Pacific Northwest Tree Improvement Research Cooperative

annual report 2001-2002

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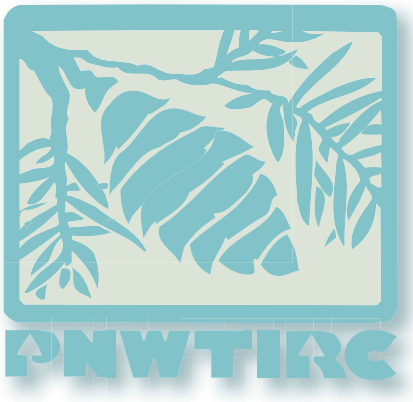

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About the PNWTIRC

The Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) was formed in 1983 to conduct research in support of operational tree improvement in the Pacific Northwest. Emphasis is on region-wide topics dealing with major coniferous species. Membership has included representatives from public agencies and private forestry companies in western Oregon, western Washington, and coastal British Columbia.

Our Mission is to:

- Create a knowledge base concerning genetic improvement and breeding of Pacific Northwest tree species
- Develop reliable, simple, and cost-effective genetic improvement methods, and apply these methods to solve tree-breeding problems
- Promote effective collaboration and communication among public agencies and private industries engaged in tree improvement in the region

All participants provide guidance and receive early access to research results. Regular and Associate members provide financial and in-kind support and are represented on the Policy/Technical Committee. This committee is responsible for making decisions on program strategy and support, identifying research problems, establishing priorities and assisting in the planning, implementation, and evaluation of studies. Because Contractual Participants provide less financial support, they have no voting rights on the Policy/Technical Committee. Liaison Members provide no financial support and have no voting rights. The PNWTIRC is housed in the Department of Forest Science at Oregon State University.

Director: Glenn Howe

Associate Director: Thimmappa Anekonda

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Highlights of 2001-2002

- Glenn Howe began as Director of the PNWTIRC and Assistant Professor of forest genetics in the Department of Forest Science at OSU in August of 2001.
- Two new members joined the PNWTIRC

  Port Blakely Tree Farms and Pope Resources (which includes their subsidiary, Olympic Resource Management).
- Six journal articles and abstracts were completed (i.e., published or in press) and the PNWTIRC staff gave eight presentations.
- The PNWTIRC *Program Description* and *Memorandum of Agreement* were updated.
- We developed 7 new SSR genetic markers and conducted detailed analyses of our full set of 22 SSRs in the *Pollen Contamination Study*. We also demonstrated the value of these markers for genotype identification. Using only three of our SSR markers, we were able to test the identity of two parent trees (i.e., field selections) that were about to be included in an operational seed orchard. Although one parent was correctly identified in the field (and included in the seed orchard), the other tree was not the desired parent, and was discarded.
- We identified 7 'high-priority' research topics to be addressed in the new PNWTIRC 5-year plan. These topics are (1) field validation of early testing for adaptability, (2) genetics of growth and yield modeling, (3) gene conservation, (4) tools for accelerating genetic gains, (5) genomics, (6) wood quality, and (7) genotype x silviculture and site interactions.
- We completed the first grafting for the *Miniaturized Seed Orchard Study* in February and March of 2002. Forward and backward selections were grafted into three orchard types (macro, mini and micro), two supplemental blocks (mini and micro), and one holding block.
- In the spring of 2002, we measured the results of the early flowering treatments that were applied in the spring of 2001 (*Early Flowering Study*). Gibberellic acid and girdling treatments were applied to 2- and 4-year-old grafts in the Vaughn and NWCTGA seed orchards. The combined GA/girdling treatment significantly increased female flowering on both the 2- and 4-year-old grafts, but only increased male flowering on the older trees. The same treatments were applied in the spring of 2002, and new treatments were applied to test different levels of GA.
- We held a workshop entitled *Genetic Improvement of Wood Quality in Coastal Douglas-fir and Western Hemlock*. This workshop was held on 27 June, 2002 in collaboration with the Northwest Tree Improvement Cooperative (NWTIC).

Message from the Director

My first year as Director of the PNWTIRC has been both rewarding and challenging. First, it's been great to interact with cooperative members and scientific colleagues in the USFS Pacific Northwest Research Station, Northwest Tree Improvement Cooperative (NWTIC), and the Department of Forest Science at OSU. It's also been rewarding to make progress on our current PNWTIRC research projects. We made good progress on the *Pollen Contamination Study*, obtained our first results from the *Early Flowering Study*, and grafted our first set of trees for the *Miniaturized Seed Orchard Study*.

Since becoming Director, a major challenge has been the loss two of members to industry mergers. Fortunately, we also picked up two new members this year I'm pleased to welcome Port Blakely Tree Farms and Pope Resources (including their subsidiary, Olympic Resource Management) to our cooperative. To accept new members, it was necessary to update our *Program Description* and *Memorandum of Agreement*—the last time we did this was in 1994! This critical step was completed in the spring of 2002. Unfortunately, the need to attract new members continues. Our discretionary operating budget will decrease by 10% in 2004 because our overhead costs (i.e., overhead paid to OSU) will increase from 0% to 10%. The good news is that our negotiated overhead rate is still well below the normal university rate of about 40%. By attracting a single new member, we can offset most of these extra costs. Therefore, I have set this as one of my goals for the next year.

Another goal is to improve technology transfer. Last year, Keith Jayawickrama and I organized a workshop entitled *Genetic Improvement of Wood Quality in Coastal Douglas-fir and Western Hemlock*. This workshop, which was co-sponsored by the PNWTIRC and the Northwest Tree Improvement Cooperative, was held 27 June, 2002 in Corvallis. We received many positive comments about the workshop. Therefore, I plan to organize similar workshops in the future. This workshop was a great way to highlight past PNWTIRC wood quality research, to hear from other scientists in the region, and to hear the viewpoints of the members. In short, I learned a lot!

Progress continued on our 5-year research plan. During the past year, we identified 14 'priority' research topics. At the annual meeting in June 2002, we reduced this list to seven 'high-priority' research topics. These topics are (1) field validation of early testing for adaptability, (2) genetics of growth and yield modeling, (3) gene conservation, (4) tools for accelerating genetic gains, (5) genomics, (6) wood quality, and (7) genotype x silviculture and site interactions. In the fall of 2003, we'll have sufficient resources to begin a major new

effort in at least one of these areas. Therefore, making final decisions on our new 5-year plan will be one of the top priorities for 2002-2003.

This has been a busy and exciting year for me. The learning curve is getting less steep, we have 'new blood' in the cooperative, and we'll soon be heading into new research territory. With your help and support, I look forward to another productive year and the PNWTIRC's 20th anniversary in the summer of 2003!

Introduction

Research Overview

Douglas-fir seed orchards cover nearly 2,500 acres in the Pacific Northwest. The cost of establishing and managing these orchards is substantial and will increase as new second-generation orchards are added. Therefore, it is a good time to rethink our approach to seed orchard design and management in the region. The PNWTIRC has three studies focused on seed orchards. The *Pollen Contamination Study* is aimed at increasing genetic gains by reducing pollen contamination, the *Early Flowering Study* is aimed at speeding genetic gains by promoting seed production on very young orchard grafts, and the *Miniaturized Seed Orchard Study* is designed to test promising alternatives to conventional Douglas-fir seed orchards.

The goal of the ***Pollen Contamination Study*** (page 11) is to help increase genetic gains by providing better methods for measuring and managing pollen contamination in seed orchards. Pollen from unimproved trees (or poorly adapted genotypes) surrounding seed orchards can significantly reduce genetic gains by fertilizing orchard seed. Pollen contamination in conventional orchards often exceeds 40% and can adversely affect both realized genetic gains and adaptability (Wheeler and Jech 1986; Adams and Burczyk 2000). We can help seed orchard managers increase genetic gains by giving them better tools to measure and manage pollen contamination. We are developing genetic markers called SSRs and associated analytical tools that will allow us to measure pollen contamination more easily and accurately. These tools will have two main uses. First, they can be used to estimate pollen contamination for specific orchards and seed orchard blocks. Most orchard managers have no idea how big the problem is for their orchard. Estimates of pollen contamination can then be used to estimate losses in genetic gains. Second, good tools for measuring pollen contamination are needed for measuring the effectiveness of techniques designed to reduce pollen contamination—techniques such as selective seed harvesting, bloom delay, supplemental mass pollination (SMP)[1], or controlled mass pollination (CMP). Our SSR markers will also be valuable for identifying genotypes in tree improvement programs (i.e., clonal ID), gene conservation, population genetic studies, and gene mapping.

Miniaturized seed orchards (MSOs) are promising alternatives to conventional seed orchards. In MSOs, the trees are planted at close spacings in clonal rows, then maintained at a height of only 2 to 4 m (Sweet and Krugman 1977, Weber and Stoehr 1998). The ***Miniaturized Seed Orchard Study*** (page 21) is designed to test three alternative MSO designs for Douglas-fir. There are two main benefits of MSOs. First, it should be easier to control pollen contamination and produce elite crosses to increase genetic gains. This is because the trees are

[1]Supplemental mass pollination is the broadcast application of pollen to non-isolated (i.e., non-bagged) female strobili.

short, easily accessible, and planted in clonal rows, making CMP and SMP much easier. Second, the costs of seed orchard establishment and management should be lower because fewer acres are needed, and the crowns are closer to the ground, thereby facilitating management techniques such as seed collection, pest management, bloom delay, etc.

The ***Early Flowering Study*** (page 17) is aimed at speeding the capture of genetic gains from seed orchards. Many first-generation orchards took 10 to 15 years to produce useful amounts of seed (Cress and Daniels 1990). The long time lag between seed orchard establishment and seed production represents a huge opportunity cost. Although flower stimulation techniques exist for Douglas-fir, we know little about whether these techniques work well on very young grafts (2+ years-old). Will they harm the newly grafted trees? Can they be applied every year? Do they have carry-over effects (i.e., can they also enhance seed production in later years)? These are some of the questions being addressed by the *Early Flowering Study*.

In addition to our seed orchard research, the PNWTIRC members are keenly interested in tackling other important research topics. New areas of research are discussed under *New Research Directions* (below).

New Research Directions

During the past year, we continued to develop our new 5-year research plan. At the annual meeting in June 2002, Glenn Howe presented the results of a recent vote on 14 'priority' research topics that were developed by the Five-year Plan Subcommittee. This subcommittee consisted of Christine Dean and Greg Johnson (currently representing Weyerhaeuser), Jeff DeBell (WA Dept. of Natural Resources), Jim Smith (Plum Creek), Keith Jayawickrama (NWTIC), Randall Greggs (Simpson), and Glenn Howe and Thimmappa Anekonda (PNWTIRC staff). This list of 14 'priority' topics was reduced to a shorter list of 7 'high-priority' topics at the annual meeting. Glenn Howe and the Five-year Plan Subcommittee will work with interested individuals to develop short preproposals for these 7 topics that will be evaluated by the full Policy/Technical Committee during the next year (Table 1). By the fall of 2003, we should have the resources needed to begin one or more new studies. Therefore, completion of the 5-year research plan will be a high priority for 2002-2003.

Table 1. Seven 'high-priority' research topics were identified by the PNWTIRC members at the 2001-2002 annual meeting.

Topic	Current ranking
Growth and yield modeling – genetic impacts	1
Wood quality	2
Genotype x silviculture/site interactions	3
Tools for accelerating genetic gains	3
Field validation of early testing for adaptability	4
Gene conservation	5
Genomics	5

Technology Transfer

The transfer of research from the PNWTIRC to tree improvement practitioners is an important function of the cooperative. Last year, we renewed our efforts to translate our past research results into practice. These technology transfer efforts include annual reports, annual meetings,

one-on-one meetings with cooperators, and PNWTIRC publications (including executive summaries of their relevance to applied tree improvement) (see *Publications and Abstracts by PNWTIRC Personnel: 2001-2002* on page 27).

We also began a new approach last year—workshops on important tree improvement topics. We held the first of these workshops on 27 June, 2002 (Table 2). This was a joint PNWTIRC/NWTIC workshop entitled *Genetic Improvement of Wood Quality in Coastal Douglas-fir and Western Hemlock*. We attracted speakers from the Pacific Northwest, France, and even New Zealand, and had more than 50 attendees. Because of the success of this workshop, we plan to continue these efforts. Additional workshop topics might include *Genomics of Douglas-fir*, *Strategies for improving cold and drought hardiness*, *How to integrate early testing into tree improvement programs?* or *Seed orchard options*.

New Members

Last year, we made a major push to attract new members—and were successful! Port Blakely Tree Farms and Pope Resources became PNWTIRC members on 1 July, 2002. Tim Truax and Mike Mosman will be representing Port Blakely

Table 2. Agenda of a joint PNWTIRC/NWTIC workshop entitled *Genetic Improvement of Wood Quality in Coastal Douglas-fir and Western Hemlock* held 27 June, 2002 in Corvallis, OR.

Speaker	Affiliation	Title of presentation
Howe	Oregon State Univ.	*Opening remarks*
Megraw	Weyerhaeuser (ret.)	*An overview of wood quality*
Briggs	Univ. of Wash.	*Wood quality and silviculture*
Cannon/Miller	Boise Corporation	*Improving wood quality: is it important to the industry?*
Johnson/Gartner	USFS PNWRS/ Oregon State Univ.	*An overview of wood specific gravity in coastal Douglas fir*
Johnson/Jayawickrama	USFS PNWRS/ Oregon State Univ.	*Genetics of wood specific gravity in coastal Douglas-fir*
Rozenberg	INRA, Orleans, France	*Wood quality research at INRA: implications for Douglas-fir tree improvement*
Jayawickrama	Oregon State Univ.	*Genetic improvement of conifer lumber stiffness and strength*
Howe	Oregon State Univ.	*Genetics of stem quality in coastal Douglas-fir*
Cartwright	BC Ministry of Forests	*Genetics of wood properties in western hemlock*
Knowles/Shelbourne	New Zealand Forest Res. Inst.	*Improving wood and stand quality of New Zealand's Douglas-fir plantations*
Jayawickrama	Oregon State Univ.	*Tree improvement recommendations and research needs*

on the PNWTIRC Policy/Technical Committee, whereas Bryan Schulze will be representing Pope Resources (Olympic Resource Management, which is Bryan's employer, is a subsidiary of Pope Resources).

It is particularly satisfying to have Port Blakely join because of their historical connection to forest genetics research in the U.S. One of their early owners (James G. Eddy) started the Institute of Forest Genetics in Placerville California in 1925, and later donated the Institute to the US Forest Service. It's pleasing to note that Port Blakely continues to support forest genetics research more than 75 years later!

Although the pool of organizations to draw from is declining, we will continue to solicit new members in the coming year. In these tough economic times, one of our main goals is to simply maintain our discretionary budget at its current level, and we will need to attract new members to do this. In July of 2004, OSU will begin charging the PNWTIRC a 10% overhead fee. Although we have been exempt from any overhead charges to date (this has been considered part of OSU's contribution), this will not be the case in the future. The other cooperatives at OSU already pay overhead, or will also be paying a reduced rate in 2004 (the normal university overhead rate is about 40%). In short, if we want to maintain the current dues structure and discretionary budget, we will need to keep our current members and attract one or two more. Please do what you can to help meet this goal.

Current PNWTIRC Research

Current PNWTIRC research focuses on Douglas-fir seed orchards. Although Douglas-fir seed orchards cover nearly 2,500 acres in the Pacific Northwest, conventional orchards have three limitations that either reduce genetic gains or increase management costs. First, pollen contamination from native trees and adjacent seed orchard blocks can reduce genetic gains. Pollen contamination in conventional orchards often exceeds 40% and can adversely affect both realized genetic gains and adaptability. Much of our effort during the past year focused on our *Pollen Contamination Study*. Second, genetic gains are delayed (and financial returns sacrificed) because of the long time lag between seed orchard establishment and the production of genetically improved seed. Many of the first-generation orchards, for example, took 10 to 15 years to produce useful amounts of seed (Cress and Daniels 1990). Our *Early Flowering Study* is designed to provide information that will help speed the capture of genetic gains from seed orchards. Finally, it may be possible to increase genetic gains and reduce management costs by establishing 'miniaturized seed orchards' (MSOs). In contrast to conventional orchards, which consist of large trees planted at wide spacings, MSOs consist of trees planted at close spacings in clonal rows, and maintained at a height of only 2 to 4 m (Sweet and Krugman 1977, Webber and Stoehr 1998). We will test this concept for Douglas-fir in our *Miniaturized Seed Orchard Study*.

In the following section, we will discuss our current PNWTIRC research projects:

- *Pollen Contamination Study*
- *Early Flowering Study*
- *Miniaturized Seed Orchard Study*

Pollen Contamination Study

Introduction

Pollen contamination[1] can reduce genetic gains if seed orchard blocks are located near native stands of trees or seed orchard blocks with trees from other breeding zones. For example, the proportion of seeds fertilized by non-orchard pollen often exceeds 40% in conventional Douglas-fir orchards (Adams and Burczyk 2000). This could reduce genetic gains by 20% or more. Therefore, improved methods for measuring and managing pollen contamination are needed.

[1]Pollen contamination is measured as the proportion of seeds fertilized by pollen coming from outside of the seed orchard block.

What are SSRs?

SSRs (Simple Sequence Repeats) are stretches of DNA composed of many short repeats (e.g., repeats of 2-3 nucleotides, such as 'AC' or 'ATC') that are aligned end-to-end (in tandem). Because the number of repeats often varies among chromosomes and individuals, SSRs are good genetic markers. For example, an SSR locus with 12 repeats of 'AC' (i.e., $(AC)_{12}$ = ACACACACACACACACACAC), might mutate to $(AC)_{13}$, or 13 tandem repeats of 'AC.' SSRs can be scored by isolating DNA, amplifying the SSR region with DNA primers and the polymerase chain reaction (PCR), then measuring the length of the resulting DNA band after it is pulled through a gel with an electric current (electrophoresis). Because the $(AC)_{13}$ DNA fragment is slightly longer than the $(AC)_{12}$ fragment, it will migrate a little more slowly through the gel. Therefore, each different SSR allele appears as a band at a different location on the gel. A good SSR marker is genetically variable (e.g., has 8-15 alleles in the test population), has a low frequency of null alleles, and amplifies a single locus in each PCR reaction.

Although pollen contamination is usually measured using genetic markers called isozymes, these markers have some important drawbacks—they are only moderately variable and it is necessary to measure many isozyme markers (loci) and many offspring to get reasonable estimates of pollen contamination. The goal of the *Pollen Contamination Study* is to develop improved, DNA-based genetic markers for estimating pollen contamination and other mating parameters in Douglas-fir seed orchards.

Simple sequence repeats (SSRs), or microsatellites, are tandem repeats of short (1-6 bp) DNA sequences (see *What are SSRs?*). SSRs have several distinct advantages compared to other codominant markers, such as isozymes. First, they are highly variable, often having more than 10 alleles per locus (Goldstein and Pollock 1997). Therefore, they should be better than isozymes for identifying the parents of seed orchard seed. Second, they are usually codominant, that is, they allow heterozygous phenotypes to be observed directly. This makes them more useful than dominant markers, such as RAPDs (Randomly Amplified Polymorphic DNAs). Finally, SSRs are abundant and widely distributed across the genome (Powell et al 1996). SSR alleles are detected by amplifying small amounts of DNA template using the polymerase chain reaction (PCR), followed by high-resolution electrophoresis of the resulting products. The potential for high throughput marker detection, combined with their high allelic variability, makes SSRs a great tool for identifying genotypes. SSRs have already been used to infer paternity through genotypic exclusion in tree populations (Dow and Ashley 1998; Streiff et al 1999; Lian et al 2001). In each of these examples, just a few loci (4-6) were needed. SSRs have been used for 'fingerprinting,' genome mapping, studies of population genetic structure, parentage analysis, and phylogenetic analysis.

Unfortunately, the development of SSR markers is still inefficient, time-consuming, and resource-intensive, particularly in organisms with large and complex genomes, such as conifers. Many attempts to develop SSR markers for conifers have yielded just a handful of useful marker loci (Pfeiffer et al 1997; Hicks et al 1998; Soranzo et al 1998). Potential disadvantages of SSRs also include their high rates of genotyping error and their high cost per genotype (Jarne and Lagoda 1996). We developed SSR markers and will use them to measure pollen contamination in a conventional Douglas-fir seed orchard.

Accomplishments for 2001-2002

We are nearing the completion of Phase 1 of the *Pollen Contamination Study*. Last year, we fully characterized 22 SSR markers (Table 3). Based on their ease of scoring and repeatability, at least 15 of these markers are suitable for measuring pollen contamination—well above our original goal of 7-10 markers! We are also making progress on collecting data and developing analytical procedures for measuring pollen contamination in the *Test Block*.

Our SSRs are the most variable informative markers reported in conifers

Last year, we reported on 15 promising SSRs plus another 62 markers that had not been fully tested (PNWTIRC Annual Report, 2000-2001). After testing these 62 markers, we had a total of 34 promising markers to consider (i.e., 15 from last year + 19 new SSRs). Based on segregation analyses, 12 of these 34 markers will not be helpful for measuring pollen contamination. They may be useful for other less-demanding applications, however.

The remaining 22 SSRs show single-locus, Mendelian inheritance. Fifteen of these markers are particularly valuable—they are easily and consistently scored as single-locus, codominant markers with a low frequency of null alleles. We are now concentrating our efforts on these 15 markers (this is more than enough for measuring pollen contamination). Another 7 SSRs are still promising, but need to be optimized to consistently obtain high-quality data for all genotypes.

The *Pollen Contamination Study* has two phases.

The objectives of Phase 1 are to:

- Develop 7-10 SSR marker loci for Douglas-fir
- Confirm the inheritance of the markers
- Measure their genetic variability
- Use the most variable markers to measure pollen contamination in a conventional seed orchard
- Optimize testing and estimation procedures

The objectives of Phase 2 are to: Use the SSR markers to determine how pollen contamination varies with:

- Flowering phenology
- Location of the ramets within the seed orchard

We will measure pollen contamination in one orchard block of the Plum Creek seed orchard in western Oregon, hereafter referred to as the *Test Block*.

SSRs will be useful for both *measuring* and *managing* pollen contamination.

Once our SSRs are available, they should be useful for:[1]

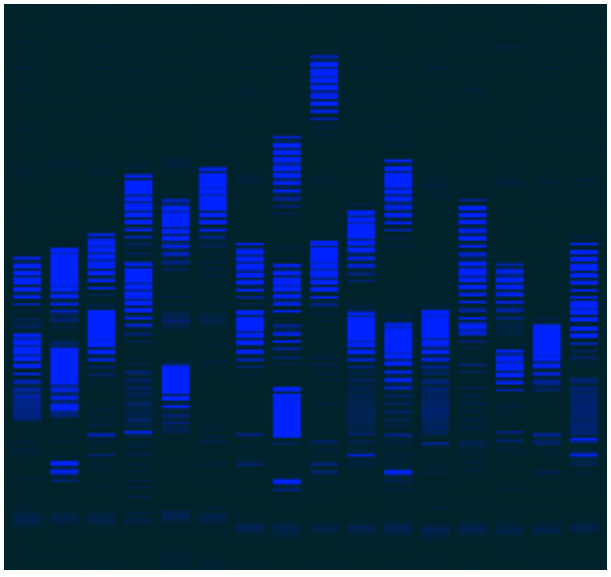
- Comparing alternative methods of supplemental mass pollination.
- Judging the effectiveness of 'bloom delay' for reducing pollen contamination.
- Measuring pollen contamination for individual ramets or clones (i.e., early vs late flowering clones; ramets on the edge vs the interior of the seed orchard).

[1] See the 2000-2001 PNWTIRC Annual Report for more detail.

Table 3. Variability of Douglas-fir SSR markers.

Locus[a]	N[b]	A[c]	H_o[d]	H_e[e]
OSU_1C3	28	28	0.929	0.968
OSU_1F9	35	33	0.943	0.973
OSU_2B6	32	28	0.813	0.957
OSU_2C2	38	12	0.711	0.752
OSU_2C3	35	25	0.943	0.955
OSU_2D4	34	30	0.912	0.968
OSU_2D6	34	30	0.912	0.975
OSU_2D9	16	8	ND[f]	ND[f]
OSU_2G4	27	19	0.778	0.937
OSU_2G12	34	16	0.824	0.914
OSU_3B2	32	27	0.938	0.962
OSU_3B9	30	25	0.900	0.930
OSU_3D5	35	19	0.943	0.931
OSU_3E3	29	31	0.897	0.969
OSU_3F1	27	20	0.741	0.936
OSU_3G9	35	22	0.857	0.926
OSU_3H4	32	25	0.875	0.957
OSU_4A7	34	30	0.912	0.960
OSU_4E9	34	24	0.853	0.923
OSU_4G2	30	16	0.900	0.920
OSU_5A8	37	7	0.595	0.805
OSU_783	33	15	0.939	0.879
Mean =	32	23	0.863	0.928

[a] To get the complete locus name, the name in the table should be preceded by 'OSUPCT_ssrPm'.
[b] N is the number of trees genotyped.
[c] A is the number of alleles detected in a sample of N trees.
[d] H_o is the observed heterozygosity.
[e] H_e is the expected heterozygosity.
[f] ND indicates that these values were not determined because only 16 trees were genotyped.



The variability of our SSRs exceeded our expectations (e.g. Figure 1). We observed an average of 23 alleles per locus in a sample of 38 trees surrounding the seed orchard (see column 'A' in Table 3). On average, each of these trees was heterozygous (i.e., had two different alleles) at 86% of the SSR loci we measured (i.e., mean H_o = 0.863, Table 3). Our markers are more variable and, therefore, have more information content than SSRs developed for other conifers (Table 4).

Identifying genotypes using SSRs—practical examples

SSRs are also great tools for identifying genotypes via genetic fingerprinting. This was demonstrated by a project we worked on with Margaret Banks (Stimson Lumber Co.) and Keith Jayawickrama (Northwest Tree Improvement Cooperative). We used three of our SSR markers to fingerprint two Douglas-fir trees that were about to be grafted into a seed orchard. Although these trees were thought to be the correct field selections that had performed so well in progeny tests, the original markings on the trees were gone, so we could not be sure. Therefore, we fingerprinted field selections plus 8 to 10 trees known to be the offspring of the desired plus trees (i.e., offspring growing in progeny tests) (Table 5). If the trees identified in the field are the correct parents, then each offspring should have at least one allele that matches an allele in the chosen parent. By measuring only 3 SSR loci, we were able to conclude that one of the parents was correct, but the other was incorrect. The correct parent was included in the seed orchard, but the incorrect one was not, thereby increasing genetic gains.

We also used our SSRs to double-check the ramets in the *Test Block* of the Plum Creek seed orchard. We genotyped all 152 ramets of the 58 clones in the *Test Block*, and found only one mistake—the SSR genotype for one ramet did not match other ramets that were labeled as the same clone. Apparently, this ramet was mislabeled when the *Test Block* was established. Actually, we were encouraged that other labeling mistakes were not found because labeling errors are often quite high in tree improvement programs (Adams et al 1988).

Figure 1. Variation of SSR marker OSU_2C3 among 16 Douglas-fir trees. Each lane represents the SSR genotype of a single, unrelated Douglas-fir tree. Thirteen of these 16 trees (81%) are heterozygous for this locus.

Table 4. Variability of dinucleotide SSRs in conifers.

Species	No. of SSRs	N[a]	A[b]	H_o (H_e)[c]	Reference
Pseudotsuga menziesii	21[e]	78	32	0.864	Pooled data (PNWTIRC)
	21[e]	46	26	0.864	Trees within the orchard (PNWTIRC)
	21[e]	32	23	0.863	Trees outside the orchard (PNWTIRC)
	50	24	8	(0.673)	(Amarasinghe and Carlson 2002)
Pinus sylvestris	7	13[d]	6.7	(0.850)	(Soranzo et al 1998)
Picea abies	7	18	13	(0.789)	(Pfeiffer et al 1997)
Pinus halepensis / P. brutia	7	50/47	2.9	0.586	(Keys et al 2000)
Picea glauca	15	14	10.2	0.520	(Hodgetts et al 2001)
Pinus strobus	16	16	5.4	0.515	(Echt et al 1996)

[a] N is the mean number of individuals genotyped per SSR locus.
[b] A is the mean number of alleles per locus.
[c] Numbers not in parentheses are observed heterozygosities (H_o). Numbers in parentheses are expected heterozygosities (H_e).
[d] Megagametophytes were sampled in this study.
[e] Results do not include data for OSU_2D9 because only 16 trees were measured for this locus (Table 3).

These examples demonstrate that we can make solid conclusions about genotype identification using only 3 of our SSR markers. At least five times as many isozyme loci would have been needed to make decisions with the same level of confidence.

Identifying genotypes using SSRs—theoretical considerations

The 'probability of exclusion' (PE) is the proportion of potential fathers[1] in a population that can be excluded from being the real father of an individual based on information from a given set of marker loci. Values near 1.0 indicate that the markers can be used to confidently eliminate a very large number of potential fathers from being the real father of the individual in question. We calculated the PE of our markers using the genotypes of the 58 clones in the *Test Block* and the single mislabeled ramet (discussed above). The PE is 0.991 using only 3 SSR markers (i.e., when nothing is known about the genotype of the mother). When the maternal genotype is known, the PE increases to 0.998. Figure 2 shows how PE increases as more markers are added.

How do our SSR markers compare to isozyme markers? Remarkably, 23 'idealized' isozymes (each with an expected heterozygosity of 0.5) would be needed to achieve a PE as large as we obtained with just 3 SSR loci (i.e., 0.991). In addition, because the heterozygosities of conifer isozyme loci are typically much lower than 0.5, 23 isozyme markers is a conservative estimate (Adams 1992).

[1]Potential fathers are trees that produced pollen and could have mated with the maternal parent to produce the offspring in question.

Table 5. Parent tree identification using SSR genetic markers. The SSR genotypes of two putative open-pollinated (OP) field selections were compared to the genotypes of known progeny growing in genetic test plantations (n = 8-10). If the putative parents in the field are correct, then all of their progeny must have at least one of the two parental alleles. Cases in which a progeny allele matches one of the alleles in the putative parent are shown in white. Allele numbers (e.g., 209 or 216) represent relative lengths of alternative SSR alleles.

Putative OP parent (i.e., tree in the field)			Progeny number (in progeny test plantation)											
ID	SSR genotype (marker name)	Progeny allele	1	2	3	4	5	6	7	8	9	10	Inferred genotype of real OP parent	Conclusion: putative parent is:
1	209, 216 (OSU_3F1)	Shorter =	205	209	202	216	209	216	216	209	209	216	209, 216	Correct
		Longer =	216	209	209	216	219	227	223	216	211	218		
1	184, 191 (OSU_3B9)	Shorter =	172	188	184	184	176	172	191	191	186	184	184, 191	Correct
		Longer =	191	191	184	184	184	191	191	191	191	191		
1	254, 256 (OSU_4A7)	Shorter =	254	254	228	256	226	226	254	254	226	254	254, 256	Correct
		Longer =	254	284	256	284	256	254	254	254	254	254		
2	204, 222 (OSU_3F1)	Shorter =	210	210	192	210	210	214	190	214			210, 214	Incorrect
		Longer =	224	214	210	214	226	214	210	220				
2	130, 190 (OSU_3B9)	Shorter =	130	188	130	130	188	130	130	130			130, 188	Incorrect
		Longer =	208	194	210	196	200	130	188	190				
2	226, 226 (OSU_4A7)	Shorter =	226	226	226	226	226	268	228	282			226, 284	Incorrect
		Longer =	226	228	244	248	284	284	284	284				

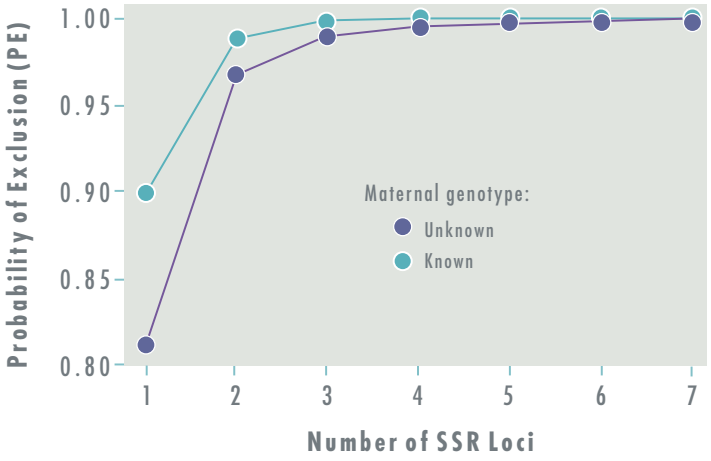


Figure 2. PE increases as more SSR markers are added. The probability of exclusion (PE) is the proportion of potential fathers in a population that can be excluded from being the real father of an individual based on information from a given set of marker loci. PEs were calculated using data from our single-locus SSR markers.

The high resolution achieved by just 3 SSR loci confirms that SSRs are powerful tools for measuring pollen contamination and identifying genotypes in Douglas-fir.

Plans for 2002-2003

Our plans for this year are to complete the *Pollen Contamination Study*. We have already genotyped all clones in the *Test Block* and a sample of 200 seed from a bulked seed crop for 3 SSR loci. DNA has been extracted from 200 seed from a second seed crop. We are currently making progress on developing analytical procedures to precisely measure pollen contamination. Based on these procedures, we will estimate pollen contamination in the *Test Block* and will compare pollen contamination levels across clones with different flowering phenologies and among ramets with different locations in the *Test Block*.

Early Flowering Study

Introduction

Early flower stimulation is important for speeding the capture of genetic gains from seed orchards. The long time lag between seed orchard establishment and seed production (often 7-10 years) represents a substantial opportunity cost in tree improvement programs. Therefore, improved methods for stimulating flower production on very young grafts is desirable. Early flowering is also valuable for shortening the generation time in breeding programs.

Flowering of Douglas-fir trees can be enhanced by girdling, application of gibberellic acid (GA), and fertilization. Nonetheless, most of these treatments have been tested and optimized for older trees—i.e., trees as young as 4 years from grafting (Ross et al 1980), but usually older (Pharis et al 1987). It would also be valuable to have proven techniques for stimulating flowering of very young grafts (2+ years from grafting). This is the goal of the *Early Flowering Study*. Methods of flower stimulation that are developed in this study will be applied in the *Miniaturized Seed Orchard Study* (see below).

The objectives of the *Early Flowering Study* are to:

- Develop improved methods for promoting early and sustained flowering on young Douglas-fir grafts
- Determine the optimum age to begin flower stimulation treatments
- Measure the impacts of early flower stimulation on ramet health

The *Early Flowering Study* addresses the following questions: Which techniques are best for maximizing very early seed production? How soon after grafting can these techniques be safely applied? How can the damage caused by flower stimulation treatments be minimized? How do flower stimulation techniques interact with the design and management of min-

Figure 3. Materials used in the Early Flowering Study. A. The experimental design in the Vaughn orchard block (2-year-old grafts in 2001) consisted of 9 clones planted at a 13 x 8 foot spacing in a completely randomized design. B. The experimental design in the NWCTGA orchard block (4-year-old grafts in 2001) consisted of 9 clones planted at a 13 x 8 foot spacing in clonal rows.

iaturized seed orchards, including close spacing, heavy pruning, and the application of growth regulators to control tree height?

Design of treatments applied in 2001

Table 6. Early flowering experiments and treatments.

Expt.	Block/year	Graft age	Number of treatments: Girdling (G)	GA	Total with control (C)	Clones	Ramets / clone
1	Vaughn/2001[a]	2	1	1 (1x)[c]	4 (G; GA; G+GA; C)	9	4
1	Vaughn/2002[b]	3	1	1 (1x)[c]	4 (G; GA; G+GA; C)	9	4
2	NWCTGA/2001[a]	4	1	1	4 (G; GA; G+GA; C)	9	4
2	NWCTGA/2002[b]	5	1	1	4 (G; GA; G+GA; C)	9	4
3	Vaughn/2002[d]	3	1	3	4 (G+1x, 1.5x, 2x GA; C)	9	4

[a] Results from the 2001 treatments are discussed in this report.
[b] The same treatments that were applied in 2001 were reapplied to the same trees in 2002.
[c] 1x represents the amount of GA applied to the trees in 2001.
[d] We used the same clones as in Experiment #1 but different ramets (i.e., the trees had not been previously treated).

We applied our first set of flower stimulation treatments in the spring of 2001 (Table 6; PNWTIRC Annual Report, 2000-2001). These experiments were designed to test the effects of girdling and GA application on both 2- and 4-year-old grafts. We used two young seed orchards for these experiments, both of which are managed by Roseburg Resources. The Vaughn seed orchard block contains trees that were grafted in early 1999 (Figure 3). The second orchard, which is owned by the Northwest Christmas Tree Growers Association (NWCTGA), contains trees that were grafted in early 1997 (Figure 3).

The same four treatments were applied to trees in both orchards. These treatments included girdling (G), $GA_{4/7}$ (GA), girdling plus $GA_{4/7}$ (G+GA) and an untreated control (C) (Table 6 and Figure 4). The trees were girdled on 18 April, 2001 and GAs were injected into the stem on May 16, near the time of bud burst. In the Vaughn seed orchard, the treatments were applied to 2-year-old grafts (i.e., grafts that had already completed two growing seasons in the field). Nine clones were selected and each treatment was randomly applied to four ramets per clone. The same treatments were tested on 4-year-old grafts in the NWCTGA orchard using nine different clones and four ramets per treatment. Results from these experiments are described below (see *Accomplishments for 2001-2002*).

Figure 4. In the spring of 2002, we reapplied the same girdling and/or $GA_{4/7}$ treatments that were first tested in 2001. A. Girdling of a 3-year-old graft in 2002. B and C. $GA_{4/7}$ application to a 3-year-old graft in 2002.

Accomplishments for 2001-2002

We obtained the first results from our *Early Flowering Study* in the spring of 2002. Thimmappa Anekonda and Mike Albrecht assessed the effectiveness of the 2001 treatments by counting seed and pollen cones. Despite using low levels of GA (i.e., Procone, Abbott Laboratories), GA increased flowering—but only in combination with girdling (these results are described in more detail below). At the same time, we found no evidence that any of the treatments had a large adverse affect on ramet health. Based on these results, we decided to reapply the same treatments to the same trees in the spring of 2002 (i.e., continue Experiments #1 and #2; Table 6). We also decided to test greater amounts of GA in combination with girdling on previously untreated trees (see *Design of treatments applied in 2002, below*).

Girdling plus GAs synergistically stimulated female flowering on the 2- and 4-year-old grafts

The girdling plus GA treatment (G+GA) produced the greatest number of seed cones on both the 2- and 4-year-old grafts (Figure 5). In contrast, neither girdling nor the GA treatments alone produced significantly more seed cones than did the control. For the 2-year-old grafts, the number of seed cones per tree in the G+GA treatment was more than 200 times that in the control treatment (7.1 vs 0.3). This difference was even greater for the 4-year-old grafts (11 vs 0.3). At these young ages, the numbers of seed cones per tree are obviously constrained by the relatively small sizes of the crowns.

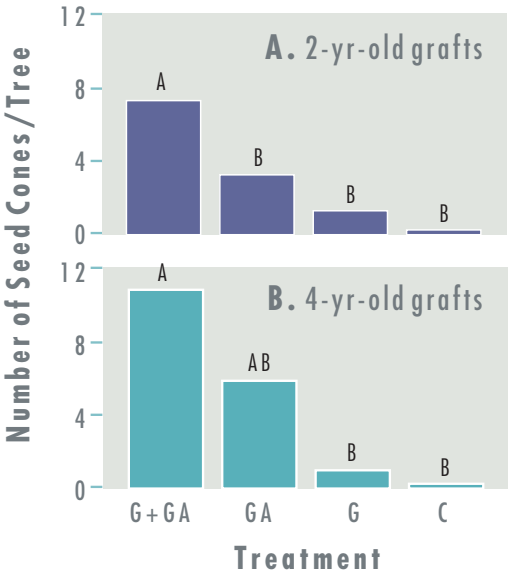


Figure 5. Effects of flower stimulation treatments on seed cone production. The girdling plus $GA_{4/7}$ treatment (G+GA) significantly enhanced seed cone production on both the 2- and 4 year-old grafts. G+GA is the girdling plus $GA_{4/7}$ treatment, GA is the $GA_{4/7}$ treatment; G is the girdling treatment, and C is the untreated control. Treatments identified by the same letter (A, B) are not significantly different at the 5% level of probability.

Girdling plus GAs synergistically stimulated male flowering on the 4-year-old, but not on the 2 year-old grafts

On the 4-year-old grafts, the girdling plus GA treatment produced significantly more pollen cones than did the other treatments (Figure 6B). In contrast, none of the treatments boosted pollen cone production on the 2-year-old grafts (Figure 6A). The differences in response between the 2- and 4-year-old grafts probably reflect differences in the age of the grafts, but this has not been confirmed.

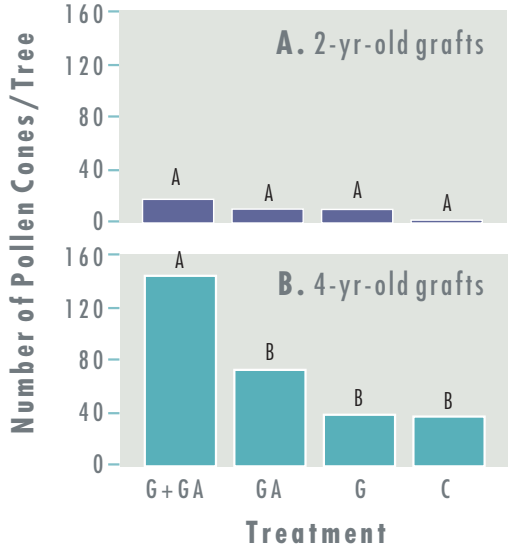


Figure 6. Effects of flower stimulation treatments on pollen cone production. The girdling plus $GA_{4/7}$ treatment (G+GA) significantly enhanced pollen cone production on the 4 year-old, but not on the 2-year-old grafts. Treatments and statistical tests are as described in Figure 5.

The girdling and GA treatments had little adverse affect on ramet health

Because aggressive flower stimulation treatments could harm very young grafts, we applied low levels of GA in the 2001 experiment. Although we observed some wind damage to the trees (i.e., from wind-rocking), no major adverse effects of any of the treatments

were observed. Therefore, we decided to test greater concentrations of GA in the spring of 2002.

Design of treatments applied in 2002

Based on our measurements of flowering and ramet health in the spring of 2002, we decided to reapply the same treatments to the same trees in both the Vaughn and NWCTGA orchards (i.e., we continued Experiments #1 and #2; Table 6). We also decided to begin a new experiment to study the effect of higher GA concentrations in combination with girdling (Experiment #3; Table 6). This experiment was conducted in the Vaughn seed orchard block using 3-year-old grafts. Three levels of GA (1x, 1.5x, and 2x relative to the 2001 GA levels) were applied to girdled trees. These new treatments were applied to the same 9 clones as were used in Experiment #1, but different ramets were used. All of the girdling treatments in 2002 were done on 11 April, 2002, whereas the GA treatments were applied on 7 May, 2002 in the NWCTGA orchard block, and on 13 May, 2002 in the Vaughn block.

Plans for 2002-2003

During 2002-2003 we will measure elongation growth on the trees treated in 2001 and 2002, count seed and pollen cones on the trees treated in 2002, apply insecticides to control seed insects, then measure both seed yield and quality for the trees treated in 2002.

Plans for future years

Early and sustained flowering is valuable for both seed orchard management and advanced generation breeding. We will design new early flowering experiments (or discontinue these experiments) after evaluating the results from the trees treated in 2002. In any case, results from all three experiments will be incorporated into the design of the *Miniaturized Seed Orchard Study*.

Miniaturized Seed Orchard Study

Introduction

Miniaturized seed orchards (MSOs) are promising alternatives to conventional seed orchards. In MSOs, the trees are planted at close spacings in clonal rows, then maintained at a height of only 2 to 4 m (Sweet and Krugman 1977, Webber and Stoehr 1998). Using this approach, seeds are produced close to the ground on many small trees, rather than on a few larger ones. In contrast, trees in conventional orchards are planted at wide spacings, ramets of the same clone are separated from one another to maximize outcrossing, and the trees are allowed to become much larger (15+ m).

MSOs have two main advantages compared to conventional orchards—increased genetic gains and reduced management costs (see *Potential Advantages of Miniaturized Seed Orchards*). For these reasons, miniaturized seed orchards are now standard for producing radiata pine seed in New Zealand (Sweet 1995). Despite these potential advantages, MSOs are unproven. The costs of MSOs could be greater than the costs of conventional orchards because of the extra work needed to keep the trees small. In addition, it is unclear how seed production will be affected by the change in seed orchard design and management.

Potential Advantages of Miniaturized Seed Orchards

Increased genetic gains:

- Enhanced ability to produce elite crosses via supplemental mass pollination (SMP) or controlled mass pollination (CMP) because trees are small and planted in clonal rows
- Reduced pollen contamination from outside of the orchard block because of more efficient SMP, CMP, or bloom delay

Decreased management costs:

- Reduced land costs because of the greater planting density
- Reduced management costs because the trees are small (i.e., more efficient seed collection and pest control)

The objectives of the *Miniaturized Seed Orchard Study* are to:

- Compare three orchard types for their (a) quantity of flowering and seed production, (b) ease and efficiency of management and (c) ramet health and seed quality
- Define the optimum age to begin flower stimulation in MSOs
- Determine whether small crowns can be maintained by controlling apical dominance with growth regulators
- Compare methods of SMP and control mass pollination in MSOs
- Determine whether clones respond differently to MSO designs and management regimes

The goal of the *Miniaturized Seed Orchard Study* is to compare three alternative spacings and management regimes on a scale large enough to evaluate realistic management costs, seed yields and seed quality (Anekonda and Adams 1999). The ramet spacings and target tree heights in our macro-, mini- and micro-orchards are shown in Table 7.

Table 7. Characteristics of three orchard types tested in the *Miniaturized Seed Orchard Study*.

Orchard type	Spacing (m)	Tree/hectare	Total # of trees	Final height (m)
Macro	6 x 4 m	416	640	4
Mini	4 x 2 m	1,250	640	2
Micro	3 x 1 m	3,333	768	2

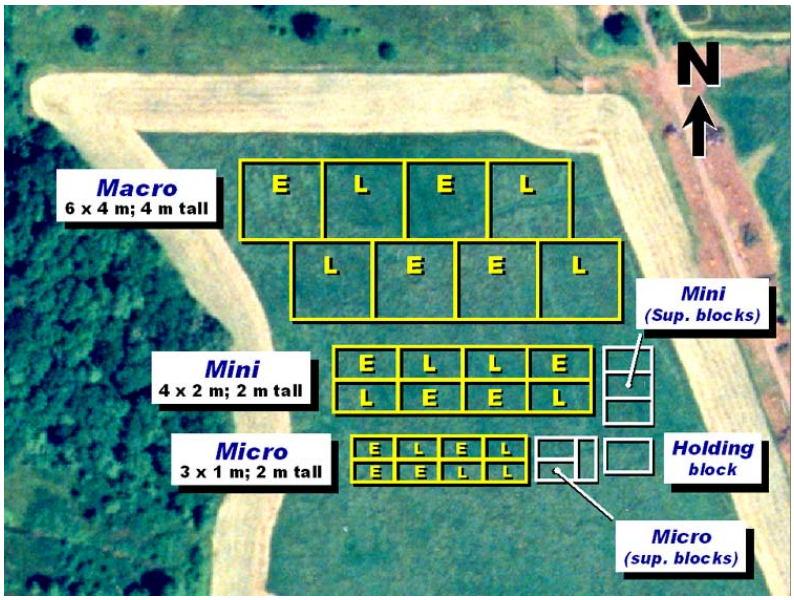


Figure 7. Aerial photo showing the field layout of the Miniaturized Seed Orchard Study on land owned by Plum Creek Timber.

Field design and management

Three orchard types are being compared at a site owned by Plum Creek Timber Company (Figure 7). Eight identical blocks (main plots) were established within each of the orchard types. Each of these blocks will contain the same 16 clones, consisting of eight forward selections (younger ortets) and eight backward selections (older ortets)[1]. For the macro- and mini-orchards, the clones are being grafted into five ramet row-plots. For the micro-orchard, the row-plots will contain six ramets each, so that every other ramet can be removed, if necessary. Additional details on the design and management of the *Miniaturized Seed Orchard Study* were reported in last year's annual report (PNWTIRC Annual Report, 2000-2001).

The MSOs are being irrigated to enhance survival and growth, increase seed yields, and provide more effective control of frosts and flower phenology. Within each orchard, the target tree height will be maintained using either mechanical or chemical pruning. Flower stimulation will be used to obtain early and sustained seed production.

Accomplishments in 2001-2002

Scions were grafted onto the rootstock in February 2002 (Figure 8). Jim Smith of Plum Creek Timber Company surveyed the scions in August of 2002 and found that only 9% of the scions were alive in the main experiment. In the supplemental blocks (which are to be used for initial testing of MSO treatments), the survival was 31%. Jerry Barnes, who grafted the trees, discussed possible reasons for the poor results at the annual meeting in June 2002, and provided the co-op with a written report discussing his conclusions (available upon request). Because of the poor survival of the grafts, we will regraft most or all of the rootstock again in the late winter of 2002-2003. Our original plan was to graft the seed orchard in both 2002 and 2003. Therefore, the poor survival in 2002 will not set us back if we obtain very high survival in 2003.

Randall Greggs established a small miniaturized seed orchard on Simpson Timber Company land in the late winter of 2002. Glenn Howe and Randall Greggs worked together to design the orchard in a way that allows us to apply cultural treatments in the future and obtain results that can augment the results from the main MSO experiment at the Plum Creek Seed Orchard. The design of the Simpson experiment is shown in Figure 9.

[1]'Forward selections' are selections from progeny tests, whereas 'backward selections' are parents that were selected based on progeny test results.

Plans for 2002-2003

We will prepare for regrafting the scions in the winter of 2003 by replacing rootstock in the Plum Creek seed orchard as needed. Jim Smith will coordinate this activity. Jim Smith will also select 16 new clones to be grafted onto the rootstock. New scions will be collected by Jim Smith and grafted in the late winter of 2003.

Plans for future years

We will periodically compare the advantages and disadvantages of each orchard type over the next 15 years (Objective 1). Flowering, ramet health, seed yield and seed quality will be monitored yearly, and the costs of labor and supplies are being recorded so that we can compare the economics of the orchard types. Because the grafting will continue in 2003, we now plan to begin flower stimulation in the mini-orchard in the spring of 2005 (Objective 2). The testing of the growth regulator treatments (Objective 3) has been delayed until we get a new Assistant Director on board. Treatments such as the application of auxins or cytokinins may be useful for maintaining small trees without mechanical pruning, but it is unclear how they will affect flowering and seed yields. We will eventually compare methods of SMP and CMP in the MSOs (Objective 4). Because these experiments must wait until we have sufficient flowering and seed production in the orchards, their timing is not yet known. Finally, we plan to monitor whether the clones respond differently to the MSO treatments (Objective 5).

Figure 8. Jerry Barnes grafted the trees in the Plum Creek miniaturized seed orchards in the winter of 2002.

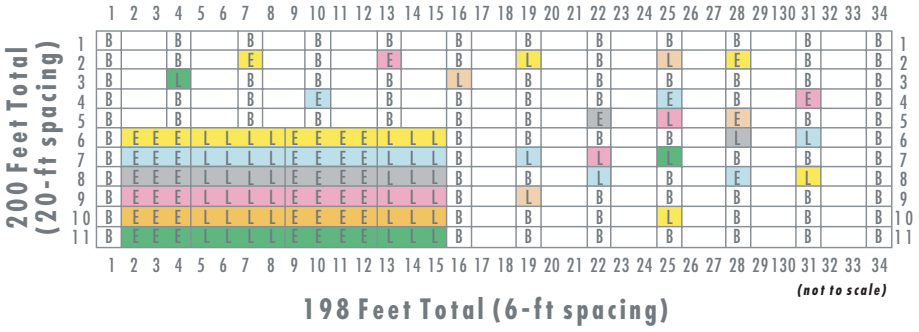


Figure 9. Design of the miniaturized seed orchard experiment on Simpson Timber Company land. In this experiment, the same clones are tested in a miniaturized orchard (i.e., the trees planted at a tight spacing in the lower left-hand corner of the figure) and in a conventional orchard (i.e., the trees plated at a wide spacing). The 6 color-coded positions represent the 6 clones tested in both orchards. 'B' represents a border tree, whereas 'E' and 'L' represent trees that will be given proposed early (E) and late (L) flower stimulation treatments.

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Activities Planned for 2002-2003

- We will hire a new Assistant Director.
- We will select a new PNWTIRC research project from our list of 7 high-priority research topics (see page 8).
- We will hire a graduate student to begin working on a new PNWTIRC research project in the fall of 2003.
- For the *Early Flowering Study*, we will measure flowering, cone production and seed yields on the grafts we stimulated in the spring of 2002.
- For the *Miniaturized Seed Orchard Study*, we will regraft the scions onto the miniaturized seed orchard rootstock. Maintenance of the seed orchard blocks will be carried out as needed.
- For the *Pollen Contamination Study*, we will submit one manuscript for publication describing our newly developed SSR markers. We will use these markers to estimate pollen contamination in the conventional Plum Creek seed orchard.

Appendix 1

Publications and Abstracts by PNWTIRC Personnel: 2001-2002

Adams, W.T., Aitken, S.N., Joyce, D.G., Howe, G.T. and Vargas-Hernandez, J. 2001. Evaluating efficacy of early testing for stem growth in coastal Douglas-fir. Silvae Genetica 50:167-175.

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Howe, G.T. 2001. Physiology and genetics of dormancy-related traits in *Populus*. *In*: Proc. Western Forest Genetics Assoc. Meeting, Davis, CA, July 30-Aug. 2, 2001.

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Appendix 2

Presentations by PNWTIRC Personnel: 2001-2002

Anekonda, T.A. 2001. Miniaturized seed orchards in coastal Douglas-fir. Presented at the Northwest Tree Improvement Cooperative workshop entitled 'Getting Genetic Gain in Operational Plantations,' Beaverton, OR, November 14, 2001.

Howe, G.T. 2001. Physiology and genetics of dormancy-related traits in *Populus*. 2001. Plenary talk, Western Forest Genetics Association Meeting, Davis, CA, August 1, 2001.

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Appendix 3

PNWTIRC Financial Support for Fiscal Year 2001-2002

Regular members[1]	$88,000
Associate members[1]	8,000
Contracts	8,000
Forest Research Laboratory, Oregon State University[2]	91,699
Total	195,699

[1] Each Regular Member contributed $8,000 and each Associate Member contributed $4,000 excluding in-kind contributions of labor, supplies, etc.

[2] The contribution from Oregon State University includes salaries, facility costs, and administrative support.

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