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Embryo Development and Hybridity Barriers in the White Pines (Section Strobus)

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The study of ovule histogenesis in a non-crossable or weakly crossable species combination establishes the time of breakdown in the developmental sequence. It also indicates the general nature of the barrier and may suggest possible ways of overcoming it or of increasing the yield of difficult crosses. In addition, it may provide new information on taxonomic affinities to supplement that obtained from crossability patterns.

Seed failure in pines can occur before, during, or after fertilization (BUCHHOLZ 1944). In the hard pines (subgenus *Pinus [Diploxylon]*)[1]), several workers have reported that hybrid failure occurred at an early stage because of the inability of pollen tubes to function normally in the nucellar tissue of the foreign species. The incompatibility is presumed to be the result of chemical differences limiting pollen tube growth in the female gametophyte. Some possible factors involved are amino acids (MCWILLIAM 1959), auxin inhibitors (HASHIZUME and KONDO 1962 a, 1962 b), and sugars (CHIRA and BERTA 1965). In some crosses, low-level gamma irradiation of the pollen may stimulate subsequent pollen tube growth and overcome inhibition (VIDAKOVIĆ 1963).

There is no evidence of pollen tube incompatibility in the soft or white pines (subgenus *Strobus* [Haploxylon]). In this group, any early seed failures appear to be associated with a lack of pollination, inherent characteristics of the seed tree, or other causes not attributable to the species cross (KRIEBEL 1967). Development of the gametophytes during the first year after pollination is normal regardless of species combination (UEDA et al. 1961). The process is identical to that of P. *strobus* L. as described by FERGUSON (1904).

The sterile crosses P. peuce GRISEB. X P. cembra L. and P. peuce X P. koraiensis SIEB et Zucc. were studied by HAGMAN and MIKKOLA (1963). In each cross the parents are in different subsections of section *Strobus*. No crosses between species of subsection Cembrae and those of subsection *Strobi* have been successful, except those involving P. lambertiana DOUGL., which may have been erroneously placed in Strobi (WRIGHT 1962). HAGMAN and MIKKOLA found that fertilization took place in ovules of P. peuce X cembra and in most ovules of P. peuce X koraiensis. Degeneration occurred inside the archegonia at the proembryo stage as defined by DOYLE (1963).

Initial work on P. strobus X cembra and P. strobus X koraiensis indicated that the developmental pattern was similar to that found in the P. peuce hybrids, though some ovules contained the earliest stages of a true embryo (KRIEBEL 1967, 1968). Embryogeny followed the typical pattern described by BUCHHOLZ (1918, 1929, 1931) up to the time of abortion. This report includes subsequent research and presents quantitative data on comparative embryogeny in hybrid ovules of viable and inviable species combinations.

Materials and Methods

Controlled pollinations were made on P. strobus in each of 3 years. The male parents included P. strobus as a control, one weakly crossable species (P. flexilis JAMES)[2]), and 2 species not crossable with P. strobus (P. cembra, P. koraiensis). Whenever possible, more than one male and female parent was used for each species cross. With the exception of P. koraiensis, each of the species used as a pollen parent was included in 2 or more years' experiments. Pollens of P. strobus and P. flexilis were freshly collected; those of other species had been stored at —12° C for 1 to 4 years.

Immature female strobili were collected during the second growing season. We had previously established that the critical period of archegonial development, fertilization, and embryo formation occurred in the last half of June in the experimental area. Therefore, the 1966 collections were made daily during the period 16 June 1966 to 30 June 1966 and on 3 dates in July and August. In the second experiment, collections were made daily from 18 June 1968 to 3 July 1968 and on 5 later dates in July. The 1969 collections, again daily, covered the period 26 June to 3 July.

The immature strobili were collected in the early morning and brought to the laboratory within 1 hour of the time of picking. The cones were weighed and measured, then dissected. The ovules were fixed in Craf V (SASS 1951) in a vacuum desiccator and kept in cold storage at 4° C. They were dehydrated with TBA (JOHANSEN 1940) and embedded in Tissuemat or Paraplast.

Each block contained 8 ovules, with occasional exceptions. The blocks were cut into 13 μ serial sections on a rotary microtome. Usually, 3 sections were mounted on a slide and 28 to 36 slides were made from a block of 8 ovules. A part of the first year's slides were stained with safranin and anilin blue; subsequently all slides were prepared with JOHANSEN'S quadruple stain (JOHANSEN 1940).

[1]) Terminology of CRITCHFIELD and LITTLE (1966) and SHAW (1914). respectively; that of CRITCHFIELD and LITTLE is used in this paper.

[2]) The size (38 ft. height at age 56), needle and cone characteristics verify the species as P. flexilis JAMES, not P. strobiformis ENGELM.

Development was studied under the microscope by examination of all serial sections made from each group of 8 ovules. The data recorded included the number of pollen tubes, the maximum stage of pollen tube development, the number of archegonia and the maximum stage of development of an archegonium or its embryo system. Abnormalities were also recorded. To date more than 2300 ovules have been examined. The average number of ovules examined per species cross for each collection day was 25 for the 1966 collections, 20 for the 1968 collections, and 35 for the 1969 collections.

Stages of ovule development were scored by number according to the following system adapted from DOYLE (1963) and DOGRA (1967):

Stage	*Description*
0	Egg nucleus at the upper or nucellar end of the archegonium, adjacent to the ventral canal cell
1	Egg nucleus in the center of the archegonium
2	Syngamy
3	First mitotic division
4	Two free nuclei visible in the archegonium
5	Four free nuclei visible in the archegonium
6	One tier of four nuclei at the base of the archegonium (primary proembryo)
7	Two tiers of four nuclei each at the base of the archegonium (primary proembryo); beginning of cell wall formation
8	Three tiers of four cells each at the base of the archegonium (internal division and secondary proembryo)
9	Four tiers of four cells each at the base of the archegonium (secondary proembryo)
10	Embryo extending into the corrosion region; elongation of the first embryonal segment (primary suspensor)
11	Embryo with the first and second embryonal segments or tubes and several tiers of apical cells
12	Embryo with the first embryonal segment and more than one additional tier of elongated embryonal segments, usually compressed; apical units separated

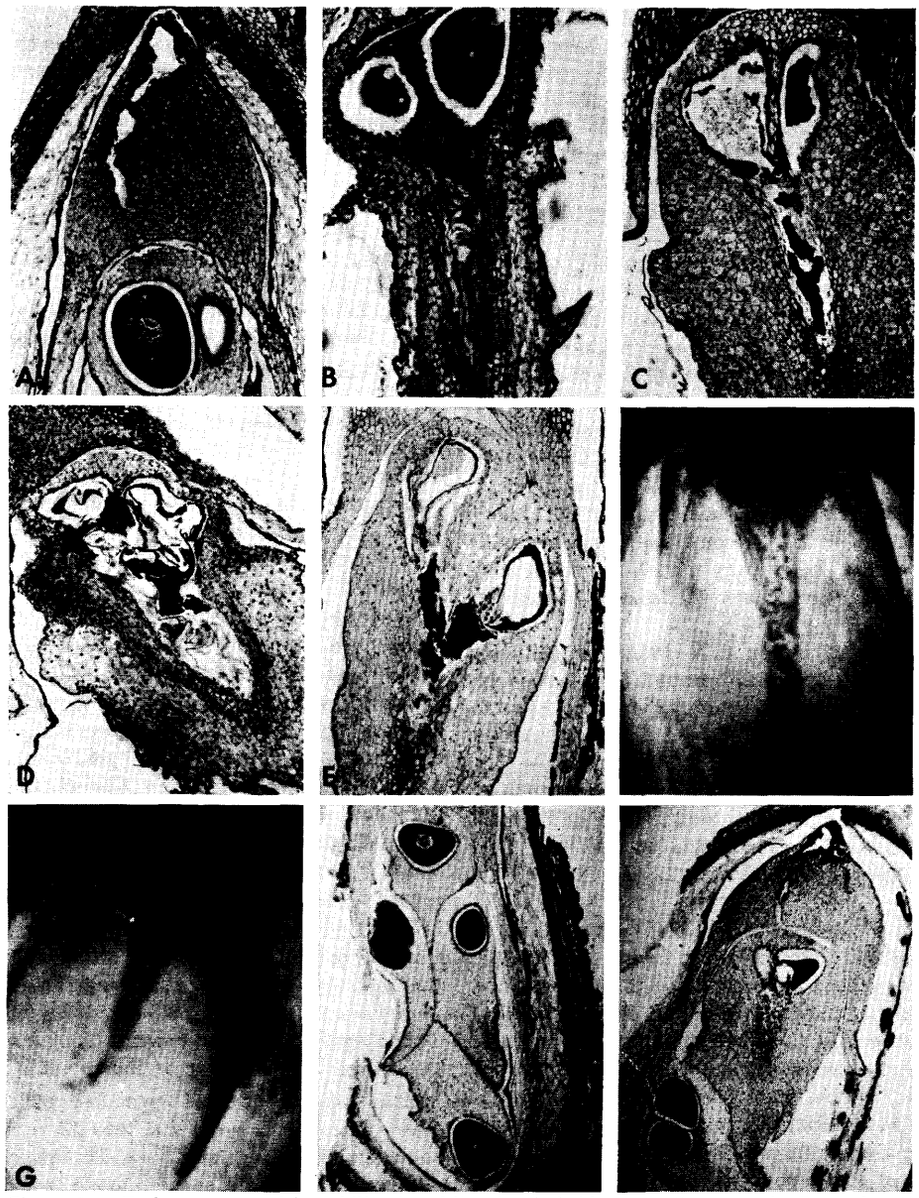

Figure 1. — Embryogeny and inviability in white pine hybrids. — A. Syngamy in *P. strobus* × flexilis, 21 June 1966. 22×. — B. Living Stage 12 embryo, *P. strobus* × *cembra*, 27 June 1969. 34×. — C. Aborted Stage 11 or 12 embryo, *P. strobus* × *cembra*, 27 June 1969. 22×. — D. Living and aborted embryos in a single ovule, *P. strobus* × *cembra*, 27 June 1969. 22×. — E. Two aborted embryos in a single ovule, *P. strobus* × *koraiensis*, 27 June 1968. 22×. — F. Radiograph of suspensor system, *P. strobus* × *strobus*, 2 July 1969. 11×. — G. Radiograph of corrosion cavity, *P. strobus* × *cembra*, 2 July 1969. 11×. — H. Ovule with 4 prothallia and 4 sets of archegonia, 26 June 1969. 11×. — I. Ovule with fertilized archegonium plus abnormally located and unfertilized archegonia, 28 June 1969. 11×.

Table 1. — Percentage of ovules containing an embryo or remnants of an aborted embryo, by collection date, year and species cross.

Collection Date	*P. strobus* × *strobus*			*P. strobus* × *flexilis*			*P. strobus* × *cembra*		*P. strobus* × *koraiensis*
	1966	1968	1969	1966	1968	1969	1966	1969	1968
June 19	0	—	—	0	0	—	0	—	0
20	0	—	—	0	0	—	—	—	0
21	7	71	—	0	0	—	0	—	0
22	0	—	—	0	0	—	0	—	0
23	0	—	—	0	0	—	13	—	0
24	57	—	—	0	40	—	3	—	0
25	60	—	—	22	70	—	41	—	13
26	—	—	62	0	84	75	—	35	42
27	88	100	61	0	83	73	80	80	38
28	—		88	15	60	88		84	54
29	100		100	0	88	83		93	33
30				77	—	100		100	—
July 1					94	100		96	83
2					100				
3					100				

X-ray analysis was used in 1968 and 1969 to supplement observations of the serial sections. The objectives were (1) elimination of the disruption of internal structure resulting from microtechnique, and (2) rapid examination of a large number of ovules. We wished to determine the degree to which these advantages might offset the disadvantage of limited resolution inherent in the projection of an image on an X-ray emulsion.

In each of the 2 years, some of the daily ovule collections from individual isolation bags were divided into 2 parts. Each part contained an average of 40 ovules. One part was analyzed by radiography to determine the proportion of ovules that contained corrosion cavities. The other part was prepared for study by microtechnique to obtain the proportion of ovules that contained embryos. The results of the 2 methods were compared by regression analysis to study the relation between the presence of a corrosion cavity and the presence of an embryo. In all, more than 1600 ovules were examined by radiography.

In 1968, the radiographs were made with Kodak Type R (single-coated) ultra-fine grain X-ray film. In 1969, they were made on 25 × 75 mm Kodak High Resolution Plates for maximum definition with 7 to 50 magnifications. The technique was as previously described (Kriebel 1966, 1970).

Each ovule was scored for the presence or absence of a corrosion cavity. In 1969, we also recorded the presence or absence of embryo material in the corrosion cavity.

Results

Living embryos of all species crosses were found in stages 10 to 12. Most hybrid ovules fixed on the last few collection dates contained aborted embryos which were deeply stained and sometimes fragmented *(Fig. 1, B—E)*. The maximum stage of development of fragmented embryos was estimated from the length of the corrosion cavity and the size and location of the embryo segments. It was evident that most aborted embryos had formed more than one embryonal segment before collapse. Some living embryos had several compressed embryonal tubes with four apical embryonal units in a very early stage of development.

Table 1 summarizes the observations on embryo formation taken from serial sections. The percentage of ovules containing one or more embryos is listed by species cross, year and collection date.

Table 2. — Percentage of ovules containing a corrosion cavity visible on the radiographs, by collection date, year and species cross.

Collection Date	*P. strobus* × *strobus*		*P. strobus* × *flexilis*		*P. strobus* × *cembra*	*P. strobus* × *koraiensis*
	1968	1969	1968	1969	1969	1968
June 20	—	—	0	—	—	0
21	7	—	0	—	—	0
22	—	—	0	—	—	2
23	—	—	0	—	—	6
24	—	—	50	—	—	0
25	—	—	53	—	—	4
26	96	—	75	—	—	46
27	—	20	75	—	100	40
28	—	70	74	80	80	30
29	—	95	83	95	83	17
30	—	100	—	90	97	—
July 1	—	100	100	100	97	62
2	96	100	96	95	87	
3	98	87	—	100	77	
—	—		—			
10	100		96			

In *P. strobus* × *strobus*, embryo development was more rapid than in the hybrids during the first 2 years, but not during the last year. Development was slow in *P. strobus* × *flexilis* ovules in the first year but more rapid in subsequent years and comparable to that of most of the other crosses. In 1966, the supply of *P. strobus* × *cembra* strobili was exhausted before the end of June. By the last collection day (27 June), 80% of the ovules contained embryos. The rate of development was about the same in 1969; by 30 June, 100% of the ovules were in stages 10 to 12. In *P. strobus* × *koraiensis*, embryogeny proceeded at a slower pace than it did in other species crosses made in the same year, or in *P. strobus* × *cembra* in other years.

Nearly all of the ovules of each hybrid that did not form an embryo were in the process of zygote or proembryo development by 1 July. In 1968 and 1969, all ovules of species crosses had at least one archegonium in some stage of embryogeny by that date. Though comparative data for 1966 are not as complete, the trends were similar.

The latest dates any living hybrid embryos were observed were 27 June 1969 for *P. strobus* × *cembra*, 1 July 1969 for *P. strobus* × *koraiensis* and late August 1968 and 1969 (maturity) for *P. strobus* × *flexilis*.

Results of the X-ray studies are presented in *Table 2*. By 1 July of each year, 96 to 100% of all ovules examined had corrosion cavities, except in *P. strobus* × *koraiensis* *(Fig. 1, F—G)*. The general trends are clear, though there are some sample irregularities. At least a part of the apparent decline in *P. strobus* × *cembra* after 1 July is due to image obscurities caused by degeneration.

X-ray examination of ovules collected in late July and early August showed that all hybrid ovules were "empty", i. e., they contained only shriveled remnants of embryo and endosperm tissue, except for about 2% of the *flexilis* hybrids. About 1% of the *flexilis* hybrids reached maturity with a fully developed embryo and a somewhat shrunken endosperm. The other 1% degenerated by the end of July.

The regression of embryo formation on corrosion cavity formation is shown in *Figure 2*. Two confidence bands were plotted for the linear regression equation $Y = 7.133 + .968\,X$. The inner band defines the confidence limits at $P = .05$ for μ, the average proportion of ovules in the population expected to contain embryos, given a certain percentage of ovules in the population containing corrosion cavities. The outer band shows the confidence limits for the prediction of an individual Y in a 40-ovule subsample, given X determined from the other half of the sample (Snedecor and Cochran 1967).

Syngamy and zygote formation apparently occurred within the span of a very few hours. Fusion of the sperm and egg nuclei was observed in only a few of the many archegonia collected during the period in which fertilization occurred *(Fig. 1 A)*. This period was estimated from the daily frequencies of archegonia in late pre-fertilization and early post-fertilization conditions. Though syngamy occasionally took place as early as 14 June, the peak periods of fertilization were estimated to be 21 to 24 June in 1966 and 17 to 22 June in 1968. In these years, fertilization and proembryo development of hybrid ovules was 2 to 7 days behind the same stages in *P. strobus* × *strobus*. The differences were smaller at the time of embryo formation than during the free-nucleate and proembryo stages.

Abnormalities of the female gametophyte were observed in all species combinations. These abnormalities consisted mainly of multiple prothallia and multiple sets of archegonia. Similar conditions in *Pinus* have been reported by

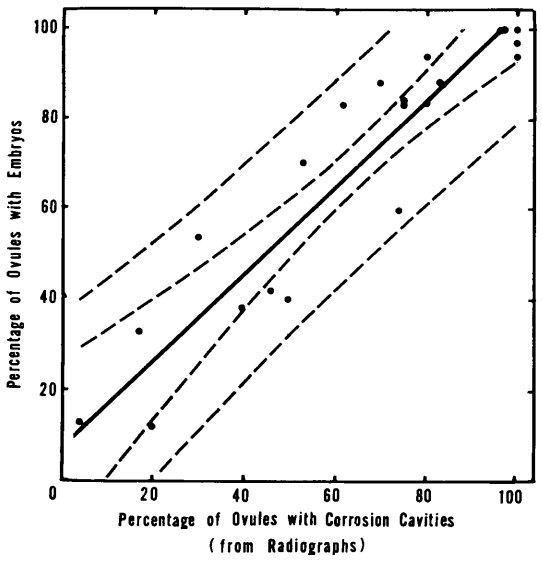

Figure 2. — Regression of the percentage of ovules containing embryos on the percentage of radiographed ovules with corrosion cavities; confidence limits are shown.

Ferguson (1904), Buchholz (1918), Sarvas (1962), Dogra (1967) and others. Usually, a separate set of archegonia was found in each prothallium; one ovule contained 2 archegonia in each of 4 prothallia *(Fig. 1, H—I)*. In some ovules, 2 or 3 sets of archegonia were found in what appeared to be a single prothallium. Percentages of ovules containing multiple prothallia and multiple archegonia are listed in *Tables 3 and 4*.

Discussion

It is evident that the crossability barrier between *P. strobus* and the 3 other white pines, one in the same subsection and 2 in a different subsection, is the result of embryo inviability. In nearly all pollinated ovules from

Table 3. — Percentage of ovules with multiple prothallia or multiple sets of archegonia, by female parent.

Female Parent Number	Total Number of Ovules	Percentage of Abnormal ovules 1966	1968	1969
1	352	0.0		
2	60	3.3		
3	128			8.5
4	64			14.1
5	32			3.1
6	116			4.3
7	168			1.7
8	342	0.5	4.3	
9	106	0.9		
10	969	0.1	3.1	
All Trees	2337	0.3	3.3	5.7

Table 4. — Percentage of ovules with multiple prothallia or multiple sets of archegonia, by species cross.

Species Cross	Total Number of Ovules	Percentage of Abnormal Ovules 1966	1968	1969
P. strobus × *strobus*	390	1.7	0.0	10.4
P. strobus × *flexilis*	1021	0.2	4.2	5.2
P. strobus × *cembra*	587	0.2		3.4
P. strobus × *koraiensis*	339		2.6	
All species crosses	2337	0.3	3.3	5.7

crosses of *P. strobus* with *P. flexilis* and *P. cembra*, and in most ovules of *P. strobus* × *koraiensis*, breakdown occurred during late embryogeny, usually from 3 to 12 days after fertilization. Since most of the remaining ovules were in the proembryo stages, nearly all pollinated ovules were in some stage of embryogeny at the time of failure.

In the crosses by HAGMAN and MIKKOLA (1963) of *P. peuce* with *P. cembra* and *P. koraiensis*, development was observed to the late proembryo stages. It is possible that further studies would show that development beyond the archegonial stages can occur in these crosses in a different environment or with different parent trees. In any case, the breakdown occurs after initiation of embryogeny and the pattern is similar to that found in the *strobus* hybrids.

We do not have embryological studies of all species crosses in the white pines, but many of the combinations not investigated are crossable to a high degree. Combined evidence from all crosses between subsections of the white pines indicates a general pattern of embryo inviability. There is no evidence of any barrier resulting from pollen tube incompatibility or irregularities occurring at the time of fertilization. In contrast, work on the hard pines to date, though limited in species coverage, shows a general pattern of pollen tube incompatibility.

Crossability patterns have improved the basis for classification of the pines. The degree of crossability, expressed in terms of the proportion of sound or viable seed obtained, is useful in determining the strength of an affinity between two species. The study of seed formation adds a new dimension to the analysis of species relationships, by providing a means of evaluating the relationships among non-crossable species and low-viability species combinations. Embryological investigations of white pines support the presently accepted division of Section *Strobus* into two subsections. But they also suggest that there may be a closer affinity among the species of Section *Strobus* than there is within Section *Pinus* or even within Subsection *Sylvestres*. More work is needed, however, especially among the various subsections of the hard pines.

Pine breeders have found that the degree of crossability is dependent on the particular selection of parent trees as well as the species combination. In the white pines, hybrid seed yields from some trees are higher than intraspecific seed yields and some species crosses can only be made on certain individuals (WRIGHT 1953). Thus, parental selection for crossability may increase yields of weakly crossable species. Embryological research suggests that white pine breeders should also test the effectiveness of parental selection for persistence of embryo viability in all sterile species combinations. The most productive approach will probably be a combination of such selection with research on the physiological basis of hybrid failure during late embryogeny.

Radiography appears to be a useful supplement to microtechnique in embryological research. In the particular application described, confidence limits were determined for sample distribution on both sides of the regression line with a probability of significance 19 times out of 20. Therefore, we could except a sample to contain more than the indicated *minimum* percentage of ovules with embryos 39 times out of 40. If the sample were enlarged to 100 ovules, the minimum would be closer to the regression line than the indicated limits. The technique may be useful where non-destructive analysis is needed, as in evaluation prior to ovule culture, or even for rapid estimation as a substitute for microtechnique when critical analysis is not required.

Summary

A study of crossability barriers in the white pines was conducted over a 3-year period. Controlled pollinations of *Pinus strobus* L. were made with pollens of *Pinus cembra* L., *Pinus koraiensis* SIEB. et ZUCC., *Pinus flexilis* JAMES and *Pinus strobus* L. Strobili were collected and fixed at daily intervals during the second growth period. Records were taken of the stage of ovule development, in order to obtain quantitative estimates of the degree of development possible in non-crossable, weakly crossable and readily crossable species combinations.

In nearly all of the ovules collected around the end of June, at least one embryo had penetrated the archegonial wall and extended tubular embryonal segments into the corrosion region before collapsing. The percentage of ovules that developed to this point was slightly lower in *P. strobus* × *koraiensis* than in the other species crosses. The rate of embryo development was about the same in *P. strobus* × *cembra* and *P. strobus* × *flexilis* as it was in *P. strobus* × *strobus*. Development was slower in crosses with *P. koraiensis*.

Both microtechnique and X-ray radiography were used for analysis of immature seed. Corrosion cavities, when present, were visible on the radiographs. Analysis of divided samples by microtechnique and radiography showed that the presence of a corrosion cavity was correlated with the presence of an early-stage embryo. X-ray analysis provided a rapid method of estimating within definable confidence limits the extent of embryo formation in a sample of immature seed.

Abnormalities were found in ovules from most trees and from all species crosses. There was no apparent relation to species combination, however.

The results, together with previous work, indicate that embryo inviability, rather than gametic incompatibility, is the critical factor limiting species crossability in the white pines.

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Analysis of Genetic Variation in 1-, 2-, and 3-year old Eastern White Pine in Incomplete Diallel Cross Experiments

By H. B. KRIEBEL, G. NAMKOONG and R. A. USANIS[1])

This paper reports the results of diallel analyses of juvenile height growth in eastern white pine *(Pinus strobus* L.), and compares the results with estimates of heritability and the components of variance of a 1-parent progeny test.

The questions discussed are: (1) How much variation in parameter estimates is caused by crossing two randomly selected sets of parent trees from the same breeding population, one set in one year and the other set the next year?; (2) How do variance components and heritabilities obtained at ages 1, 2 and 3 compare for the families resulting from each year's diallel crosses and how high is the genetic correlation between responses in different years?; (3) How can a diallel analysis be made on a large number of families when there are missing cells and family size varies?; (4) What is the effect of a high level of environmental uniformity in the progeny test on the heritabilities obtained?

Materials and Methods

All crosses were made in a young stand of native white pine in central Ohio. The parent trees could be considered a random selection with respect to vigor. Actually, reproductive fertility was the most important consideration in tree selection, because female cone production was necessary for the wind pollination study and a moderate or greater level of bisexuality was required for the diallel cross experiments.

The inclusion of reciprocals and unpollinated controls in the diallels, along with a need for replication on each tree, limited the number of suitable parents to 10 in 1962 and 9 in 1963. From 5 to 7 bags of each cross combination were pollinated on each tree. The reciprocals were included in both experiments to estimate a reciprocal-maternal component of variance for the three ages. Though seed weight is known to influence early growth rate (SPURR 1944), the significance of this component of variance has not been investigated previously in eastern white pine.

Fresh pollen was used for all crossing. The flowers were isolated in synthetic sausage-casing bags prior to local pollen dissemination. The conelets were subsequently protected from insects during both growing seasons by white cloth bags. There were, nevertheless, some cone losses from wind- and man-caused branch breakage before maturity. Such losses are difficult to avoid in *P. strobus* because female cone production is restricted to the upper part of the crown. As a result, some cross combinations yielded too small a quantity of seed for inclusion in the experiments and family size varied. Thus, the diallels were irregular and incomplete.

Seed from the 1962 diallel crosses was collected in late August, 1963 and that from the 1963 crosses in late August, 1964. Wind-pollinated seed was collected from 20 trees in the same breeding population in August, 1962.

After cone collection, the seeds were air-separated and stratified for 90 days at 5° C on Perlite in petri dishes. We then placed them in trays in a specially constructed germinator and put them in a warm greenhouse. As the seeds germinated, we sowed them in individual small clay pots in a sterilized potting mixture consisting of 2 parts Wooster silt loam, 1 part sand, and 1 part German peat moss.

In the 1962 diallel experiment (1963 seed), the pots were placed in a greenhouse during the early spring of 1964 in 2 randomized blocks with families as plots. They were moved to a lath house in June and kept there during the first summer. In the autumn, each family in each block was divided in two, and the 4 resulting replications were planted in a nursery, where they remained during the next 2 growing seasons. In the 1963 diallel experiment (1964 seed), the trees were grouped in 4 replications from the start. They were also started in the greenhouse, but were subsequently kept in pots in the lath house until the end of the third growing season. During the intervening winters, the trees, still in the 4 replications, were kept in a hotbed at temperatures slightly above freezing. The seedlings were repotted at the end of the first growing season without major disturbance to the original soil around the roots.

In the test of wind-pollinated progenies, the seedlings were also kept in pots throughout the first three years. The family groups were unreplicated during the first and second years, then divided into 4 blocks prior to the third growing season. The pots were changed to a larger size at the end of the first year as in the 1963 diallel experiment.

The basic measurement used for analysis of variance was total plant height, measured for each tree at the end of each of the first 3 growing seasons. In the diallel experiments, the group of trees selected for crossing each year was considered to be a sample from a general breeding population. The progenies were obtained by using each

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