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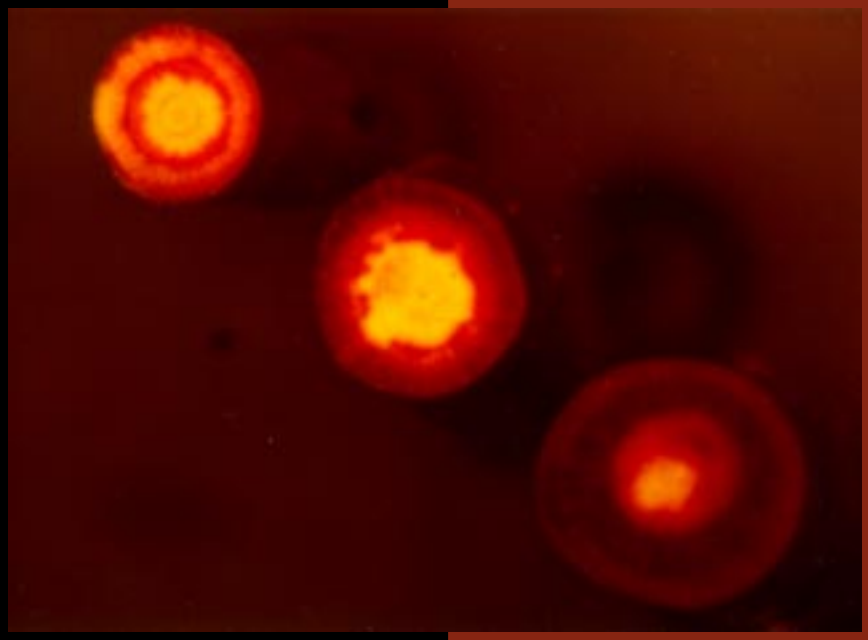

Pacific Northwest Tree Improvement Research Cooperative

ANNUAL REPORT
1998-99

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COLLEGE OF FORESTRY

PNWTIRC

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The Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) was formed in 1983 in response to the need for genetics research in support of operational tree improvement programs in the Pacific Northwest. There are several types of memberships in the Cooperative. Regular members contribute directly to the Cooperative through both an annual membership fee and by supplying in-kind support including land, labor and equipment for research projects. Associate members, landowners with less than 100,000 acres, pay a smaller annual fee and do not necessarily participate in research projects to the same degree as Regular Members. Liaison members contribute to discussions on key matters but have no voting rights.

The Policy/Technical Committee guides the activities of the cooperative. It is responsible for making decisions on overall program strategy and support, identifying research problems, establishing priorities, and assisting in the planning, implementation and evaluation of studies. This committee is comprised of representatives of each cooperator.

The PNWTIRC is housed in the Department of Forest Science at Oregon State University. Tom Adams is the Leader of the Cooperative, and Thimmappa Anekonda is the Associate Director. Christine Lomas is a graduate student funded fully by the Cooperative.

This report was written by Tom Adams, Thimmappa Anekonda, and Christine Lomas.

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ANNUAL REPORT
1998-99

PNWTIRC

CONTENTS

HIGHLIGHTS OF 1998-1999

- Greg O'Neill completed his Ph.D. dissertation in May, and Christine Lomas her M.S. thesis in October of 1999.

- The Cooperative staff has moved to brand new facilities in Richardson Hall.

- Shoot damage due to drought ranged widely among 39 full-sib families in the Seedling Drought Physiology Study, suggesting that selection among families for increased seedling drought hardiness would be quite effective. There appears to be little genetic association between drought hardiness in seedlings and growth potential in favorable moisture regimes.

- A calorimetric investigation based on a subset of families from the Seedling Drought Physiology study found that respiration parameters were generally reduced under moisture stress, but that families can differ in the degree of their response. Family differences in respiration traits expressed when seedlings are grown under well-watered conditions may be useful for predicting drought hardiness.

- The magnitudes of annual growth ring variables (e.g., earlywood width, latewood density, latewood proportion) are sensitive to moisture availability in the growing season in which a ring is produced. X-ray densitometry analysis of increment cores from sapling-age trees in one progeny test site showed that families differed widely in their response to past summer droughts.

- Nine potential microsatellite (SSR) marker loci were identified for use in Douglas-fir seed orchard studies. Preliminary analysis revealed as many as 10-12 different variants (alleles) at some loci in DNA samples from less than 20 individuals.

- A plan was approved for a 15-year study to evaluate alternative miniaturized seed orchard designs in Douglas-fir.

INTRODUCTION

In the mountainous terrain of the Pacific Northwest, ensuring adaptation of improved varieties to cold and drought is a high priority of tree breeders. This year, the last of the data from the three-year Seedling Drought Physiology Study were collected and analysis begun in earnest. This completes data collection associated with eight years of research on the genetics of cold and drought adaptation in coastal Douglas-fir. In addition to a better understanding of the inheritance of cold and drought hardiness traits, a major objective of this research is to develop methods of screening genotypes for hardiness to stress environments. We think we have come a long way in eight years, but there is still a mountain of drought hardiness data to be analyzed and interpreted. In addition, our ultimate goal is to test the ability to predict adaptability of families to cold and drought stress in the field based on the results of family screening trials in nurseries and characteristics of planting environments. Thus, planning for field verification of our screening methods will begin in the coming year.

Christine Lomas completed the analysis of family responses to drought applied in the second growing season of the Seedling Drought Physiology Study and defended her M.S. thesis in October. Results of her analysis not presented in last year's annual report are summarized in the next section. We also report on a preliminary assessment of responses to an even harsher drought applied in the third growing season.

In addition to the more "conventional" approaches to examining drought hardiness in the Seedling Drought Physiology Study, Thimmappa Anekonda used calorimetry methods to examine relationships between dark respiration traits and drought hardiness in a small subset of families from the seedling drought physiology study. In June, Thimmappa reported results from this investigation in an invited paper delivered to the International Society for Biological Calorimetry. A summary of this research is also given below.

Another unique approach to assessing drought hardiness is to retrospectively measure response to summer drought by examining annual growth rings of trees in field progeny tests. This is the approach used in the Field Drought Study. Although the analysis of growth ring data proved to be challenging, the results are interesting. Annual ring data collected from full-sib families growing on a single test site show that genetic variation in responses to summer drought can be detected in saplings using this method. (see "Response of Saplings to Drought").

After returning from a brief period working on a tree improvement education project in Peru, graduate student Greg O'Neill buckled down to complete his Ph.D. dissertation on the genetics of seedling cold hardiness in Douglas-fir. Most of the results from his dissertation were summarized in previous annual reports (PNWTIRC Annual Reports 1995-96 to 1997-98), so we don't repeat them here. Look for complete reports from this research in upcoming scientific journal articles! Greg is now in a post-doctoral position at the University of British Columbia working with Dr. Sally Aitken.

The Cooperative's launch into seed orchard research got started in a big way this year with the initiation of the Pollen Contamination Study. Dr. Kostya Krutovskii worked half time on the development of hypervariable molecular markers for this study and much progress was made (see next section). We intend to apply these markers to evaluating pollen contamination in one Douglas-fir orchard this coming year.

In addition to launching the Pollen Contamination Study, a proposal to evaluate the micro-orchard concept in Douglas-fir was completed and accepted by the Cooperative's membership. The "Micro-Orchard Project", which will actually compare three different orchard designs, has a proposed life of 15 years, and thus, is the first long term study initiated by the Cooperative. Although the plan is approved, final selection of the experimental orchard site is yet to be made. This decision is expected by year's end. Layout and planting of the experimental orchards is scheduled for spring of 2001. A summary of the proposed study plan is presented later in this report.

The Cooperative staff moved into new offices on the third floor of Richardson Hall this spring. Moving into this brand new building was disruptive in a busy time of the year, but the up-graded laboratory and office facilities are worth it. The view of Mary's peak from our west-facing windows is fantastic! Stop by for a tour.

Finally, after 15 years with the smaller "booklet" format for our annual report, we decided to go to a larger, bolder layout. We hope you enjoy our new look!

CURRENT PNWTIRC RESEARCH

SEEDLING DROUGHT PHYSIOLOGY

The Seedling Drought Physiology Study was initiated in 1996 with the main goal to develop efficient screening methods for drought hardiness traits in coastal Douglas-fir. Because Douglas-fir breeding programs in the Pacific Northwest are expanding the current breeding zones into fewer and larger zones, genotypes adapted to a wide range of habitats within each zone, including stress environments, will be needed. Development of genetically improved planting material for drought-prone sites should aid the breeding zone restructuring effort. Such an effort, however, requires accurate estimates of the heritability of drought hardiness traits as well as interrelationships between drought hardiness and economic characteristics, particularly stem growth. With these considerations in mind, the main objectives of this study are as follows:

1) To assess the impact of drought on seedling growth and physiology.

2) To evaluate the genetic control of drought hardiness and interrelationships between drought hardiness and seedling growth potential.

3) To understand the extent to which the relative performance of families for seedling growth is influenced by summer soil moisture availability.

MATERIALS AND METHODS

This study included 39 full-sib families of coastal Douglas-fir from southwestern British Columbia (Vancouver Island and the coastal mainland) grown in nursery beds at Oregon State University. The experimental design was a split plot replicated in five blocks. Main plots consisted of alternative watering regimes applied during the second (1997) and third (1998) growing seasons. In the second growing season, the three regimes were well-watered (control), mild drought, and moderate drought implemented from mid-June through early September. Although measures of soil water availability (i.e., predawn xylem water potential) in the second growing season indicated that the drought treatments were effective in limiting mid-summer soil moisture (i.e., –1 to –2 MPa and –2 to –3 MPa in mild and moderate treatments respectively, versus >-1 MPa in control), impacts of drought on growth and survival of seedlings were relatively limited (see PNWTIRC Annual Report 1997-98; Note that the mild and moderate drought treatments in year 2 were called “moderate” and “severe” droughts, respectively, in this report). Part of the reason for the limited impact is that drought treatments were not initiated until mid-June, when much of the seedling growth was already completed. In order to achieve higher levels of drought stress, drought was again applied in the third growing season in main plots that had received mild drought in the second year. In this case, however, the drought was initiated earlier (May 1) and greater xylem water potential deficits were achieved by mid-summer (i.e., -3 to –4 MPa). The remaining main plots were well watered in the third growing season. Thus, the three treatments in the third year were control (well watered both years), recovery (moderate drought in year two followed by well-watered in year three), and severe drought (mild drought in year 2 followed by severe drought in year three).

At the time of planting, each family was represented by 8 trees in two randomly-located 4-tree row plots within each main plot, with seedling spacing of 8 cm x 8 cm. At the end of the second growing season, every other seedling in all row plots were harvested. Thus, treatments in the third year were applied to only one-half of the original number of seedlings.

Heights and diameters of all seedlings were measured at the end of the second and third growing seasons in all treatments. Drought injury to all seedlings was scored visually in 10% damage classes, based on intensity of yellowing and browning of shoots in the mild and moderate drought treatments during the second year and in the severe drought treatment the third year. Little or no damage was observed in the well-watered treatments both years.

Xylem cavitation is collapse of xylem conduits resulting in impaired movement of water up the stem.

Graduate student Christine Lomas undertook a portion of the main study, and assessed physiological responses to drought, measured by xylem cavitation and hydraulic conductivity. She participated in measuring these traits on seedlings in both years, but only the results for the second growing season are included in her MS thesis. Xylem cavitation is collapse of xylem conduits resulting in impaired movement of water up the stem. Drought stress is a primary factor that leads to xylem cavitation. Xylem cavitation was assessed using an anatomical method where safranin dye was allowed to pass through 3 cm-long basal stem segments of freshly harvested seedlings. The portion of stem cross-sectional area that was not stained by the dye represents the proportion of cavitation. We estimated this proportion separately for each of the two annual growth rings (R1, and R2) in the second year, and in the three growth rings (R1, R2, and R3) in the third year, using 10% classes. In the second-year, cavitation was assessed on 10 seedlings per family in all three treatments (3 treatments x 39 families x 10 trees = 1170), and in the third-year it was assessed primarily in the severe drought treatment (39 families x 10 seedlings = 390), with a few additional measurements made in the control and recovery treatments (2 treatments x 50 seedlings = 100).

Hydraulic conductivity is the quantity of water transported through a given length of stem under a constant pressure. The magnitude of hydraulic conductivity is a function of stem diameter and the proportion of functioning xylem conduits (i.e., lack of xylem cavitation). Hydraulic conductivity was assessed on a random sub-sample of 20 seedlings per treatment in the second year and in the same 490 trees assessed for cavitation in the third year. Because of the enhanced drought in year 3, many trees were heavily damaged in the severe treatment. Preliminary analysis indicated that trees with a damage score greater than 6 had 100% cavitation and zero hydraulic conductivity (essentially were dead or nearly so). Thus, only “live” trees (damage scores < 6) were assessed for cavitation and hydraulic conductivity in the severe treatment. Means in the severe treatment were subsequently calculated by taking into account the proportions of live and dead trees. The 50 seedlings measured for cavitation and hydraulic conductivity in the control and recovery treatments of year 3 were sampled at random. Because there was little or no stem damage in these treatments, simple arithmetic averages of the 50-tree samples were used to estimate treatment means.

The effects of drought on second-year growth, shoot damage and stem cavitation were addressed in last year’s annual report. Here, we present results on the influence of the harsher drought applied in the third growing season on stem growth, damage and cavitation, and on hydraulic conductivity (Objective 1). We also evaluate the recovery of seedlings in the third year that were subjected to the moderate drought treatment in the second year. Finally, we present preliminary results addressing Objectives 2 and 3 above.

Results

Impact of drought on third-year growth and drought hardiness

The severe drought applied during the third growing season was highly effective, significantly ($p<0.05$) reducing height and diameter growth in the drought treatment relative to the control by an average of 27% (Figure 1). In year 2, the mild and moderate drought treatments reduced growth by 6.2 and 10.8%, respectively, indicating that we were much more successful in inflicting drought stress in the third year. After a well-watered third growing season, seedlings that received moderate drought in year 2 (recovery treatment) had nearly the same diameter as the control trees, which were well-watered both seasons. Mean height of seedlings in the recovery treatment, however, was significantly less than in the control seedlings (Figure 1). Thus, although the impact of the moderate drought on height growth was relatively limited in year 2, it had a lasting effect, at least through the next growing season.

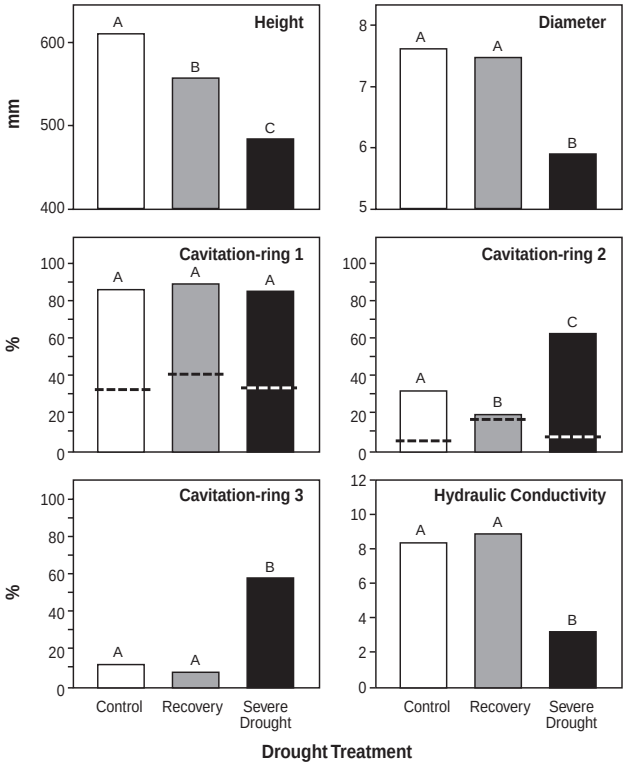

Figure 1. Average height, diameter, xylem cavitation, and hydraulic conductivity of Douglas-fir seedlings in the third growing season under three drought treatments (control, recovery, and severe drought). When letters over bars differ, mean values differ significantly between treatments ($p<0.05$). The dashed line indicates the mean % cavitation observed in the particular annual ring at the end of the second growing season.

The severe drought in year 3 had variable effects on the magnitude of cavitation in the three annual growth rings (Figure 1). Nearly all of the first ring was cavitated and mean cavitation was about double that observed in the previous year, regardless of the treatment. Percent cavitation in the control and drought treatments, but not in recovery, increased substantially over the previous year in the second growth ring, with mean cavitation greatest in the severe drought treatment. Cavitation in the current or third-year growth ring was substantially greater for seedlings under severe drought (39%) than for seedlings in either the control (12.6%) or recovery (8.3%) treatments. Findings from the previous year also showed that the current-year's growth ring is more sensitive to cavitation due to drought than earlier growth rings. Thus, for the purposes of assessing genetic response to drought, it is best to use percent cavitation in the growth ring formed during the drought period. It is interesting that mean cavitation in ring 2 was significantly lower in the recovery than in the control treatment. Perhaps the moderate drought in year 2 preconditioned xylem vessels produced in that year (e.g., by resulting in smaller cells or thicker cell walls), so that they were less susceptible to non-drought-induced cavitation the following year.

Mean hydraulic conductivity of seedlings in the severe drought treatment was less than 1/2 of that in the control and recovery treatments. Both xylem cavitation and reduced stem diameter contribute to this substantial reduction in water conducting capacity.

Genetic control of drought hardiness and interrelationships between drought hardiness and growth

Correlations between family means in both years (moderate drought in year 2 and severe drought in year 3) suggest that stem damage has a fairly strong genetic association with both

stem cavitation and hydraulic conductivity. Thus, for the purposes of this preliminary genetic analysis we refer only to shoot damage as a measure of drought hardiness. The inheritance of xylem cavitation, hydraulic conductivity, and other measures of drought hardiness based on differential growth in drought versus control treatments will be presented next year when we have analyzed these traits more fully. Because of the very limited damage in the mild drought treatment in year 2, only shoot damage results for the moderate drought in this year will be addressed.

Family means for shoot damage ranged more than three-fold when seedlings were grown in either the moderate or severe drought treatments (Table 1). Estimated individual tree heritabilities for shoot damage, however, were low in both cases, on par with what is observed for stem diameter in the control and recovery treatments, but only about 1/3 to 1/2 the magnitude of h^2 for stem height. Although the estimated heritabilities for shoot damage were low, wide variation among family means suggests that selection of families for increased seedling drought hardiness would be quite effective. The reliability of shoot damage as a trait for assessing drought hardiness of families is supported by the significant ($p < 0.01$), positive correlation (0.43)between family means for damage scores in years 2 and 3. Thus, mean shoot damage of families was relatively consistent over the two years, despite the large difference in severity of the drought treatments.

Because the families in this study constitute a pedigreed mating design it is possible to partition the variation among families into general (GCA) and specific combining ability (SCA) components (Table 1). GCA variance is genetic variation resulting from differences in the average performance of parents as reflected in their offspring produced by random mating

Table 1. Estimates of treatment means, family ranges (over 39 families), individual-tree heritabilities (h^2), and percentages of total family variance due to general (GCA) and specific combining ability (SCA) for percent shoot damage under two drought treatments (mild drought in year 2, severe drought in year 3), and for seedling height and diameter in the control (well-watered, years 2 and 3) and recovery (moderate drought in year 2, well-watered in year 3) treatments.

				Family[a]		% of total family variance	
Treatment	Year	Trait	Mean	range	h^2	GCA	SCA
Moderate	2	Shoot damage (%)	16.9	10.0-36.7	0.12	43	57
Severe	3	Shoot damage (%)[b]	38.5	18.5-59.0	0.12	100	0
Control	2	Height (mm)	353	300-441	0.28	41	59
		Diameter (mm)	5.5	4.6-6.7	0.07	24	76
	3	Height (mm)	614	515-730	0.39	66	33
		Diameter (mm)	7.6	6.1-8.9	0.15	46	54
Recovery	3	Height (mm)	557	491-696	0.28	82	18
		Diameter (mm)	7.5	6.7-9.10	0.11	50	50

[a] Family differences were significant ($p<0.05$) for all traits.

[b] Shoot damage was scored twice in 1998, once on August 13th and again on September 7th. Damage values in this table represent the first score. The second score, on average, was 10% higher than the first score and the second score was compared with the respiration measurements in the next section.

with all other parents in the breeding population. SCA variance is the remaining genetic variation among families due to the inability to predict the performance of offspring from specific two-parent crosses based on the average performance of the parents. The observation that a large percentage of the total family variance is due to GCA (Table 1) indicates that good gains in both seedling drought hardiness and growth traits can be expected when offspring of selected parents is produced by random mating (e.g., in wind-pollinated seed orchards). Substantial SCA for most traits, on the other hand, means that additional gains can be achieved by crossing specific pairs of parents with particularly outstanding offspring. The potential for enhanced gains through mass production of specific crosses is one reason why the cooperative is exploring the economic viability of micro-orchards in Douglas-fir (see "Micro-orchard Study Plan" later in this report).

Although generally negative, estimated correlations of family means for shoot damage due to drought with both height and diameter in the control treatment were never greater than $|0.21|$ and non-significant in both years (Table 2). Thus, there is no evidence in seedlings that selection for fast stem growth under optimal conditions will result in decreased drought hardiness. These results also suggest that genotypes with both seedling drought hardiness and rapid growth potential when there is no moisture stress, can readily be found in Douglas-fir breeding populations.

Although the estimated heritabilities for shoot damage were low, wide variation among family means suggests that selection of families for increased seedling drought hardiness would be quite effective.

Table 2. Estimated correlations between family means for percent shoot damage due to summer drought and seedling growth (height and diameter) under well-watered (control) conditions.

Year (drought treatment)	Height [a]	Diameter [a]
2 (Moderate)	-0.07	0.00
3 (Severe)	-0.21	-0.21

[a] In all cases the estimated correlation coefficients are not significantly ($p<0.05$) different from zero.

Extent to which the relative performance of families for seedling growth is influenced by summer soil moisture availability.

Family means for height and diameter in the control treatment were positively and significantly ($P<0.01$) correlated with their corresponding traits when seedlings were grown in the drought and recovery treatments (Table 3). Although, the estimated family-mean correlations were only moderate (mean 0.61), genetic correlations are likely to be stronger. Thus, the drought treatments applied in this study had little influence on the relative performance of families for stem growth. There are two reasons for this result: 1) Total height and diameter in the drought treatments were largely due to growth in previous years when seedlings were well watered; and 2) even when drought was applied early in the season, much stem growth probably occurred before soil moisture became limiting. For example, in the most severe drought treatment (year 3), water was withheld starting on May 1 but predawn xylem water potential (measures soil moisture availability) was still only mild (-1.5 MPa) in late June and did not reach it lowest levels (~-3.5 MPa) until mid July. Presumably, differential growth response of families to drought conditions would be more evident if drought were applied in sequential growing seasons.

Table 3. Estimated correlations of family means for growth (height and diameter) in the control treatment with growth in drought (years 2 and 3) and recovery (year 3 only) treatments the same growing season.

Year	Treatment	Height [a]	Diameter [a]
2	Mild drought	0.63	0.69
	Moderate drought	0.85	0.57
3	Recovery	0.64	0.62
	Severe drought	0.54	0.67

[a] All estimated correlation coefficients are significantly different from zero ($p<0.01$).

These results indicate that total height or diameter under drought may not be very useful measures of drought hardiness in short term seedling trials. Height or diameter increments or some other measures of growth during the time that drought is applied may be better, but we have not explored the options fully. It may turn out, that direct measures of drought damage like visual shoot damage scores, percent cavitation, or reduction in hydraulic conductivity will be more suitable for assessing drought hardiness at the seedling stage. Stay tuned!

Genetics of Dark Respiration and its Relationship with Drought Hardiness

Background

Summer drought typically involves both decreasing moisture availability and increasing temperature stress, therefore, an addendum to the "Seedling Drought Physiology Study" was established to explore temperature-stress effects on seedling physiology. This study was jointly funded by the PNWTIRC and by a grant to Thimmappa Anekonda from the OSU Research Council. Originally, calorimetry methodology was applied to the coastal (var. *menziesii*) and rocky mountain (var. *glauca*) varieties of Douglas-fir to determine the potential for detecting genetic differences in sensitivity of dark respiration to temperature stress. Because the results on the varieties were deemed promising, calorimetry techniques were subsequently applied to 12 of the 39 families in the Seedling Drought Physiology Study. A manuscript on results of these 12 families has already been accepted for publication; here we present a summary of the results.

During respiration in the dark, calorimetry can simultaneously measure the rate of loss of heat from plant tissue (i.e., the metabolic heat rate, q) and the rate of carbon dioxide production (R_{CO_2}) at defined temperatures. R_{CO_2} in dark respiration is also an indirect measure of the rate of chemical energy produced (i.e., ATP synthesized) within plant cells. In a way, q measures loss of energy and R_{CO_2} measures available energy for growth (biomass formation) from dark respiration. The difference, R_{CO_2}-q, is defined as specific growth rate (R_{SG}), i.e., the rate of incorporation of carbon into new growth per mass of tissue. The ratio of q to R_{CO_2} (q/R_{CO_2}) is a measure of respiration efficiency. When energy loss is large (i.e., high q/R_{CO_2}) or R_{SG} is low, which is expected under stress environments, plant cells lose the ability

to produce energy at a rate sufficient to maintain cellular structure or formation of new biomass, and plant growth rate is thus limited.

The relationship between q (or R_{CO_2}) and temperature (T) can be represented in a regression plot, where the natural logarithm of q (or R_{CO_2}) is plotted against the reciprocal of absolute temperature in kiloKelvin. The slope of this plot is the temperature coefficient of metabolic heat rate (μ_q) (or temperature coefficient of CO_2 production rate, μ_{CO_2}). Relative μ_q and μ_{CO_2} values have been shown to be related to adaptation of plants to climatic temperature. Thus, to investigate metabolic responses to temperature stress and potentially identify additional screening traits for drought hardiness, the above respiratory parameters were measured on detached shoots of a subsample of the full-sib families with the following objectives:

(1) To examine the influence of drought on dark respiration.

(2) To assess the extent of genetic variation in dark respiration traits among families of coastal Douglas-fir in a single breeding population.

(3) To evaluate genetic relationships between dark respiration traits and drought hardiness.

Materials and Methods

The 39 families included in the main study were first ranked for drought hardiness based on visual shoot damage resulting from the moderate drought treatment in the second growing season (1997), when mean damage ranged from 10 to 37%. To assess the influence of drought on dark respiration (Objective 1), 10 seedlings (5 blocks x 2 seedlings/block) from the two most drought-hardy and two least drought-hardy families (in year 2) were sampled from the control and severe drought treatments during mid-June in the third growing season (1998) and subjected to calorimetric measurements of q and R_{CO_2} at 17 and 25°C (N=80; 2 treatments x 4 families x 10 seedlings/family). In addition, q was measured for these four families in the temperature range of 20 - 55°C in 5° increments (N=80). This wide range of temperatures made it possible to evaluate impacts of temperature stress on dark respiration. Family means for stem damage in 1998 were 63% and 37% for the two least drought hardy families in 1997 (315 and 323, respectively), and were 30% and 46% for the most drought hardy families (135 and 114, respectively).

To evaluate genetic variation in dark respiration traits (Objective 2) and the relationships between these traits and drought hardiness (Objective 3), two seedlings from the control treatment of each block were also sampled in the third growing season from each of 12 families covering the range of visual drought injury in 1997 (i.e., 10 seedlings/family for calorimetric analyses at 2 test temperatures, 17 and 25°C; N=120). The range in mean drought injury among these 12 families in 1998 was 29% to 68%.

One actively growing, fresh lateral shoot-apex (~100 mg) was collected at 8 am from a primary branch in the uppermost whorl of each seedling and placed in an open 15-ml centrifuge tube containing cold, half-strength Hoagland's solution and 1% sucrose. The tubes were maintained near 5°C during the period of storage prior to calorimetric measurements. Fresh samples were collected every day of measurement during June 11 - July 17, 1998. Sample collection order and measurements within replicates were randomized.

Measurements of metabolic heat rate (q) and CO_2 production rate (R_{CO_2}) were made using a multi-cell, differential scanning calorimeter. Temperature coefficients of metabolic heat rate (μ_q) and of CO_2 production rate (μ_{CO_2}), the ratio of metabolic heat rate to CO_2 production rate (q/R_{CO_2}), and specific growth rate (R_{SG}) were derived from the original variables.

RESULTS

Opposite effect of drought on the temperature dependence of R_{CO2} and q seems to indicate that ATP energy synthesis and ATP use reactions of the respiratory pathway have responded differently to moisture stress.

Despite the relatively low moisture stress experienced by seedlings in the drought treatment during the calorimetric measurements, means of respiratory traits measured at 17 and 25°C (over 4 families) were generally lower for shoot tips sampled from seedlings grown in the drought than in the control treatment (Table 4). The only exceptions are q/R_{CO_2} at 25°C, where the difference was not significant, and μ_q, which is significantly greater in droughted seedlings. The finding that μ_{CO_2} decreased under drought, while μ_q increased relative to the control treatment, is one of the most intriguing of the calorimetry results. This opposite ef-

Table 4. Estimated family means for respiration parameters measured at 17 and 25°C in four coastal Douglas-fir families grown under control and drought treatments. Family means for percent shoot damage due to third year drought were: 135(30%), 114(46%), 315(63%), and 323(37%).

	Respiration		Family					
T, °C	traits [a]	Treatments	135	114	315	323	Mean [b]	
17	q	Control	1.80	1.84	2.10	1.78	1.88	**
		Drought	1.17	1.31	1.53	1.17	1.29	
	R_{CO_2}	Control	4.27	5.59	4.80	4.52	4.79	ns
		Drought	4.45	3.24	5.10	3.99	4.17	
	q/R_{CO_2}	Control	430	351	503	455	435	*
		Drought	281	416	341	335	343	
	R_{SG}	Control	28	42.7	34.1	31.1	34.0	ns
		Drought	37	21.9	40.5	32.1	32.8	
25	q	Control	3.71	3.55	3.70	4.20	3.79	**
		Drought	3.08	3.24	2.86	3.04	3.06	
	R_{CO_2}	Control	11.1	9.83	10.3	10.8	10.49	**
		Drought	8.1	9.43	6.96	7.88	8.12	
	q/R_{CO_2}	Control	392	384	382	426	396	ns
		Drought	403	387	419	405	403	
	R_{SG}	Control	83.7	71.4	74.9	74.7	76.2	*
		Drought	56.8	70.4	46.7	55.1	57.5	
17 & 25	μ_q	Control	8.42	7.21	7.70	9.33	8.17	*
		Drought	10.7	9.85	8.04	10.4	9.86	
	μ_{CO_2}	Control	9.75	6.96	8.77	12.5	9.41	ns
		Drought	7.19	11.0	5.76	7.85	8.16	

[a] See text for description of these traits. Units of measurement are: q in μWatt/mg dry wt; R_{CO_2} in pica mol/second/mg dry wt; q/R_{CO_2} in kilo Joules/mol; R_{SG} in pica mol/second/mg dry wt; μ_q and μ_{CO_2} in kiloKelvin.

[b] Significance of difference between control and drought treatment means: ns=not significant; *=$p<0.05$; **=$p<0.01$.

fect on the temperature dependence of R_{CO_2} and q seems to indicate that ATP energy synthesis and ATP use reactions of the respiratory pathway have responded differently to moisture stress. Significant reduction in R_{SG} values at 25°C for all families grown under drought, relative to the control, suggest that there was a considerable reduction in rate of energy generation, which in turn appeared to have reduced the incorporation of carbon into structural biomass.

When q was measured over the broad range of temperatures (20-55°C), patterns of change in q (heat loss) with increasing temperature were similar over families and moisture (i.e. control vs. severe drought) treatments; Figure 2). Sensitivity to drought, as reflected in mean q, however, varied widely among the four families. While q differed little between the control and drought treatments in families 135 and 114, q in the drought treatment was depressed significantly relative to the control at almost all temperatures in families 315 and 323. It is interesting that the family most sensitive to drought in terms of reduced q (family 315) also had the highest stem damage score. Nevertheless, the remaining families do not support a particular pattern. It is obvious that a larger sample of families is needed to fully assess the impact of drought on temperature response in q.

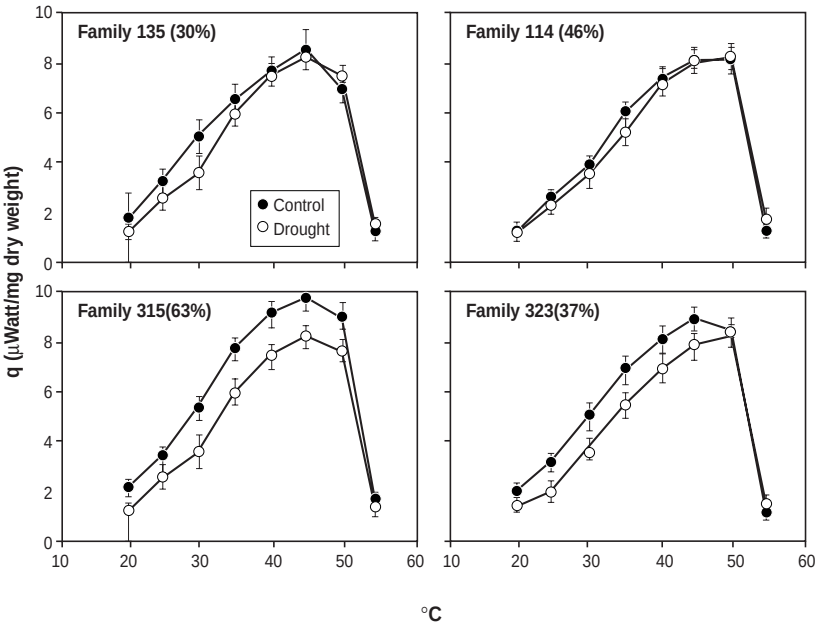

Fig. 2. Change in family means of metabolic heat rate (q) with increasing temperature (°C) for four families grown under control and drought treatments. Mean percent damage to shoots from the drought treatment is given for each family in parentheses. Error bars are the standard errors of family means at each measurement temperature.

Individuals from the wider sample of 12 families grown in the control treatment differed markedly in respiration traits when tested at 17 and 25°C, with coefficients of variation (CV) never less than 35% (Table 5). CVs and ranges in family means were also large, with family differences significant ($p<0.05$) for q at 25°C and for μ_{CO_2}. Thus, genetic variation in respiration traits is apparent.

Third-year shoot damage was moderately and negatively associated with μ_{CO_2} ($r^2 = 0.34$; $p<0.05$),

Table 5. Estimated sample means, and ranges and coefficients of variation (CV) among individual seedlings (119) and among family means (12) for respiration traits measured at 17 and 25°C in 12 coastal Douglas-fir families grown under the control (well-watered) treatment.

	Respiration	Individuals			Families	
T,°C	Traits[a]	Mean	Range	CV	Range	CV
17	q	1.84	0.56- 4.26	39	1.42-2.14	11
	R_{CO_2}	5.06	1.80-10.10	39	4.27-6.49	13
	q/R_{CO_2}	413	83-967	48	314-506	16
	R_{SG}	36.4	2.05-93.0	60	20.6-53.9	24
25	q	3.96	1.17-8.11	35	3.39-5.05	14 **
	R_{CO_2}	11.1	1.96-30.7	39	9.53-15.3	16
	q/R_{CO_2}	392	101-868	38	311-452	9
	R_{SG}	80	14.1-224	51	61.8-107	16
17 & 25	μ_q	9.36	0.22-21.2	47	7.22-12.3	16
	μ_{CO_2}	9.48	0.07-23.1	49	4.79-12.9	25 **

[a]See text for descriptions of traits. For measurement units refer to Table 1.

**Family means differed significantly ($p<0.01$). Families were not significantly different ($p>0.05$) for the remaining traits.

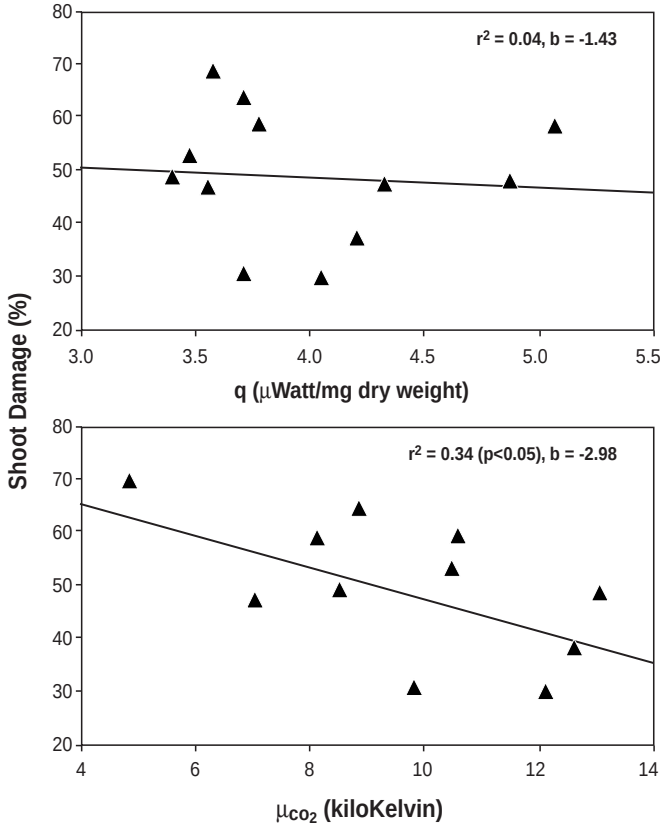

Fig. 3. Family means for 3rd year shoot damage in the severe drought treatment plotted over q at 25°C and μ_{CO_2} in the control treatment.

indicating that families with the highest μ_{CO_2} in the control treatment are the least susceptible to damage from summer drought (Fig. 3). That is, the families that are the most responsive to an increase in temperature from 17°C to 25°C, in terms of increased energy production, are the most hardy to severe drought. This result suggests that the response of families to severe drought at the seedling stage may be predictable on the basis of simple calorimetric measurements made on seedlings grown in well-watered conditions. No association, however, was found between shoot damage and q at 25°C (r^2=0.04). Obviously, further testing with larger samples of Douglas-fir families is required. In particular, it would be interesting to compare calorimetric parameters of droughted and control seedlings at different times during the course of the growing season as the impact of moisture stress intensifies.

Conclusions

This study measured genetic variation in respiration parameters and explored the possibility of using calorimetry to predict drought hardiness in coastal Douglas-fir families. The following conclusions were reached:

1) On average, q, R_{CO_2}, and R_{SG} values were reduced under moisture stress. μ_q values, however, increased and μ_{CO_2} decreased in response to drought.

2) Family means of q in the most drought sensitive family were significantly higher in the control than in drought treatment across the temperature range of 20 to 50°C.

3) Two respiration (R_{SG} at 17°C and μ_{CO_2}) traits showed a high degree of genetic variation among families.

4) Respiration traits determined from seedlings grown without moisture stress may be useful for predicting seedling drought hardiness.

Response of Saplings to Drought

As with our earlier cold hardiness studies, drought hardiness is being investigated in both seedling experiments and in older (sapling-age) trees in field progeny tests. The objective of the field study is to determine whether annual growth ring variables, including size and density of annual rings and their earlywood and latewood components, can be used to retrospectively assess sensitivity of Douglas-fir genotypes to summer drought. The study was designed to be implemented in two stages (PNWTIRC Annual Report 1995-96). In stage 1, the goal was to determine the degree to which components of annual rings are sensitive to summer moisture availability. If ring variables showed consistent responses to drought, the study was to proceed to stage 2, where the goal was to determine the extent to which responses to drought are under genetic control.

Results of stage 1, which consisted of Andy Bower's M.S. Thesis, were presented in last year's annual report. In summary, Andy found that a number of annual ring variables are sensitive to drought during the growing season, with latewood traits showing the most consistent trends. Based on these encouraging results, we initiated stage 2 by examining family variation in relationships of annual ring variables with summer moisture availability on a single test site.

Because the sample of trees in stage 1 came from multiple families and test sites, and because age of growth rings, as well as soil moisture availability influence ring variables, rather complicated multiple regression models were used previously to evaluate the relationships between ring variables and soil moisture (PNWTIRC Annual Report 1997-98). For genetic analysis we required a simpler approach that would allow straightforward assessment of family differences in drought sensitivity. Below we describe this procedure, applying it first to a re-analysis of the stage 1 data and then to the family data of stage 2.

Stage 1

Estimation of annual ring variables and soil moisture deficit

Increment cores were extracted June 1996 from trees growing on eight progeny test sites in coastal British Columbia in order to sample a wide variety of soil moisture conditions. Two cores were taken at breast height from 18-19 year-old trees, with two trees sampled from each of eight full-sib families in four of the sites and two trees from each of another set of eight families at the remaining sites. The cores were subjected to x-ray densitometry analysis which gives a profile of densities across the increment core, and subdivides the core into annual rings. From the density profile of each annual ring, four ring growth variables (ring width, earlywood width, latewood width, latewood proportion) and three ring density variables (ring density, earlywood density, latewood density) were derived. Typically, data for 10 growth rings were available from each tree.

Soil moisture conditions when each annual ring was formed (i.e., for each year and site) were summarized by estimating the soil moisture deficit (SMD) for the particular growing season and test site location. These estimates were calculated by Dr. David Spittlehouse (Research Branch, British Columbia Ministry of Forests) using a water balance model based on inputs of climate, soil type and topography. The greater the SMD, the greater the growing season drought stress. The mean and standard deviation of SMD over years for two of the eight sites proved to be near zero; these sites were dropped from further analysis.

Assessing the relationships between annual ring variables and SMD

The goal is to examine associations between ring variables produced in any particular year with the SMD for the corresponding growing season. The problem is that factors other than soil moisture availability also influence ring variables. In particular, ring variables in young trees change in a consistent fashion with increasing distance from the pith. Relationships between ring variables and tree age at the time rings were formed are illustrated for two traits (ring width and density) in Figures 4 and 5. Here, each trait is plotted on tree age and fitted with a quadratic regression equation for three individual trees (dashed lines). The mean regression curves for all trees measured in stage 1 are also shown (solid line). On average, ring width steadily increases with increasing age, but tapers off in later years (Figure 4), while ring density has nearly the opposite trend (Figure 5). There is also wide variation in the trends

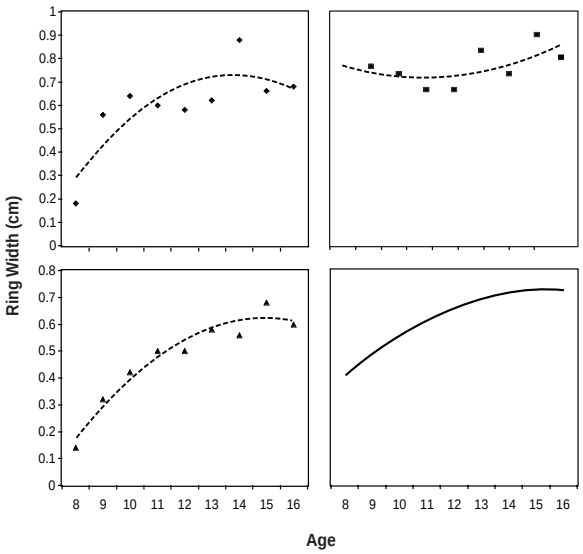

Figure 4. Annual ring width regressed on age when the ring was produced for three representative trees (dashed lines + data points) and for all trees measured in stage 1 (solid line).

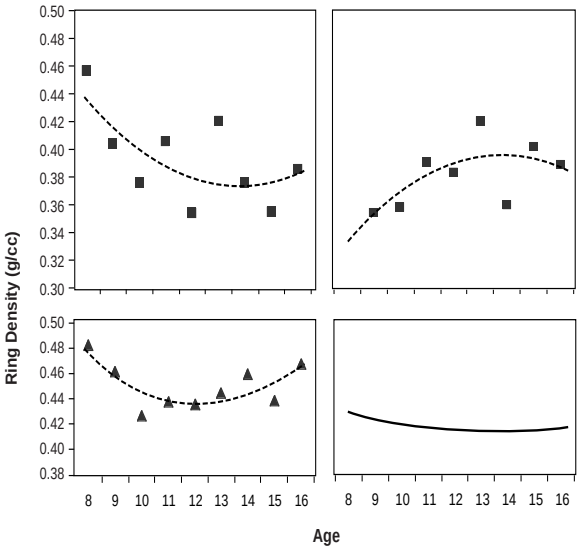

Figure 5. Annual ring density regressed on age when the ring was produced for three representative trees (dashed lines + data points) and for all trees measured in stage 1 (solid line).

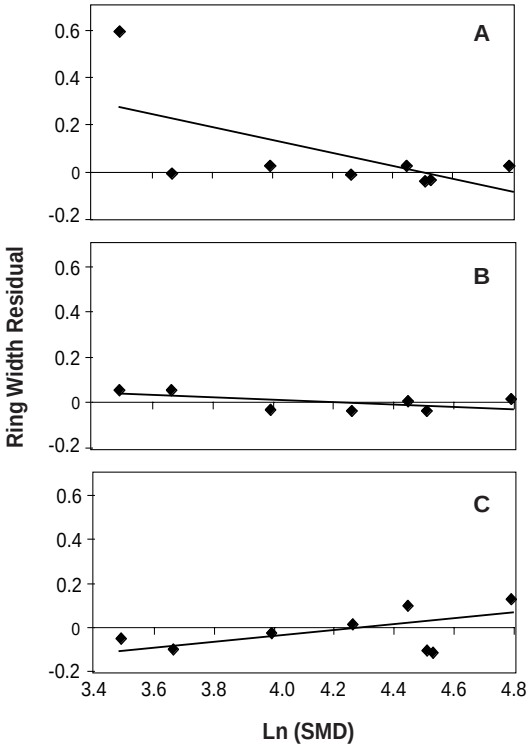

Figure 6. Linear regression of age-model residuals for ring width on ln(SMD) for three representative trees.

among individual trees, although the quadratic model seems to do a good job in fitting the relationships.

To quantify the relationships between ring variables and SMD, annual ring values for each tree were first regressed on tree age using quadratic equations ("age models") as done in Figures 4 and 5. Residuals from the age-model regressions (i.e., age-adjusted ring variables) were then regressed on SMD. Preliminary regressions showed that age-model residuals either increased or decreased exponentially with increasing SMD, but the relationships could be made linear by using ln(SMD) as the independent variable. Slopes of the linear regression of age-model residuals on ln(SMD) for individual trees (which we will call "drought response coefficients", DRC) measure their sensitivity to drought. This is illustrated for three trees in Figure 6. Ring width residuals in tree A decrease with increasing SMD (i.e., DRC < 1), while those in tree B are nearly insensitive to SMD (DRC = 0), and those in tree C increase with increasing SMD (DRC > 0).

Individual-tree DRCs for each of the seven ring variables, combined over the six remaining test sites, were subjected to analyses of variance. From these analyses, average DRCs for individual test sites and for all sites combined were estimated.

RESULTS

With the exception of latewood width, mean DRCs for all traits were significantly different from zero ($p < 0.05$) at one or more test sites, indicating that sensitivity of annual ring variables to soil moisture availability during the growing season is detectable (Figures 7 and 8). Averaged across all sites, ring growth (i.e., ring width) decreased with increasing SMD, but this result was mostly due to site 52 where the mean SMD over all years was 2-3 times greater than at any other test site. It seems reasonable that when summer moisture stress is

particularly high that diameter growth will be negatively impacted. Nevertheless, on sites 54 and 59 estimated DRCs are marginally positive indicating that ring growth increased with increasing SMD on these sites. Perhaps on soils with lower average SMD, individual SMDs are never high enough to depress growth, while other factors associated with increasing SMD, such as sunnier and warmer spring days, have a positive influence on growth. We will see in the next section, however, that when many more trees are sampled on site 59, mean DRC is negative, so sampling error may be responsible for the positive DRC estimated for at least this site.

Total ring density decreased with increasing SMD at site 52, probably because high summer SMD shuts off ring growth before denser, late-season wood can be produced (Figure 8). Earlywood density was not consistently associated with SMD across sites, with significant mean DRCs being negative in two cases (sites 54 and 55) and positive in one (site 62). Latewood DRC, however, was positive for all sites and significantly so in four of them. Thus, increasing latewood density appears to be the most consistent response of annual rings to increasing SMD.

Latewood DRC, however, was positive for all sites and significantly so in four of them. Thus, increasing latewood density appears to be the most consistent response of annual rings to increasing SMD.

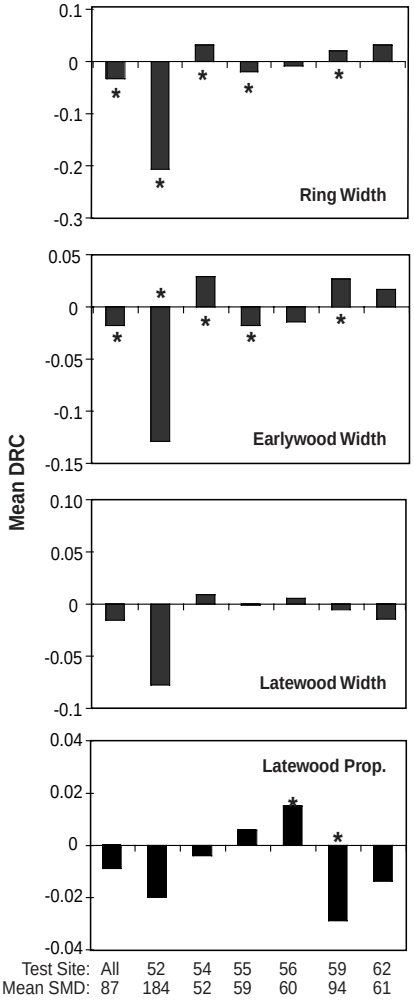

Figure 7. Mean drought response coefficients (DRC) of annual ring growth traits for six progeny test sites and for all sites combined.

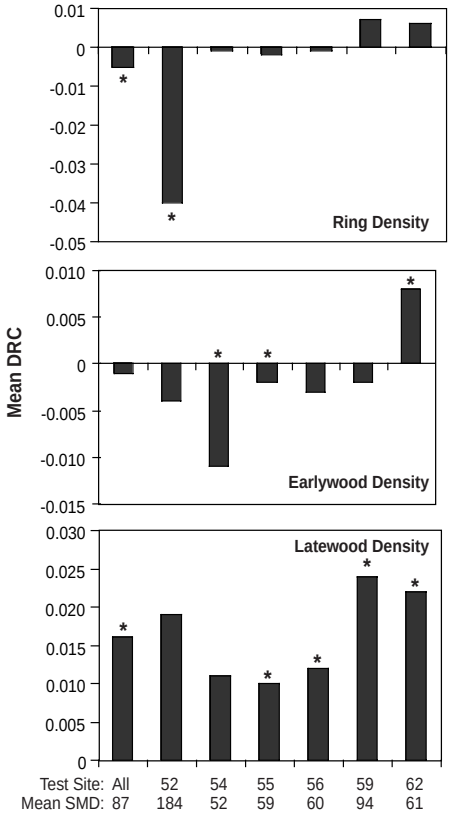

Figure 8. Mean drought response coefficients (DRC) of annual ring density traits for six progeny test sites and for all sites combined.

STAGE 2

METHODS

Freda Creek (site 59) was chosen for intensive family sampling because: 1) It contains the same 39 full-sib families included in the Seedling Drought Physiology Study; 2) It has the highest mean level and range of SMD values of the four sites sampled with these families; and, 3) It is a fairly uniform site. At the time of planting, the 39 families were arranged in each test site according to a completely randomized design with four replications of four-tree row plots. One increment core was extracted in winter 1997 at breast height from all surviving trees (total 473) in these families.

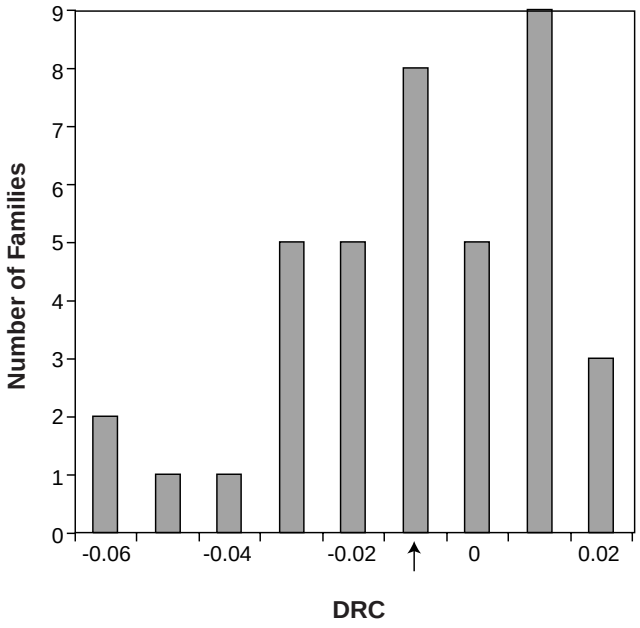

Figure 9. Distribution of mean drought response coefficients (DRC) for earlywood width among 39 families at Freda Creek. The arrow indicates the mean DRC over all families.

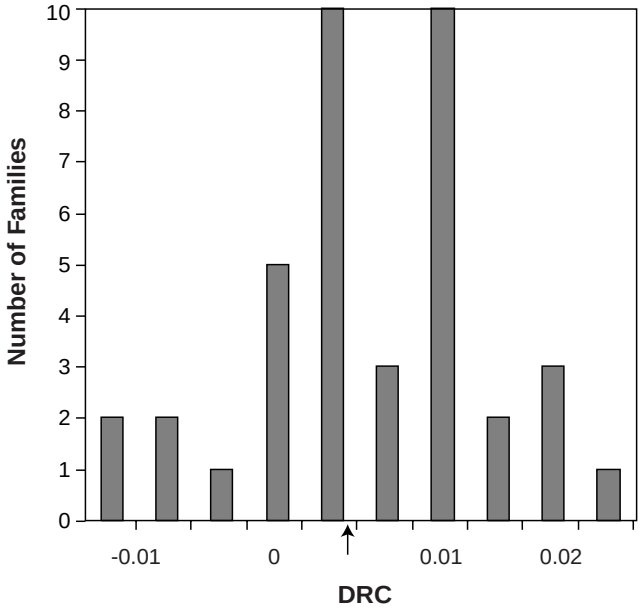

Figure 10. Distribution of mean drought response coefficients (DRC) for ring density among 39 families at Freda Creek. The arrow indicates the mean DRC over all families.

X-ray densitometry profiles revealed an unforeseen problem. Pruning of the lower limbs (to 3m) in winter of 1994-95 profoundly influenced subsequent rings in these trees, with ring width in 1995 reduced by 50% relative to the previous year, and ring density sharply increased. Because of these culturally induced impacts, rings after 1994 were not used in the analyses, leaving a maximum of 10 useful rings (1985-94). In addition, trees that did not have the minimum number of rings required for regression (5 rings) were not utilized. Ninety percent of the surviving trees remained, giving an average sample of 11.1 trees per family (range 7-16).

RESULTS

Averaged across all families, DRC was negative for all annual ring growth traits, differing significantly ($p < 0.05$) from zero for ring width, earlywood width and latewood width, but not for latewood proportion. Families differed significantly in mean DRC at the 5% level for earlywood width and latewood proportion and at the 10% level for ring width and latewood width. Mean DRC for all ring density traits, on the other hand, were positive and differed significantly ($p < 0.05$) from zero when averaged over all families. Families, however, differed significantly ($p < 0.05$) in mean DRC for ring density, but not (i.e., $p > 0.10$) for earlywood density and latewood density).

We limit the remaining discussion to earlywood width and ring density because family differences in DRC were substantial for both these traits (Figures 9 and 10). When selecting families for drought hardiness, it seems reasonable to choose those with zero or positive mean DRC values for earlywood width because these families were not unfavorably impacted by summer drought stress. It is more difficult to interpret DRC for ring

density. Families with either strongly positive or strongly negative mean DRC for this trait may be unfavorably sensitive to summer drought, either because they respond to increasing moisture stress by slower growth and increased ring density (DRC > 0) or because they cease ring growth prematurely before denser wood is produced (DRC < 0). If these assumptions are correct, families with the lowest absolute values for ring-density DRC are the most hardy to summer drought.

The next step in the analysis of the stage 2 data is to evaluate the strength of inheritance of DRCs and genetic interrelationships among DRCs for different ring variables. It is also of interest to determine the degree to which DRCs are related to stem growth and overall wood density. Preliminary analyses suggest that DRCs are only weakly associated with these important economic traits, but this needs to be confirmed. Finally, the presence of the same set of families in both the field and nursery (i.e., in the Seedling Drought Physiology study) will make it possible to compare drought hardiness rankings measured in the nursery with sensitivity to drought over a number of years in the field.

Pollen Contamination

The aim of this research is to develop a set of hypervariable microsatellite (or simple sequence repeat, SSR) marker loci and apply these markers to evaluating pollen contamination in Douglas-fir seed orchards. For nearly 20 years allozymes (biochemical genetic markers) have been used to estimate pollen contamination in a variety of conifer species. These estimates typically show that 40% or more of orchard seed results from non-orchard (i.e., contaminant) pollen sources. Although these studies have been quite informative in revealing the seriousness of pollen contamination in many seed orchards, the number of allozyme loci available for contamination studies is limited, and these loci possess only limited variation. This means that even with large samples of seeds (often > 300), there is low statistical power to detect real differences in levels of pollen contamination and the impact of cultural treatments on pollen contamination, unless the effects of these treatments are substantial.

The precision and efficiency of pollen contamination studies could be greatly enhanced with the availability of SSR marker loci, which are considerably more variable than allozymes. SSR markers would also be useful for a variety of other practical applications such as evaluating the effectiveness of supplemental mass pollination, or for certifying the identities of clones or parents of controlled crosses.

SSRs are stretches of DNA consisting of tandemly repeated sequences of nucleotides whose unit of repetition is usually between 1 and 6 base pairs. Most SSR sites are non-coding (i.e., they don't code products of value to the cell), and because of their tandem repeat nature, are especially subject to mutation. Most commonly, mutations occur through "polymerase slippage" during DNA replication, and result in an increase or decrease in the number of repeats by one dinucleotide unit. For example, an SSR locus with a repeat sequence of 12 TA dinucleotides (i.e., $(TA)_{12}$ = TATATATATATATATATATATA) might mutate to $(TA)_{13}$, or 13 tandem repeats of TA. Because the DNA fragment consisting of $(TA)_{13}$ is slightly larger than $(TA)_{12}$, it will migrate slower through a gel medium subjected to an electrical current (electrophoresis), which is the means by which SSR molecules of different size are identified.

Each different SSR appears as a band with a different degree of migration on the gel. Although the greater mutation rate of SSR loci is the main reason why these markers are more variable than allozymes, SSR mutation rate is still relatively low, such that SSR alleles can be considered stable for the purposes of most marker studies.

The process of identifying SSR sites is to determine the exact sequence of DNA in and around (i.e., on both sides of) candidate SSR sites using an automated sequencer machine. From information on the sequence of nucleotides in candidate SSR sites, pairs of primers (small pieces of synthetic DNA 20 –30 nucleotides long) can be constructed that are exactly complementary to the DNA flanking each side of the repeat region. The primer pair for each SSR is then used in a process called the polymerase chain reaction (PCR), whereby the specific sequence between the priming sites (which contains the SSR) is amplified by DNA replication. In this manner, the amount of SSR is made large enough to be identified on gels through electrophoresis. Often, an iterative process of testing slightly modified versions of the primer sequence and PCR protocols is necessary in order to optimize the expression of SSR bands on gels. In addition, not all SSR loci are hypervariable. Thus, once primers for specific SSR sites are produced, it is necessary to screen a number of individuals to confirm that all variants can be readily scored on gels and to quantify levels of polymorphism.

This project is being done in cooperation with Dr. Steven Strauss of the Forest Science Department at OSU and the first phase of the effort is under the direction Dr. Kostya Krutovskii from his laboratory. The first phase, which is to develop the SSR markers, has four specific objectives:

a) To design and test PCR primers able to amplify highly variable SSR markers in both the chloroplast and nuclear genomes of Douglas-fir.

b) To evaluate levels of polymorphism in the SSR markers so that the most variable markers may be selected for further use.

c) To confirm the inheritance and fidelity of these markers.

d) To utilize the selected markers to evaluate pollen contamination in a Douglas-fir seed orchard and to optimize testing and estimation procedures.

The intention of phase 2 of the study is to apply the markers to a more detailed analysis of pollen contamination in a second crop year of the same orchard. The goal of this analysis will be to evaluate the degree to which pollen contamination varies with flowering phenology of clones or position of stems in the orchard. In phase 3, we wish to apply these markers to a broader array of seed orchards to better understand how levels of pollen contamination are influenced by various orchard conditions (e.g., orchard size, age, background pollen levels, cultural treatments to enhance orchard flowering).

This year, much headway was made in meeting the objectives of phase 1. This work is summarized below.

Materials and Methods

Materials come from one orchard block in a central Oregon seed orchard complex. Dormant vegetative buds were collected in the winter of 1997-98 from all 58 clones in this block (2-3 ramets of each, totaling 155), 61 clones in surrounding orchard blocks (one ramet each),

and from 44 trees in surrounding natural stands. Seeds were also sampled from the bulked 1997 crop in this orchard block (for contamination estimation). In addition, seeds from many controlled crosses among the parents in the block are available for inheritance analysis.

DNA was isolated from buds using a standard protocol developed in Steve Strauss' laboratory. DNA fragments containing SSR regions were amplified using the MJR PCT-100 ThermoCycler. Primer testing, marker identification and characterization of levels of variation were done with limited samples of 12 to 19 clones.

RESULTS

SSR loci in both the chloroplast (cpDNA) and nuclear genomes (nDNA) were investigated. The chloroplast genome has advantages for paternity analysis because it is haploid and because chloroplasts are inherited through pollen in Douglas-fir. Gene sequences in the chloroplast genome of conifers have remained relatively constant through evolutionary time, thus, we were able to take advantage of primer sequences of cpDNA from other species that are published or otherwise available from colleagues. We mainly used the *Pinus thunbergii* cpDNA sequence, which is the only complete cpDNA sequence available in conifers, to search for SSR regions. Sixteen promising cpDNA primer pairs for SSR sites were designed and synthesized, and then tested with the DNA isolated from 12 individuals. We also tested an additional primer pair which amplifies a minisatellite "hotspot" cpDNA region originally identified by Dr. Valerie Hipkins in Steve Strauss' laboratory, and subsequently utilized as a hypervariable genetic marker by Dr. Michael Stoehr and others in a study of a Douglas-fir seed orchard in British Columbia. Minisatellites are analogous to microsatellites, but the tandem repeats are made up of longer nucleotide sequences.

So far, we have found nine primer pairs that amplify SSR loci from the nuclear genome that appear polymorphic and give clear band patterns

Most of the designed primers worked and amplified cpDNA fragments of the predicted size. However, with the exception of the minisatellite locus, either no variation was detected or the variation was difficult to interpret, probably because it was due to single nucleotide deletions or insertions. Thus, after thorough testing, we concluded that only the minisatellite locus could be readily used as a marker. Fortunately, in a sample of only 19 clones, this marker proved to be extremely variable, giving 10 different alleles which are easily separated and recognized on gels because of their broad range in molecular size (574 to 1109 base pairs).

For the nuclear genome, we took advantage of previous work by Drs. John Carson and John Hobbs at the University of British Columbia who had designed specific primers to amplify 108 nuclear SSRs in Douglas-fir. Forty-nine of the more promising primer pairs were generously made available to us to try out. Because these primers had not been completely tested, we had much work to do to improve PCR protocols and primer sequences. So far, we have found nine primer pairs that amplify SSR loci that appear polymorphic and give clear band patterns. These pairs have been fluorescently labeled with Operon dye, and 6 have been tested with DNA isolated from 13-19 clones. These six marker loci show a very high level of variation (Figure 11), with the number of putative alleles ranging from 5 to 12. We need to test these markers on a larger sample of clones and verify their inheritance in formal genetic tests. We also need to test more of the primer pairs.

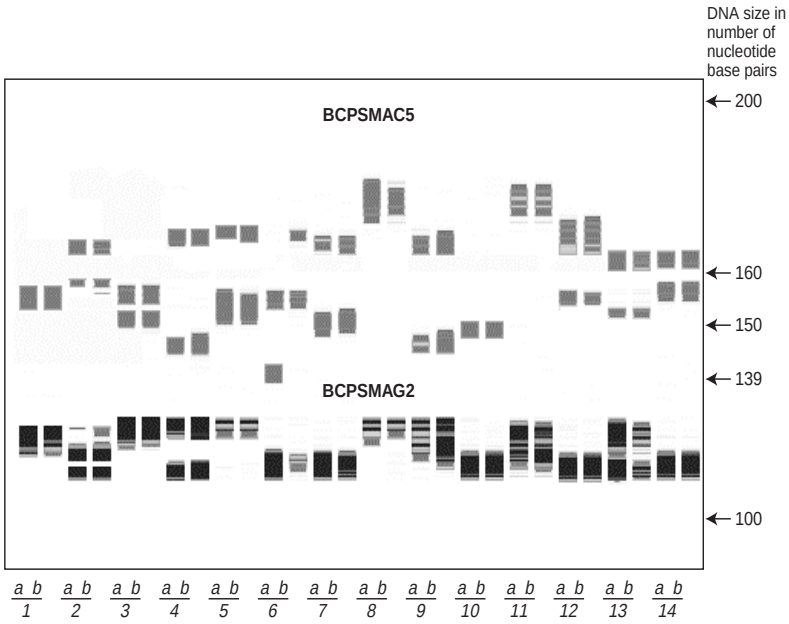

Figure 11. Gel image showing variation in DNA fragment size among 14 seed orchard clones (two ramets per clone: a and b) at two microsatellite marker loci BCPSMAC5 and BCPSMAG2. Band patterns for the a and b ramets of the same clone should be identical. This is not the case for BCPSMAC5 of clone 6, indicating that a and b are most likely ramets of different clones, probably resulting from a labeling error in the orchard.

The polymorphism available in the six putative SSR loci and one minisatellite locus already identified is impressive (Table 6). Expected heterozygosity is the probability that two alleles randomly drawn from a population will differ. Compared to the typical polymorphic allozyme locus, mean heterozygosity estimated for these SSR loci is about four times greater and the number of alleles is 3-4 times greater. The power of these SSR loci for paternity analysis (i.e., for correctly identifying male parents of offspring) is best evaluated with exclusion probabilities. Exclusion probability is the proportion of individuals from a population that can be excluded as the father of a particular offspring because they have genotypes that are incompatible with the offspring genotype at one or more loci. Thus, if paternity was investigated in a population containing 100 potential males, around 50 of the males could be excluded as the father of any particular offspring on the basis of any one of the SSR loci in Table 6, and all but one (the correct parent!) could be excluded if all seven marker loci were utilized in the analysis of paternity.

As already indicated, one goal in the coming year will be to complete the development and testing of SSR primers and to verify their polymorphism and inheritance. We will then use these markers to estimate pollen contamination in the sampled orchard block (phases 1 and 2). These materials will also be used to assess alternative methods for assessing pollen contamination, which hopefully will lead to efficient strategies for evaluating pollen contamination on a broad sample of orchards (phase 3).

Table 6. Measures of variation (Number of alleles and Expected heterozygosity) in six putative nuclear SSR marker loci and in one chloroplast ministaellite “hotspot” locus (cpSSR#17), and estimates of exclusion probabilities.

Marker	Clones sampled	Number of Alleles	Expected heterozygosity	Individual locus exclusion probability	Cumulative exclusion probability:
BCPSMAC5	18	12	0.8897	0.5581	0.5581
BCPSMAG2	18	10	0.8746	0.5348	0.7944
BCPSMAC8	15	9	0.8828	0.5369	0.9048
BCPSMAG38	13	9	0.8492	0.5096	0.9533
BCPSMAG10	19	6	0.7624	0.4510	0.9744
BCPSMAG2	17	5	0.7469	0.4363	0.9856
cpSSR#17	19	10	0.7624	0.4597	0.9922

MICRO-ORCHARD STUDY PLAN

BACKGROUND

Seed orchards are the main source of genetically superior seed needed for reforestation. Seed orchard acreage in the Pacific Northwest has more than doubled in the past 10 years with a steady annual increase exceeding 12.5%. Douglas-fir orchards comprise 74% (2, 440 acres) of the total orchard acreage in the region. According to current projections, Douglas-fir orchards will provide seed to establish 11 million acres of improved plantations by the year 2000.

With the oldest Douglas-fir orchards in the region exceeding 30 years of age, there is considerable experience in the management of "conventional" orchards, where large numbers of clones (or individuals from improved families) are planted at wide spacing in spatial patterns designed to encourage cross-pollination by wind, and where trees are allowed to achieve relatively large size (15+m tall). Early serious problems due to graft incompatibility and infrequent large flower crops have largely been solved with the development of graft-compatible rootstock and flower stimulation treatments.

There is no question that large quantities of seed can be produced in conventional Douglas-fir seed orchards, but the genetic quality of this seed is much less than desired. Pollen contamination, even in older, mature orchards with heavy pollen production, can exceed 30-40%, and mating among orchard parents is uncontrolled with wind pollination. Supplemental mass pollination (SMP, artificial application of pollen of specific males without bagging) is often touted as one means to help control mating and minimize contamination, but the actual proportion of seeds fertilized by SMP has been only modest, at best, in conventional orchards (<30%). In addition, large trees in conventional seed orchards pose serious challenges to the most important orchard management practices: application of insecticides and fungicides, controlling pollination and SMP, and harvesting cones.

Many organizations are currently moving into the second generation of breeding and considering the establishment of new orchards. Because second-generation orchards will be established using expensive first-generation selections, it is necessary that seed orchard designs ensure high genetic quality of seed. A major concern is whether traditional seed orchard designs are suited for producing desired genetic gains in timber volume and wood quality traits.

A radically different approach to seed orchard design and management is to emulate fruit-tree orchards where production is spread among many, small, easily managed trees, rather than concentrated in fewer, large trees. In addition, ramets can be established in clonal rows or blocks to facilitate artificial crossing and other management treatments. Miniaturized seed orchards are now standard practice for radiata pine in New Zealand, where seed is produced primarily by controlled pollination (CP), but research is ongoing to bring SMP to acceptable levels of success as a means of reducing seed production costs.

Douglas-fir may be more amenable than radiata pine to management in miniaturized seed orchards because it is naturally more fecund, more responsive to $GA_{4/7}$ induction treatments, and cone induction and crown pruning may be simpler because it has only a 1 1/2-year cycle from initiation of flower bud primordia to seed cones (rather than 2.5 years) (personal communication with Joe Webber, British Columbia Ministry of Forests, Victoria, BC). The po-

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tential advantages of miniaturized Douglas-fir seed orchards are several: early seed production, capacity to optimize genetic gains and versatility of breed production (by controlling mating and eliminating pollen contamination), and increased management efficiency (due to small size of trees in clonal rows). Nevertheless, management intensity and costs are greater than in conventional orchards and the quantity of seed that can be produced is not yet clear. What is needed is a study that makes it possible to compare alternative ramet spacings and management regimes on a scale large enough to evaluate realistic management needs and costs.

The main goal of the "Micro-orchard study" is to establish seed orchards with alternative, miniaturized orchard designs and compare them for quantity of flower and seed production, ease and efficiency of management, and tree health and seed quality. The study plan, which was accepted by the Cooperative's membership at this year's annual meeting is summarized in the following pages.

Objectives

1. To compare three alternative miniaturized seed orchard types for: a) quantity of flower and seed production, b) ease and efficiency of management, c) ramet health and seed quality.
2. To define the optimum age to begin flower stimulation.
3. To evaluate the ability to control crown structure by chemically managing apical dominance.
4. To compare methods of supplemental mass pollination and control pollination.
5. To determine the degree to which clones differ in their response to management treatments.

Previous Work and Present Outlook

Although basic techniques of flower stimulation through Gibberellic acid ($GA_{4/7}$) treatments and stem girdling have been well worked out in Douglas-fir, the timing of these treatments in young grafts, and tree spacing, tree height, crown form, and pruning regimes needed to optimize early and sustained flower and seed cone production have not been determined. In radiata pine, for example, miniaturized seed orchards originally were designated "hedged" orchards with grafts planted at 500/ha and pruned to a total height of around 2 m. The first four years in these orchards after establishment were devoted to developing a crown structure capable of producing abundant seed- and pollen-cone production in response to applications of $GA_{4/7}$. In a more recent variant of this concept, called the "meadow" orchard, ramets are planted at ten times the density (5000 stems/ha), with cone induction beginning only one year after establishment. Because of earlier flowering and larger numbers of stems, seed yields are expected to be greater in meadow orchards, but the projected life of these orchards is only five years.

Only a few studies on miniaturized seed orchards are currently underway in coastal Douglas-fir: Weyerhaeuser Company, British Columbia Ministry of Forests, and CEMAGREF in France. In all three of these studies, ramets have a single "micro" orchard spacing which falls

between the densities of the hedged and meadow orchards of radiata pine in New Zealand. There is no direct comparison of management needs, costs and seed yields at different ramet spacings, or with conventional orchards in these studies.

Compared to flower stimulation, there have been few investigations on ramet height and crown architecture control in Douglas-fir seed orchards. Both the BC Ministry of Forests and CEMAGREF studies include top pruning to two different target heights (2 and 3 m). Results to date in the BC study show that after three seed crops, 2 m trees, with less than half the crown volume, produce as many or more seeds than 3 m trees. Both of these studies should continue to provide useful information on the influence of top-pruning on flower and seed yields.

The goal of crown architecture control is to maximize filled seed yields on as small and compact a crown as possible.

The goal of crown architecture control is to maximize filled seed yields on as small and compact a crown as possible. A continual struggle, however, is to keep trees at the target height. Even though the central stem is top-pruned, vigorous lateral branches will turn up to take over dominance. The need to keep these laterals at bay is a major contributor to the cost of pruning. In intact trees, auxins produced in the leader limit apical dominance of laterals by suppressing cytokinin production in the laterals. Thus, it is conceivable that effective lateral branch control might be achieved by managing apical dominance in Douglas-fir by applying auxins at the top of the main stem after top pruning. Indeed, limited results in the literature suggest that this is possible. Actual success of auxin treatments in Douglas-fir seed orchards, optimal treatments, and the impacts of auxin treatment on flower and seed yields, however, are yet to be determined.

PROCEDURES

EXPERIMENTAL TREATMENTS

Experimental treatments that address each study objective are discussed below.

Objective 1. Orchard types

Three orchard types are proposed for comparison: Mini, Micro, and Macro (Table 7). All three orchard types will be managed aggressively for early and sustained flower and seed production and the leading shoots of ramets will be pruned to keep them short. In addition, lateral branches will be pruned to keep them from growing upwards beyond the target height, or they will be controlled by auxins (see Objective 3). The mini orchard is akin to the meadow orchard concept being applied to radiata pine in New Zealand. Trees are kept small to optimize pollination control (i.e., either by control pollination or SMP) with cones set only on the tops of ramets. The micro orchard follows the model of the orchard design currently being tested by the BC Ministry of Forests, Weyerhaeuser Company, and CEMACREF. Cones are produced on fewer ramets and larger crowns than the mini orchard, but pollination control is less optimal. The macro orchard serves as a control in the sense that spacing is more like conventional orchards, but ramets will be limited to 4 m to keep their height reasonable for management. Flower and seed production, seed quality, and ramet health will be routinely inventoried over time to evaluate orchard yields and health. Person-hours and supplies and equipment costs will be monitored to evaluate the economics of each orchard type.

Table 7. Planting density, spacing, and target height for the three orchard types.

Orchard type	Ramets/ha	Spacing (m x m)	Target height (m)
Mini	3, 333	3 x 1	2
Micro	1, 250	4 x 2	2
Macro	416	6 x 4	4

Objective 2. Define optimum age to begin flower stimulation

Flower stimulation treatments will contrast "early" versus "late" initiation of $GA_{4/7}$ applications (Table 8). The timing of late treatments in the micro and macro orchards is more in line with current thoughts on when (i.e., tree age) to "safely" begin flower stimulation in Douglas-fir. The question here is whether earlier stimulation will not only initiate flowering earlier, but will also lead to greater seed production in the early years of an orchard's life without harmful effects on ramet health. There will be a factorial combination of orchard type x stimulation treatments (3 orchard types x 2 stimulation ages (early vs. late) = 6 total treatments).

Table 8. Planned early and late flower stimulation ($GA_{4/7}$) treatments for the three orchard types.

Orchard type	Stimulation (Number of years after grafting)[a]	
	Early	Late
Mini	2	4
Micro	4	6
Macro	4	6

[a]For example, the first stimulation treatment would be applied in the mini orchard the second spring after grafting.

Objective 3. Evaluate the ability to control crown structure by chemically managing apical dominance

Treatments will compare the effectiveness of three concentrations (10%, 5%, and 1%) of auxins (IAA and IBA) applied artificially to the cut surface of the main stem after top pruning, on lateral shoot habit (upright vs. lateral growth), number and length of lateral branches, and cone production. This experiment will be done first on a subset of 4 clones in supplemental blocks (not part of the main study) of both the mini and micro orchard types. The need for lateral-branch pruning is likely to differ substantially between these two size classes of ramets. Thus, the effectiveness of the selected auxin treatments will be investigated in both orchard types. If results from the supplemental blocks are promising, we will to extend the investigation to include the main study.

Objective 4. Compare methods of SMP and control pollination

This experiment will compare the efficiency of one or more SMP regimes with one or more control pollination regimes in each orchard type, with regards to efficiency (cost) and

effectiveness. Because these treatments won't begin until five years after establishment (2007), their exact nature will be decided later.

Objective 5. Determine clonal variation in response to management treatments.

We plan to evaluate clonal variation in terms of ramet health, and flower and seed production in response to the orchard types, timing of flower stimulation, effectiveness of auxin, and SMP treatments.

Design of experimental orchards

Three orchard blocks, one for each orchard type, will be established in nearby locations on the same site (Figure 12, Table 9). Because orchard type will be confounded with environmental differences between the block locations, it is important that the three sites be matched as closely as possible. We assume that it won't be possible to replicate the orchard types in the traditional way (i.e., in different blocks) because of the difficulties of managing orchard types of widely divergent stem spacings. If replication of orchard types in two or more blocks is feasible, we will do this. If each orchard type is represented by a single block, the block will consist of 8 replications (main plots) of 16 clones in 5-ramet rows (clonal subplots). Initially, the two stimulation treatments (early versus late) will be assigned randomly to main plots, providing 4 replicates of each treatment in each orchard type. The experimental design is intended to allow maximum flexibility for future (adaptive) experimentation. Eight replicate main plots make it possible to apply two treatments with four replicates or four treatments with two replicates. Sixteen clones seem adequate to give an accurate assessment of average effects of treatments and to evaluate individual clone interactions.

The design also includes two small supplemental blocks within the mini and micro orchards (Figure 12). These blocks are intended initially for the auxin treatments, but may also be used for other experiments that we don't wish to risk on trees in the main orchard blocks. Each supplemental block will contain 216 trees (27 replications of 4 clones in row plots with 2 ramets each).

This study is scheduled to continue for 15 years. Layout of orchard blocks and planting of graft compatible rootstock is planned for 2001, with grafting of clones in 2002-2003. Flower stimulation treatments will commence in 2004 (Objective 2) as will the first auxin treatments (Objective 3). Flower stimulation is expected to occur every other year, with control-pollination and SMP trials conducted in the intervening (flowering) years (Objective 4). The plan allows for a total of four complete cycles of flower stimulation and pollination trials.

To evaluate the ease, efficiency, and costs of orchard management (Objective 1), a record will be kept of all work hours and tangible direct and indirect costs associated with land, equipment, and supplies utilized in maintaining each of the orchard types.

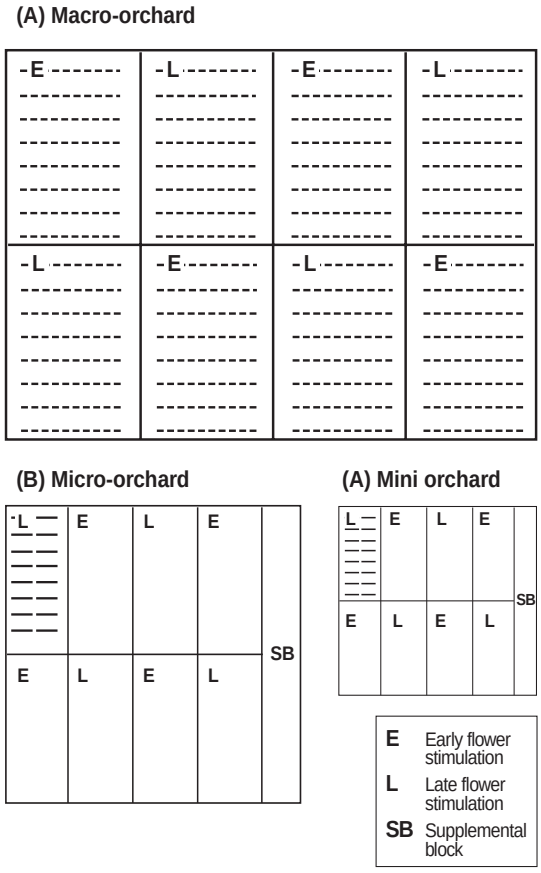

Figure 12. Design of experimental orchards

Table 9. Number of stems and land area required for the three orchard types and two supplemental blocks of this study.

Orchard Type	Spacing	Main Plots		Total Orchard	
		Stems	Area (m^2)	Stems	Area (m^2)
Macro[a] (4m target ht.)	6m x 4m = 24 m^2	80	1, 920	640	15,360
Micro[a] (2m target ht.)	4m x 2m = 8 m^2	80	640	640	5,120
Mini[a] (2m target ht.)	3m x 1m = 3 m^2	80	240	640	1,920
Supplemental[b] block for Micro	4m x 2m = 8 m^2	8	64	216	1,728
Supplemental[b] block for Mini	3m x 1m = 3 m^2	8	24	216	648
Total				2,176	24,7763[c] = 2.5ha or 6.20 acres

[a] Each orchard type block contains 8 replicate main plots, each containing 16 clones in 5-ramet row-plots.

[b]Each supplemental block contains 27 replicate main plots, each containing 4 clones in 2-ramet row-plots.

[c] The total land area required is a little larger than indicated because of unused space between and around blocks.

Data collected on seed production, ramet health and seed quality will be subjected to analysis to determine the significance of orchard type (Objective 1), timing of stimulation (Objective 2), auxin treatments (Objective 3), SMP treatments (Objective 4), and clone x treatment interactions (Objective 5).

Currently four members of PNWTIRC have offered to help establish and manage the experimental orchards on their land. We are in the final stages of selecting one of these sites.

Activities Planned for 1999-2000

A major effort this year will be to complete the data analysis for the Seedling Drought Physiology Study and to interpret the results. The goal will be to identify the most promising methods of screening for drought hardiness at the seedling stage, and to evaluate the inheritance of drought hardiness traits and the extent of genetic association between drought hardiness and stem growth potential. Likewise, analysis of the Field Drought study data will be completed. It will be interesting to determine whether drought hardiness of families evaluated at the seedling stage is related to sensitivity to drought assessed in the field. We also plan to begin designing a study to test the validity of seedling cold and drought hardiness screening for predicting adaptability to stress environments in the field.

We expect to make good headway on the pollen contamination study. Dr. Krutovskii will again work part-time on this study and a pre-doctoral student from Turkey is expected to also work on the project for 5-6 months. Our goal is to identify a set (7-10) of hypervariable SSR marker loci by confirming their polymorphism in a large sample of orchard clones and their inheritance in controlled crosses. We will then proceed to evaluate the utility of these markers by estimating pollen contamination in seed crops from one orchard.

A decision will be made on the site for the Micro-Orchard Study, hopefully, by the end of the year. We will then begin to plan in earnest pre-planting treatments and the specific layout of the orchard blocks.

Finally, an old friend and ex-student funded by the Cooperative returned to Corvallis in the fall. Dr. Jesus Vargas-Hernandez, who in 1990 completed a Ph.D. study on the genetics of wood density in Douglas-fir, will spend a sabbatical year with us. Jesus is a Professor in the Collegio de Postgraduados near Mexico City. He will be taking a fresh look at the early testing studies we completed a number of years ago to further evaluate the role of early testing for stem growth in Douglas-fir tree improvement programs. This is especially timely given our interest in using seedling screening trials to evaluate hardiness of families to cold and drought stress.

APPENDIX 1

Publications and Abstracts by PNWTIRC personnel: 1998-99

ADAMS, W.T. and J. BURCZYK. 1999. Magnitude and implications of gene flow in gene conservation reserves. In: (T. Boyle, A. Young, and D. Booshier, eds.) Forest Conservation Genetics: Principles and Practices. CSIRO, Canberra, Australia (in press).

ANEKONDA, T.S., R.S. CRIDDLE and L.D. HANSEN. 1999. Influence of age on dark respiration in eucalypts. Thermochimica Acta (in press)

ANEKONDA, T.S., R.S. CRIDDLE, M. BACCA and. L.D. HANSEN. 1999. Contrasting adaptation of two Eucalyptus subgenera is related to differences in respiratory metabolism. Functional Ecology. 13: 675-682.

ANEKONDA, T.S. and W. T. ADAMS. 2000. Genetics of dark respiration and its relationship with drought hardiness in coastal Douglas-fir. Thermochimica Acta (in press)

ANEKONDA, T.S., W. T. ADAMS and S.N. AITKEN. 2000. Cold Hardiness testing for Douglas-fir tree improvement programs: Guidelines for a simple, robust, and inexpensive screening method. Western Journal of Applied Forestry (in press)

BALDUMAN, L.M., S.N. AITKEN, M. HARMON and W.T. ADAMS. 1999. Genetic variation in cold hardiness of Douglas-fir in relation to parent tree environment. Can. J. For. Res. 29: 62-72.

DOEDE, D.L. and W.T. ADAMS. 1998. The genetics of stem volume, stem form, and branch characteristics in sapling noble fir. Silvae Genet. 47: 177-183.

LOMAS, M.C. 1999. Physiology and genetics of drought hardiness in coastal Douglas-fir seedlings. M.S. Thesis, Oregon State University, Corvallis. 101 p.

O' NEILL, G.A. 1999. Genetics of fall, winter, and spring cold hardiness in coastal Douglas-fir seedlings. Ph.D. Thesis, Oregon State University, Corvallis. 84 p.

APPENDIX 2

PNWTIRC FINANCIAL SUPPORT FOR FISCAL YEAR 1998-99

Regular members	$96,000 [a]
Associate members	12,000
Contracts	8,000
Forest Research Laboratory, Oregon State University	116,176 [b]
Total	232,176

[a] Each Regular member contributed $8,000 and each Associate member $4,000, These figures do not include in-kind contributions of labor, equipment, etc.

[b] This figure includes salaries, student tuition, use of facilities and administrative support.

The Pacific Northwest Tree Improvement Research Cooperative (PNWTIRC) was formed in 1983 in response to the need for genetics research in support of operational tree improvement programs in the Pacific Northwest. There are several types of memberships in the Cooperative. Regular members contribute directly to the Cooperative through both an annual membership fee and by supplying in-kind support including land, labor and equipment for research projects. Associate members, landowners with less than 100,000 acres, pay a smaller annual fee and do not necessarily participate in research projects to the same degree as Regular Members. Liaison members contribute to discussions on key matters but have no voting rights.

The Policy/Technical Committee guides the activities of the cooperative. It is responsible for making decisions on overall program strategy and support, identifying research problems, establishing priorities, and assisting in the planning, implementation and evaluation of studies. This committee is comprised of representatives of each cooperator.

The PNWTIRC is housed in the Department of Forest Science at Oregon State University. Tom Adams is the Leader of the Cooperative, and Thimmappa Anekonda is the Associate Director. Christine Lomas is a graduate student funded fully by the Cooperative.

This report was written by Tom Adams, Thimmappa Anekonda, and Christine Lomas.