The Detection of Good Selfers for Haploid Induction in Douglas-Fir[1])

By R. F. PIESCH[2]) and R. F. STETTLER[3])

Introduction

The value of haploid tree material and the ways to produce it have been discussed in several papers (STETTLER 1966, STETTLER, BAWA and LIVINGSTON 1969, STETTLER and BAWA 1969). This paper is a further contribution to the topic and presents data on the selection of suitable genetic material for haploid-induction experiments in Douglas-fir, *Pseudotsuga menziesii* (MIRB.) FRANCO. Several features make Douglas-fir an useful species for haploid studies: (a) it is basically diploid ($2n = 26$); (b) it has a short period for seed maturation — four to five months; (c) it can be propagated vegetatively; (d) its chromosomes are easy to count; (e) in some trees, selfing occurs with good success, suggesting past inbreeding and, thus, reduced heterozygosity. Douglas-fir is commercially important, and findings concerning its breeding methods are of economic significance. Rather than using randomly selected trees for haploid induction, we decided to base our initial studies on good selfers, i. e., individuals with a relatively high selfing performance. The absence of deleterious recessives in good selfers would increase the probability of haploid success. Previous selfing studies in Douglas-fir (ALLEN 1942, DUFFIELD 1950, ORR-EWING 1954, 1957 a, 1957 b, 1965, 1969, WHEAT 1965, SZIKLAI 1966, and SORENSEN 1969) showed a wide range of selfing performance, from nearly complete self-sterility to nearly cornplete self-fertility, with an average selfing yield (ratio of selfed seed per cone/outcrossed seed per cone) of about 10 percent. The best selfers ranged from 20 to 30 percent; one exceptional tree had a selfing yield of 96.8 percent (ORR-EWING 1957 b).

To increase the chance of finding good selfers we attempted to select "putative" selfers, i. e., trees giving indirect evidence of above-average selfing. Putative selfers were trees well isolated from other Douglas-fir, but which showed heavy cone crops for at least two previous years. The assumption was that cone production implied seed production. Previous studies have suggested that the dispersal distance of conifer pollen is minimal for single trees, but may be many miles for large stands, especially during years of heavy pollen production (WRIGHT 1952, SARVAS 1955, SILEN 1962, and LANNER, 1966).

Finally, there was some indication that trees from different geographic origin vary in their selfing ability. Studies in British Columbia most strongly suggested this (ALLEN 1942, ORR-EWING, 1954, 1957 b, and SZIKLAI 1966). SORENSEN (1969), however, found no significant difference among five locations in Oregon in the selfing yield of sampled trees. In view of the different breeding histories of different populations, we felt that putative selfers should be selected in more than one population.

The purpose of this study was to screen three well-separated Douglas-fir populations for good selfers, ideally trees carrying genetic markers, which would render haploid-induction experiments more efficient.

Materials and Methods

Three areas, Silvana, Olympia and Cle Elum, Washington were chosen for the selfing test (*Table* 1). All three are prairie or farming areas, containing many isolated Douglas-fir trees. In each area, isolated, heavy cone-producing trees (putative selfers) and stand trees (controls) were selected. Isolation minima were 200 yards to the closest neighbor tree or half a mile to the closest stand in the case of well-isolated trees, and 100 or 400 yards, respectively, for semi-isolated trees.

A total of 33 trees were selected and all were both selfed and outcrossed. Two selfing techniques were used: self-shaker — enclosing intact male strobili with the female strobili, and shaking the pollination bags at the time of pollen release; and self-syringe — removing the male strobili before bagging, and applying self-pollen with a hypodermic syringe. Fifteen trees were selfed by the shaker method only, the remaining 18 by both the shaker and syringe methods. The outcross pollen was a mix from four pollen parents, two each from the other two study areas.

In the fall, the mature, dry cones were dissected and the seeds extracted by hand. Only round and apparently filled seeds were kept. X-ray photography was then used to

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Table 1. — Description of Study Areas.

General Locality	Specific Location	Ecological Description	Number of Test Trees: Putative Selfers*)	Stand Trees	Total
Silvana	Snohomish Co., Wash. T.32N. R.4E.	Farmland. Isolated trees occurring as single remnants of original forest. Surrounding forests composed mostly of Douglas-fir, western hemlock, western red cedar and red alder.	9	2	11
Olympia	Thurston Co., Wash. T.16N. R.3W. T.18N. R.1W.	Prairie probably maintained by past recurrent natural fires Isolated trees remaining. Surrounding forests composed mostly of Douglas-fir.	9	2	11
Cle Elum	Kittitas Co.. Wash. T.20N. R.17E.	Present forest-sagebrush transition Eastern edge of the Douglas-fir range of central Washington. Surrounding forests composed mostly of ponderosa pine with scattered Douglas-fir.	9	2	11

*) includes both "well-isolated" and "semi-isolated" trees.

separate the hollow from the filled seeds. Following stratification, all the selfed and outcrossed seeds and up to 50 open-pollinated seeds per tree were sown in pots in "Cornell Soil Mix", i. e., 50 percent peat, 50 percent vermiculite, by volume, plus fertilizers. The pots, placed in a completely randomized design on two benches in the greenhouse, were watered every second day, and once a month a complete HOAGLAND's Solution (HOAGLAND and ARNON 1938) was included in the watering process. No artificial lighting was used and greenhouse temperatures for the six months of seedling growth averaged 32 to 35 C during the day, 21 to 24 C in the evening, and 16 C at night, with occasional daytime extremes to 41 C. At weekly intervals, the seedlings were inspected for the detection of deviant traits.

Results and Discussions

At the time of cone collection in September, 31 of the original 33 trees remained in the study. Two trees at Silvana were felled inadvertently during the summer by utility company personnel.

Selfing Performance:

Both hand-pollination techniques — self-syringe and outcross — produced very low seed yields. No explanation can be offered for this phenomenon since commonly accepted standards were observed with respect to techniques and timing of hand pollinations. Because of the failure, only self-shaker seed yields will be used to estimate selfing performance in the following discussion.

Table 2 presents the selfing yields for the 31 trees. Fertilization in Douglas-fir takes place after the cone and potential seeds have reached nearly full size (ALLEN 1942). Accordingly, seed-yield comparisons should be based on round seeds only (LYONS 1956). The assumption is that only round seeds are potentially fertile, whereas flat seeds are already flat at the time of fertilization and remain so. In *Table 2*, the numbers of filled seed per cone were adjusted to 100 round seed per cone. This adjustment puts the

Table 2. — Selfing Performance of the 31 Test Trees.

Area and Tree Number	Type of Tree	Total Number of Self-Shaker Cones	Adjusted Number of Filled Selfed Seed Per Cone[1])	Estimated Selfing Yield[2])
Cle Elum				
C-5	Semi-isolated	47	45.36	.655
C-11	Stand tree	7	18.21	.263
C-2	Well-isolated	85	12.98	.187
C-6	Semi-isolated	36	12.00	.173
C-8	Well-isolated	62	5.93	.086
C-9	Well-isolated	47	5.61	.081
C-1	Well-isolated	37	3.36	.048
C-7	Semi-isolated	98	2.95	.043
C-10	Semi-isolated	160	2.73	.039
C-3	Well-isolated	61	2.05	.030
C-4	Stand tree	115	1.39	.020
Mean				.148
Silvana				
S-1	Semi-isolated	30	11.95	.173
S-9	Well-isolated	97	11.24	.162
S-2	Semi-isolated	58	8.84	.128
S-5	Well-isolated	16	3.97	.057
S-10	Semi-isolated	98	3.96	.057
S-7	Stand tree	24	2.26	.033
S-6	Semi-isolated	23	1.81	.026
S-11	Semi-isolated	37	1.31	.019
S-4	Well-isolated	4	0.00	.000
Mean				.073
Olympia				
0-10	Semi-isolated	165	8.16	.118
0-9	Well-isolated	45	5.97	.086
0-1	Well-isolated	42	3.86	.056
0-5	Semi-isolated	87	2.57	.037
0-4	Semi-isolated	20	1.69	.024
0-3	Semi-isolated	55	1.58	.023
0-11	Semi-isolated	40	0.45	.006
0-2	Well-isolated	78	0.28	.004
0-8	Well-isolated	64	0.22	.003
0-7	Stand tree	109	0.11	.002
0-6	Stand tree	153	0.05	.001
Mean				.034

[1]) Adjusted to 100 round seed per cone.
[2]) Adjusted number of self-shaker seed per cone/outcrossed seed per cone. Outcrossing data based on SORENSEN (1969) where Grand Mean of 35 trees equaled 69.2 filled seed per 100 round seed.

fertilization potential of all trees on more comparable terms.

Selfing yields are customarily expressed as percentages of outcrossing yields. Occasionally, open-pollinated data are substituted for outcrossing, but only under specific conditions is such a substitution valid. The use of open-pollinated data for estimating selfing performance was not satisfactory in this study because most trees were isolated and, as a consequence, their open-pollinated seeds may have been possibly or entirely the result of selfing.

Since cross-pollination had failed in our trees, a standard outcrossing figure was taken from Sorensen (1969). The figure was a mean determined from breeding studies conducted in 1964—66 on 35 Douglas-firs at five locations in western Oregon. Although we have no way of judging the applicability of Sorensen's figure to the performance of our trees in 1966, the 1966 cone crop in Oregon was comparable to that in Washington. Successful outcrossings performed in 1968 on seven of our 31 trees gave an average of 56.4 filled seed per cone (Livingston unpubl. data), thus somewhat less than Sorensen's figure of 69.2. If the self-shaker technique, as compared to the direct application of self pollen, may underestimate the selfing performance by 29 percent (Sorensen 1969), we can assume that the application of Sorensen's figure will give a conservative estimate. Finally, any errors will probably apply to all trees equally and will not affect the relative rankings.

Table 2 shows several trees to be well above the mean selfing performance of their area, particularly tree No. C-5, which stands out with an estimated selfing yield of 65.5 percent. Generally, putative selfers ranked higher than stand trees. The exception, stand tree No. C-11, had only seven cones on which to base an estimate. No obvious difference was found, however, between well-isolated and semi-isolated trees. The means of the three areas differed, the Cle Elum trees giving twice as high a value as those from Silvana, and four times as high as those from Olympia. These area means, 14.8, 7.3 and 3.4 percent, respectively, fell within the range of values found in previous studies.

Our criteria for selecting putative selfers were apparently successful. However, they may not be applicable to all stands or under all conditions. Isolated trees may not be available in certain populations; heavy cone crops on isolated trees may be the result of parthenocarpy. Since parthenocarpy is common in Douglas-fir, the selection scheme could be made more reliable by checking open-pollinated cones for sound seed. The reliability could be further increased by conducting the selection during a medium, or poor, crop year during which pollen contamination from neighboring stands would be minimal. Lastly, a given tree may show variable selfing performance in different years and shift its rank relative to others (Orr-Ewing 1968, personal communication). Unquestionably, a mean figure determined from several years' selfings would more adequately represent the tree's genetic potential for haploid performance.

The germination percentages of both self- and open-pollinated seeds were uniformly high. The only two large selfed families showing poor germination were S-9 (79%) and 0—10 (73%), both of which contained putative lethal markers. Germination and survival data may contribute to the selection of genetic material suitable for haploid induction studies, particularly as the early emphasis on the study of induction stimuli may shift to the perpetuation of haploids or homozygotes. Good selfers carrying post-embryonic lethals may be perfectly tolerable in the early phase but highly undesirable later on.

Several models could be used to extrapolate from the selfing performance to the putative haploid performance. However, a discussion of their applicability will be given when data on the haploid performance have been evaluated (Stettler, Bawa, and Livingston 1969, Livingston unpubl. data).

Genetic Markers:

The progenies in five selfed families showed various forms of discontinuous variation following germination, with normal: deviant ratios ranging from 1.75:1 to 10.42:1. These deviant forms will be referred to as mutants. The gene(s) that they carry, presumably in homozygous condition, will be referred to as marker gene(s) or simply marker(s). *Table 3* gives the descriptions and relative frequencies of the mutants. The numbers of seedlings per class are presented and the observed ratios are calculated to facilitate all possible interpretations of the data. The reason for this is that most seed lots had less than 100 percent germination and the non-germinants may have represented either normal or mutant seedlings, or both.

The "yellow-green", "albino" and "mottled" mutants were easily recognizable and more distinct than the "yellow" and "curly" mutants. Assuming that non-germinants were randomly distributed among normal and mutant classes, the albino and yellow-green conformed to a 3 : 1 ratio at the .95 probability level (Chi-square test). The yellow mutant conformed to both a 3 : 1 and 9 : 7 ratio, depending on whether the non-germinants represented normal or mutant seedlings. The mottled mutant conformed to a 15 : 1 ratio, assuming that the non-germinants represented normal seedlings. A 3 : 1 ratio could result from either the segregation of a single marker gene, or two pairs of marker genes assorting independently but being epistatic and giving a 12 : 4 (= 3 :1) ratio. Both the 9 : 7 and the 15 : 1 ratios could result from two pairs of marker genes assorting independently and showing different degrees of epistasis.

Only two of the five putative markers could be classified as post-germination lethals. The mottled mutants died shortly after the cotyledons reached full size. The albino mutants died as soon as the food reserves in the seed were exhausted. Some seedlings in the same family (S-9) were virescent, and at germination were not distinguishable from the albinos. They began to noticeably produce chlorophyll shortly after germination. Some of them still survived at the age of 6 months. The yellow-green mutants, easily distinguishable from the normal after 4 weeks of age, were as vigorous as their normal full sibs.

Several causes have been suggested for marker traits deviating from expected marker-gene ratios. One suggested cause that applies to the yellow and curly mutants is that deviant seedlings may be difficult to identify, perhaps because the mutant trait is sensitive to the environment. In fact, a strong environment interaction was apparent with the yellow mutant. The mutant was not detected until fertilizer (including iron) was reapplied. Before the fertilizer treatment, general iron deficiency symptoms were noted throughout most seed lots. All lots responded to the treatment except the mutants in the S-1 family.

Figure 1 illustrates the detection time for the five mutants. Some were apparent early, others later, and some for only a short time, e. g., virescent seedlings. This clearly suggests that frequent and critical inspection as well as an awareness of possible genotype/environment interactions are necessary for detection of mutants.

Table 3. — Description and Relative Frequency of Mutant Seedlings.

Tree Number	Description of Mutant	Description of Normal (= wildtype)	Number of Seedlings Per Class a) N	M	NG	Observed Ratios N + NG:M	N:M	N:M + NG
S-1	*Yellow* Youngest needles light yellow to nearly white, needles curled or swirled	Youngest needles blue-green and straight	97	53	34	2.48:1*	1.83:1	1.11:1**
S-2	*Curly* Primary needles curly at tips and tightly clumped around apex	Primary needles straight and radiating from apex	193	23	9	8.80:1	8.40:1	6.04:1
S-7	*Yellow-green* Cotyledons, primary and secondary needles yellow-green	Cotyledons, primary and secondary needles blue-green	14	8	4	2.25:1*	1.75:1*	1.17:1
S-9	*Albino* Cotyledons, light yellow to white, hypocotyls, pink to light red, some seedlings virescent	Cotyledons blue-green, hypocotyls dark red	193	50	65	5.16:1	3.86:1*	1.68:1
0-10	*Mottled* Cotyledons turning mottled brown after reaching normal size, no primary needles produced	Cotyledons remain blue-green until primary needles produced	73	7	30	14.72:1***	10.42:1	1.98:1

a) N = Normal M = Mutant NG = Non-Germinant.
* Conforms to a 3:1 ratio with a probability of .95 (Chi-square).
** Conforms to a 9:7 ratio with a probability of .95 (Chi-square).
*** Conforms to a 15:1 ratio with a probability of .95 (Chi-square).

Carriers of genetic markers add considerably to the efficiency of haploid induction trials. They are especially helpful in early haploid induction experiments, where the emphasis is placed on developing successful induction techniques, and on detecting responsive females. Large numbers of seedlings can be screened with minimum labor. Even post-germination lethal markers such as albinos can be useful as long as seedling survival is not crucial. At later stages of a haploid program, however, vigorous recessives such as the yellow-green mutant are more desirable.

Seedling Dormancy:

We found strong genotype/environment interactions with respect to seedling dormancy. Inadvertently, a block of 16 pots did not receive fertilizer in the soil-mix preparation. At age 2 months, the seedlings within the block were noticeably smaller, and more of them had dormant apical buds than the surrounding seedlings. The block contained seedlings from several trees from all three study areas. A Chi-square test showed that the lack of fertilizer had caused a significant increase in dormant seedlings in the Cle Elum progenies but not in the Silvana or Olympia progenies.

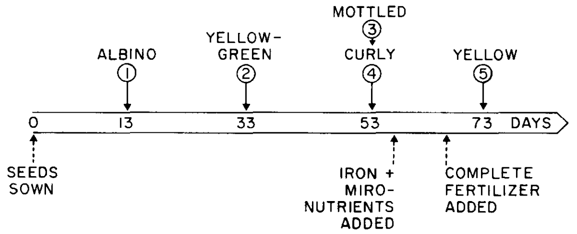

Figure 1. — The detection times of putative markers.

Dormancy was also influenced by an "edge-effect" (outside rows on each bench). Again, edge position caused a significant increase in dormant seedlings in the Cle Elum progenies but not in the Silvana or Olympia progenies.

Summary and Conclusions

A selfing study was conducted in 1966 for the purpose of identifying good selfers as well as carriers of marker genes for future haploid-induction trials with Douglas-fir. In each of three areas in the State of Washington, Olympia, Silvana and Cle Elum, nine isolated trees that had shown evidence of heavy cone crops in the past (putative selfers) were selected. In addition, two stand trees in each area were chosen as controls. All 33 trees were both self- and cross-pollinated. The seeds were extracted and all round seeds were X-rayed to separate filled from empty seeds. All filled control-pollinated seeds and up to 50 filled open-pollinated seeds per tree were stratified and then sown. Several of the trees tested proved to be relatively good selfers, producing many sound and germinable seeds. Progenies from five trees showed variable proportions of deviant seedlings, suggesting the presence of genetic markers.

The following conclusions can be drawn from the study:

(1) Douglas-fir varies greatly in its selfing potential. Relative selfing yields ranged from nearly zero to as high as 65 percent filled seed in individual trees.

(2) The "shaker" method of selfing (enclosing intact male strobili with the female strobili, and shaking the bags at the time of pollen release) may be used successfully and with results comparable to selfing by syringe. The self-shaker technique may be especially useful when large numbers of trees are involved.

(3) The selection scheme for putative selfers was successful in the three areas tested. The stand trees generally did not perform as well as the isolated trees. Because of the possibility of parthenocarpy, the selection scheme could be improved by checking the full or even viable seed yield of isolated trees. Furthermore, it is reasonable to expect better results from applying the scheme in poor crop years than in good crop years.

(4) There were differences in the average selfing potential among the three areas. Compared with the results from other selfing studies, Cle Elum was above average and Olympia below average in selfing potential. Thus, in the search for good selfers, it would seem advisable to test trees in several populations.

(5) Five different mutants (presumably expressions of marker genes) were detected over a 2-month period; one was not detected until a general fertilizer treatment was given. Therefore, in the search for marker genes, frequent and critical seedling inspection is most necessary throughout the first few months following germination. Also, due to genotype/environment interactions, certain markers may become apparent only under certain conditions.

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Genetic Variation in Southern Rocky Mountain White Fir

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White fir (*Abies concolor* [Gord. and Glend.] Lindl.) is a high-elevation tree of the Sierra Nevada Mountains and southern Rocky Mountains. It is an important part of the landscape in the southern Rockies but of minor importance for timber because of the inaccessibility of most of the fir forests.

Although white fir has not been planted extensively within its native range, it is a common ornamental in eastern United States and Europe. Its regular crown and soft blue foliage are much admired in private gardens and parks. The same traits make it a desirable Christmas tree and many eastern growers have experimented with it with varying degrees of success. Up to 20 years have been needed to produce salable Christmas trees in Michigan. The present work was undertaken to discover seed sources which could grow much faster than those used in the past.

There are several reports of natural hybrids between white fir and another western species, grand fir (*A. grandis* [Dougl.] Lindl.). Intermediates are common where the ranges of the two overlap in northern California and southern Oregon and such hybrids have also been reported from Europe. But, until recently, such reports comprised the sum total of genetic knowledge of these two species.

The Sierra Nevada and Rocky Mountains populations of white fir have occasionally been recognized as distinct varieties, var. *lowiana* and var. *concolor* respectively. In several other species, the Sierra-Cascade forms have proven less suitable to the harsh winters of the Northeast than the Rocky Mountain forms. Therefore the present study was confined to interior sources. A study of the Sierra-Cascade populations, similar to the one reported here, was undertaken at the University of California and has been reported by Conkle, Libby and Hamrick.[2])

Through the courtesy of the U.S. Forest Service and other agencies seeds were obtained from natural stands in Utah, Colorado, Arizona and New Mexico in the autumn of 1961. Although we desired cones from several trees per

[1]) Professor of Forestry, Resident Forester of the W. K. Kellogg Forest and Resident Forester of the Fred Russ Forest of Michigan State University, respectively. This work is part of the NC-51 project "Improvement of Forest Trees through Selection and Breeding" supported in part by regional research funds from the U. S. Department of Agriculture. Approved for publication as Journal Article Number 5034 of the Michigan Agricultural Experiment Station.

[2]) Conkle, M. T., W. J. Libby and J. L. Hamrick. 1967. Winter injury among white fir seedlings. Pacific Southwest Forest and Range Experiment Station Res. Note 138. Dr. Libby shared most of his seedlots with us and they were sown in Michigan in 1964, two years later than the present study. All suffered repeated severe winter injury if they grew above the snow cover. Therefore they have not been field planted. The only direct comparison possible is between those trees and four Rocky Mountain seedlots retained as windbreaks in the nursery. In foliage characteristics the trees from southern California resemble Rocky Mountain white fir but those from northern California and Oregon do not. The northern Sierra trees have much greener foliage which is 2-ranked. The differences seem large enough to warrant continued separation of var. *lowiana* and var. *concolor*.