15th, the NS parent trees from April 23rd to August 2nd and the QC parent trees from April 28th to August 7th.

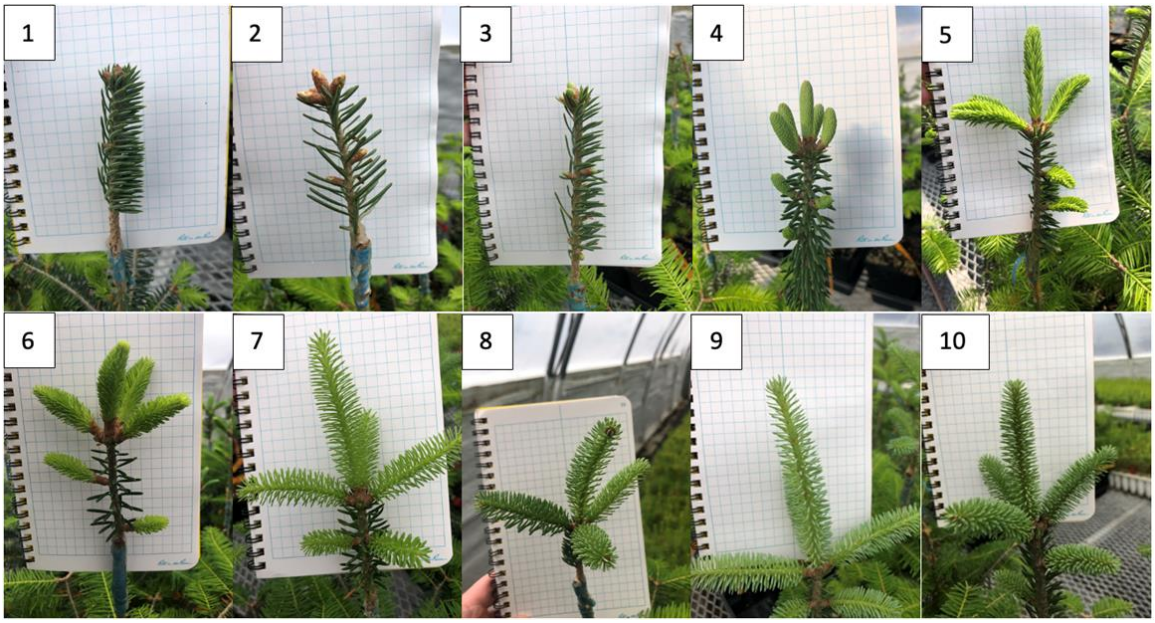

Figure 2. Bud development growth stages used to classify all grafts completed in 2022 and followed during the 2023 growing season. **Stage 0** was used for individuals still in dormancy (not shown). **Stage 1:** Slight bud swelling. **Stage 2:** Swollen, transparent bud scales that are green and white but still closed. **Stage 3:** Bud scales broken with tips of green needles emerging. **Stage 4:** New growth elongated to twice the length of the initial bud, needles are not yet spread. **Stage 5:** First spread of the needles, looks like a paint brush or feather at the end of the shoot, individual needles are not yet spread apart. **Stage 6:** Shoot elongation and most needles spread, basal needles not yet spread. **Stage 7:** Differentiation of shoot, all needles more or less spread, terminal bud green or light brown. **Stage 8:** Terminal bud is dark brown. **Stage 9:** Internodal buds are present along the new growth shoot, orange/red in appearance. **Stage 10:** New growth shoot is completely lignified; the stem is brown with no bright green flesh left.

Balsam fir bud development can be described as three distinct but overlapping phases. The first phase occurs in the spring from bud dormancy until bud burst (Stage 0-3). The second phase is from bud burst to the end of shoot elongation (Stage 3-8) and the third phase is the maturation of buds and development of shoot primordia for the next year of growth (Stage 8-10) [34]. This study focused on the first two phases of shoot growth and bud development, however for the first

phase it was stages 0-2 because the second stage was the first obvious sign of bud movement and easily identifiable. These two phases are structured to capture the bud flush timing of the samples and then separately, the overall shoot growth, and development. Occasionally, a graft would proceed through multiple stages between weekly observations, so stage 3 was used when stage 2 was not present. Stage 3 occurred when bud scales separated, and the new shoot primordia were exposed. When stage 8 was not present then stage 9 was used, which is when internodal buds begin to form along the new shoot.

Field Grafting

In 2020/2021, travel was restricted due to COVID, so an alternate grafting plan was developed and implemented for the spring of 2021. The original plan was to do field grafting in each of the three provinces. The modified plan focussed on evaluating the effects of rootstock on timing of budflush but the grafting was only done in Nova Scotia. The experimental design adopted was:

Three sets of eight trees were selected at each of two sites, New Germany and Stanburne, Nova Scotia. Each set of eight trees comprised four early flushing and four late flushing trees. These were all 'wild' or natural trees. Trees were identified as early or late flushers.

Five scions were collected from the third whorl from each tree (Figure 1). Within each set of eight trees, as much as practicable, one shoot from each of the four early flushers were grafted onto a shoot in the second whorl from each of the four late flushing trees in the set, AND vice versa (four lates onto the four earlies in the set). The fifth scion was re-grafted onto the parent tree from which it came (Figure 4). A total of 240 field grafts were done (Figure 5). All grafting was done By Matt and Robin Wright, Dana Eagles and Colin Hunter (Figure 6). The grafting technique used in 2021 was the whip-graft method.

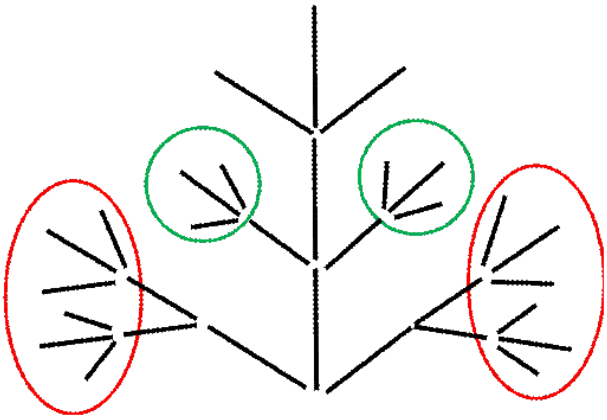

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Figure 4. Schematic of the positions from where the scions were collected from each tree (red ovals) and where they were grafted (green circles).

Figure 5. Photos of two trees showing the top of a tree with tagged grafts (left) and a closeup of several grafts (right).

Joel Tremblay and Matt Wright conducted survival assessments on the field grafting on June 26, 2021, and again on July 16, 2021. The weather post-grafting was exceptionally hot and dry, and overall and survival was low: Sixteen and twenty-five grafts out of 120 survived at the Rosebud and Stanburne sites respectively. The low survival precluded a meaningful comparison of early and late flushing and the effects of rootstock on scion development in 2022.

In 2022, the same experimental design as used in 2021 was followed. however the grafting method used was side-veneer grafting.

Figure 6. Photos of Colin Hunter (top left) and Dana Eagles (top right) the two growers who field grafted with Matt and Robin Wright, and one of the more vigorous surviving grafts (bottom).

Statistical Analyses

Microsoft Excel Version 2310 was used to calculate means and standard deviation when comparing the duration required to complete each growth phase between each genotype [35]. The duration data was normally distributed and could not be normalized using any of the following transformations: square, square root, square root max, $\log_{10}$, $\log_{10}$ squared or scaling. The Kruskal Wallis Rank Sum non-parametric test was then chosen to complete the analyses because it does not assume that the distribution of the data is normal. The Kruskal Wallis test was used to test for significant differences between grafted genotypic groups. This test compared the number of weeks required to complete the first and second growth phases between the early and late flushing groups, as well as between provinces. These tests were completed in R version 4.1.0 [36]. The Kruskal Wallis Rank Sum test equation is as follows:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1)$$

where H is the test statistic which is compared against the chi-square distribution *k-1* of the degrees of freedom where *k* is the number of groups, *N* is the total number of observations, n_i is the number of samples in each group, R_i is the total sum of ranks in each group. The two early and two late flushing genotypes were combined within each of the three provinces. So, each of the three provincial groups had two flushing groups within, one early and one late.

Provincial comparisons had more than two levels, so a post-hoc pairwise multiple-comparison Dunn's test was applied to determine at which level significance existed. The Dunn's test formula is as follows:

$$z_i = \frac{\bar{W}_A - \bar{W}_B}{\sigma_i}$$

where $\bar{W}_i$ is the mean rank for each group i which is shown as "*A*" and "*B*", which are the different groups, flush and province, and σ_i is the standard deviation of the numerator given by the following formula:

$$\sigma_i = \sqrt{\left(\frac{N(N+1)}{12} - \frac{\sum_{i=1}^{r} t_i^3 - t_i}{12(N-1)}\right)\left(\frac{1}{n_A} + \frac{1}{n_B}\right)}$$

where N is the total number of observations across groups of flush type and province, r is the number of tied ranks, and t_i is the number of observations tied at the i^{th} value [37,38].

Results for the field grafting work were assessed but not analysed statistically.

Results

Parent Tree and Graft Development Averages

All grafted material remained at the NB location throughout the assessments, because of this, the NS and QC grafts are not directly comparable to their respective parent trees located in different

provinces. The NB samples were comparable since they were both located at the Kingsclear provincial tree nursery. Figures 7 to 9 show the average number of weeks required for both the grafts and parent trees of each genotype to complete both growth phases for the 2023 growing season. The results for the NS and QC trees regardless of location support the early and late flushing traits of the genotypes. The separation between the NB trees is less distinct and support the need of detailed historical observations like what was used to select the NS and QC samples.

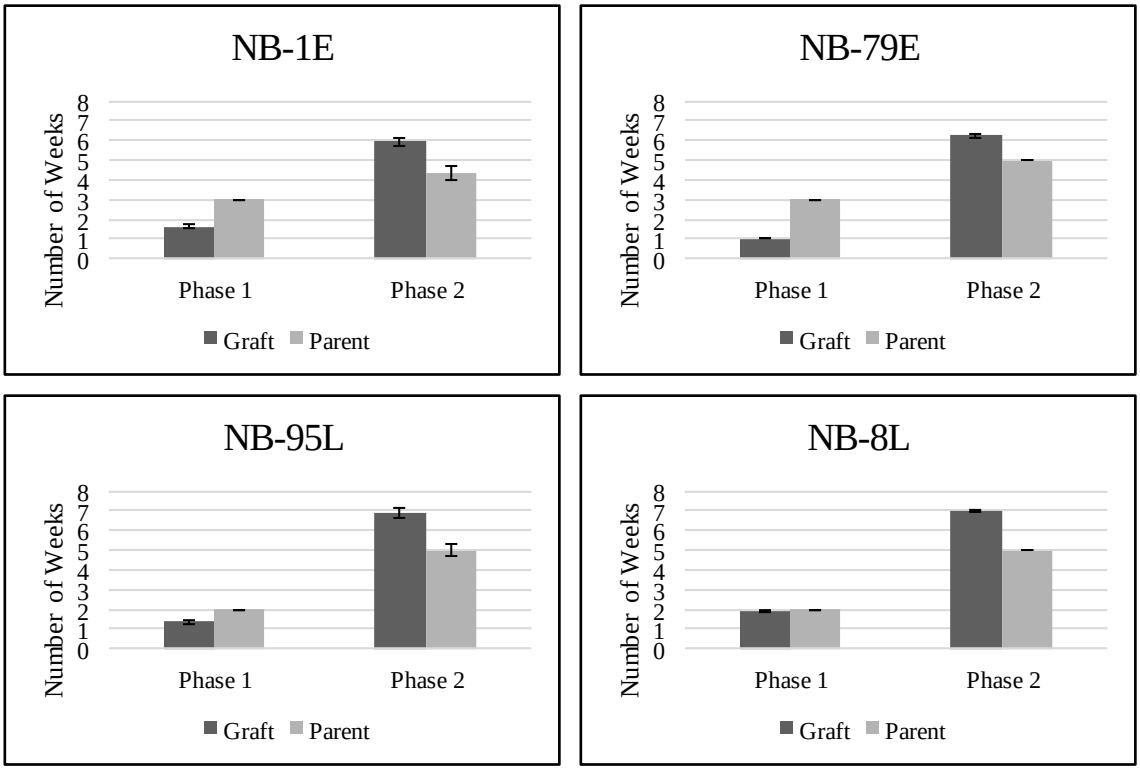

Figure 7. Comparison of average shoot development durations between New Brunswick grafts and parent trees for both growth phases. Error bars indicate standard error of the mean.

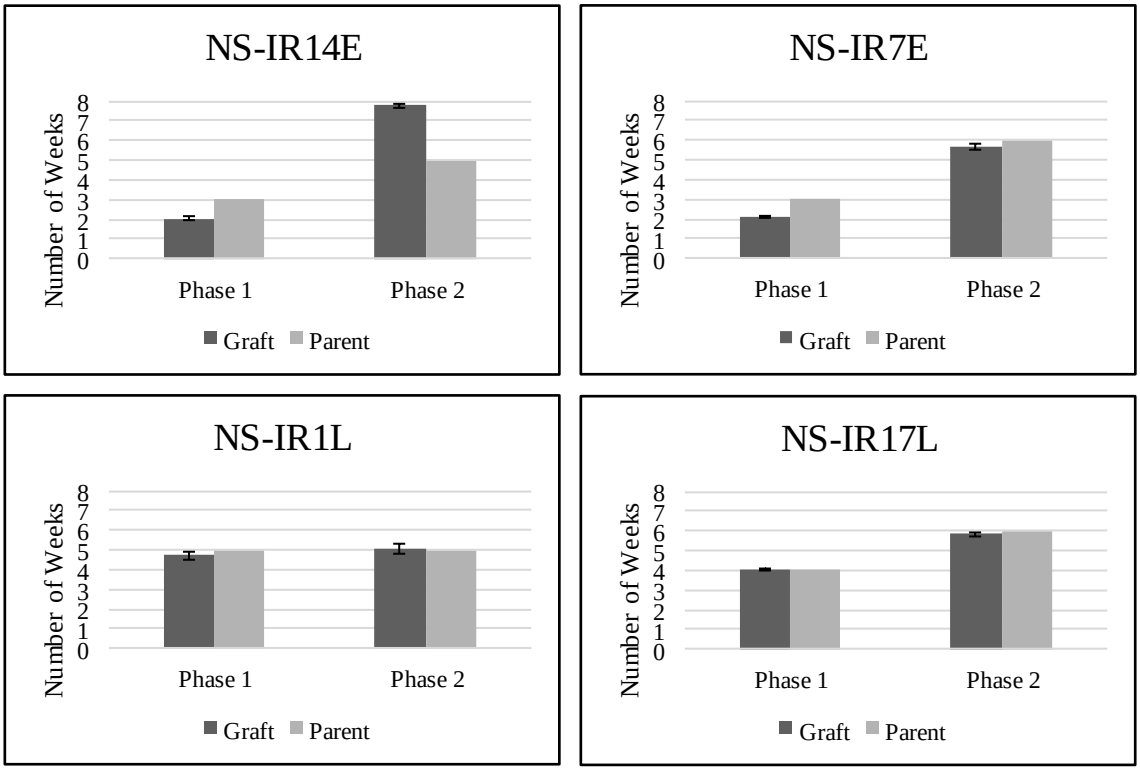

Figure 8. Comparison of average shoot development durations between Nova Scotia grafts and parent trees for both growth phases. Error bars indicate standard error of the mean. Standard error was only possible for the grafts because parents had single observations.

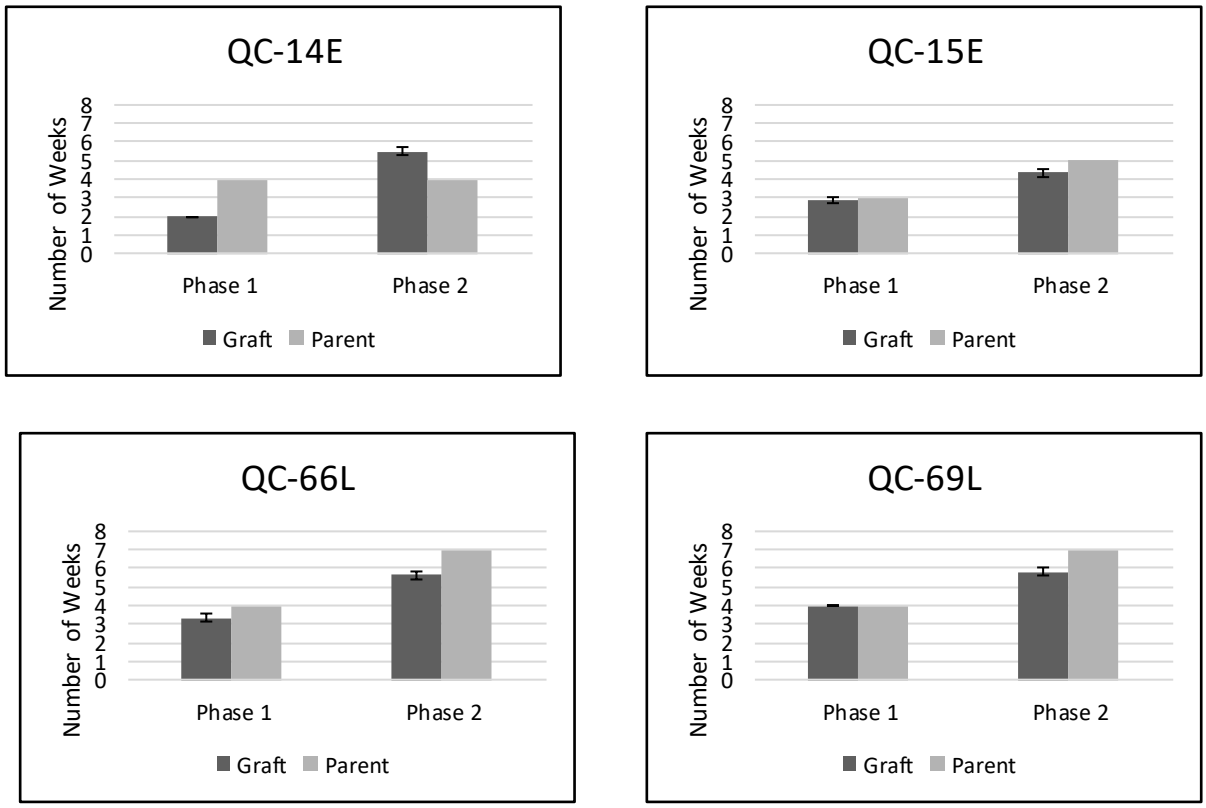

Figure 9. Comparison of average shoot development durations between Quebec grafts and parent trees for both growth phases. Error bars indicate standard error of the mean. Standard error was only possible for the grafts because parents had single observations.

Graft Bud Flush and Shoot Development

Greenhouse grafts

Observations for all samples began in the last week of April and the first week of May 2023 and bud and shoot development varied among provinces, clones, and flush types. The mean number of weeks required to complete the first growth depicts the separation between early and late flushing genotypes well in the first growth phase (Table 2). The NB samples had the least amount of separation between clones and required a maximum of two weeks to all complete growth phase 1. The NS samples had the largest separation where late flushing clones required approximately twice as long to complete growth phase 1 and a maximum of six weeks. The QC samples had a more distinct separation between early and late flushing genotypes as well where late flushing samples required up to twice the amount of time and a maximum of 4 weeks. For the second growth phase which featured all shoot elongation the separation between genotypes is much smaller. The NB grafts varied by one week and took a maximum of eight weeks to complete this phase. The NS grafts had relatively no separation besides one outlying early flushing genotype that on average required two weeks longer and a maximum of eight weeks. The QC grafts also have very little separation in the later part of their growth and required a maximum of six weeks.

Table 1. Comparison between early and late flushing genotypes of mean number of weeks required to complete each growth phase.

Clone ID	Growth Phase 1		Growth Phase 2	
	Mean	STDEV	Mean	STDEV
NB-1E	1.64	0.50	5.93	0.73
NB-79E	1.00	0.00	6.26	0.45
NB-8L	1.95	0.23	6.95	0.23
NB-95L	1.35	0.49	6.88	1.17
NS-IR14E	2.05	0.38	7.77	0.43
NS-IR7E	2.11	0.32	5.67	0.69
NS-IR1L	4.71	0.83	5.07	0.83
NS-IR17L	4.07	0.27	5.86	0.36
QC-14E	2.00	0.00	5.50	0.55
QC-15E	2.83	0.41	4.33	0.52
QC-66L	3.33	0.52	5.67	0.52
QC-69L	4.00	0.00	5.80	0.45

Shoot development duration for growth phase 1 were significantly different among provinces, and between early and late flush types (Table 3). The province variable was further analysed with a Dunn test, where the only nonsignificant value was the comparison between NS and QC, which suggests that grafts between these provinces developed at a similar rate (Table 4).

Table 2. Kruskal Wallis Rank Sum test comparing bud development time (weeks) among provinces and bud flush type (early and late) for growth phase 1.

Stage comparison	Chi Sq	df	P value
Bud development vs province	77.86	2	<0.001
Bud development vs Flush	28.79	1	<0.001

Table 4. Dunn test comparing bud development time (weeks) between provinces for growth phase 1.

Comparison	Z	P.unadj	P.adj
NB - NS	-8.00	1.24E-15	<0.001
NB - QC	-6.30	<0.001	<0.001
NS - QC	-0.62	0.53	0.53

The time required for grafts to complete growth phase 2 did not differ significantly between the two bud flush types (Table 5). There was another significant difference between the provincial groups and when analysing further with a Dunn test, the NB and NS comparison was the only one to produce a significant value (Tables 5 and 6).

Table 3. Kruskal Wallis Rank Sum test comparing bud developing time (weeks) among provinces and flush type (early and late) for growth phase 2.

Stage comparison	Chi Sq	df	P value
Bud development vs province	23.82	2	<0.001
Bud development vs Flush	0.09	1	0.76

Table 4. Dunn test comparing bud development time (weeks) between provinces for growth phase 2.

Comparison	Z	P.unadj	P.adj
NB - NS	1.78	0.074	0.074
NB - QC	4.88	<0.001	<0.001
NS - QC	3.61	<0.001	<0.001

A Kruskal Wallis test was used to compare the early and late flushing ramets in both growth phases within each province (Table 7). All comparisons within each province produced a significant value except for New Brunswick in growth phase 1.

Table 5. Kruskal Wallis Rank Sum test comparing early and late flushing grafts for both growth phases within each province.

Province	Growth Phase	Chi Sq	df	P value
New Brunswick	1	3.62	1	0.057
	2	14.93	1	<0.001
Nova Scotia	1	60.26	1	<0.001
	2	18.86	1	<0.001
Quebec	1	13.27	1	<0.001
	2	9.85	1	0.001

Field grafts

Graft survival after one full growing season post-grafting (grafted in 2022 and assessed in 2023) was 94% (Table 8). Of the total surviving grafts, a total of 14 additional grafts were classed as being weak. Development was assessed approximately weekly in 2023 using the same classification system described for the greenhouse grafts.

Table 8. Graft survival at the end of the 2023 growing season for the 2022 grafts. (#living/total number grafted).

New Germany	Late flushing clones				Early flushing clones			
Set A	6/6	6/7	4/4	6/7	4/4	3/4	3/4	3/4
Set B	5/5	4/4	3/3	6/7	5/7	3/3	3/3	4/4
Set C	6/6	7/7	7/7	6/7	3/3	4/4	3/3	3/3
Survival (%)	100	94.7	100	100	85.7	90.9	90.0	90.9
Stanburne (Rosebud)								
Set A	7/7	7/7	7/7	7/7	8/8	7/7	7/7	5/7
Set B	8/8	8/8	8/8	8/8	7/7	8/8	7/7	7/8
Set C	7/7	4/6	6/6	8/8	3/5	6/6	8/8	7/8
Survival (%)	100	90.5	100	100	90.0	100	100	82.6

Discussion

This study focused on the first two phases of shoot growth and bud development known to occur in balsam fir [34]. This method was able to capture the magnitude of difference between the early and late flushing genotypes, as well as their overall development. As observed in the field, some samples would occasionally proceed through multiple development stages in between assessments. A more frequent assessment schedule would have been beneficial and added to the understanding of the growth and development and perhaps significance of separation between early and late flushing samples, but this was not feasible during this study.

When assessing the samples, the stage of development was determined by a visual inspection. This method while practical for use in the field, had limitations depending on the size of the tree and the need for other means of gaining a closer view of the crowns such as binoculars or a camera.

Auxins are known to directly influence many biological processes within plants, including growth, development, and environmental responses [39,40]. Auxins can be found in different concentrations throughout and within different plant tissues which can influence timing and how different parts of a plant develop [41]. Because dissimilar auxin concentrations can cause a gradient of development in the crown of a tree, when assessing parent trees for development stage, only the top third portion of the tree where all scion material in the study originated from was assessed. A study on the effects of scion material harvested from different positions in the crowns on Fraser fir reported that scions from the uppermost crown position had a 90% success rate, compared to 50-70% for material harvested lower in the crown [42]. However, a study using Apache pine (*Pinus engelmannii*) and Apache pine crossed Arizona pine (*Pinus arizonica*) did not find any significant difference in success rates when grafting scion material from two different positions in the crown [24]. So, conifers of different species may differ in their response(s).

Yearly climatic variation can influence many aspects of balsam fir growth [43] including flush timing, growth duration, elongation, and bud set. Detailed bud and shoot development were only monitored for one growing season for the grafts and parent trees in this study. Bud and shoot development were expected to differ between the NS and QC parent trees and grafts growing in different provinces. The grafts, however, for each province remained at the same location throughout the entire assessment period so no variation in treatment was present.

Although bud flush and development observations were made weekly, the specific dates did not coincide exactly for the three sites. Due to the variation in people's schedules and other unforeseen issues such as weather events, the data was reported on a weekly basis. The weekly observation dates of the parent trees from NS and QC were fitted to the closest date that matched the grafted ramets assessed in NB. The parent trees in NB were assessed at the same time as the grafted ramets so no adjustment was needed. The largest variation was a period of five days which occurred twice for the QC parent trees.

Two specific development phases were used for the assessments. Growth phase 1 was used to capture the flush timing of each graft and consisted of the period from dormancy (stage 0) to the first obvious sign of bud flush where bud scales were swollen, slightly expanded, white and not yet broken (stage 2). Growth phase 2 was used to capture the overall growth of the new shoot. This was the period between stage 2 and the cessation of shoot growth and the beginning of terminal bud development (stage 8) where the terminal bud started to turn dark brown. Having these two development phases separate from each other allowed for the observation and analysis of the different early and late bud flush timings and then growth thereafter. The specific stages of 2 and 8 were chosen because they are easily identifiable in the field. Development after stage 8 consists of the third phase of growth which focuses on lignification, bud formation and development of next year's shoot primordia [34].

Initial growth of the grafts provided good information, but not enough time has elapsed to speculate on growth multiple years post-grafting. Planting these grafts in the three participating provinces will provide insight into the long-term performance of these genotypes in different geographic areas.

Early and Late Flushing Grafts

There is a lack of literature available regarding specifically the influence of grafting on bud flush timing within conifers. However, it is known that timing of bud flush in *Abies* is under strict genetic control, and it is possible to selectively manage for this trait [16-18]. The results produced from this study suggest that the grafted material retained their early or late flushing trait from their respective parent tree regardless of the rootstock.

Greenhouse grafts

Every comparison between the early and late flushing groups within each province produced a significant result except one: for the NB comparison for growth phase 1 (Table 3.7). These results indicate that the selections of trees for early and late flushing were correct, and they also support the value of relying on multiple years of historical observations, such as what was available for the NS and QC samples, but not NB.

The average development time for growth phases 1 and 2, was compared between parent trees and their respective grafts (Figures 3.4-3.6). The NB parent tree samples exhibited the least amount of separation between early and late flushing samples which was expected. However, the grafts did show a slightly clearer separation than the parent trees. All four of the NS samples clearly showed the separation between the early and late flushing samples for both the parents and grafts. Similar to NB, the QC grafts exhibited a clearer separation between the early and late flushing grafts than the parents. This comparison yielded two key results: (1) that multiple years of bud flush monitoring are critical in determining the flush timing of a tree; and (2) grafts of the parent trees behaved like mass clone populations and exhibiting their respective early and late flushing traits.

When comparing the mean duration times on a genotypic level, the results from growth phase 1 clearly show the degree of separation between the identified early and late flushing trees from the three provinces (Table 3.2) The maximum required durations between genotypes to complete each phase varied much more in the first growth phase which can be attributed to the significance of the bud flush timing traits in each. For the second growth phase it was relatively close between genotypes within each province, however an outlier result is an early flushing genotype from NS requiring up to eight weeks to complete shoot elongation. These results suggest that the initial flush timing in growth phase 1, does not have a significant impact on the time needed to complete growth phase 2. Since all samples received the same treatment throughout the entire experiment it is possible that the later season growth is more dependent on the tree's individual genetics [44] and responses to the environmental conditions in which they reside.

The time required for the grafted clones to reach the end of growth phase 1 differed significantly among province and flush types (Table 3.3). If the influence of the rootstock were to significantly affect the flush timing of the grafted material, then no difference amongst groups or random results would have been expected from the random genetic mixes of the rootstocks. This indicates that scion development was under strong genetic control and that scions continued to express the traits of their respective genotypes regardless of the rootstock.

When comparing the time required for clones to reach the end of growth phase 2, only the province variable produced a significant result, flush type did not (Table 3.5). This suggests that the materials from different provinces still grew independently of one another but that, regardless of the early or late bud flush timing, the grafts developed at a similar rate in the later portion of their growth.

The paired differences among provinces test for growth phase 1 resulted in statistically significant values when comparing solely with the NB group (Table 3.4). This supports a lack of separation between early and late flushing samples in the NB compared to the other two provinces. This trend continued when comparing growth phase 2, but only for the NB and NS test (Table 3.6). These results and that of table 3.5 where the early or late flush type did not have a significant influence during the second growth phase, further supporting that flush timing does not have a significant effect on the shoot elongation and development of the scion.

Field grafts

The intention is to follow graft development in 2024. At that time a detailed comparison of shoot development will be done to confirm that early and late flushing clones retain their development patterns when field-grafted regardless of the orientation of the branches onto which they were grafted.

Conclusions

With the predicted effects of climate change for the Atlantic Canadian region, Christmas tree improvement programs in this region will need to expand their focus from not only conventional aesthetic quality traits but to include additional traits to continue growing balsam fir in a warming climate, specifically, later bud flush to avoid late spring frost damage.

The objective of this study was to determine the influence of the rootstock on grafted balsam fir bud flush timing and development by grafting and monitoring a series of identified early and late flushing trees. The hypothesis for this experiment was that the rootstock material will not change the relative phenology or bud flush timing of the grafted scion. The results from this study show that it is possible to select late flushing trees and after identification, the integrity of the late flushing trait is retained after grafting. Enabling successful grafting, and out planting for Christmas tree improvement programs focused on developing climate change adapted stock for growers to use. The influence of the rootstock in this study cannot be ruled out for certain, however if an influence was present, it was relatively minor in terms of bud flush timing. When monitoring a specific tree to determine bud flush timing, historical data proved to be valuable. However, microsite influence could also be a factor, so by grafting multiple ramets and still observing similar bud flush timing as the parent tree, this can support selections.

The field grafting trial further demonstrated that time of bud flush appeared to be under strong genetic control. Scions from trees identified as early and late flushers retained their relative timing of flushing whether grafted onto early or late flushing parents. Reciprocal grafting back onto the original parent trees likewise developed in-line with the parent.

An additional outcome from this study was the establishment of three clonal seed orchards utilizing all early and late flushing grafts as well as a select amount of plus tree grafts dedicated for future research.

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