

## CLONAL-ROW VS. RANDOM SEED ORCHARD DESIGNS: MATING PATTERN AND SEED YIELD OF WESTERN HEMLOCK (*TSUGA HETEROPHYLLA* (RAF.) SARG.)

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Received February 5, 2003; accepted July 7, 2003

### ABSTRACT

Comparisons of the mating pattern (selfing rate and level of correlated matings) and seed yield between clonal-row and the traditional random design in a western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) seed orchard were conducted. The study included: (1) a reproductive phenology survey to determine the duration of pollination season, (2) controlled selfing trial to determine clonal propensity to selfing, (3) seed yield comparison between seed orchards designs and among clones within seed orchards, and (4) determination of selfing and correlated matings rates using allozyme markers. Results indicated that the orchard has an extended, 22-day, pollination period and clones were characterized based on their reproductive activity as early, medium, and late. The majority of the 25 self-pollinated clones yielded very low seed set, however, few clones showed higher selfing propensity with percent of filled seed ranging from 8 to 29%. Seed orchard design had no significant effect on seed yield ( $p = 0.27$ ). Outcrossing rate estimates were significantly different between the clonal-row ( $t = 0.899$ ) and random ( $t = 0.970$ ) seed orchards, and both estimates significantly departed from complete outcrossing (*i.e.*,  $t = 1.0$ ), indicating that selfing is higher in the clonal-row design. Estimates of correlated mating substantially varied between the two seed orchard designs with 35 and 8% for the clonal-row and random seed orchards, respectively, demonstrating genetic quality differences of seed produced from the two seed orchard designs. It was concluded that if clonal-row design is considered for advanced generation seed orchards, then a slight modification involving staggering the clonal-rows will allow each clone to be flanked by four other clones. Together with the use of effective pollen management such as carefully timed, clone-specific, multiple supplemental-mass-pollination applications may reduce selfing and correlated matings.

**Key words:** *Tsuga heterophylla* (Raf.) Sarg., seed orchard design, seed yield, reproductive phenology, mating system, pollen management.

### INTRODUCTION

The success of tree improvement programs in British Columbia is evident from the establishment of operational advanced-generation seed orchards. Research on first-generation seed orchards concluded that traditional wind-pollination was efficient for seed production; however, most of the assumptions required for maximum genetic efficiency were not fulfilled (EL-KASSABY 1989). Replacing wind-pollinated, first-generation seed orchards with advanced-generation is expected to increase the genetic worth of seed crops. The transition from first- to advanced-generation seed orchards requires the consideration of alternative seed orchard designs that will enable the maximization of genetic worth of future seed crops per unit time (NAM-KOONG *et al.* 1966). WILLIAMS and ASKEW (1993) presented several options for advanced-generation seed orchards and demonstrated that the traditional random

design, commonly used in first-generation seed orchards is not efficient in capturing the potential for advanced-generation selections.

Establishment of new seed orchards with advanced-generation selections, and replacement of older ones is a common practice. This model is usually associated with delayed seed production, which depends upon the time by which advanced-generation orchards' clones reach their sexual maturity. WILLIAMS and ASKEW (1993) defined the concept of seed orchard's economic gain as the difference between the genetic values of an advanced-generation seed orchard's crop and that of a previous generation orchard. Economic gain must be taken into account when considering the delay of time till the advanced-generation orchards to reach sexual maturity, and the practice of genetic upgrading of the existing first-generation orchards by roguing and/or by using supplemental-mass-pollination (SMP). Newly established orchards may then conceivably produce

seed crops with lower genetic gains than older up-graded first-generation mature seed orchards. Thus, the discrete, static, generation-type orchards provide gains associated with the degree of development of the breeding program and the opportunity to enhance this gain through roguing and/or SMP (ASKEW 1992).

The arrangement of clones within orchards is assumed to be random, so that minimum inbreeding and maximum outcrossing through the mixing of pollen occur. If seed crops is to be collected separately by individual clone, then randomization of ramets belonging to different clones within the seed orchard will impose additional effort to locate these ramets during crop management activities. In general, seed orchards' management efficiency, with respect to reproductive bud assessment (to determine the expected crop size) and reproductive phenology assessment (to determine optimum receptivity time) for SMP application will require additional effort under any clonal randomization scheme. GREENWOOD (1983) has advocated clonal-row seed orchard design as an alternative that should be given serious consideration. Previous evaluation of this seed orchard design (*i.e.*, clonal-row) was restricted to seed yields only (BRAMLETT & BRIDGWATER 1987).

Orchard designs that allow placement of ramets of individual clones in rows would ease the management efficiency, however, mating patterns are expected to be affected. Thus, the genetic evaluation of this type of seed orchard design is necessary due to the non-random placement of clonal ramets in the seed orchard layout.

In this paper, the mating-system patterns (selfing and correlated matings) and seed yield attributes are compared in a western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) seed orchard consisting of two sections with different clonal arrangements: random and clonal-row. Western hemlock provided a unique opportunity for this study for the following reasons: (1) the species has: a – unique pollination biology characterized by true pollen competition (*i.e.*, increased chance of success of unrelated pollen) and a long receptivity period (COLANGELI & OWENS 1989, 1990) and b – discrete positioning of male and female reproductive buds on the tree where most male cones are restricted to the lower branches (COLANGELI & OWENS 1988), (2) wide reproductive phenology differences among clones/ genotypes (*i.e.*, higher chance of selfing and correlated mating), (3) orchard tree height is kept at  $\leq 3$  m, facilitating pollen management, (4) all western hemlock orchards rely on supplemental-mass-pollination (SMP) as a standard pollen management practice, and (5) pollen contamination in the study area is extremely low, thus seed yield and mating pattern

results do not suffer from confounding factors (EL-KASSABY & RITLAND 1986).

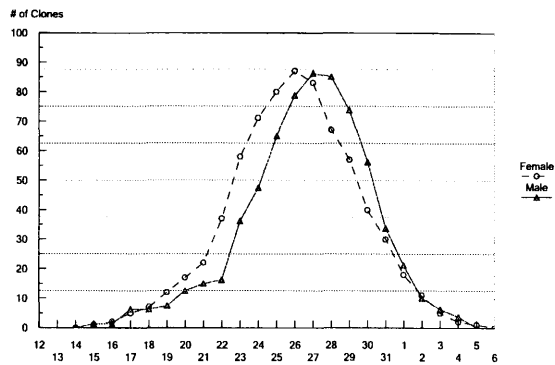
## MATERIALS AND METHODS

### Seed orchard description

This study was conducted in a 1.1 ha clonal western hemlock seed-orchard located in Sannichton, British Columbia (B.C., latitude 48° 35' N, longitude 123° 24' W, elevation 50 m). The orchard consists of two unequal adjacent sections: the larger section (0.87 ha) is planted in a random single-tree mix (random) and the smaller section (0.22 ha) is planted in a clonal-row arrangement (clonal-row). The orchard was established in 1977; however, newly grafted trees of the same genotype are being planted throughout the orchard for mortality replacement. The replacement grafts varied in their reproductive maturity based on the time they were included in the seed orchard. The orchard comprises 102 clones (average number of ramets/clone = 7.5) selected from elevations between 5 and 770 m on western Vancouver Island and the coastal mainland of B.C. Trees are spaced 3 m apart (within and between rows) and tree height is kept at approximately 3 m by top pruning. The standard cone crop management of this orchard involves application of supplemental-mass-pollination (SMP) usually following reproductive phenology assessments to identify receptive trees. Management of western hemlock seed orchards cone crops in the Saanich Peninsula area of southern Vancouver Island, where this orchard is located, usually requires SMP due to the relative positions of male and female reproductive buds on the tree (*i.e.*, most male cones are restricted to the lower branches (COLANGELI & OWENS 1988)), SMP treatments during the pollination season involved the use of six pollen mixtures, originating from 12 to 51 (average = 21.7) male donors. Pollen mixes consisted of equal quantities of pollen from each male donor. SMP was performed by using a hand-operated insecticide sprayer with a tubular wand. Pollen was pumped through the device until it was observed emitting from the end of the wand. The amount of pollen applied was not completely controlled, but was assumed sufficient to saturate receptive seed-conelets.

### Reproductive phenology

Reproductive-bud phenology data for the entire orchard (every sexually active tree) were collected by monitoring trees every second day throughout the 1992 pollina-



**Figure 1.** Diagram of reproductive phenology development of western hemlock seed orchard throughout the 1992 (March - April) pollination season showing the number of clones with receptive seed- and pollen-shedding cones.

tion season. The reproductive phenology classification stages for seed- (female) and pollen-cones (male) of COLANGELI & OWENS (1988, 1989) were used. The critical stage (duration) of seed-cone receptivity started after bud burst (stage 3) and ended after ovuliferous scales had covered the bracts (stage 8). The time between the start and end of pollen-shedding was used to identify the critical stage for male reproductive cones (stage 8). Reproductive phenology was used to identify receptive trees for SMP treatment and to develop a reproductive phenology development/choreography curve (EL-KASSABY *et al.* 1984) that depicted the extent of the pollination season, showing the number of reproductively active tree or clones on any given day (Fig. 1).

### Selfing/inbreeding rate

In order to determine clonal differential propensity for selfing, a total of 25 clones were selected for this experiment. It should be re-emphasized that the discrete positioning of male and female reproductive buds on western hemlock trees (*i.e.*, most male cones are restricted to the lower branches while female cones are located throughout the tree (COLANGELI & OWENS 1988)) affected clonal selection for selfing determination. The availability of clusters of seed- and pollen-cones on several branches on the same tree was the main selection feature. Prior to the beginning of the pollination season, isolation bags with polyethylene windows were placed on four branches of each selected ramet/clones. Seed- and pollen-cone development was monitored through the polyethylene windows and self-pollination was promoted by shaking the branches several times during pollen-shedding and seed-cone receptivity. The pollination bags were

removed after the end of the pollination season, and branches were labelled. In the fall, prior to seed shedding, cones were collected, placed in open paper bags and stored in an open-sided, freely-ventilated shed for 12 weeks prior to seed extraction. Dry cones were hand-dissected and all scales were examined for developing seeds. Filled and empty seeds were determined using X-ray methods, and percent of filled seeds (an estimate of actual selfing rate) was determined for each clone (by dividing the number of filled seeds by the total number of seeds). A total of 74,452 seeds was examined.

### Seed yield

Seed yield between the clonal-row and random seed orchard designs was compared by collecting a random sample of 45 cones from each one of the 66 (clonal-row; average: 3.7 ramets/clone, range: 1 to 9) and the 57 (random; average: 1.2 ramets/clone, range: 1 to 3) ramets that covered the same seed orchard area. Seed extraction method and the determination of the number of filled seed per tree (ramet) for each clone were similar to that reported in the selfing experiment above. The number of filled seed per cone was determined by dividing the total number of filled seed by the number of cones sampled from each tree. An unbalanced nested analysis of variance (ANOVA) model was used to compare the filled seed set of the two orchard designs using the GLM procedure (SAS Institute 1993). Preliminary analysis indicated a significant difference ( $P < 0.0001$ ) in the number of seeds per cone among clones caused by variation in cone size, thus the total number of seeds per cone was used as a covariate for filled seed per cone comparison (SAS Institute 1993).

### Outcrossing rate and correlated matings

Seed samples were collected from 18 and 20 clones representing the clonal-row and randomized seed orchard designs, respectively. Forty seeds per ramet from each clone were soaked in distilled water for 24 hr and the megagametophytic ( $1n$ ) and corresponding embryonic ( $2n$ ) tissues of each seed were assayed electrophoretically for 10 enzyme loci: phosphoglucose isomerase (*Pgi1*, *Pgi2*), phosphoglucosyltransferase (*Pgm1*), aspartate amino-transferase (*Aat1*, *Aat2*), malate dehydrogenase (*Mdh4*), aconitase (*Aco*), glutamate dehydrogenase (*Gdh*), and glucose-6-phosphate dehydrogenase (*G6pd1*, *G6pd2*) using two gel-electrode buffer systems (Tris citrate pH 7.0 (SICILIANO & SHAW 1976) and Tris citrate: lithium borate pH 8.5 (RIDGEWAY *et al.*

**Table 1. Probability value (*P*) for the 1:1 segregation ratios of 10 polymorphic loci in western hemlock seeds.**

Locus	# of trees	Genotype	Observed ratio	Pooled $G^1$ ( <i>P</i> )	Heterogeneity $G^2$ ( <i>P</i> )
<i>Pgi1</i>	7	1:2 <sup>3</sup>	143 : 131	0.433	0.691
<i>Pgi2</i>	2	1:2	37 : 43	0.502	0.823
<i>Pgi2</i>	3	1:3	73 : 55	0.112	0.074
<i>Mdh4</i>	10	1:3	213 : 191	0.284	0.381
<i>Pgm1</i>	7	1:2	129 : 149	0.230	0.148
<i>Pgm1</i>	3	1:3	59 : 61	0.855	0.048
<i>G6pd1</i>	5	1:2	88 : 95	0.608	0.369
<i>G6pd1</i>	2	1:3	40 : 40	1.000	1.000
<i>G6pd2</i>	10	1:3	200 : 197	0.880	0.801
<i>Aco</i>	7	1:3	166 : 106	0.003	0.911
<i>Gdh</i>	18	1:3	367 : 358	0.738	0.043
<i>Aat1</i>	1	1:3	21 : 19	0.752	-
<i>Aat2</i>	1	1:3	22 : 26	0.564	-

<sup>1</sup> Pooled *G* values indicate the overall deviation from 1:1 ratio.

<sup>2</sup> Heterogeneity *G* values indicate the amount of heterogeneity in the segregation ratio among trees.

<sup>3</sup> Allozymes were numbered starting with "1" for most common alleles; faster and slower alleles were given even and odd numbers, respectively.

1970)). The staining as well as allelic designation methods used followed those of CONKLE *et al.* (1982) and O'MALLEY *et al.* (1980) (see Table 1 for allelic designation). The Mendelian inheritance of the studied loci has been examined (megagametophytes of the heterozygous trees are expected to segregate in a 1:1 ratio) using the heterogeneity and pooled *G*-tests, modifications of the  $\chi^2$  contingency test (STEEL & TORRIE 1980) on pooled data from trees sharing the same locus' allelic combination at each locus in their megagametophytic tissue (Table 1).

Seven loci (*Pgi1*, *Mdh4*, *G6pd2*, *Aco*, *Gdh*, *Aat1*, and *Aat2*) were diallelic while the remaining three loci (*Pgi2*, *Pgm1*, and *G6pd1*) were triallelic (Table 1). With the exception of *Aco*, the segregation analyses for all the observed pooled allelic combinations did not differ significantly from the expected 1:1 ratio (Table 1), indicating that these allozymes exhibited distinct, co-dominant expression and simple Mendelian segregation in their mode of inheritance. Segregation ratios among the seven *Aco* heterozygote trees showed consistent bias for one allele. This was confirmed by the observed non-significant ( $P = 0.911$ ) heterogeneity test among the segregation ratios of the seven trees that showed segregation (Table 1). The *Aco* locus was not included in the mating system parameter estimations. The heterogeneity *G*-test among trees' segregation ratios was significant for two allelic combinations (*Pgm1* and *Gdh*) (Table 1); however, close examination of these allelic combinations on an individual-tree basis indicated lack of systematic bias towards any specific allele (data not given).

Multilocus estimates of outcrossing rate ( $t_m$ ) were

estimated for each seed orchard design as well as for individual clones within each seed orchard using the maximum likelihood procedure of RITLAND and EL-KASSABY (1985). The correlated matings ( $r_p$ , the percentage of seeds attributed to full-sib matings) were estimated following the maximum likelihood bootstrap method of RITLAND (1989).

## RESULTS AND DISCUSSION

Understanding the pollination mechanism of a species, its reproductive phenology and their interaction are fundamental prerequisites for effective seed orchard management. The "Velcro-like" pollination mechanism of western hemlock has been recognized as unique among the *Pinaceae* (COLANGELI & OWENS 1989). Western hemlock's pollen is characterized by roughly sculptured pollen grains that adhere to the epicuticular wax layer of the exposed ovuliferous bract. Pollination starts with pollen adherence to the bract followed by the elongation of the ovuliferous scales, thus trapping the pollen between the bracts and the scales (*i.e.*, a passive pollination mechanism in which the anatomical structures do not participate in directing the pollen towards the nucellus). Trapped pollen grains then germinate and pollen tubes grow towards the micropyle where true pollen competition occurs (COLANGELI & OWENS 1989). This type of pollination mechanism effectively extends the receptivity period of seed-cones. In fact, COLANGELI and OWENS (1989) recorded the period between seed-cone bud burst and closure in western hemlock and concluded that it is the longest among the

studied conifers. They also observed year-to-year variation of the duration between bud burst and cone closure for the same genotypes, indicating that it is sensitive to environmental factors. COLANGELI and OWENS (1989) indicated that the period between bud burst and ovuliferous scale elongation (4 to 7 days) also represents the pollination period contributing to maximum seed yield. In addition, they reported that the duration of pollen shed ranged from 4 to 10 days. This indicates that SMP could be an effective pollen management tool and that the timing and extent of pollination season depend on the local environmental conditions of the seed orchard.

The pollination season of the studied seed orchard (encompassing seed-cone receptivity and pollen-shedding) covered 22 days and extended from March 15<sup>th</sup> to April 5<sup>th</sup> (Fig. 1). Maximum seed-cone receptivity coincided with the peak of pollen shedding (only one day difference) (Fig. 1). Receptive seed-cone bearers outnumbered pollen producers only during the first half of the pollination season, while during the second half the reverse was noticed (Fig. 1). The reproductive phenology choreography of the seed orchard's clones (relative number and their timing) indicates that the number of early and late pollen-shedding clones, and subsequently the amount of pollen available for pollination is not adequate to fertilize receptive seed-cones. Although western hemlock has an extended pollination period, increasing the chance for pollination and fertilization, it should be emphasized that pollination success is related to the proportion of bracts exposed for pollen to adhere (COLANGELI & OWENS 1989). SMP is used as a standard pollen management practice due to the following reasons: (1) low number of pollen-shedding clones during the early and late phenological classes, (2) relatively short pollen release period, (3) position of pollen-cones on the tree, (4) the within-orchard spatial distribution of pollen-shedding clones relative to receptive clones, and (5) lack of background pollen in the seed orchard site. The duration of the pollination season and the distribution of western

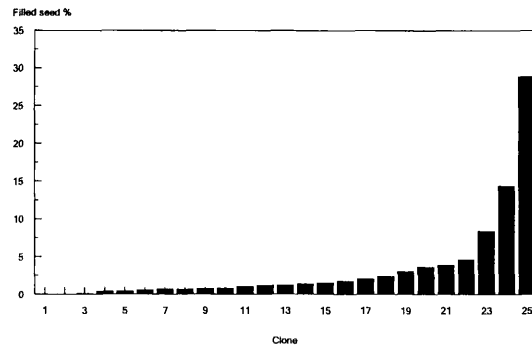


Figure 2. Percent of filled seeds for 25 studied western hemlock clones after selfing. Clones are presented in increasing order of their selfing rate.

hemlock clones observed in this seed orchard, is similar to other species' seed orchards that were studied in the Sannich Peninsula that also were characterized by relatively few pollen donors during the early and late phenological classes and extended pollination periods (e.g., Douglas-fir (*Pseudotsuga menziessii* (Mirb.) Franco), EL-KASSABY *et al.*, 1984 and Sitka spruce (*Picea sitchensis* (Bong.) Carr.), EL-KASSABY & REYNOLDS 1990).

For seed orchards, to be effectively managed, obtaining base-line genetic information of their constituent clones is essential. Clonal genetic information such as breeding value, reproductive phenology (see above), reproductive output mode, and selfing ability could be used to maximize the genetic worth of seed orchard crops (WOODS *et al.*, 1996). Prior information regarding clones' selfing propensities would be important for seed orchard design and consideration during pollen management (e.g., SMP application).

Results from the selfing trial indicated that the overall selfing rate (mean = 3.4 %, SD = 6.2) expressed by filled seed percent, is generally low in western hemlock (Fig. 2). However, it should be emphasized that a few clones showed higher propensities for selfing (8-29 %) (Fig. 2). These clones should be the targets of increased attention during the SMP application.

Table 2. Analysis of covariance for filled seed per cone comparison between clonal-row and random seed orchard designs of western hemlock.

S. O. V.	D. F.	S. S.	F	P
Orchards	1	8.105	1.23	0.273
Clones/orchards	63	1212.505	2.91	0.0001
Covariate <sup>1</sup>	1	480.907	72.81	0.0001
Residual	56	369.882		

<sup>1</sup> Covariate = total number of seeds per cone.

**Table 3. Estimates of multilocus ( $t_m$ ), and correlated paternity ( $r_p$ ) for the western hemlock clonal-row and random seed orchard designs (standard deviations are given in parentheses).**

Parameter	Clonal-row	Random
$t_m$	0.899 (0.037)	0.970 (0.021)
$r_p$	0.349 (0.166)	0.077 (0.032)
$N^1$	18	20
$n^2$	736	796

<sup>1)</sup> # of clones sampled.

<sup>2)</sup> # of seed censused.

The seed yield (filled seed per cone) comparison showed no significant difference ( $P > 0.05$ ) between the clonal-row and random seed orchard designs, after adjusting for cone size (Table 2). As expected, differences among clones within the seed orchard were observed and were highly significant ( $P < 0.0001$ ) (Table 1). These results indicate that when SMP is applied, seed orchard design has no effect on seed yield; however, the differing genetic quality of seed yield between the two seed orchards would be informative for providing insight of within-seed orchard pollen dynamics even with only one SMP application (see below).

Multilocus outcrossing rate estimates ( $t_m$ ) were 0.899 (SE = 0.037) and 0.970 (SE = 0.021) for the clonal-row and random seed orchards, respectively (Table 3). These estimates differed significantly from  $t = 1.0$  and the difference between the two seed orchard designs was significant, indicating that selfing was higher in the clonal-row than the random (Table 3). Higher selfing in the clonal-row seed orchard design is expected due to the proximity of ramets of the same clone. However, the observed selfing rate in the clonal-row seed orchard was higher than expected even after the application of unrelated pollen through SMP. It seems that the within-orchard pollen cloud and the SMP application were therefore adequate to saturate receptive ovules as indicated by the seed yield comparison, although, related pollen from other ramets of the same maternal clones were successful in fertilization even after the application of unrelated pollen. Multiple SMP applications over the extended receptivity period of each clone will increase the diversity of pollen mixture and thus increase the chance for outcrossing via pollen competition.

Individual clone outcrossing rate estimates varied among clones within both seed orchard designs (random: 0.37 to 1.0, clonal-row: 0.54 to 1.0). The percentage of clones with complete outcrossing ( $t = 1.0$ ) in the

random and clonal-row seed orchards was 45 and 28, respectively. Additionally, the percentage of clones with outcrossing rates that is  $> 0.9$  in the random and clonal-row seed orchards was 80 and 61, respectively, confirming that the chance of inbreeding is higher in the clonal-row design. Furthermore, the pollination mechanism of the species with its extended receptivity permits self-pollen to adhere to the epicuticular wax layer of the exposed bract and compete for space with unrelated pollen, indicating that one SMP application was not effective to saturate most of the receptive sites with optimal (*i.e.*, unrelated) pollen. It is also noteworthy that one clone in the randomized seed orchard had an outcrossing rate of  $t_m = 0.37$  indicating higher propensity for selfing even with clonal randomization and SMP application supporting the results of the controlled selfing experiment. It should also be mentioned that the observed within seed orchard design differences in outcrossing rate is a reflection of the genetic differences among clones.

The correlation of outcrossed paternity (*i.e.*, % of full-sib seeds) varied significantly between the clonal-row (0.349) and random (0.077) seed orchards, as the first value is five-fold higher (Table 3). Each clone in the clonal-row seed orchard is spatially contiguous with two different clones, thus higher chances for correlated mating. Again, these results were surprising after SMP was applied, indicating that one application is not adequate as a pollen management option. Slightly modifying the original clonal-row seed orchard design, in which each clone is flanked by additional two other clones, should reduce both selfing and correlated mating. This can be accomplished by staggering adjacent rows so that each individual clone will be flanked by four unrelated clones.

The advantage of management ease in clonal-row seed orchards should be weighted against the results reported above (*i.e.*, increased selfing and correlated matings). For western hemlock, pollen management strategy that is dependent on SMP is justified due to the unique reproductive biology attributes of the species. However, the development of effective pollen management systems that rely on multiple SMP applications with proper timing to ensure the saturation of receptive sites should improve the genetic quality of the crop.

The replacement of first-generation seed orchards with second- and/or advanced-generations is expected to result in increased genetic worth of seed crops. This replacement requires consideration of alternative seed orchard designs that maximize the genetic worth of future seed crops and facilitate management.

## ACKNOWLEDGEMENTS

I would like to thank S. Barnes and C. Cook for technical assistance, K. Ritland for providing the correlated mating computer program, D. Lindgren for stimulating discussion, and G.R. Askew and J. Krakowski for valuable comments and suggestions.

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