

AN INVESTIGATION INTO THE EFFECTS OF SELF-POLLINATION ON
PSEUDOTSUGA MENZIESII (MIRB.) FRANCO

by

Alan Lindsay Orr-Ewing

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We accept this thesis as conforming to the
standard required from candidates for the
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Alan L. Orr-Ewing

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COMMITTEE IN CHARGE

DEAN H. F. ANGUS, *Chairman*

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B. C. BINNING

G. S. ALLEN

K. COLE

R. W. WELLWOOD

K. GRAHAM

External Examiner—DR. J. W. DUFFIELD

Industries Forest Nursery, Nisqually, Washington

AN INVESTIGATION INTO THE EFFECTS OF SELF-POLLINATION ON *PSEUDOTSUGA MENZIESII* (MIRB.) FRANCO

ABSTRACT

The genetic systems which prevent self-fertilization in the Angiosperms are based on the incompatibility of pollen and style. Past studies have also shown that many of the Gymnosperms, including *Pseudotsuga*, produce little or no viable seed after self-pollination. The causes of this seed failure were not known.

Controlled self- and cross-pollinations were accordingly made on eight *Pseudotsuga menziesii*, sample cones were removed at various dates and the ovules embedded. The remaining cones were collected at maturity. The yield of viable seed from different trees varied considerably. The investigation was divided into two parts, a cytological study of development within the ovules of two trees which produced very little viable seed and the effects of self-pollination on the progeny.

It was found that neither pollen germination nor its subsequent development up to the time of syngamy were inhibited. Proembryo and early embryonic development were normal but nearly all the embryos collapsed before they had reached an advanced stage. It is concluded that embryo collapse is probably an inbreeding effect caused by the action of lethal and semi-lethal genes.

It was found that inbreeding resulted in progeny which were usually smaller and less vigorous than those from both controlled cross- and wind-pollinations.

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GRADUATE STUDIES

Field of Study: Genetics

Genetics.....A. H. Hutchinson, *et al*
Histology and Cytology.....A. H. Hutchinson
Silvics and Silviculture.....G. S. Allen

Other Studies:

Forest Ecology.....E. C. Stone and A. W. Sampson
Forest Management.....P. M. Barr
Biometrics.....E. Harris
Forest Research.....R. N. Colwell and E. C. Stone

ABSTRACT

Extensive investigations have shown that the genetic systems which prevent self-fertilization in many of the Angiosperms are based upon the incompatibility of pollen and style. Past studies have also shown that many of the Gymnosperms, including Pseudotsuga, produce little or no viable seed after self-pollination. The causes of this seed failure, however, had not been determined.

It was considered that a cytological study of development within the ovules after the self-pollination of Pseudotsuga menziesii should show whether seed failure was caused by comparable incompatible systems. Controlled self- and cross-pollinations were accordingly made on two trees at Vancouver in 1952 and on three trees each, at Victoria and Lake Cowichan in 1954. Cone samples from both pollinations were removed at various dates from each tree and the ovules embedded for later sectioning. The remaining cones were collected at maturity and the seed extracted. It was found that the yields of viable seed varied greatly in individual trees and the investigation was accordingly divided into two parts. The cytological study was confined to two of the trees which had produced very little viable seed after selfing. The second part of the investigation was concerned with the effect of self-pollination on the progeny.

The cytological study showed that neither germination of the pollen nor its subsequent development until the time of syngamy were in any way inhibited and both compared favourably with development

in the ovules from cross-pollinated cones. Proembryo formation and early embryonic development were normal but the embryos in both trees collapsed approximately ninety days after self-pollination. This collapse appeared to be caused by some failure in the vital relationship between the young embryos and their surrounding gametophytes. The latter appeared healthy and comparable in every respect with those in which embryos from cross-pollination were growing vigorously.

It is considered that embryo collapse after self-pollination is probably an inbreeding effect caused by the action of lethal and semi-lethal genes when brought together in a homozygous state. This explanation could account for the very variable effects of self-pollination on the trees studied.

Some viable seed was also obtained from cones isolated and not pollinated. The seedlings are diploid and the possible causes of agamospermy are discussed.

A study of the inbred progeny shows that self-pollination usually results in seedlings which are smaller and less vigorous than those from controlled cross-pollination. The seedlings from wind-pollination were intermediate and the small size of some suggests that self-pollination may have occurred.

The practical aspects of self-pollination in relation to both the natural and artificial regeneration of forest land are briefly discussed.

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INTRODUCTION

The mating systems of plants and animals have interested breeders for many years, particularly in respect of the relative merits of inbreeding and outbreeding, the one favouring reproduction by closely related parents and the other by parents not closely related. An even closer form of inbreeding is possible in hermaphroditic plants as self-pollination results in fusion of the male and female gametes from the same plant. It was soon appreciated, however, that certain plants produced no viable seed at all after self-pollination and, according to East (1929), Kölreuter in 1746, was the first person to make this very significant observation. Darwin (1900, page 439) later carried out extensive selfing and crossing experiments with many plants and was able to demonstrate that selfing not only resulted in greatly reduced seed yield but also reduced vigour in the progeny. He concluded that, generally, cross-fertilization was beneficial and self-fertilization injurious, at least with the plants with which he had experimented. It was not until comparatively recently, however, that the full significance of these earlier findings was appreciated. According to Dobzhansky (1951, page 300), the appearance of sexual reproduction was perhaps the most important advance in the evolution of life and the simplest form of retrogression of sexuality is self-fertilization. He explains that the danger of self-fertilization is inherent in hermaphroditism and that many hermaphrodites have developed various devices to prevent selfing, although some still reproduce facultatively or obligatorily by this means. Many plant species

have developed floral mechanisms, such as protandry and protogyny, to prevent self-fertilization but it has only been realized comparatively recently that several very efficient genetic systems have also been evolved for the same purpose. These systems have been extensively investigated in many of the Angiosperms and, as explained by Bateman (1952), their primary function is to prevent self-fertilization. He states that they differ from the many devices that interpose a mechanical barrier to self-pollination in that they act at a later stage by interposing a physiological barrier between self-pollination and self-fertilization.

The effects of inbreeding on the progeny have also been extensively investigated and Darwin's conclusions that selfed progeny had less vigour than those that had been crossed, have been confirmed with many different species. Later investigators, in addition, have been able to appreciate the genetic interpretation of inbreeding and have used this to great advantage.

There have, however, been remarkably few detailed studies on either self-incompatibility or the effects of inbreeding in the Gymnosperms. It is true that there have been some minor studies which have involved the selfing of a few trees and a comparison of the yield of viable seed with that from either wind or controlled cross-pollination. In a few of these studies, the seeds have been sown and the development of the inbreds compared with those of the controls. Such investigations have been almost exclusively confined to the Pinaceae and the most interesting will be briefly summarized.

One of the earliest studies was that of Sylven (1910), who

selfed five Picea abies, and found that only four of the trees produced any viable seed, and that the yield was less than that obtained from wind-pollination. These seeds were sown and it was found that the mortality rate of the inbreds was much the higher for the first few years after germination. Langlet (1940) later reported that at twenty-seven years of age, the average height and diameter of these trees were less than half of those resulting from wind-pollination, but that the effects of inbreeding varied from tree to tree. There had also been considerable mortality among the inbreds. Kolessnikoff (1929) selfed a number of Pinus sylvestris and obtained no viable seed at all. Dengler (1932) carried out extensive selfing studies both with this species and with Pinus montana and found that either no viable seed was produced or that the yield was much below that obtained from seed from controlled cross-pollination. He suggested that some repeated inhibition mechanism must occur, either at syngamy or during early growth of the embryo, and that it must check normal development. Dengler (1939) later reported that development of the inbred trees varied greatly at the age of seven years; some were comparable to the crossbreds whereas others were poorly shaped and stunted in growth. These trees were examined again by Scamoni (1950), who reported that the mortality of the inbreds had been heavy and that the majority were greatly inferior in growth and form to the crossbreds.

In 1938, Langner (1951) selfed seven Larix leptolepis and Larix europaea and found that only three of these trees produced any viable seed. Langner (1952) later found that there had been

considerable mortality among the inbreds and that growth in both height and diameter was markedly inferior to that of the individuals from controlled cross-pollination. He also observed that there was much variation in the effects of inbreeding in the case of individual trees, the progeny of one tree showing much more evidence of inbreeding depression than did the others. Langner concluded that this parent tree must have contained a large number of recessive genes which were responsible for the crippled growth of the seedlings and that these genes were exposed by selfing. Von Gothe (1952) selfed other trees of the same species and obtained no viable seed from Larix europaea and only a small amount from Larix leptolepis. Von Gothe (1953) later reported that the inbreds lacked vigour and were all killed by frost the first winter.

These studies all indicate that selfing results in the production of little or no viable seed and of progeny that is usually inferior in both growth and vigour. The effects of selfing, however, appear to be variable since other studies have shown that it does not always result in reduced seed yields or in weak progeny. Piatnitsky (1936), for example, selfed five species of Larix, and reported that there was no evidence of "self-sterility" in four of these trees, and germination of the inbreds was only lower in one tree compared to that from wind-pollination. Syrach Larsen (1937, page 72) reported that there was no evidence of inbreeding depression in the four-year-old seedlings from self- and controlled cross-pollination in Thuja plicata; the yield of viable seed from selfing in this study, however, was substantially below that obtained after cross-pollination. Forshell (1953) found that

selfing in Pinus sylvestris usually produced less viable seed than did controlled cross-pollination; in the case of one particular tree, however, selfing resulted in more viable seed than did crossing.

The evidence from past studies definitely suggests that there is some impediment to normal seed development following selfing, and that it usually results in progeny which is neither vigorous nor well formed. The question naturally arises as to whether the effects of selfing on the production of viable seed could be caused by incompatibility systems similar to those found in the Angiosperms. It is interesting, therefore, to observe that there are two opinions as to whether such systems are possible in the Gymnosperms. Whitehouse (1950), for example, stated that the two main outbreeding devices in the Gymnosperms are dichogamy and dioecism and that, although these both occur in the Angiosperms, other outbreeding devices are found which are based on the incompatibility of pollen and style. He wrote: "These are worthy of special attention since no comparable mechanisms are known or indeed appear to be possible in Gymnosperms". Whitehouse later considered the possibility of a physiological antagonism developing between the cells of the carpel and the pollen tubes of similar genetic constitutions. He added, however;

Nevertheless, it appears unlikely that such an antagonism could attain sufficient selective value in a Gymnosperm, since the thickness of nucellar tissues which the pollen-tubes must penetrate to reach the embryo-sac is hardly sufficient to be capable of interposing a physiological barrier.

Bateman (1952) did not agree with these conclusions and stated:

Reference to Coulter and Chamberlain (1910), shows that it is typical of Gymnosperms for pollen tubes to be compelled to grow through a well-developed nucellus before fertilization is possible. Since in Capsella ... and Parthenium ... a single layer of cells in the stigma is sufficient to inhibit self-pollen, the massive nucellus of most Gymnosperms should be quite capable of acting as a physiological sieve for pollen tubes. The absence of a style in the Gymnosperms is not therefore a sufficient explanation of the absence of self-incompatibility from this phylum (if it is indeed absent).

Bateman also added this footnote: "In a private communication Dr.

A. Gustafsson claims to have found self-incompatibility in Pinus sylvestris, thus proving that the absence of a style does not prevent Gymnosperms from evolving self-incompatibility". It can be appreciated, therefore, that there was considerable scope for a cytological study to determine whether or not such an incompatibility system exists. According to Sears (1937), incompatibility systems in the Angiosperms can occur in three distinct stages.

- (1) Pollen germination may be decreased, in which case the inhibitory reaction occurs only in the stigma.
- (2) Pollen germination is normal but pollen tube growth is inhibited in the style.
- (3) The pollen tubes grow normally, reach and fertilize the ovules but no seed develops.

A cytological study, therefore, should determine whether or not any of these stages were present in a Gymnosperm.

Past studies by Allen (1942), Duffield (1950), and Meyer (1951), had all indicated that the selfing of Pseudotsuga menziesii had invariably resulted in little or no viable seed being produced. The

species was, therefore, considered "self-sterile", so that it was a very suitable choice for an investigation of this nature. It should be noted that the term self-incompatible has been used throughout the following pages in place of that of self-sterile as it is the more correct. Lewis (1954) has recently pointed out that:

The term self-sterility was originally applied to a mating system in hermaphrodite plants, all individuals of which produce normally functioning male and female gametophytes and which fail to produce zygotes after self-fertilization. But the discovery that certain cross-pollinations also failed to produce zygotes, and the association of the term sterility with the lack or inviability of one or other of the two gametophytes has lead to the term incompatibility.

Several authors, quoted in this dissertation, have used the older term "self-sterility", and in such cases it has been retained.

Furthermore, since no studies on the development of any inbred seedlings had previously been conducted, this represented another new field of investigation.

This dissertation, therefore, has been divided into two parts; the first deals with the cytological study and the second part with field studies of the progeny from the trees investigated. In addition, reference is made in both parts to the possible occurrence of Agamospermy.

PART 1.

I. CONTROLLED BREEDING TECHNIQUES

Isolation of the Female Cone

It should be noted that throughout this investigation, the microsporangiate and megasporangiate strobili have been referred to as male and female cones respectively. McLean and Cook (1951, page 673) have stated that although these reproductive organs are sporophytic in origin and, therefore, in no way sexual in character, it is customary to refer to them in this manner.

The first requirement for controlled breeding is that the female cone must be effectively isolated from any contamination by unknown sources of pollen. The development of the cones, therefore, has to be carefully checked in order that they are isolated at the right time. Pseudotsuga is monoecious and the male and female cones are often found on the same branchlet, although there is a general tendency for the female cones to be more numerous in the upper half of the crown. The appearance of the male and female cone buds in late February is shown in figure 1, plate I, the female buds are both larger and more pointed than the male buds and occur lateral to the terminal vegetative bud. A more advanced stage is shown in figure 2; the male buds are now considerably swollen and the female buds should be isolated at this time for later controlled pollination. In figure 3, the male cones have emerged from the bud scales and will soon mature, while the female buds are much enlarged and the cones are beginning to emerge. Exposure of female

buds as late as this stage would involve some risk of contamination since pollen from more precocious trees could now settle on the female cone. There are no specific dates for isolation since development of both male and female cones varies greatly from tree to tree and even on the same tree. Duffield (1950) found that the opening of Pseudotsuga cone buds was apparently more sudden and synchronized over a single tree than in Pinus. No such synchronization was observed in the trees in this investigation, however, and it was found that the female buds at the top of the tree and on southerly aspects invariably had to be isolated first. The dates of both isolation and pollination are given in table I, which shows that neither of the operations could be completed in one day, owing to the varied state of development of the female cones in different parts of the tree. Constant inspection of the female cones is the only safe method of insuring that isolation and pollination are completed at the correct time. There was also some variation in development between localities, the trees A, C, and D, located near Victoria, had to be isolated earlier than those at Cowichan Lake.

Viscose sausage casing was used for isolating the female buds and the bags were constructed in the manner recommended by Duffield (1950); figure 2, plate II, illustrates a cone isolated by this means. These bags are cheap, light, and transparent, and development can be readily observed; they are also permeable to moisture and can withstand the variable spring weather. There is no chance of pollen contamination, provided that the cones are isolated at the right

time and that the bags are not removed too soon after pollination; these conclusions are supported by those of Nienstaedt and Kriebel (1955). The one disadvantage of the viscose bags is that they readily absorb heat which results in some cone mortality.

Duffield (1950) noted that Pseudotsuga was more susceptible to heat injury within the bags than were most species of Pinus; Nienstaedt and Kriebel (1955) found that the internal temperatures were high compared with those within other types of bags. This heat injury can be substantially reduced by restricting the selection of cones to the north side of the tree and by avoiding south-facing aspects. The mortality is shown in table I, and in some trees it was quite serious. In the majority of cases, the female cones died prior to emergence from the bud scales; in other instances, they died soon after pollination. The cone is at a critical stage of its development at the time of year when isolation and pollination takes place and some mortality must be expected.

Each isolation bag was identified with a light aluminum tag which indicated its number and the number of cones contained, and whether they were to be self- or cross-pollinated, or to be left unpollinated. This information was recorded on the special form illustrated in figure 1, plate II, which was developed from the one described by Cumming and Righter (1948). Care was taken to remove all male buds which might otherwise have been isolated with those of the female. Male cones were left inside the bags only in the case of female cones which were to be self-pollinated.

Pollination

Pollen for controlled breeding must be available at the time when the female cones are most receptive; branchlets with male cones, therefore, were removed at the time that the female cones were isolated. These were brought indoors and placed in water in a warm room to accelerate development of the cones. Paper was spread beneath each container and the pollen was readily collected when the cones matured. In order to avoid any possible contamination, this operation was conducted in a separate room for each pollen lot. The pollen was finally placed in flasks which were lightly plugged with absorbent cotton and stored at 4° C. until required. In the meantime, the isolated female cones were periodically checked so that they could be pollinated when most receptive. It was considered that the stage illustrated in figure 4, plate I, was the most favourable for pollination as the ovuliferous scales were fully extended.

Pollinations were made by means of a hypodermic syringe fitted with a rubber bulb and a pollen agitator as recommended by Duffield (1950). The operation is illustrated in figure 3, plate II. The end of the needle was inserted through the viscose casing bag and a cloud of pollen blown directly on to the cones, then the small hole in the bag was sealed with scotch tape. The pollen parent and date of pollination were recorded on the special form (figure 1, plate II) and a coloured chicken ring was slipped on the branchlet at the base of the bag. This showed that the enclosed cones had been pollinated

and the colour indicated the type of pollination. In this investigation, red rings indicated self-pollination and yellow cross-pollination; green rings indicated that the cones had been purposely left unpollinated. The coloured rings greatly simplified later sampling and prevented mistakes. It can be seen from table I that pollination of the cones of any particular tree generally required several days to accomplish and was not completed until all the cones had reached the receptive stage; this varied in different parts of the tree. When the scales on the cone had closed and it had begun to invert, the isolation bag was removed and replaced by a protective kraft bag. Figure 5, plate I, illustrates a cone whose scales have closed so that pollination is no longer possible. The dates on which these bags were removed also varied for the different trees as indicated in table I. The kraft bags were necessary for protection against insects and squirrels and had no adverse effect on the development of the cone. Ordinary kraft paper bags were used but the seams were all stapled and reinforced with waterproof glue; one of these bags is illustrated in figure 4, plate II. Figure 6, plate I, shows a sixty-foot tree with the isolation bags in place.

TABLE I. DATES OF ISOLATION AND POLLINATION OF CONES IN 1952 AND 1954.

Tree	No. of bags	No. of cones isolated	Dates of isolation	Dates of pollina- tion	Dates of bag removal	No. of cones collected
1*	47	100	11-15/3	30/3-7/4	27-28/4	74
2*	30	105	15-23/3	14/4	27/4	76
A	73	310	22/3-13/4	16/4-3/5	1-9/5	224
C	57	202	25/3-6/4	13-16/4	24/4-3/5	104
D	71	271	26/3-10/4	15-26/4	4-8/5	126
F	47	280	29/3-20/4	29/4-5/5	14-15/5	212
H	58	278	31/3-20/4	21-29/4	14/5	179
J	48	268	1-2/4	21-29/4	13-14/5	220

* Trees 1 and 2 were located in Vancouver and pollinated in 1952.

Pollen Germination Tests

In a controlled breeding study, the pollen should always be tested prior to use in order to ensure that it is viable, since sterile pollen is not uncommon. No previous germination studies had been conducted with Pseudotsuga pollen and different types of media had to be tried before a satisfactory one was found. The method recommended by Righter (1939) for germinating Pinus pollen was not satisfactory since Pseudotsuga pollen germinates poorly in distilled water. Various concentrations of both sucrose and agar were tried and the most satisfactory medium was finally found to be two per cent bacto-agar. This was heated and then poured into the petri dish to be used for the germination test. When the agar had cooled, pollen from one particular lot was lightly scattered on it in five different places and a few drops of water were placed on the inside of the lid in order to maintain a high humidity. The petri dish

and its contents were then incubated for twenty-four hours at 26° C. and at the end of this period, the five areas of pollen were examined under a low-power microscope. A field of fifty pollen grains was selected at random in each area and the amount of germination was checked. Pollen was considered to have germinated when it had swollen and the outer exine layer had been ruptured; there was usually some protrusion of the pollen tube as well. The vigour of different pollen lots varied considerably as clearly illustrated in figures 7 and 8, plate II. These two pollen lots from trees D and F respectively were collected in 1954 and the germination test was conducted one year later. Only one pollen grain has germinated in the pollen visible in figure 7; the pollen from this tree was never particularly vigorous and its viability declined far more rapidly in storage than did any of the others collected at the same time. It is quite logical, however, to expect that there will be a considerable variation in pollen viability from one tree to another and the need for germination tests when artificial crosses are to be made is indicated.

Figure 5, plate II, illustrates typical Pseudotsuga pollen grains; these are wingless, bright yellow, about eight microns in width and roughly elliptical in shape. Their general appearance is very similar to that of a coffee bean, and the characteristic depression on the one side is probably an adaptation for wind dissemination.

Pollen Viability After Storage

No previous work had been carried out on the storage requirements

of Pseudotsuga pollen although storage may be often necessary, since controlled crosses can not always be made the same year that pollen is collected. Some exploratory work was, therefore, carried out between 1952 and 1955. Periodic germination tests were conducted during this period on several different pollen lots which were stored in lightly stoppered flasks at 4° C. No attempt was made to control either temperature or humidity as the purpose of the test was to find out how long Pseudotsuga pollen would remain fully viable if stored in an ordinary refrigerator. The results showed a gradual decline in germination capacity of pollen over a three year period. This is illustrated in figure 6, plate II; there is still some viability in this two-year-old pollen but it is very low and considerable desiccation is evident.

In 1954, pollen from three two-year-old lots was used for artificial pollination. The results are shown in table 2 and it can be seen that although all the pollen lots showed some germination, only one viable seed was produced. Some cones were also removed from the parent trees a few weeks after pollination in order to check on pollen development. A few ovules were sectioned and in one, ungerminated pollen was found twenty-three days after pollination, while in another, an ungerminated pollen grain was found thirty-two days after pollination. This clearly indicated that the results from the germination tests used do not necessarily indicate the extent to which the pollen is capable of fertilization.

TABLE 2. RESULTS OF POLLINATION WITH TWO-YEAR-OLD POLLEN.

Tree	Pollen parent	Pollen germin.*	No. of cones collected	No. of cleaned seeds	Germin.	Cutting tests of ungermin. seed
A	3	120/250	8	1	-	Seeds empty
	4	128/250	22	6	-	Seeds empty
D	2	54/250	1	-	-	
	5	191/250	5	8	1	1 with embryo
F	4	128/250	2	4	-	Seeds empty

* This test was made April 1954 and was based on five samples

In 1955, a similar test was conducted with pollen which had been stored for one year and, in this case, a direct comparison could be made since the same lots were used on the same trees in 1954. The results are shown in table 3 and indicate, that although neither of these pollen lots showed any significant decrease in germination, there was a highly significant decrease in the amount of viable seed produced. There is little doubt that Pseudotsuga pollen loses its fertilization potential rather quickly and that improved storage techniques should be sought. These results show that the artificial germination tests used do not necessarily indicate whether or not the pollen is capable of fertilization. Duffield (1954) has recently pointed out the little that is actually known about the physiology of gymnosperm pollen and the unreliability of such tests. He adds:

However, it ... seems reasonable to assume provisionally that pollen which cannot be made to germinate by usual means or which, on germination, shows unusually poor tube growth, is likely to be ineffective in causing

fertilization. Herein lies the present practical value of pollen testing as a routine procedure in breeding operations.

TABLE 3. RESULTS OF POLLINATION WITH FRESH AND ONE-YEAR-OLD POLLEN

Tree	Pollen parent	Year of pollin.	Pollen germin.*	Cone no.	No. cleaned seeds	Seeds germin.	Cutting test of ungermin. seed
F	H	1954	240/250	33	420	400	-
		1955	244/250	4	67	2	9 with embryos
J	C	1954	230/250	41	1051	397/420	-
		1955	240/250	13	555	39	96 with embryos

* This test was based on five samples

PLATE I

- Figure 1. Appearance of the male and female cone buds of Pseudotsuga, Victoria. February 23, 1954. Natural size.
- Figure 2. Later development, the male buds are considerably swollen and the cones will soon emerge through the bud scales. Natural size.
- Figure 3. The female buds should be isolated before this stage is reached, as the cones are emerging and the male cones are almost mature. Natural size.
- Figure 4. Pollination should take place at this stage. Natural size.
- Figure 5. The cone scales are closed and pollination can no longer take place. Natural size.
- Figure 6. A sixty-foot tree with the isolation bags in position. March, 1954.

PLATE I

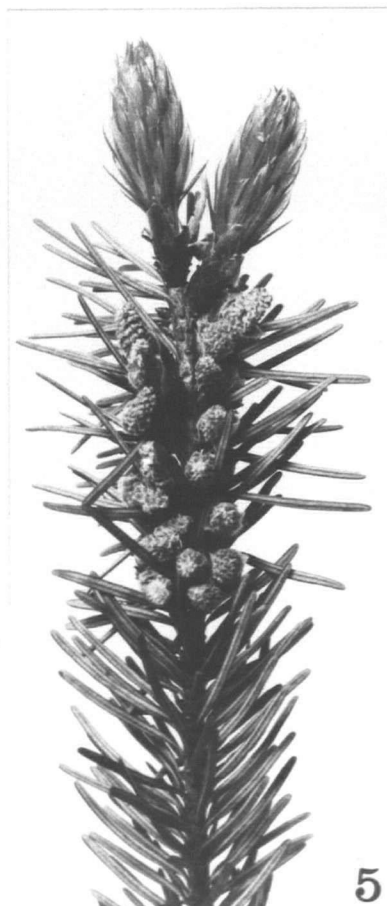
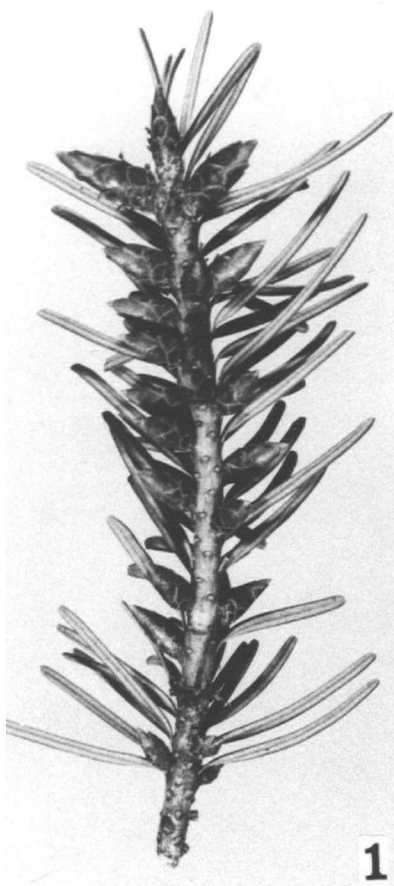


PLATE II

Figure 1. A pollination record form.

Figure 2. An isolated female cone; ample space has been left in the bag for future development, and a small strip of absorbent cotton has been stapled into the end of the bag to prevent pollen contamination. Approx. 1/6 natural size.

Figure 3. A young cone being artificially pollinated with the modified hypodermic syringe.

Figure 4. The kraft bag used for protection against squirrels. Approx. 1/6 natural size.

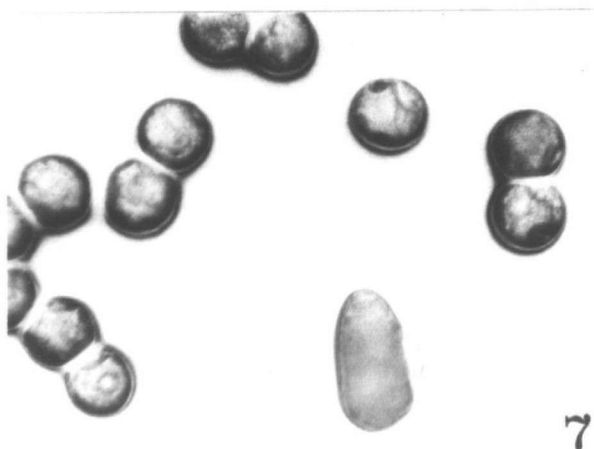
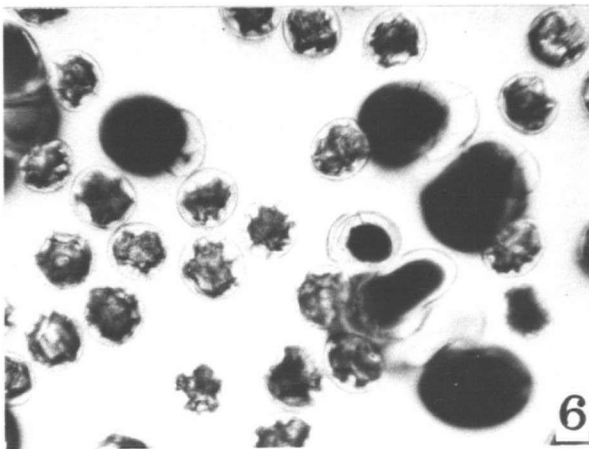
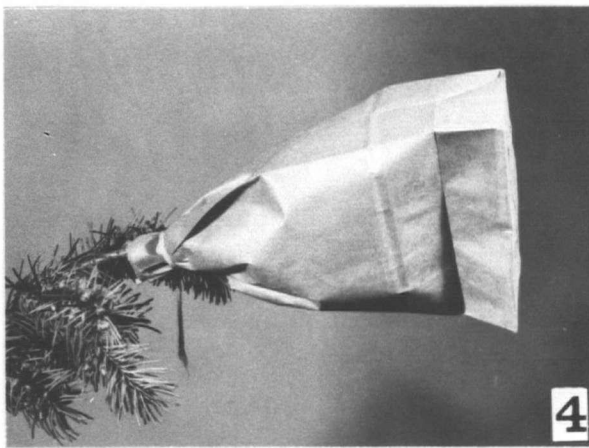
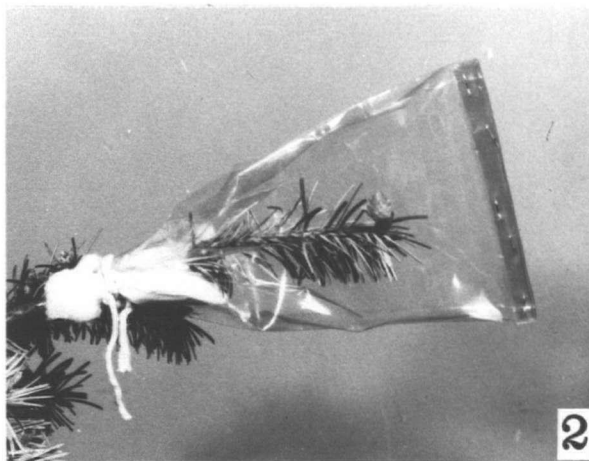
Figure 5. Pollen grains of Pseudotsuga. X 92.

Figure 6. Poor germination of two-year-old pollen from tree 1. Nov. 1954. X 92.

Figure 7. Poor germination of one-year-old pollen from tree D. March, 1955. X 92.

Figure 8. Vigorous germination of one-year-old pollen from tree F. March, 1955. X 92.

PLATE II

[illegible]

II CYTOLOGICAL INVESTIGATIONS WITH TREE 1

General

The effects of self-pollination on seed viability cannot be determined until controlled pollinations have been conducted and a germination test made on the seed. In 1952, Orr-Ewing (1954) self-pollinated two Pseudotsuga near Vancouver and found that one of these trees (tree 1) produced very little viable seed, only two seeds germinating from thirty-five cones. The germination of seeds resulting from cross-pollination, however, was considerably higher. It was evident that a cytological examination of the ovules from this tree would show whether a true self-incompatibility system was responsible for the low yield of seed. Such a system, however, could be expressed at several different stages of development, as has previously been demonstrated by Sears (1937) in the Angiosperms.

It was possible, therefore, that the pollen grains of Pseudotsuga might not germinate after self-pollination or, again, that the development of the pollen tube might be inhibited. Bateman (1952) pointed out that the massive nucellus of most gymnosperms could act as a physiological sieve for pollen tubes. This could also occur in Pseudotsuga since the pollen tubes have to grow through the apex of the nucellus to reach the archegonium. The cytological investigation was, therefore, divided into the following distinct studies. Firstly, a study of pollen germination and development to the time that the pollen tubes reached the archegonium. Secondly, a study of subsequent development from the time the two sperm nuclei

were discharged into the archegonium and up to formation of the proembryo. Finally, should there be found no self-incompatibility system operative in these first two stages, a study of the development of the embryos would be indicated.

Microtechnique

These studies involved a considerable amount of cone sampling at different dates from the time of pollination. The usual procedure was to remove two cones at each date, one of these had been self-pollinated and the other cross-pollinated. The ovules were quickly dislodged from the ovuliferous scales, and the integuments also were removed in the case of collections made towards the end of May when they had become hard and made sectioning difficult. The ovules were then placed in either of two killing-and-fixing solutions and air was removed with a suction pump. Carnoy's fluid with chloroform (Johansen 1940, page 40), and Allen-Bouin III (Sass 1940, page 19), were both used and found equally satisfactory. The former had been used by Allen (1945), in an earlier investigation with Pseudotsuga, and the latter was recommended by Johansen (1954), who also advised the substitution of propionic for glacial acetic acid. The ovules were left in Carnoy's fluid for 12 hours, washed quickly in 95 per cent ethyl alcohol and finally stored in 70 per cent alcohol. When the Allen-Bouin III solution was used, the ovules were placed in the solution for 24 hours and then washed in tap water and brought up in stages to 70 per cent alcohol. They were stored for a short

time in a refrigerator and then dehydrated and embedded by Johansen's tertiary butyl alcohol method (1940, page 130). Anhydrous isopropyl alcohol, however, was used in the place of absolute ethyl alcohol, again on Johansen's recommendations (1954). Parawax was used for preliminary infiltration, with tissuemat replacing it for the final embedding. The embedded ovules were finally labelled with the date, tree number, the type of pollination, and the killing-and-fixing solution used.

Sections were cut at twelve microns on a rotary microtome, affixed with Haupt's adhesive (Johansen 1940, page 20) and finally stained with either Heidenhain's iron haematoxylin or Feulgen's stain. Both stains gave consistently good results. The best schedule for haematoxylin involved mordanting for thirty minutes in a 4 per cent solution of iron alum, followed by staining in 1 per cent haematoxylin for ten minutes, and then destaining in 2 per cent iron alum. Coleman's (1938) recommendations were followed for the preparation of Feulgen's stain. The material was hydrolized for ten minutes in 1 N. hydrochloric acid, stained in Feulgen for ten minutes and finally washed in three changes of sulphur dioxide water. A very dilute solution of fast green dissolved in absolute ethyl alcohol and clove oil was used for the counterstain. After dehydration in xylene, the sections were finally mounted in Permount.

Pollen Germination and Subsequent Development

Sections were made from self-pollinated ovules which had been collected at different dates and these were compared with sections from cross-pollinated ovules collected at approximately the same

time after pollination. Particular care was taken to obtain clear sections of the micropyle so that the pollen grains could be counted and checked for germination. If germination had occurred, their development was recorded under three headings.

- (1) The generative cell had not divided.
- (2) The generative cell had divided to form the body and stalk cells.
- (3) The body cell was dividing to form the two sperm nuclei.

A summary of the results is shown in table 4 and it was obvious that neither pollen germination nor its subsequent development were inhibited after self-pollination since only twelve pollen grains were found not germinated. The remaining one hundred and ninety-four pollen grains had all germinated and were in various stages of development. One of these is illustrated in figure 1, plate III, twelve days after self-pollination; the generative cell has divided to form the body and stalk cells and the vestigial prothallial cells can be seen. Another germinating pollen grain, seventeen days after self-pollination, is illustrated in figure 2. The pollen tube is prominent and the tube, body, stalk, and prothallial cells are all visible.

It was also evident that the nucellus did not act as a physiological sieve for pollen tubes since numerous tubes were observed growing towards the archegonia. One of these is illustrated in figure 3, fifty-four days after self-pollination. A comparison of pollen development after self- and cross-pollination

showed that there were no differences between the two at this stage.

Some sections were also made from the self- and cross-pollinated ovules of tree 2, the other Pseudotsuga investigated in 1952; these were removed only five days after self-pollination but in some ovules pollen germination had already occurred. The higher temperatures within the bags undoubtedly must have accelerated pollen germination in the micropyle. The earliest germination recorded by Lawson (1909) for Pseudotsuga in California was seven to ten days, whereas Allen (1943) in British Columbia found that pollen remained about three weeks in the micropyle before germination. Both these earlier investigations, however, were made from ovules collected after wind pollination.

TABLE 4. POLLEN DEVELOPMENT AFTER SELF- AND CROSS-POLLINATION
TREE 1.

Type of pollin.	Days after pollin.	No. of ovules sampled	No. of pollen grains	Germin.	Stage of pollen development		
					Gener- ative tube cells	Body & stalk cells	Divis. of body cell
Self	12	15	37	36	17	19	-
"	17	2	9	9	2	7	-
"	26	15	84	76	4	72	-
"	27	13	76	73	0	70	3
		45	206	194			
Cross	9	10	63	46	19	27	-
"	21	13	30	28	8	20	-
		23	93	74			

Fertilization and Proembryo Development

Sections were made from fifteen self-pollinated ovules which had been removed fifty-four days after pollination; the stages of development in each archegonia have been summarized in table 5. Syngamy and the subsequent divisions of the zygote were observed in eleven of the ovules; in two others, sperm nuclei had been discharged into the archegonia and were moving towards the egg nuclei. In the remaining two ovules, pollen tubes were either penetrating the nucellus or had already reached the archegonia. Sections were made also from four ovules removed fifty-eight days after self-pollination and early proembryo development was observed in three of these. Development after self-pollination again compared very favourably with those from the cross-pollinated ovules. Eleven of the latter were removed fifty-five days after pollination and syngamy and later development were observed in only three of them. In five other ovules, pollen tubes were either penetrating the nucellus or had reached the archegonium.

Figure 4, plate III, shows the two sperm nuclei moving towards the egg nucleus in one of these self-pollinated ovules and figure 5 shows the sperm nucleus fusing with the nucleus of the egg. The subsequent division of the zygote is illustrated in figure 6 and in figure 7, the two free nuclei have divided and three of the four resulting daughter nuclei can be seen moving towards the basal part of the archegonium. The early development of the proembryo is illustrated in figure 8. It is quite evident from these figures that no physiological barrier to self-fertilization and development

of the proembryo had occurred.

It was again apparent that the bags accelerated development as syngamy was observed fifty-four days after pollination, whereas Allen (1943) found that two months was the usual time in the case of wind-pollination.

TABLE 5. DEVELOPMENT IN ARCHEGONIA AFTER SELF- AND CROSS-POLLINATION
TREE 1.

Type of pollin.	Days after pollin.	No. of ovules sampled	No. of archegonia	Archegonia with these stages			
				Sperm nuclei present	Syngamy	1st & 2nd zygotic divis.	Proembryo development
Self	54	15	57	6	13	8	-
"	58	4	14	-	1	-	5
Cross	55	11	40	5	1	1	1

PLATE III

Figure 1. A germinating pollen grain twelve days after self-pollination. April 14, 1952. X 480.

Figure 2. Vigorous pollen tube growth seventeen days after self-pollination; note the tube, body, stalk, and prothallial cells.
April 19, 1952. X 480.

Figure 3. A pollen tube penetrating the nucellus fifty-four days after self-pollination.
May 26, 1952. X 480

Figure 4. The two sperm nuclei in the archegonium; one of these will unite with the egg nucleus.
May 26, 1952. X 345.

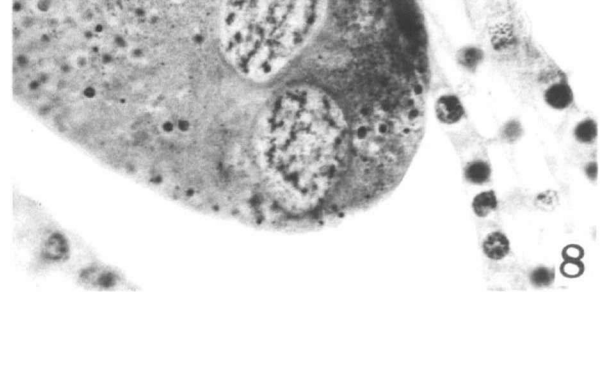
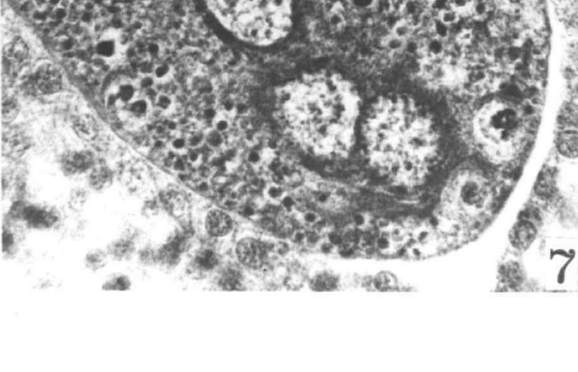
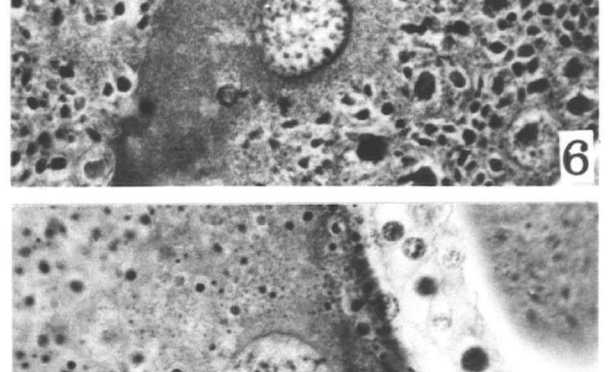
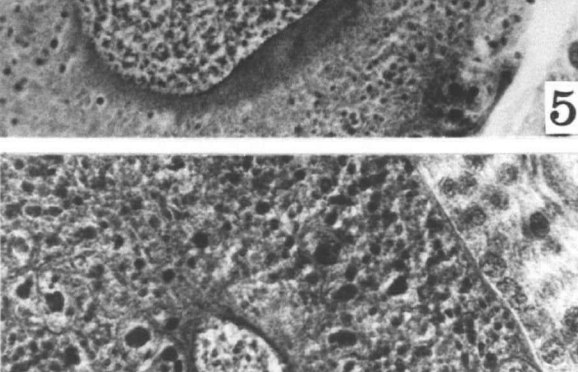
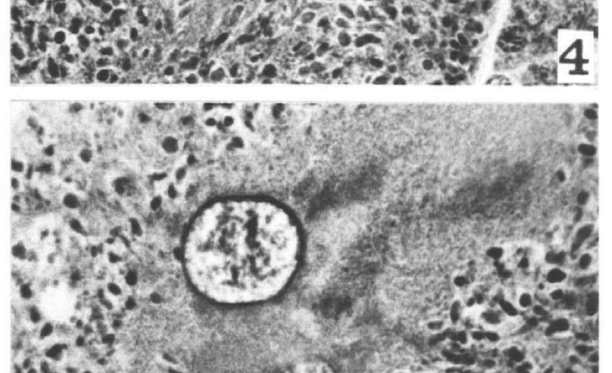
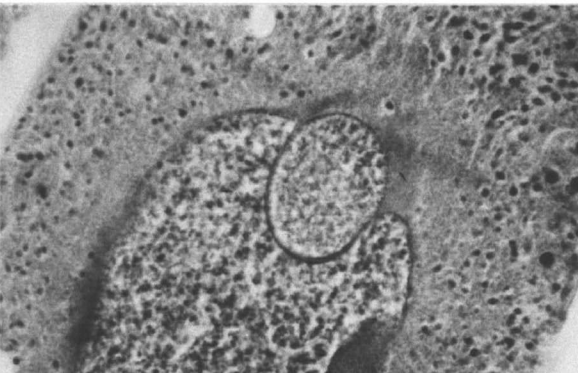
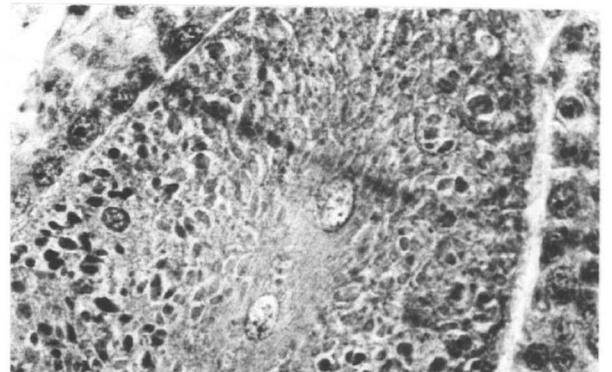
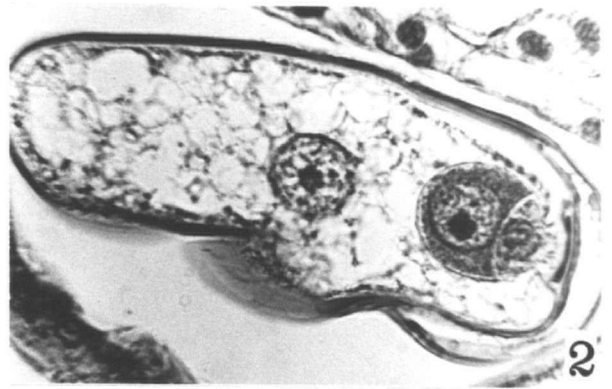
Figure 5. Fusion of the male and female nuclei, note the disparity in size.
May 26, 1952. X 345.

Figure 6. First division of the zygote.
May 26, 1952. X 345.

Figure 7. Three of the four free nuclei moving towards the base of the archegonium after the second division of the zygote. May 26, 1952. X 345.

Figure 8. Early development of the proembryo, fifty days after self-pollination. June 1, 1952. X 345.

PLATE III



Embryo Development

The results of the examination of the previous stages of development had indicated that the impediment to normal seed development must occur after self-fertilization and at some time during development of the embryo. Sections were made, therefore, from self-pollinated ovules which had been removed at seven different lengths of time after fertilization. For each of these collections, the number of embryos in each ovule and the number of nuclei in the terminal embryo were recorded. The number of nuclei used was that obtained by counting those visible in the embryo in one focal plane. The terminal embryo was considered to be the dominant one of the ovule throughout this investigation. It was usually the largest and Buchholz (1918) found that, in Pinus, it was the successful one in the struggle for supremacy among the embryos. He added that only very exceptionally was the successful embryo found to be the second one instead of the terminal. The terminal embryo, of course, has the advantage of being at the apex of the corrosion cavity whereas the other embryos are forced to develop behind it. Buchholz (1946) pointed out that these other embryos became ensnared in the suspensors of the terminal embryo and were gradually pushed back towards the micropyle.

For each collection, the condition of the terminal embryo was carefully checked for any signs of collapse. It was fully realized that killing-and-fixing could produce artifacts and that embedding and sectioning could damage the young embryos to the

extent that the material might be misinterpreted. The procedure throughout this investigation, therefore, was to classify embryos as "collapsed" only when this condition was quite obvious. The results of the examinations for each collection date are summarised as follows:

(1) June 9, sixty-eight days after self-pollination.

Fertilization was observed in all of the ten ovules sectioned; eight contained vigorous young embryos which were perfectly normal in appearance. One such embryo is illustrated in figure 1, plate IV, which shows a female gametophyte of normal appearance with sharply stained nuclei and active mitosis in evidence.

It was interesting to find syngamy in the archegonia of four ovules which contained young embryos, particularly since only the proembryo stage was present in two other ovules. This variation in development within the same ovule and cone is not in agreement with the conclusions of Buchholz (1945), who concluded that fertilization occurred in Pinus on nearly the same day within all the cones of a number of individuals of the same species growing on the same site. In tree 1, however, syngamy was first observed on May 26th, two weeks prior to this date. It was also quite evident that fertilization in Pseudotsuga takes place over a considerable period among different trees growing on the same site.

TABLE 6. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JUNE 9.
TREE 1.

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	1	2
2	1	2
3	2	3
4	2	4
5	2	2
6	2	2
7	2	3
8	3	4

(2) June 16, seventy-six days after self-pollination.

Eight ovules were sectioned and six of these contained embryos. In two of these ovules, however, the first signs of embryo collapse were noted. This early state of collapse was characterized by cell shrinkage, excessive vacuolization, and very dense staining with haematoxylin. The entire cell contents were commonly stained densely as shown in figures 2 and 3, plate IV. Both of these collapsing embryos were small but still comparable in size to the other terminal embryos and it is significant that mitosis was observed in only one of these normal embryos.

TABLE 7. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JUNE 16.
TREE 1.

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	2	7
2	3	7
3	4	8
4	3	9
5	3	X
6	3	X

X signifies early embryo collapse

(3) June 21, eighty days after self-pollination.

Thirty-one ovules were sectioned; twenty-five of these contained embryos and the remainder had not been fertilized. Embryo collapse had definitely increased as witnessed by the collapse of seven terminal embryos. In addition, some of the remaining embryos lacked vigour and mitosis was observed in only seven of them; in three of these mitosis appeared abnormal with considerable clumping of the chromosomes. Careful examination of the ovules throughout this investigation showed that collapse was general whenever the terminal embryo had collapsed, and that there was never any question of a second embryo taking its place. Some of the sound embryos had grown considerably since the last date and figure 4, plate IV, illustrates one of the largest of these. Another terminal embryo which had stained abnormally and was on the point of collapse is illustrated in figure 5; two collapsed embryos are shown in figure 6.

The cells of the gametophyte at this date showed evidence of becoming filled with densely staining material, and this was true regardless of the condition of the embryos.

TABLE 8. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JUNE 21.
TREE 1.

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	1	4
2	2	7
3	2	8
4	4	11
5	5	11
6	3	12
7	3	12
8	2	14
9	3	14
10	3	15
11	2	16
12	3	19
13	2	24
14	5	24
15	3	26
16	2	30
17	4	33
18	4	34
19	1	X
20	1	X
21	1	X
22	2	X
23	2	X
24	3	X
25	4	X

X signifies early embryo collapse

(4) June 28, eighty-seven days after self-pollination.

This sample was interesting since it could be compared with one resulting from controlled cross-pollination eighty-four days previously. Twenty-one self-pollinated ovules were sectioned and

in only three of these could the terminal embryos be considered normal. These embryos were small, no mitosis was observed in them, and they were in marked contrast to those resulting from cross-pollination. The largest of these latter embryos is illustrated in figure 8, plate IV; it showed a considerable number of mitoses and eighty-two nuclei in one focal plane, compared with twenty-nine nuclei in the largest of the embryos resulting from self-pollination. Figure 7 illustrates a selfed embryo in a state of collapse; its cells and nuclei are abnormally large and the cell contents show considerable shrinkage. Fifteen of the remaining ovules contained embryos in the advanced state of collapse (see figure 1, plate V). This condition is characterized by complete cell collapse and erratic staining of the nuclei. The remains of three collapsed embryos can be seen in figure 2; the gametophyte tissue, however, appears to be perfectly normal, its nuclei stain sharply and its cells contain a considerable amount of deeply staining material.

The data in table 9 indicate that a considerable difference exists in the development of the dominant embryos in different ovules of the same cone. In the embryos from the cross-pollinated ovules, for example, the number of nuclei visible in one optical plane varies from thirteen to eighty-two. Similar variation in embryo size was reported for Pinus by Buchholz (1946), who suggested that it may be due to genetic variation and, therefore, may be a measure of inherent embryonic vigour.

TABLE 9. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JUNE 28. TREE 1.

Ovule No.	<u>Self-pollination</u>		Ovule No.	<u>Cross-pollination</u>	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	4	11	1	2	13
2	3	19	2	1	16
3	1	29	3	1	16
4	1	X	4	4	40
5	1	X	5	4	40
6	2	X	6	2	82
7	2	Y			
8	2	Y			
9	2	Y			
10	3	Y			
11	3	Y			
12	3	Y			
13	3	Y			
14	3	Y			
15	3	Y			
16	3	Y			
17	3	Y			
18	4	Y			
19	4	Y			
20	4	Y			
21	4	Y			

X signifies early and Y advanced embryo collapse

(5) July 7, ninety-six days after self-pollination

The completeness of embryo collapse in tree 1 after self-pollination is well illustrated by this sample for, although the twenty-four fertilized ovules which were sectioned all contained embryos, everyone of these had collapsed. Figures 3 and 4, plate V, show the same section under different magnification; it is evident that the gametophyte cells are still full of dark-staining material, yet the terminal and other embryos have all collapsed. A zone of apparently depleted cells surrounds the corrosion cavity but such

was invariably present at this stage regardless of the type of pollination. The occurrence of a similar zone in Larix was reported by Schopf (1943). Other collapsed terminal embryos appear in figures 5 and 6; here the nuclei stain rather faintly and the gametophyte cells are beginning to collapse.

TABLE 10. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JULY 7.
TREE 1.

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	1	Y
2	1	Y
3	2	Y
4	2	Y
5	2	Y
6	2	Y
7	2	Y
8	2	Y
9	2	Y
10	2	Y
11	2	Y
12	2	Y
13	3	Y
14	3	Y
15	3	Y
16	3	Y
17	3	Y
18	3	Y
19	3	Y
20	3	Y
21	4	Y
22	4	Y
23	4	Y
24	5	Y

Y signifies advanced embryo collapse

(6) July 13, one hundred days after self-pollination.

Of the six ovules sectioned, the embryos of five had collapsed

completely; the gametophyte tissue was beginning to break down and the dark staining material in its cells was diminishing. One of these terminal embryos is illustrated in figure 7, plate V; collapse is complete and there is very little staining. In the remaining ovule, however, a well developed embryo was found; it is illustrated in figure 8 and was definitely past the stage where embryo collapse usually occurred.

TABLE 11. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JULY 13.
TREE 1.

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	1	too numerous to count
2	2	Y
3	2	Y
4	2	Y
5	3	Y
6	4	Y

Y signifies advanced embryo collapse

(7) July 21, one hundred and eight days after self-pollination.

Of the four ovules examined, two contained collapsed embryos; these were very difficult to find since the gametophyte cells did not stain and their nuclei had disappeared.

Studies were discontinued beyond this date because embryo failure appeared to be complete. The results, which are summarized in table 12, showed that no barrier to early embryo development existed, as the ninety ovules examined contained two hundred and thirty-five embryos. The very low yield of viable seed from this

tree, however, indicated that very few embryos developed to maturity.

TABLE 12. THE NUMBER OF EMBRYOS AFTER SELF-POLLINATION. TREE 1.

Date of collection	No. of ovules sectioned	No. of ovules with embryos	No. of embryos	Average no. of embryos per ovule
9/6	10	8	15	1.9
16/6	8	6	18	3.0
21/6	31	25	67	2.7
28/6	21	21	58	2.8
7/7	26	24	63	2.6
13/7	6	6	14	2.3
	102	90	235	2.6

PLATE IV

- Figure 1. Vigorous young embryo sixty-eight days after self-pollination. June 9, 1952. X 345.
- Figure 2. Young terminal embryo collapsing seventy-six days after self-pollination. June 16, 1952. X 345.
- Figure 3. The characteristic dense staining of the embryo cells in the early collapse state. June 16, 1952. X 890.
- Figure 4. Terminal embryo eighty days after self-pollination. June 21, 1952. X 345.
- Figure 5. Terminal embryo on the point of collapse. June 21, 1952. X 345.
- Figure 6. Two collapsed embryos; note the dense staining and cell shrinkage. June 21, 1952. X 345.
- Figure 7. Terminal embryo, eighty-seven days after self-pollination. June 28, 1952. X 345.
- Figure 8. A vigorous terminal embryo eighty-four days after cross-pollination, compare with figure 7. June 28, 1952. X 345.

PLATE IV

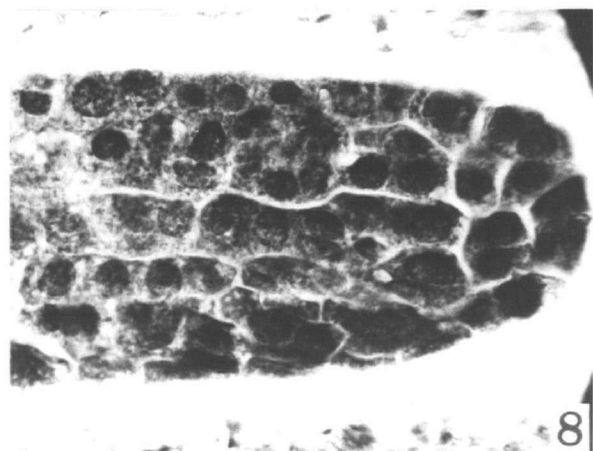
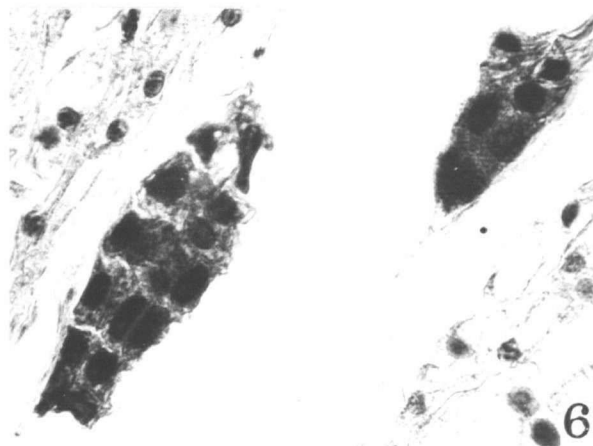
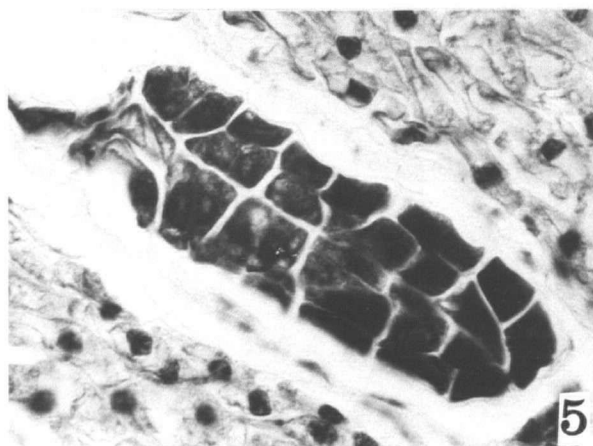
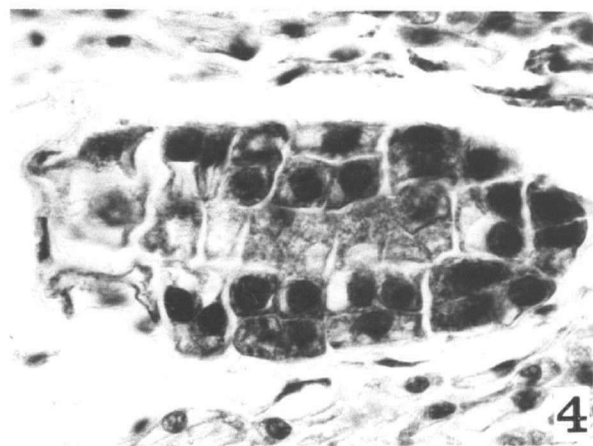
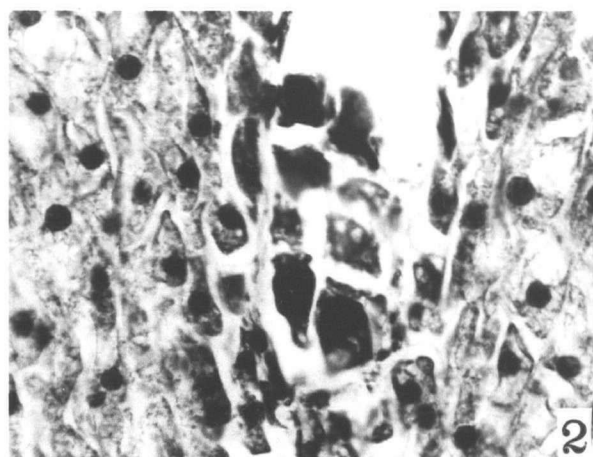
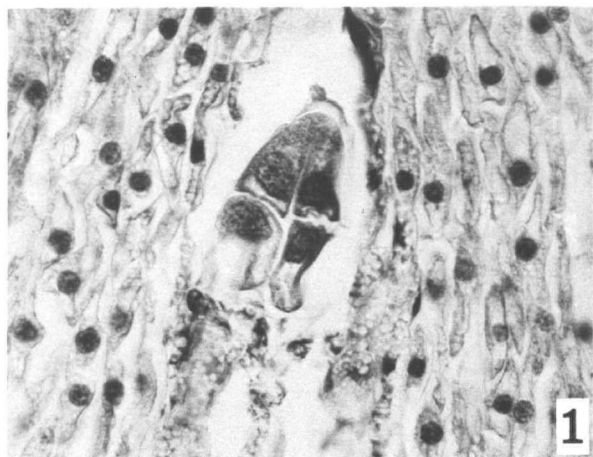
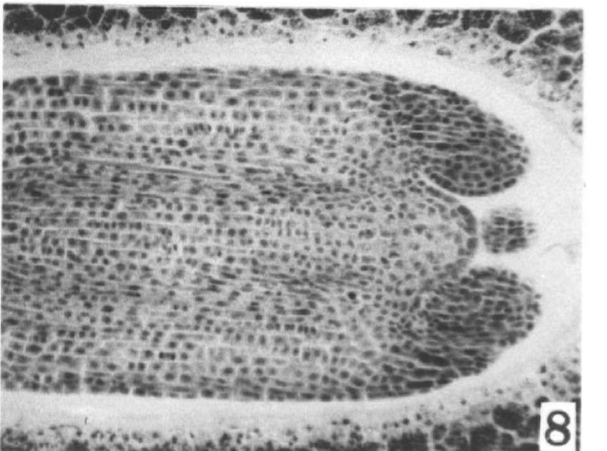
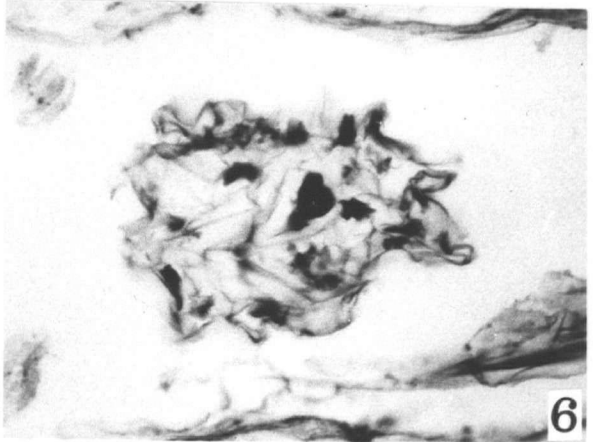
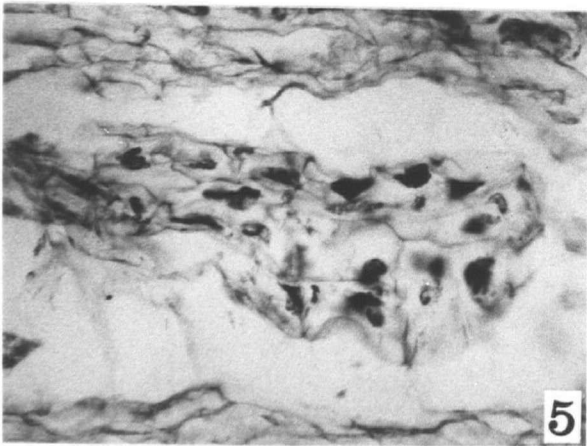
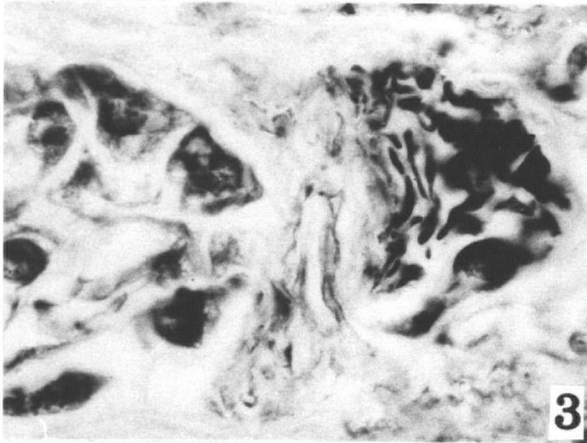
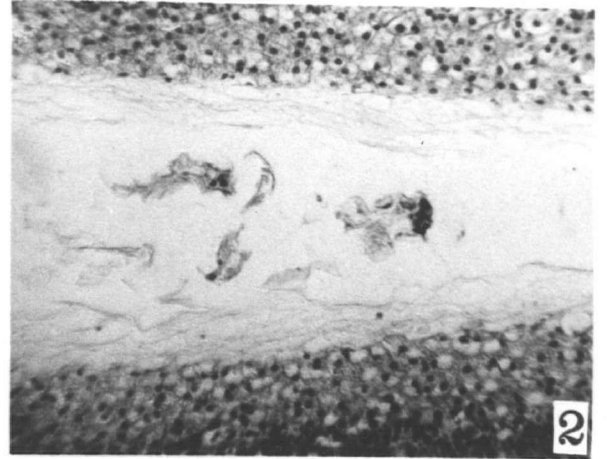


PLATE V

- Figure 1. Terminal embryo eighty-seven days after self-pollination and in the advanced state of collapse. June 28, 1952. X 345.
- Figure 2. Three collapsed embryos; note the condition of the gametophyte. June 28, 1952. X 92.
- Figure 3. Two collapsed embryos ninety-six days after self-pollination. July 7, 1952. X 345.
- Figure 4. The same two embryos under lower magnification; note the food material in the cells of the gametophyte. X 92.
- Figure 5. A collapsed terminal embryo; note the erratic staining. July 7, 1952. X 345.
- Figure 6. A collapsed terminal embryo. July 7, 1952. X 345.
- Figure 7. A collapsed terminal embryo one hundred days after self-pollination. There is little staining and the gametophyte cells are breaking down. July 13, 1952. X 345.
- Figure 8. A late embryo; note the cotyledons and plumule primordia. July 13, 1952. X 92.

PLATE V



III CYTOLOGICAL INVESTIGATION WITH TREE A

Comparative Ovule Abortion After Self-Pollination

The controlled self-pollination of 1952 had shown that there was a considerable variation in the amount of viable seed produced by two different trees. This variation was investigated further in 1954, as it was considered desirable to obtain some comparable results from other trees. Six more trees were therefore selected for controlled self-pollination; three of these were near Victoria and the remainder farther north at Lake Cowichan. The female cones were isolated and self- and cross-pollinated in the manner already described. Sample cones were later removed at different dates from each tree and the ovules killed-and-fixed prior to embedding.

The effect of self-pollination on these six trees was naturally not known at this time and would not have been known until their cones had been collected and the seed germinated. It was essential, however, that there should be some quick guide as to the effect of self-pollination on the different trees in order that a cytological study could be made on the most suitable tree. It was found that a very useful guide was obtained by examining the condition of the ovules when the integuments had been removed prior to killing-and-fixing. There are two unproductive regions near the tip and base of the cones of Pseudotsuga and the ovules in this region do not develop past the rudimentary stage. Similar regions on the cones of Pinus resinosa Ait have been described by

Lyons (1956). Such ovules were discounted as their condition was not related to the effects of pollination. The condition of the ovule¹ in the productive region, however, received particular attention. A healthy ovule is firm and white in colour (see figure 8, plate IX) but towards the end of June and the beginning of July, some of the ovules became limp and discoloured. This condition increased in intensity and the ovules finally collapsed and shrivelled up completely as shown in figure 8, plate IX. Such ovules have been termed aborted by Lyons (1956) and this definition will be used to distinguish between collapse of the ovules and collapse of the embryos.

Ovule abortion can be caused either by lack of pollination and fertilization or by the prior collapse of the embryo; it was previously found that an ovule will not persist to maturity if there is no embryo. The numbers of healthy and aborted ovules were recorded for each tree as soon as the first signs of abortion were observed. Table 13 shows these results and it can be seen that ovule abortion in cones that had intentionally been left unpollinated and abortion in cones that had been pollinated, occurred at approximately the same time. The dates of this abortion occurred several weeks after fertilization in all six trees. Ovule abortion after self-pollination was much more noticeable in trees A, C, and D than in trees F, H, and J, and these latter trees were clearly of little value for the cytological investigation. It can be seen that self-pollination

¹For convenience, ovule here refers to the intra-integumentary tissues and does not include the integuments.

resulted in complete ovule abortion for tree A and there was little doubt that much of this must have been caused by the prior collapse of the embryo. Tree A was, therefore, selected for the cytological investigation and the results later showed that this supposition was perfectly correct.

TABLE 13. COMPARATIVE OVULE ABORTION IN POLLINATED AND UNPOLLINATED CONES

Tree	Date of cone removal	Days after pollin.	Self- pollinated cones		Cross- pollinated cones		Cones not pollinated*	
			Healthy Ovules	Aborted Ovules	Healthy Ovules	Aborted Ovules	Healthy Ovules	Aborted Ovules
A	28/6	71	37	5	no collapse		no collapse	
	2/7	75	35	8	46	3	no collapse	
	7/7	78	3	19	18	16	all collapsed	
	14/7	85	0	37	24	17	0	41
	21/7	92	0	51	31	14	0	29
C	7/7	83	0	53	27	18	no	
	14/7	92	17	22	35	20	samples	
	26/7	104	2	22	25	12		
D	28/6	74	4	15	19	0	no collapse	
	1/7	77	1	24	22	10	no collapse	
	7/7	80	3	30	11	22	21	5
	14/7		no samples				0	39
F	11/7	68	no collapse		no collapse		39	3
	14/7	71	39	2	41	5	3	35
	17/7	78	46	1	27	5	1	37
	27/7	83	41	6	35	3	0	43
	13/8	105	19	28	42	17	0	43
H	14/7	76	74	1	47	8	29	41
	17/7	79	50	0	22	29	1	69
	22/7	84	62	3	58	0	0	67
	27/7	89	37	11	49	3	0	66
	13/8	106	7	55	42	5	0	66
J	11/7	81	no collapse		42	11	9	42
	15/7	85	40	1	28	19	0	42
	17/7	87	31	5	38	9	0	61
	27/7	89	25	13	14	32	0	46
	13/8	114	12	42	43	6	0	45

* These cones had been isolated and intentionally left unpollinated.

Fertilization and Proembryo Development

The results of the investigation with tree 1 had shown that fertilization occurred in the normal manner after self-pollination

but that the subsequent collapse of the embryos was the cause of the low yield of viable seed. It seemed probable that fertilization would proceed normally after self-pollination in tree A, but it was thought desirable that this assumption should be checked. Sections were, therefore, made from eight ovules which had been removed fifty-one days after self-pollination. Syngamy and later stages of development were found in five of these ovules and sperm nuclei were seen in the archegonia of the remainder. The first division of the zygote in one of these ovules is illustrated in figure 2, plate VI.

In all of the eight cross-pollinated ovules sectioned, syngamy and later stages of development were observed. The development within the archegonia of the self- and cross-pollinated ovules is shown in table 14 which indicates that development in the latter was more advanced. The cross-pollinated sample, however, had been pollinated two days earlier which could account for the more advanced development. The results definitely indicated that there was no physiological barrier to self-fertilization and supported the findings already reported for tree 1.

TABLE 14. DEVELOPMENT IN ARCHEGONIA AFTER SELF- AND CROSS-POLLINATION
TREE A

Type of pollin.	Days after pollin.	No. of ovules sampled	No. of archeg- onia.	Archegonia with these stages			
				Sperm nuclei present	Syngamy	1st & 2nd zygotic divisions	Proembryo develop- ment
Self	51	8	38	10	6	3	1
Cross	53	8	36	-	1	1	20

Embryo Development

The results of the previous examination showed that the impediment to normal seed development must occur after self-fertilization and during development of the embryo. The subsequent examinations, therefore, replicated those conducted with tree 1, comparisons with samples from controlled cross-pollination could also be made at most dates.

(1) June 16, fifty-nine days after self-pollination.

Of the fifteen ovules sectioned, fertilization had occurred in thirteen. Vigorous young embryos were observed in ten of these ovules; one of these is illustrated in figure 3, plate VI; again, several stages of development were found within the same ovules as occurred in the case of tree 1. Syngamy, for example, was present in three ovules which also contained embryos; syngamy, proembryos, and embryos were all seen in another ovule. Figure 1, plate VI; shows a sperm nucleus lying within that of the egg, nine days after syngamy was first recorded from this tree.

TABLE 15. DEVELOPMENT OF EMBRYOS AFTER SELF-POLLINATION. JUNE 16.
TREE A

Ovule No.	No. of embryos per ovule	No. of nuclei in terminal embryo
1	3	2
2	3	2
3	1	2
4	2	2
5	4	2
6	4	2
7	2	2
8	4	4
9	4	4
10	3	4

(2) June 20, sixty-eight and sixty-five days respectively after self- and cross-pollination.

Of the ten self-pollinated ovules sectioned, three contained embryos, the remaining seven had not been fertilized. The terminal embryos were growing vigorously and there was no sign of any collapse.

On the other hand, of the fifteen cross-pollinated ovules sectioned, fourteen contained young embryos, one of which is illustrated in figure 4, plate VI.

TABLE 16. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JUNE 20. TREE A

Ovule No.	<u>Self-pollination</u>		Ovule No.	<u>Cross-pollination</u>	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	1	7	1	4	2
2	3	7	2	1	2
3	2	11	3	3	3
			4	5	4
			5	5	4
			6	3	4
			7	3	4
			8	3	4
			9	2	4
			10	1	4
			11	4	4
			12	5	4
			13	3	5
			14	4	6

(3) June 23, sixty-eight days after both self- and cross-pollination.

Of the fourteen self-pollinated ovules sectioned, eleven contained embryos. The first signs of embryo collapse, however, appeared in this collection; three of the ovules contained terminal embryos in the early state of collapse. The remaining eight ovules contained sound terminal

embryos and mitosis was observed in three of them; one such embryo is illustrated in figure 5, plate VI.

Fifteen cross-pollinated ovules were sectioned and thirteen of them contained embryos. In one of the ovules the terminal embryo had collapsed but the remainder were perfectly normal. These embryos, moreover, were already developing more rapidly than those from self-pollination and the number of nuclei observable in one focal plane was generally higher; one such embryo is illustrated in figure 6.

The contents of the gametophyte cells had previously not shown any unusual concentration of stainable material but from this date onward, the cell contents began to increase in stainability. This occurred without relation to the type of pollination.

TABLE 17. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JUNE 23. TREE A

Ovule No.	Self-pollination		Ovule No.	Cross-pollination	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	3	6	1	3	5
2	3	7	2	3	6
3	3	7	3	3	8
4	4	7	4	3	8
5	3	9	5	5	9
6	4	10	6	2	9
7	4	10	7	5	11
8	4	11	8	3	13
9	1	X	9	4	14
10	1	X	10	4	14
11	2	X	11	5	15
			12	4	17
			13	3	X

X signifies early embryo collapse

(4) June 28, seventy-one days after both self- and cross-pollination

Of the fifteen self-pollinated ovules sectioned, ten contained embryos. In six of these ovules the terminal embryos were in the early state of collapse as shown in figure 8, plate VI. Collapse was again characterized by the dense staining and shrinkage previously observed in the collapsing embryos of tree 1; the embryo in figure 8, for example, is very similar in appearance to that in figure 2, plate IV. Another embryo which is on the point of collapse can be seen in figure 7; staining is particularly dense at the apex.

Twenty-one cross-pollinated ovules were sectioned and nineteen of these contained embryos. In six ovules the terminal embryos were in the early state of collapse (see figure 1, plate VII). Their appearance is very similar to that of embryos collapsing after self-pollination. The remaining ovules contained healthy embryos which were considerably more developed than those from self-pollination; in one focal plane, their nuclei numbered between fifteen and forty-six compared with eight and sixteen for those resulting from self-pollination. The presence or absence of mitosis in these terminal embryos was also suggestive; it was observed only in two embryos resulting from self-pollination compared to eleven resulting from cross-pollination. The cones from which both groups of ovules were taken were removed from adjacent branches so that the position on the tree could hardly have effected development within the ovules.

TABLE 18. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JUNE 28. TREE A

Ovule No.	<u>Self-pollination</u>		Ovule No.	<u>Cross-pollination</u>	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	2	8	1	3	15
2	4	9	2	5	16
3	2	13	3	2	18
4	3	16	4	3	20
5	1	X	5	4	20
6	2	X	6	3	21
7	2	X	7	6	25
8	4	X	8	2	26
9	4	X	9	3	32
10	4	X	10	5	34
			11	4	35
			12	2	40
			13	5	46
			14	2	X
			15	3	X
			16	3	X
			17	4	X
			18	4	X
			19	4	X

X signifies early embryo collapse

(5) July 2nd, seventy-five days after both self- and cross-pollination.

Of the thirty-four self-pollinated ovules sectioned, thirty-three contained embryos; in five of these the terminal embryos had collapsed. The majority of the terminal embryos in the other ovules were small and only one showed evidence of cell division. One such embryo is illustrated in figure 2, plate VII; it may be compared with figure 4 which shows an embryo resulting from cross-pollination and collected on the same date. A larger embryo resulting from self-pollination is illustrated in figure 3 but it is still not

comparable in vigour with that in figure 4.

Of the thirty-one cross-pollinated ovules sectioned, twenty-eight contained embryos. There was no collapse in any of the terminal embryos which were in striking contrast to those resulting from self-pollination. The number of nuclei visible in one focal plane ranged between twenty and one hundred and thirty-eight, compared with seven and sixty-four in the case of the embryos resulting from selfing.

TABLE 19. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JULY 2. TREE A

Ovule No.	<u>Self-pollination</u>		Ovule No.	<u>Cross-pollination</u>	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	5	7	1	5	20
2	4	8	2	4	32
3	4	9	3	6	39
4	5	10	4	4	41
5	3	13	5	4	44
6	5	15	6	5	49
7	3	15	7	6	50
8	1	15	8	5	52
9	4	16	9	5	54
10	5	16	10	4	56
11	5	16	11	3	56
12	4	18	12	2	62
13	5	18	13	4	65
14	3	20	14	2	69
15	2	20	15	3	69
16	2	21	16	4	71
17	5	22	17	4	74
18	2	23	18	3	78
19	6	24	19	4	86
20	5	26	20	6	87
21	3	27	21	3	88
22	3	29	22	5	88
23	3	30	23	4	90
24	4	31	24	3	97
25	4	31	25	5	98
26	5	37	26	5	103
27	3	38	27	4	126
28	4	64	28	4	138
29	1	X			
30	3	X			
31	3	X			
32	4	X			
33	4	X			

X signifies early embryo collapse

(6) July 8, eighty and seventy-five days respectively after self-
and cross-pollination.

Only three self-pollinated ovules were sectioned at this date

since they were the only sound ones observed when the seed coats were removed; none of the other thirty-eight aborted ovules was embedded. The three ovules all contained embryos in the advanced state of collapse and one of these is illustrated in figure 5, plate VII. There was no sign of collapse in the gametophyte cells, but the dark-staining cell contents were disappearing.

Sixteen cross-pollinated ovules were sectioned. Fifteen of these contained terminal embryos with a number of visible nuclei ranging from thirty to one hundred and twenty-eight; numerous mitoses were also observed in all of these embryos. There was intense competition among the embryos in most of these ovules; it was not unusual to find three or four developing in the same ovule, all with approximately the same number of nuclei.

TABLE 20. DEVELOPMENT OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
JULY 8.. TREE A

Ovule No.	<u>Self-pollination</u>		Ovule No.	<u>Cross-pollination</u>	
	No. of embryos per ovule	No. of nuclei in terminal embryo		No. of embryos per ovule	No. of nuclei in terminal embryo
1	2	Y	1	3	30
2	2	Y	2	1	33
3	3	Y	3	2	39
			4	6	48
			5	4	52
			6	2	54
			7	6	57
			8	2	67
			9	4	80
			10	2	93
			11	1	94
			12	3	95
			13	1	101
			14	5	112
			15	3	128

Y signifies advanced embryo collapse

(7) July 14, Eighty-two days after cross-pollination.

No self-pollinated ovules were sectioned at this date because they had all aborted. All of the five cross-pollinated ovules sectioned contained rapidly developing embryos. One of these is illustrated in figure 6, plate VII, and it can be seen that there is already a considerable amount of embryonic organisation.

(8) July 21, one hundred and five and eighty-nine days respectively after self- and cross-pollination.

Two collapsed self-pollinated ovules were sectioned and both contained embryos. One of these terminal embryos is illustrated in figure 7, plate VII; it can be seen that there is very little staining in the gametophyte cells which have begun to break down. An ovule in which fertilization had occurred, however, could still be distinguished from an unfertilized one at this date since the latter was in a much more advanced state of collapse.

Three cross-pollinated ovules were sectioned; all of these contained embryos in advanced stages of development, one of which is illustrated in figure 8.

The cytological study was not continued past this date since all of the self-pollinated ovules had aborted in the remaining samples collected on July 27th and August 8th. The results which are summarised in table 21 again showed that no barrier to early embryo development existed as the seventy-two ovules examined contained two hundred and thirty-one embryos. A test of Chi square, moreover, showed that there was no significant difference between the number of embryos produced after self- or cross-pollination.

TABLE 21. NUMBER OF EMBRYOS AFTER SELF- AND CROSS-POLLINATION
TREE A

Date of collection	Self-pollination			Cross-pollination		
	No. of ovules with embryos	No. of embryos	Average no. of embryos per ovule	No. of ovules with embryos	No. of embryos	Average no. of embryos per ovule
16/6	10	30	3.0	-	-	-
20/6	3	6	2.0	14	46	3.3
23/6	11	32	2.9	13	47	3.6
28/6	10	28	2.8	19	67	3.5
2/7	33	122	3.7	28	116	4.1
8/7	3	7	2.3	15	45	3.0
14/7	-	-	-	5	20	4.0
21/7	2	6	3.0	3	7	2.3
	72	231	3.2	97	348	3.6

PLATE VI

- Figure 1. The sperm lying in the egg nucleus fifty-nine days after self-pollination. June 16, 1954. X 890.
- Figure 2. First division of the zygote fifty days after self-pollination. June 7, 1954. X 345.
- Figure 3. Vigorous young embryo fifty-nine days after self-pollination. June 16, 1954. X 345.
- Figure 4. Young embryos sixty-five days after cross-pollination. June 20, 1954. X 345.
- Figure 5. A terminal embryo sixty-eight days after self-pollination; note the mitoses in the cells.
June 23, 1954. X 345.
- Figure 6. A terminal embryo sixty-eight days after cross-pollination, compare its development with that in figure 5. June 23, 1954. X 345.
- Figure 7. A terminal embryo about to collapse, seventy-one days after self-pollination. June 28, 1954. X 345.
- Figure 8. A collapsed terminal embryo after self-pollination; note the characteristic dense staining.
June 28, 1954. X 345.

PLATE VI

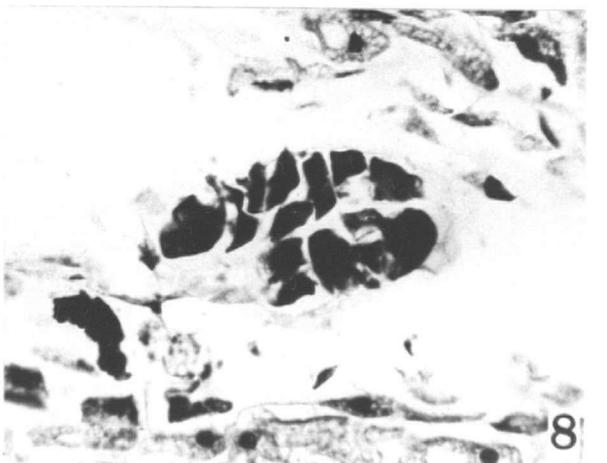
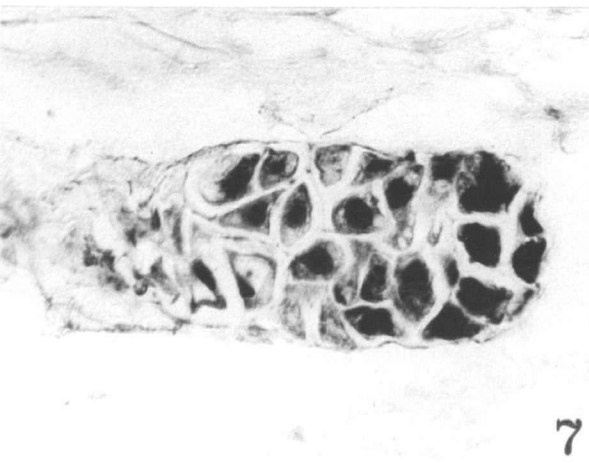
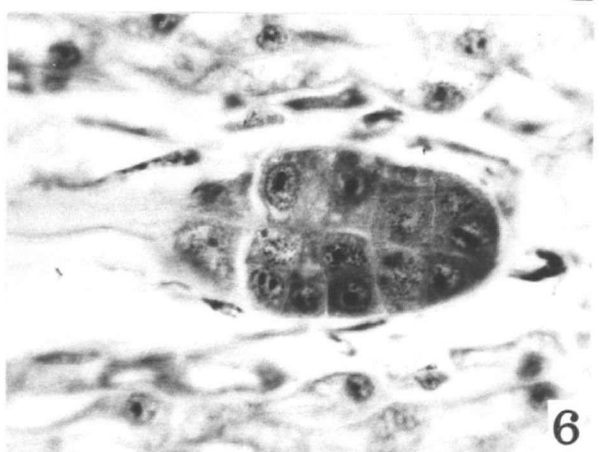
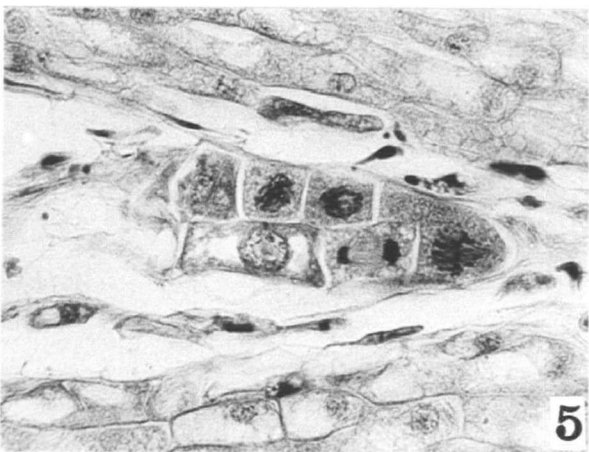
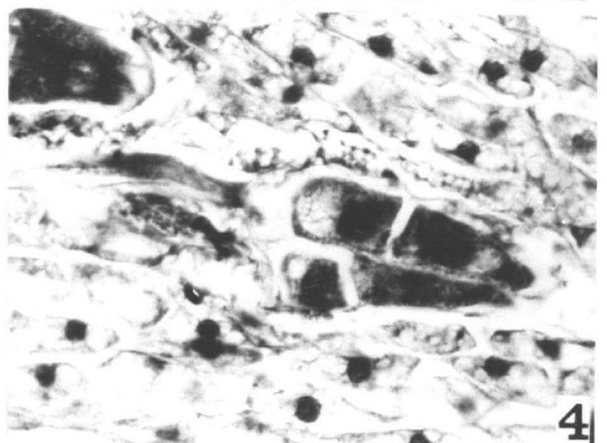
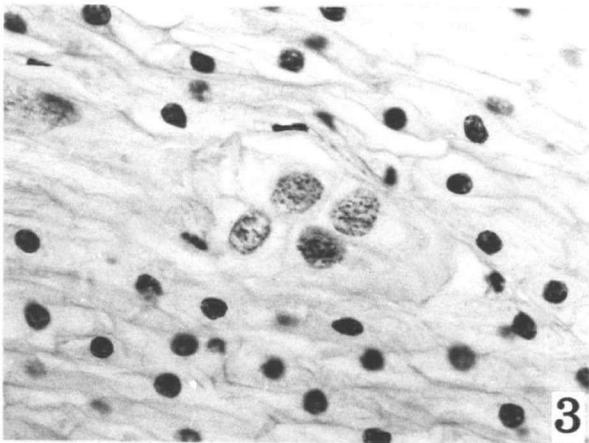
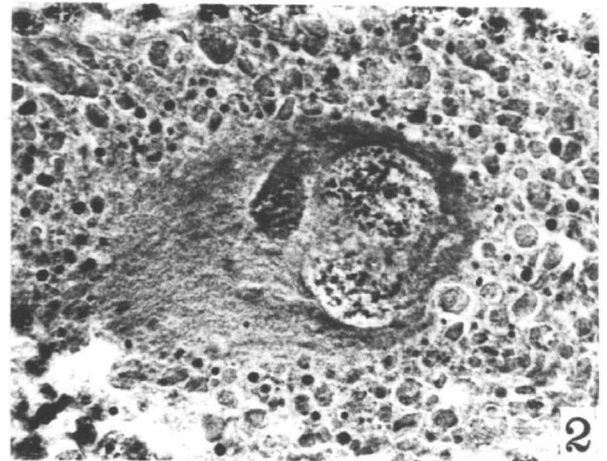
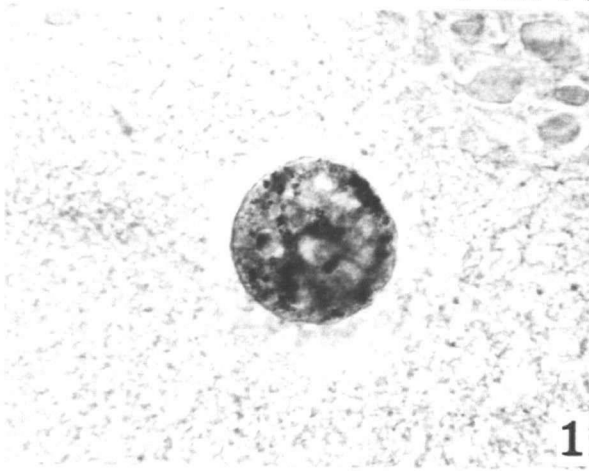
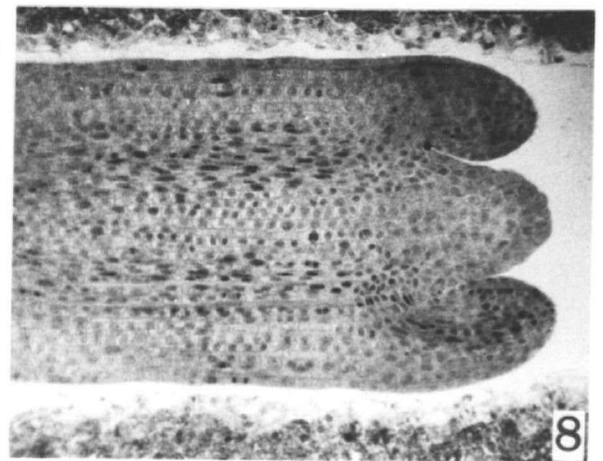
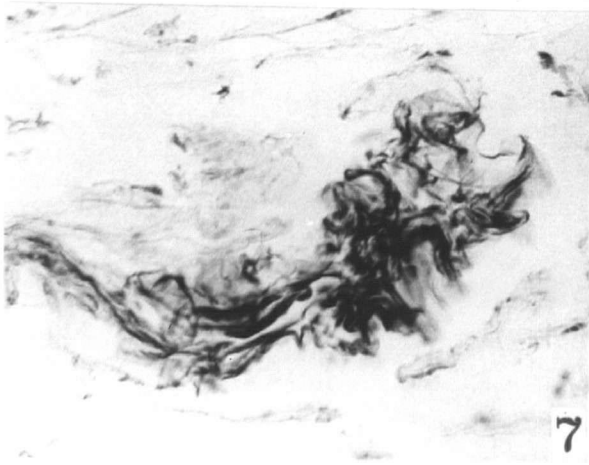
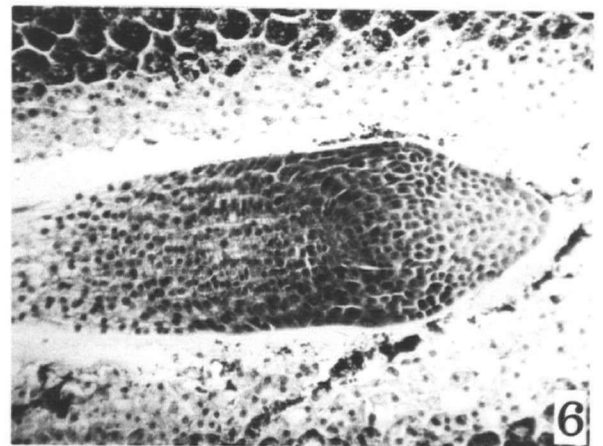
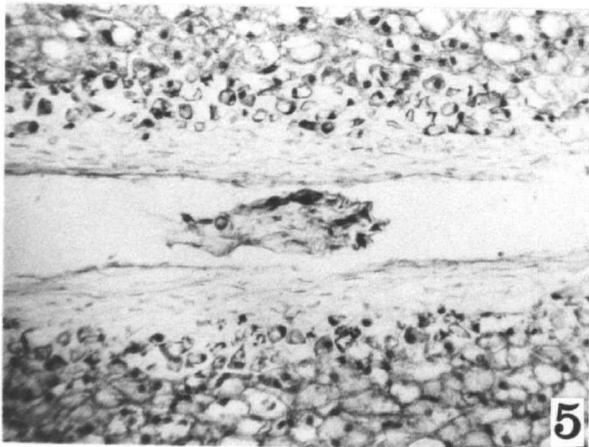
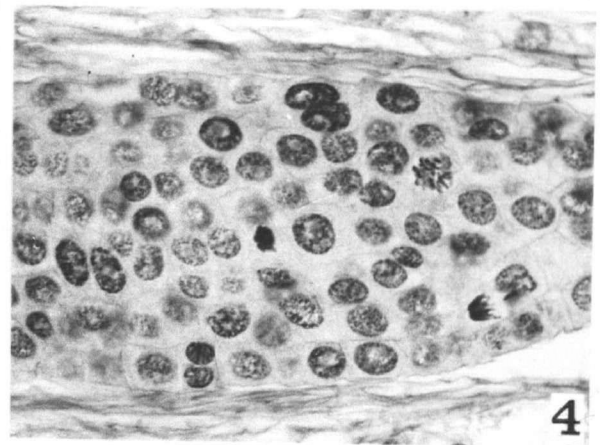
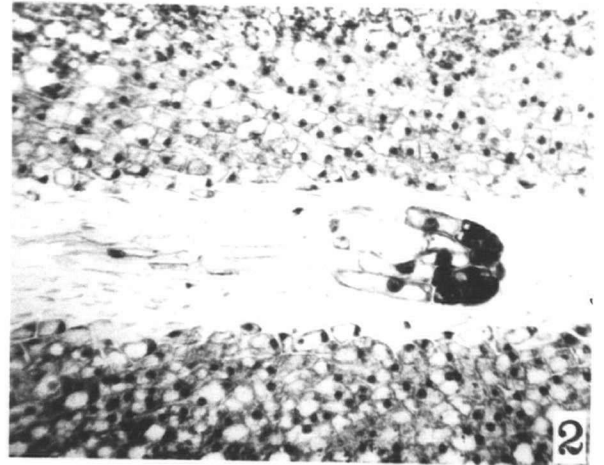
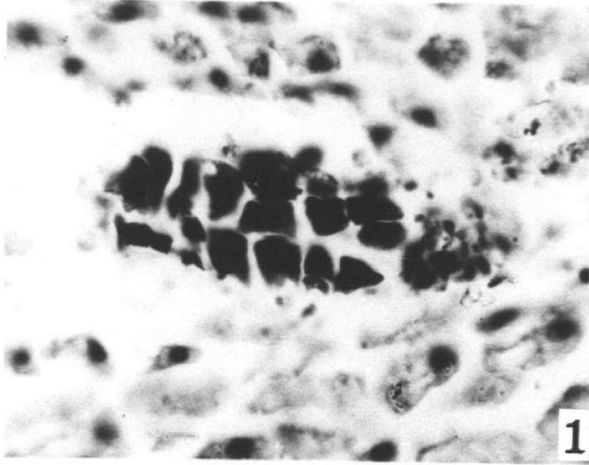


PLATE VII

- Figure 1. A collapsed terminal embryo seventy-one days after cross-pollination; note the similarity with figure 8, plate VI. June 28, 1954. X 345.
- Figure 2. A terminal embryo seventy-five days after self-pollination. July 2, 1954. X 92.
- Figure 3. A terminal embryo after self-pollination, compare development with figure 4. July 2, 1954. X 345.
- Figure 4. A terminal embryo seventy-five days after cross-pollination. July 2, 1954. X 345.
- Figure 5. A collapsed terminal embryo eighty days after self-pollination. July 8, 1954. X 92.
- Figure 6. A terminal embryo eighty-two days after cross-pollination; note the depleted cell layers of the gametophyte. July 14, 1954. X 92.
- Figure 7. A collapsed terminal embryo, one hundred and five days after self-pollination. July 21, 1954. X 345.
- Figure 8. A terminal embryo eighty-nine days after cross-pollination. July 21, 1954. X 92.

PLATE VII



Comparison of Embryo Collapse in Trees 1 and A

It was quite evident that the low viability of seed after self-pollination in both trees 1 and A was due to some inhibition to embryo development. Pollen germination and fertilization all compared favourably with that following cross-pollination, and the early development of the embryos was also quite normal. These embryos, however, were soon completely outgrown by those resulting from cross-pollination. This is vividly illustrated in plate VIII, which shows the comparative embryo growth following both kinds of pollination. The points on the graphs are based on the mean number of nuclei visible in one focal plane in the terminal embryos; the very rapid growth of those resulting from cross-pollination is in striking contrast to that of the embryos resulting from self-pollination. A few sections were made from the self-pollinated ovules of tree D in which ovule abortion had also been high. The normal stages of fertilization were observed and subsequent embryo collapse occurred at approximately the same time as in tree A. It seems reasonable to conclude, therefore, that embryo collapse is the cause of the low seed yields after self-pollination in Pseudotsuga.

There were some interesting differences between the course of embryo collapse in trees 1 and A. It should be remembered, however, that the investigation on these two trees was carried out in different years and that trees 1 and A were growing in Vancouver and Victoria respectively. Syngamy in both trees

occurred between fifty-one and fifty-four days after self-pollination, but the subsequent development of these embryos was much slower in tree 1 and they never reached the size of those in tree A. In both cases, very few viable seeds were produced, but there would appear to have been a higher degree of embryo collapse in tree 1, as only two viable seeds were produced from thirty-nine cones in contrast to sixteen from eighty-two cones in tree A. Development of the embryos resulting from crossing also was slower in the case of tree 1 than of tree A; the largest embryo from tree 1, eighty-seven days after pollination, for example, showed only eighty-two nuclei in one focal plane whereas the nuclei in an embryo of similar age from tree A were too numerous to count. The characteristic dense staining and cell shrinkage of the embryo in the early collapse state, however, were common to both trees and the physical appearance of the cells of the gametophyte was identical. No collapse was observed in these cells prior to the collapse of the embryo which invariably occurred in the trees investigated when the cells of the gametophyte were full of stainable material.

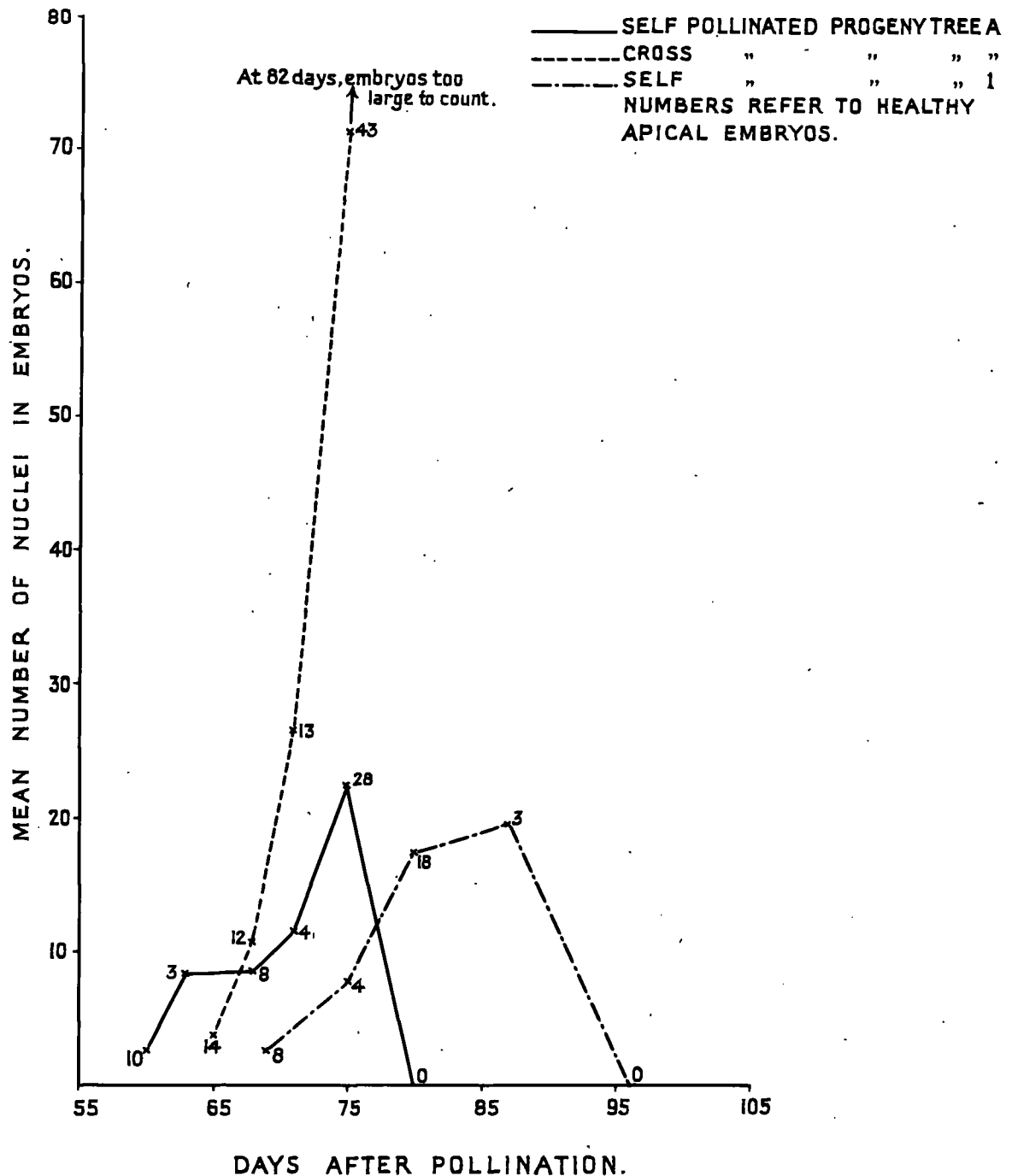
PLATE VIII

Comparative embryo growth following self-
and cross-pollination of trees 1 and A.

The number of nuclei refers to the maximum
number visible in one focal plane, and not
to the total number contained by the embryo.

PLATE VIII

COMPARATIVE EMBRYO GROWTH FOLLOWING SELF AND CROSS POLLINATION.



IV THE POSSIBLE CAUSES OF EMBRYO COLLAPSE AFTER SELF-POLLINATION

Self-Incompatibility

An important question is posed; is embryo collapse after self-pollination part of a genetic system of incompatibility or is it the result of some different cause? The conclusions of several well known investigators of the incompatibility systems in the Angiosperms definitely suggest that embryo collapse is not part of such a genetic system since fertilization is able to take place.

East (1929), one of the early investigators of incompatibility, stated:

Various species of hermaphroditic animals and plants exist in which a union between the male and female gametes produced by a given individual is difficult or even impossible, although each type of gamete is functional in other unions. This phenomenon is usually called self-sterility.

Mather (1943) pointed out that: "Incompatibility may be defined as the failure, following mating or pollination, of a male and a female gamete to achieve fertilization,...." According to Lewis (1944):

Sexual reproduction is essentially the formation of a new individual resulting from the fusion of two gametic nuclei. Any hindrance to this fusion, within the regular mating group, except when it is due to a defect in the nuclei themselves, is said to be due to incompatibility.

Bateman (1952) stated: "The primary function of self-incompatibility is the avoidance of self-fertilization". It will be recalled that Sears (1937), classified the incompatibility systems of the Angiosperms into three distinct stages (page 6 of this dissertation). In one of these which concerned Gasteria, the pollen tubes grew

normally, reached and fertilized the ovules, but no seed developed. Mather (1944), however, did not consider that this is a true self-incompatibility system and pointed out:

It is said that in some plants, like Gasteria ... incompatibility manifests itself as a breakdown of development after fertilization. Such a situation can, however, scarcely be described as due to incompatibility in the present sense, for it omits the essential selective advantage of conservation of female gametes, and hence must have arisen in some other way. In fact, it is difficult to see how such a system could arise by direct selection.

Lewis (1954) later wrote:

An extreme case was described by Sears in Gasteria in which the incompatible pollen tubes grow at the same rate as the compatible tubes, reach the ovary, and fertilize the endosperm, but the egg nucleus is not fertilized. Unfortunately nothing is known about the incompatibility system in this species.

If these definitions of incompatibility are accepted there is clearly little justification for applying this term to embryo collapse after self-pollination in Pseudotsuga, since fertilization is in no way inhibited. Embryo collapse, therefore, must be ascribed to some other cause. Bateman (1952) pointed out, however, that there is no reason why a true incompatibility system should not be present in the Gymnosperms. It is, therefore, interesting to note that Forshell (1953) recently claimed that such a system does exist in Pinus sylvestris and that fusion of the sex cells is partly inhibited. She states that her theory of incompatibility is not contrary to what is known about the genes of self-sterility in the Angiosperms in which pollen-tube growth, after self-

pollination, is restrained by these genes. Her study is the only other known investigation on self-incompatibility in the Gymnosperms and her conclusions have been carefully considered as it is unlikely that the behavior of two species of the Pinaceae should be radically different. Forshell's conclusions, apparently, are not based upon cytological evidence but on an earlier study by Branscheidt (1939), and on X-ray photographs of seeds resulting from both self- and cross-pollination of Pinus sylvestris.

In his study, Branscheidt (1939) succeeded in growing pollen of Taxus baccata on an artificial medium and followed the complete development from germination to the maturity of the male sex cells. Under these artificial conditions, the pollen tubes did not go through the customary rest period which is usually six weeks in duration. Branscheidt referred to a previous study on Taxus baccata by Jäger¹ who found that the several archegonia in the same ovule were often at different stages of development. A young developing archegonium, for example, could be found near one from which the embryo has already developed. Branscheidt concluded that the immature archegonium or gametophyte must produce some inhibiting substance which causes the rest period of the pollen tube and that this substance must be eliminated when the archegonium is mature. Forshell added that, in Pinus sylvestris, this inhibiting effect of the archegonia does not prevent the pollen tubes from growing after self-pollination since a large number of seeds are found which are either empty or contain only gametophyte tissue. Such seeds can

¹Jäger L. Beiträge zur Kenntnis der Endosperm-bildung und zur Embryologie von Taxus baccata. Flora 86. 1899.

develop only if the integument and gametophyte tissue are stimulated by a pollen tube. The archegonia, however, do prevent fusion of the male and female nuclei in those ovules where the inhibiting effect does not cease following the rest period of the pollen tube. Forshell pointed out that the reason why self-sterility is not complete and why a large or small number of embryos are produced after self-fertilization in Pinus sylvestris, is explained by Branscheidt. He concluded that as the several archegonia in an ovule were at different stages of development, the inhibiting substance would be eliminated at different times for each archegonium. Furthermore, several pollen tubes at different stages would surround archegonia which were also in different stages of development. Forshell, therefore, stated that the formation of viable seed after self-pollination is due to the archegonia losing their inhibiting effect, either partly or completely at a late stage so that they can then be fertilized. The "self-sterility" of Pinus sylvestris is, therefore, not complete according to this concept.

There appears to be little evidence to support this hypothesis. Branscheidt (1939) based all of his conclusions on the previous study by Jäger and on the finding that pollen, growing on an artificial medium, did not go through the rest period which was usual under natural conditions. Neither he nor Forshell presented any experimental evidence that an inhibiting substance is produced by the archegonia. Forshell, moreover, presented no cytological evidence to support her statements that fusion of the male and female nuclei is prevented in certain ovules. She, therefore, had no proof that there

was less fertilization after self-pollination than after cross-pollination. Anhaeusser (1953), recently conducted a much more intensive study on pollen germination and development on artificial media in the Gymnosperms. He believed that Branscheidt did not select a typical representative in regard to the rest period of the pollen tubes and delay in fertilization since the interval between pollination and fertilization is only six weeks. A much clearer picture of the problem would have been given by a genus such as Pinus.

Regarding the X-ray photographs of the seed from self- and cross-pollination in Pinus sylvestris, there is certainly evidence that there were fewer embryos in the self-pollinated ovules as compared with those cross-pollinated. These photographs, however, appear to have been taken when the embryos were at a late stage of development as the cotyledons in some cases were already developed. This investigation with Pseudotsuga, however, has shown that the embryos are just as numerous after self-fertilization as after cross-fertilization but that they collapse at an early age before organisation of the surviving embryos occurs. The collapsed embryos gradually disintegrate and are soon very difficult to find. Photographs by X-ray taken shortly after fertilization in the case of Pinus could have presented a totally different picture since at that time, there may have been just as many embryos in the self-pollinated ovules as there were in the cross-pollinated.

A recent study on Pinus monticola by Bingham and Squillace (1955), also suggests that it is unlikely that a self-incompatibility

system is operative in Pinus. They self-pollinated several trees for successive years and in one tree, the yield of viable seed varied considerably from year to year. Such variation would be unlikely if any known incompatibility system were present. The fact that certain Gymnosperms can produce variable amounts of viable seed after self-pollination must make the terms "self-sterile" and "self-incompatible" superfluous. It is, therefore, suggested that neither of these terms should be used in their present sense for the Gymnosperms until well substantiated evidence has been presented.

Inbreeding Effect

The most likely explanation of embryo collapse is that it is caused by inbreeding which results in the increased homozygosity of recessive deleterious genes. Dobzhansky (1952, page 220), demonstrated that in time, any normal breeding population will build up a mass of deleterious recessive genes by mutation. Many of these genes, moreover, are lethal or semi-lethal but they are sheltered from selection pressure by the normal dominant alleles in a heterozygous organism. The situation, however, is completely changed by inbreeding because many of these recessive genes will then be homozygous and consequently exposed. It is logical to assume that there will be both lethal and semi-lethal genes in Pseudotsuga, and that they are capable of destroying the embryo soon after fertilization when in a homozygous state. The number of such genes will undoubtedly vary greatly from tree to tree and the effects of self-pollination in terms of embryo collapse will vary accordingly. The degree of collapse may again be dependent upon certain

recombinations which explains why repeated self-pollinations of a tree do not always give the same result. This has already been demonstrated in Pinus monticola by Bingham and Squillace (1955). The exact conditions which bring about embryo collapse, however, will probably remain obscure. Wardlaw (1955, page 10), pointed out that growth from the zygote or spore to the adult state is characterized by an orderly sequence of development in which factors in the hereditary constitution are involved. These factors or genes, moreover, determine and control metabolic processes and are, therefore, involved in all growth and cellular activity. Wardlaw stated that the time of action of particular genes is of special importance in embryogenesis as only by their activation can orderly development follow. He added, however; "But how the genes act, individually and collectively, is a problem that is as difficult as it is important".

It is evident, therefore, that any drastic change such as inbreeding a normally outbreeding organism could very easily upset the orderly sequence of development in the young embryo. Brink (1952) pointed out that the early embryo is dependent upon the endosperm for certain metabolites which, initially, the embryo is quite incapable of synthesizing. It later becomes progressively less dependent by acquiring for itself the synthetic capabilities previously limited to the nurse tissue. The cause of embryo collapse in Pseudotsuga would appear to be from some failure in this vital relationship between the early embryo and the gametophyte

tissue. These embryos regularly collapsed at an early stage of development and, consequently, before they were less dependent upon the gametophyte. Every case of collapse, moreover, occurred in ovules whose gametophyte cells were full of dark-staining material apparently of little or no value to the embryos. Embryos from cross-pollination, however, were able to develop in the normal manner under what appeared to be identical conditions in the gametophyte. Wardlaw (1955, page 12), pointed out that in the case of plants, very little is known of the chemical composition of the zygote, the young embryo, the surrounding tissues and the metabolic processes involved in growth and development. At the present time, therefore, it can be concluded only that embryo collapse in Pseudotsuga is more likely to be the result of inbreeding than of some unknown incompatibility system.

There is other evidence from the Gymnosperms that embryo development is dependent upon the inheritance of the parents. Buchholz (1945) effectively demonstrated that hybrid vigour, the antithesis to inbreeding, can be observed in the early development of embryos from a hybrid pine as compared with the development of those from the parent trees. He decided, however, that this hybrid vigour was nothing that might be detected by a morphological study of the embryos themselves since its nature was definitely a physiological vigour of growth. This same physiological vigour of growth was definitely exhibited in the early development of the embryos from cross-pollination as compared with those from self-pollination during the cytological investigation of tree A.

Embryo collapse and its possible connection with inbreeding

has been reported in some Angiosperms. Brink and Cooper (1940), for example, found that in Medicago sativa, the endosperm grew faster after cross-pollination than after self-pollination. An embryo from cross-pollination was accompanied, at a given early stage of development, by an endosperm more advanced than that associated with an embryo from self-pollination. They suggested that the reduced rate of early endosperm growth was an inbreeding phenomenon associated with the increasing homozygosity of numerous recessive genes, each having a small adverse effect on development. Cooper and Brink (1940) later concluded that the collapse of the fertile ovules in Medicago sativa, which occurred so freely after self-pollination, could be due to some of the genes conditioning pollen-tube behaviour, these being also responsible for ovule collapse. On the other hand, it could be due to recessive genes at other loci which, when brought together in a homozygous state by inbreeding, adversely affect the early development of the zygote. Cooper and Brink (1940) pointed out that although the evidence was limited, it indicated that the collapse of these fertile ovules was an inbreeding effect rather than a direct manifestation of self-incompatibility.

Further cytological investigations on the effects of self-pollination in other Gymnosperms are obviously desirable in order to augment present limited information.

V PARTHENOCARPY AND AGAMOSPERMY

The development of non-pollinated cones with fully formed but empty seeds has been termed parthenocarpy and appears to have been first investigated in the Gymnosperms by Kurdiani (see Hummel, 1930). He concluded that it was a damaging influence in the forest as the development of cones and empty seed took nutrients away from the tree which would otherwise have been used for growth or for the formation of flower primordia the following year. Kurdiani found that parthenocarpy was of common occurrence in Taxus, Thuja, Larix, Abies, Picea, and Juniperus. It was found that the non-pollinated cones of Pinus sylvestris, however, usually dropped off although in later studies, Pinus eldarica produced cones of normal size but the seed coats were absent. Syrach Larson (1937, page 73), later found that parthenocarpy was also of normal occurrence in Chamaecyparis, Cryptomeria, Tsuga, and Pseudotsuga and it is obviously widespread in the Gymnosperms, a fact, however, which is still not always recognized. Anderson (1947), for example, making controlled crosses on Picea, noted:

The most peculiar finding of all is that the isolated control flowers grew out into perfectly normal cones ... Formerly it was generally expected that a certain number of egg-cells must be fertilized in the female inflorescence to ensure cone development.

Again, Isaac (1949, page 9), discussing flowering and seed formation in Pseudotsuga stated: "...if not successfully pollinated or fertilized, the conelet like the male flower, withers and disappears".

The possibility that parthenocarpic cones of some of the Gymnosperms might also produce viable seed is of considerably more

interest as, although its occurrence has not been definitely established, there are indications that it may occasionally occur. This phenomenon, previously known as parthenogenesis, is of fairly common occurrence in many of the Angiosperms. It is now better known as apomixis, which Stebbins (1950, page 380) defined as covering all types of asexual reproduction which tend to replace or to act as substitutes for the sexual method. Gustafsson (1946, page 7) further subdivided apomixis into two main groups, agamospermy where reproduction is from seeds and vegetative reproduction where it is from vegetative formations. The various ways in which agamospermy can arise were described in detail but no reference was made to the Gymnosperms.

There have, however, been several reports that viable seeds have occasionally been found in parthenocarpic cones. Dengler (1932), for example, isolated cones on Pinus sylvestris and obtained one parthenocarpic cone that contained two seeds which germinated before extraction. Allen (1942) isolated cones of Pseudotsuga menziesii and germinated the three viable seeds obtained from two of the trees. The cones, moreover, had been isolated a week before they had emerged from the bud scales and before pollination had begun. Orr-Ewing (1954) obtained twenty-two filled seeds containing embryos from isolated cones on another tree of the same species. Twelve of these seeds germinated and seven of the seedlings were alive in 1955. One isolated cone was also removed a few weeks before fertilization would normally have occurred and forty-eight of the ovules were sectioned and stained, but there was no trace of any

pollen. It is improbable that these seedlings could have resulted from normal pollination since the female buds were all isolated before the cones had emerged.

It is evident, however, that direct cytological evidence must be obtained before agamospermy can be accepted as an established fact; evidence to date is still by no means conclusive but, on the other hand, it can not be lightly dismissed. The earliest report of agamospermy, (or parthenogenesis as it was then called), was by Saxton (1909), in an investigation of Pinus pinaster. He found some ovules with proembryos in all stages of development but either there was no trace of pollen tubes in the nucellar cap or the tubes extended through only a part of it. Saxton claimed that, in one section, the egg nucleus had begun to divide before the pollen tube had reached the archegonium and no sign existed of either the second sperm or the tube nuclei. The number of chromosomes in the egg nucleus could not be counted but Saxton thought that they were less than the diploid number. Coulter and Chamberlain (1917, page 300) supported these findings, and wrote:

It has long been expected that parthenogenesis would be found to occur among the Pinaceae and recently Saxton has reported that Pinus pinaster ... is parthenogenetic ... The series was close enough, and the comparison with stages in the normal embryo complete enough to make the conclusion reasonably safe.

It must be admitted, however, that this study is open to considerable criticism since it was based on sections from ovules which had not been isolated from pollination. Pollen tubes and their nuclei are not always easy to locate and without the necessary control, normal fertilization could easily have occurred and been overlooked.

There is, however, stronger evidence that the ventral canal cell nucleus may occasionally fertilize the egg nucleus which would result in a diploid embryo. Chamberlain (1935, pp. 336 and 340), for example, wrote:

It is a curious fact that the first two cases of fertilization described for conifers were abnormal. Strasburger, as early as 1878, ..., figured two gamete nuclei of about equal size in Picea vulgaris. Nearly 20 years later, Coulter described two fusing gametes of about equal size in Pinus laricio. There can hardly be any doubt now that the "sperm", in both these cases, was an enlarged ventral canal nucleus, since normal fertilization has been observed so often in both these species, and the sperm nucleus has been proved to be very small in comparison with that of the egg.

Chamberlain later added: "My own work on Pinus made this interpretation practically a certainty". Hutchinson (1915), reported that in Abies balsamea the ventral canal cell nucleus occasionally broke through the cell wall and was seen moving towards the egg nucleus. Johansen (1950, page 18), referring to an early report in Ginkgo, stated: "Fusion of the ventral canal nucleus with the nucleus of the archegonium has been described, but whether this can result in the development of a normal embryo is unknown although not beyond the realm of possibility". The most conclusive evidence has been presented by Sedgewick (1924), in his investigation of Encephalartos, a genus in the Cycadaceae. He stated:

Fertilization was not observed, and apparently fertilization by a sperm is not necessary in Encephalartos for the successful development of embryos. In greenhouse material and material collected in the field, it is found that the ventral canal nucleus instead of degenerating, has enlarged and is approaching the egg.

He added, however, that the two nuclei were not found in contact but were very close together. Sedgewick later stated that:

An additional reason ... is based on Chamberlain's field observations. In collecting Encephalartos ... , he procured only one staminate cone in the neighbourhood of plants bearing ovulate cones. A further reason is found in careful study of all the preparations. In not a single free nuclear proembryo has a sperm sheaf or ciliated band been found.

He then added:

The cytoplasmic sheaf of the sperm, and especially its ciliated spiral band, are so conspicuous that failure to find them in complete serial sections is practically a proof that they are not present ... , therefore, it is safe to conclude that E. villosus and E. friderici guilielmi can produce normal embryos in the absence of pollination and fertilization by a sperm.

Chamberlain (1935, pp. 122) added: "The fact that embryos have been found in conservatory material where no male cones were present indicates that such a fertilization might occur". Chamberlain also stated that the reported hybrids between Ceratozamia longifolia and Ceratozamia mexicana, also support this conclusion as three-year-old pollen was used and cycad pollen does not retain its vitality for more than a month. He added: "It is possible that the mere irritation of dead pollen in the pollen chamber might stimulate development; but it is certain that the three year old pollen was dead".

Another possible manner in which agamospermy could occur was described by Johansen (1950, page 79), for Ephedra campylopoda:

Both microgametes enter an archegonium; one fuses with the ventral canal nucleus and the other with the archegonial nucleus ... moreover, nuclei from the cells surrounding the archegonium may become freed to migrate into the latter and the union of the archegonial nucleus with such a free nucleus was actually observed in an unfertilized archegonium. Whether such an occurrence can give rise to an embryo is not known.

These varied reports suggest that agamospermy could occur and

it was hoped that a more detailed cytological study could be made on tree 2 from which the viable seeds from isolated and unpollinated cones were first obtained. It produced no cones, however, in either 1953 or 1954, and, therefore, some cones were isolated and left unpollinated on five of the trees artificially pollinated in 1954. Sample cones were removed at regular intervals from the time when fertilization would normally be expected to occur. The ovules were then embedded and some were later sectioned and stained. In the fall, the remaining cones were collected, and the seed extracted and cleaned. The purpose of cleaning the seed was to remove as much of the empty seed as possible and it can be seen from table 22 that very little seed remained. The results of the germination test, however, showed that this seed was all empty.

TABLE 22. GERMINATION TEST OF SEED FROM UNPOLLINATED CONES

Tree	No. of unpollinated cones	No. of seeds	No. of cleaned seeds	Germination	Cutting test of ungermin. seed
A	38	1419	2	Nil	Empty
D	35	966	7	Nil	Empty
F	53	2368	9	Nil	Empty
H	30	1821	0	Nil	Empty
J	56	2719	5	Nil	Empty

The results of the cytological study proved more interesting. The ovules aborted in the five trees three to four weeks after fertilization would normally have occurred. There were two exceptions, however, two healthy ovules were collected from trees A and J at the end of July and the beginning of September,

respectively. Both ovules were later sectioned and each contained diploid embryos which are illustrated in figures 5 and 6, plate IX. It is realized that the possibility of pollen contamination can not be entirely discounted, but it is considered to be very remote as a detailed record was kept of the date of isolation of these cones on both trees where these embryos were found. The cone from which the embryo originated on tree A was isolated on March 22nd, and pollen dissemination from this tree was not observed until May 1st; the earliest dissemination in the vicinity began on April 10th. The other cone on tree J was isolated on April 1st; the earliest pollen dissemination from this tree did not take place until May 5th which was earlier than that from the other two trees, F and H. In both cases, isolation took place at least two weeks before the young cones had emerged from the bud scales. The viscose casing bags were securely tied to the branchlets and were not removed until the cones scales had closed completely. The greatest care was taken to remove any male buds present on the branchlets prior to isolation and it is certain that all were picked off.

Unfortunately, none of the sections from the unpollinated samples gave any conclusive evidence as to how these two embryos originated. However, one particularly interesting section was obtained from an unfertilized ovule which had been removed at the time when fertilization was taking place in pollinated cones. This section, illustrated in figure 7, plate IX, was stained with Feulgen and showed what appears to be an enlarged ventral canal cell nucleus in close proximity to that of the egg, with chromatic figures

visible in both nuclei. The series of sections of this ovule were carefully checked and there was no other trace of the ventral canal cell nucleus. It is presumed, therefore, that it may have broken through the cell wall although no sign of the remnants of this cell wall could be found. It is also possible that the egg nucleus had divided and that these two nuclei are the result of this division. Both nuclei are very similar in appearance to those shown by Coulter and Chamberlain (1917, figure 298, page 267) for Pinus laricio and interpreted by them as the ventral canal and egg nuclei. The cytological evidence from this investigation with Pseudotsuga is by no means conclusive but it indicates a possible manner in which agamospermy could arise and result in a diploid embryo.

One interesting feature of the unfertilized ovules in Pseudotsuga in this study was the comparatively long time that elapsed before they finally aborted. Figure 1, plate IX, for example, illustrates an ovule with archegonia and egg nuclei, the jacket cells still stain sharply and there is considerable mitosis both in these and in the cells of the gametophyte. This ovule, however, was removed on June 20th, at least two weeks after fertilization was first noted in the tree. Figure 2, plate IX, illustrates an ovule removed on June 23rd, the archegonium has finally broken down and its former position is marked by a dark staining and shrunken mass. The nuclei of the gametophyte, however, still stain sharply and there are numerous mitoses. Figure 3, plate IX, illustrates another ovule removed on June 28th; it is aborting and the cell walls are breaking down while the nuclei

stain with abnormal intensity. Ovule abortion in unpollinated cones generally occurs about four weeks after the date of normal fertilization, the nuclei at first stain very intensely and finally do not stain at all. Some ovules, however, do not abort until much later; figure 4, plate IX, for example, shows an intact ovule removed on July 14th, nearly six weeks after the normal date of fertilization. Figure 3 and 4 show that there is a definite corrosion cavity in these unpollinated ovules which forms even though fertilization has not occurred. Buchholz (1918) reported that he examined many ovules of Pinus in which the gametophytes had well developed corrosion cavities without any traces of embryos, indicating that the archegonia may excrete the digestive enzyme even though the eggs have not been fertilized. Schopf (1943) found that the gametophytic tissue below the archegonium in Larix broke down at the regular time whether an embryo was present or not. He noted that "In the "automatic" corrosion cavities lacking embryos, the break-down of tissue left a residue very similar to that formed in the old archegonia of fertile gametophytes". This residue is clearly visible in figure 2 and 4, plate IX, and was commonly but not invariably found in unfertilized ovules. It was never present, however, in those that had been fertilized and was one means of distinguishing between them. The more significant but later difference between the two was the complete absence of stainable material in the cells of the gametophyte in unfertilized ovules. Stainable material began to accumulate in the cells of fertilized

ovules a few weeks after syngamy and the cells soon became filled with dark material, as illustrated in figure 4, plate V. The cells of the unfertilized ovule in figure 4, plate IX, however, are almost empty. Allen (1943), in his investigation of Pseudotsuga, found that prior to elongation of the primary suspensor cells, cells in the region of the main axis of the ovule began to accumulate granules of reserve food material. This starch zone, moreover, became more and more extensive and was formed whether or not the ovule was fertilized. The zone was also observed in this investigation but it did not persist.

There seems little doubt that fertilization must in some way stimulate the gametophyte to provide an adequate store of food material. It is possible that the embryo itself is the centre of this stimulus as the cells of the gametophyte are only full of dark staining material if embryos are present. If they are not present, the whole ovule finally aborts. This explanation would certainly seem applicable to Pseudotsuga. It, however, would not appear to be applicable to Pinus as Forshell (1953) stated that neither the integument nor the female gametophyte in Pinus sylvestris can develop without the stimulus of the pollen tubes. Forshell referred to Goebel (1932, page 1799) who pointed out that the conveyance of the male gametes to the egg cell is by no means the only function of the pollen tubes. He added that in the Coniferae, the ovules are in most cases pollinated before the megaspores have developed and that the germinating pollen grains stimulate their further development. Goebel, however, presented no experimental

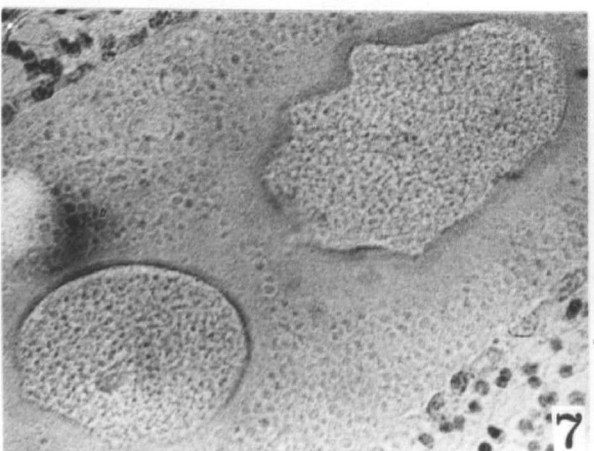
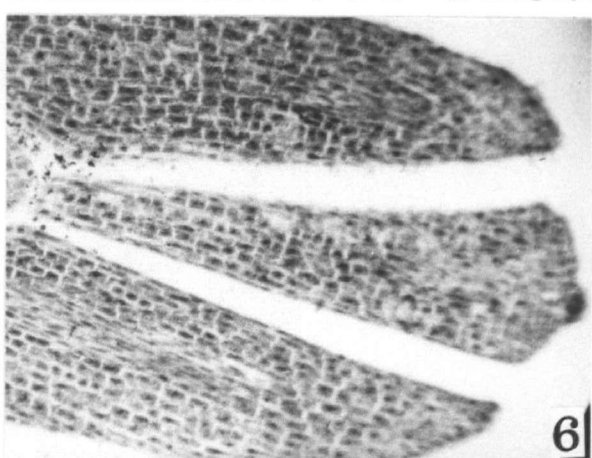
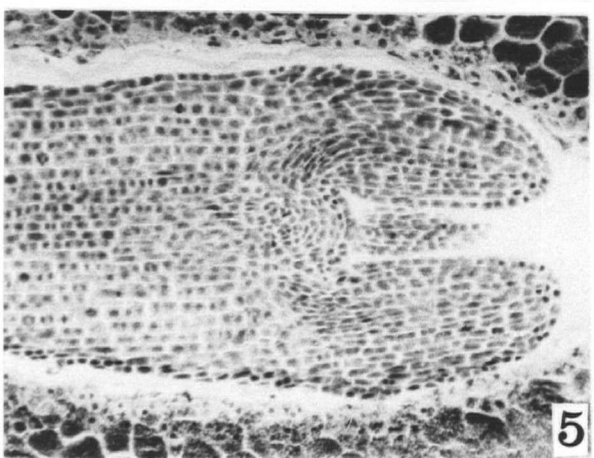
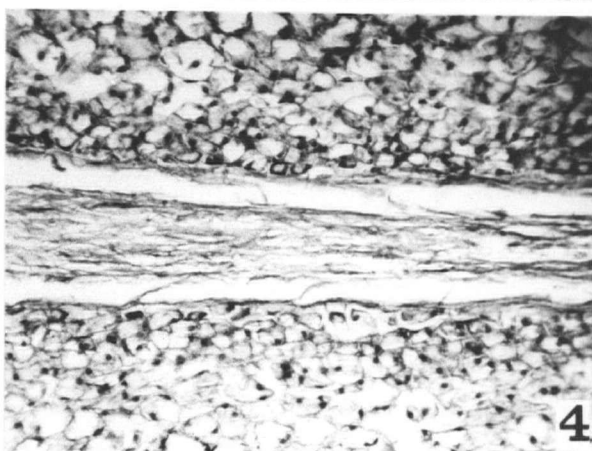
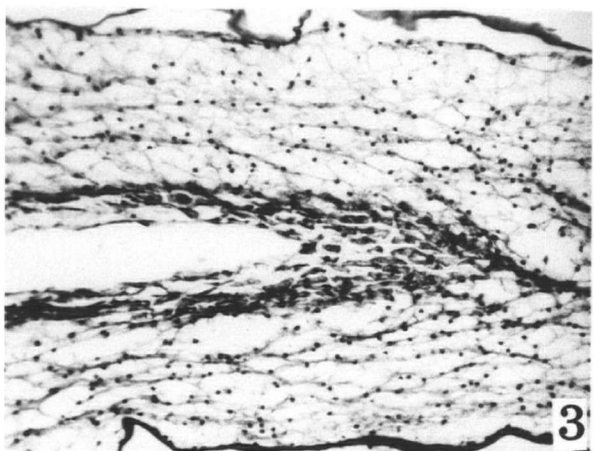
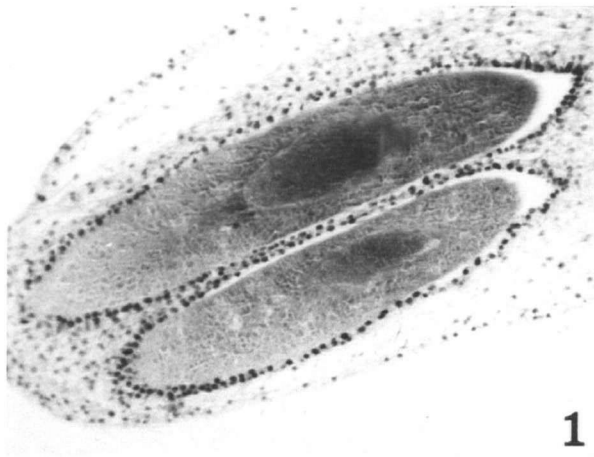
evidence to support this assumption. It is interesting to note that Forshell (1953) isolated some young cones of Pinus sylvestris and that they usually did not develop. A few of these young cones did develop to maturity and either produced fully formed but empty seed or seed containing embryos. Forshell, however, considered that this was the result of faulty isolation of the cones, in other words some uncontrolled pollination had taken place.

It does indeed seem strange why the behaviour of these two genera in the Pinaceae should be so different and further studies with the genus Pinus would seem desirable. There is ample evidence, however, to show that in Pseudotsuga the female gametophyte can develop up to the time of fertilization, irrespective of whether pollination has occurred or not. There is also no question that the integument can develop without the stimulus of pollination.

PLATE IX

- Figure 1. Two archegonia with unfertilized egg nuclei; note the sharply staining jacket cells. Tree A.
June 20, 1954. X 92.
- Figure 2. The remains of an archegonium in an unfertilized ovule; note the empty cells of the gametophyte and the cell break-down in the corrosion cavity. Tree D.
June 23, 1954. X 345.
- Figure 3. An unfertilized ovule in the first stages of abortion.
June 28, 1954. Tree A. X 92.
- Figure 4. An unfertilized ovule, note the empty cells of the gametophyte. Tree A. July 14, 1954. X 345.
- Figure 5. An embryo found in an ovule from an unpollinated cone of tree A. July 27, 1954. X 92.
- Figure 6. An embryo found in an ovule from an unpollinated cone of tree J. September 1, 1954. X 92.
- Figure 7. A nucleus thought to be that of the ventral canal cell approaching the egg nucleus. Tree 1.
June 1, 1954. X92.
- Figure 8. A healthy ovule compared with others which have aborted.

PLATE IX



PART 2.

I. RESULTS OF THE 1953 PROGENY TEST

Comparative Height and Diameter Development

These results are related to the comparative development of the seedlings resulting from the self, controlled cross and wind-pollination of trees 1 and 2. The earlier information pertinent to the cone and seed numbers, germination and the development of the seedlings to the fall of 1953 has already been published (Orr-Ewing, 1954).

In the spring of 1954 the seedlings were removed from the pots and planted out in a random design in seed beds at the B. C. Forest Service nursery at Duncan and kept shaded and watered during the summer months. The heights and diameters were measured in the late fall and the results were similar to those of the previous year. There were highly significant differences in height between the inbred seedlings and those resulting from both cross and wind-pollination on tree 2. The two inbred seedlings obtained from tree 1, also were both smaller than those that resulted from cross- and wind-pollination on the same tree.

In the spring of 1955 the seedlings were all carefully moved to a permanent location in the nursery and planted out at eight-foot spacing. The heights and diameters were measured in the fall; the difference between the heights of the inbreds and the seedlings from controlled cross-pollination on tree 2 was again found to be

highly significant. The development of the seedlings from tree 2 between 1953 and 1955 is graphically illustrated in plate XIII. These graphs show that the seedlings from cross-pollination are superior to the inbreds in both height and diameter whereas those from wind-pollination are intermediate between them. This is not unexpected since, in the case of wind-pollination, the pollen parent or parents are unknown, and uncontrolled self-pollination can easily occur. Statistical comparisons of the inbred seedlings with those from other types of pollination on the same tree are by no means the sole purpose of inbreeding studies, although they serve to emphasize the practical aspects of self-pollination in forestry practice. It is quite evident that the inbreeding of a naturally outbreeding species, such as Pseudotsuga will ordinarily result in some loss of vigour and that the seedlings will usually compare unfavourably with those from cross-pollination. Mather and Edwardes (1943) have pointed out that when an outbreeding system is changed artificially to inbreeding, the inbred lines are much more variable and less vigorous than the outbreeding population from which they came. The polygenic systems controlling heights, vigour and fertility are adjusted to outbreeding, the system in force, and not to inbreeding. The frequency distributions and coefficients of variation of both the heights and diameters of the progeny of these trees are shown in table 23 and 24. These indicate that much more variation existed among the inbred seedlings of tree 2 than among those produced by cross-pollination. Considerable variation among

the seedlings from wind-pollination was found and it is very probable that a considerable amount of uncontrolled self-pollination took place in this tree. Some of the variation in the seedlings from wind-pollination is illustrated in figures 3 and 4, plate XI. These two seedlings were the same age and had been grown under identical conditions, yet their heights were six and thirty-four inches respectively.

TABLE 23. FREQUENCY DISTRIBUTIONS AND COEFFICIENTS OF VARIATION BY HEIGHT CLASSES

Tree	Pollin.	Height classes in inches (at age three years)							Total No.	Mean ht.	C.V.
		2-6	7-11	12-16	17-21	22-26	27-31	32-36			
1	Self		1	1					2	11.6	-
	Cross				6	5	2		13	23.0	-
	Wind					2	1		3	22.8	-
2	Self	5	14	9	5	4			37	13.1	48.3
	Cross			5	14	21	12	4	56	24.1	20.6
	Wind	3	5	9	12	9	5	1	44	19.0	36.6
	Non		5	2					7	10.2	28.7

TABLE 24. FREQUENCY DISTRIBUTIONS AND COEFFICIENTS OF VARIATION BY DIAMETER CLASSES

Tree	Pollin.	Diameter classes in millimeters (at age three years)							Total No.	Mean diam.	C.V.
		3-6	7-10	11-14	15-18	19-22	23-26				
1	Self	1		1					2	9.5	-
	Cross	1		2	4	7			13	18.1	-
	Wind				1	2			3	18.7	-
2	Self	9	8	13	3	4			37	10.6	45.9
	Cross	1		7	16	27	5		56	18.2	19.7
	Wind	3	8	12	7	12	2		44	14.7	36.9
	Non	2	2	3					7	9.1	29.4

Variation in Form

Shull (1952, page 24) stated that in his early inbreeding studies with Zea mays, the difference between the self- and cross-fertilized rows were obvious. A very different explanation of the facts, however, was forced on him when it was found that several self-fertilized rows differed widely from each other in morphological characteristics, indicating that they belonged to distinct elementary strains. He then realized that selfing merely served to purify the strains (since the homozygous lethals died) and that his comparisons were not properly between self- and cross-fertilization but between pure strains and their hybrids.

It is appreciated that distinct elementary strains can hardly be expected to appear after only one generation of inbreeding but much variation in form already can be found in the inbreds from tree 2. Figures 1, 2, 3 and 4, plate X, show four distinct forms, figure 1 illustrates a dwarf form which was only two inches high and it is interesting to find that Jones (1924, page 310) reported similar dwarf forms when inbreeding Zea mays. Figure 2, illustrates a more vigorous but bushy form with no true leader, while the seedling in figure 4 is much larger and has a wide spreading form with considerable branch growth. This branching habit, moreover, was apparent in 1953. The seedling in figure 3, on the other hand, has a much better form and compared favourably with any of those from cross-pollination. Figures 3 and 4 both demonstrate that inbreeding does not always result in reduction of vigour. This is

in agreement with results from the inbreeding of Zea mays (Hayes et al, page 77, 1955). The amount of variation will depend entirely upon the genetic background of the parent trees which, in this investigation, is not known; and could only be evaluated by an analysis of the progenies when they were older. East and Jones (1919, page 139) have pointed out:

... that inbreeding has but one demonstrable effect on organisms subject to its action - the isolation of homozygous types. The diversity of the resulting types depends directly upon number of heterozygous hereditary factors present in the individuals with which the process is begun; it is likely, therefore, to vary directly with the amount of cross-breeding experienced by their immediate ancestors.

The parent trees of the seedlings in both the 1952 and 1955 progeny tests were all from second growth stands and they could have resulted from either selfing, related or unrelated crosses.

Inbreeding is the more effective method of exposing certain recessive characters which would rarely appear as a result of outbreeding. One such abnormal form, exposed by the self-pollination of tree 2, is illustrated in figure 5, plate XII. This small seedling developed with twin terminal buds, both of which have since produced shoots; figures 6 and 7 illustrate the subsequent development in 1954 and 1955. This abnormal form was not observed in any of the seedlings from cross-pollinations. Inbreeding also resulted in the appearance of other forms which were inherently weak and unable to survive once they were planted out. One type of seedling common to both the 1952 and 1955 progeny tests seemed unable to develop beyond the cotyledon stage and never produced

true leaves; one of these is illustrated in figure 2, plate XII. Inbreeding, therefore, can be expected to result in increased mortality when it occurs in normal outbreeding species such as Pseudotsuga. It was found that the mortality of the inbreds in this progeny test was considerably higher than that of the seedlings resulting from controlled cross-pollination. Twelve out of a total of forty-nine inbred seedlings have died since 1953 as compared with only three out of a total of fifty-nine crossbreds. The mortality of the seedlings from wind-pollination was also high, eleven dying out of a total of fifty-five, this suggests that some uncontrolled inbreeding must have occurred.

Variation between Progenies

It is hardly possible to compare the progenies of trees 1 and 2 owing to the small number of seedlings from the former tree. One striking difference, however, has been consistently observed and it is quite independent of the type of pollination. The progeny from tree 1 regularly all flush at least a week before any of those from tree 2. This was observed in the springs of 1954 and 1955 in spite of transplanting. This early flushing habit has made the progeny of tree 1 particularly susceptible to late spring frosts which have already caused some damage. It was not surprising to find that the parent tree also flushed at least a week earlier than tree 2.

Seedlings from Unpollinated Cones

It will be recalled that twelve seeds obtained from the isolated and unpollinated cones of tree 2 germinated and the earlier information on their development has been published (Orr-Ewing,

1954). Only seven of these seedlings now remain and as can be seen from the graphs in plate XIII, they are inferior in both height and diameter to all the other progenies of tree 2. The number of samples is admittedly small, but it can be concluded that the heights are significantly less than those of the seedlings from both cross- and wind-pollination and apparently less than the inbreds. The seedlings are extremely uniform and show little variation in either height or diameter (see tables 23 and 24). This uniformity is illustrated in figures 1 and 2, plate XI; according to Warmke (1952), it is characteristic of apomictic offspring as they are not subject to the effects of synapsis, segregation and recombination. Smears from the root tips have indicated that these seedlings are all diploid. It can not definitely be concluded that these seven seedlings are apomictic in origin although the evidence does suggest that this may be the case.

II RESULTS OF THE 1955 PROGENY TEST

Seed Extraction and Cleaning

The cones from the six trees were collected in the fall of 1954; those from trees A, C, and D were removed ten to twelve days earlier than those from the trees at Lake Cowichan. The cones were air-dried for several weeks and the seed was then extracted and dewinged. It was carefully cleaned to remove as much empty seed as possible and then counted before being stored in sealed containers at 4° C. Great care was taken throughout to ensure that no contamination occurred in any of the seed lots. The results of the seed extraction and cleaning are shown in table 25; the average number of seeds is based on the number found in the productive region of the sample cones removed for the cytological investigation. The flat and misshapen seeds, which are often found at the tip and base of a cone, are not included in this figure. The number of seeds remaining after cleaning indicates that much less seed is obtained after self-pollination than after either controlled cross- or wind-pollination. Very few cleaned seeds were obtained after the self-pollination of trees A, C, and D; this, however, was not unexpected since the amount of ovule collapse had also been very high. Selfing, however, had much less effect on trees F, H, and J; in tree F there was actually a higher percentage of cleaned seeds after self-pollination than after cross-pollination. In every other case, however, controlled cross-pollination yielded more filled seed.

TABLE 25 RESULTS OF SEED EXTRACTION AND CLEANING AFTER DIFFERENT POLLINATIONS. 1955.

Tree	Type of pollin.	No. of Cones	Aver. no. seeds per cone	No. of seeds	No. of cleaned seeds	Per cent of cleaned seed
A	Self	82	37.4	3066	90	2.9
	Cross	47		1756	634	36.1
	Wind	47		1756	456	26.0
C	Self	47	45.4	2134	56	2.6
	Cross	25		1135	598	52.7
	Wind	53		2406	856	35.6
D	Self	16	27.6	442	6	1.4
	Cross	24		662	217	33.0
	Wind	73		2015	1375	68.2
F	Self	72	44.7	3218	959	29.8
	Cross	33		1475	420	28.5
	Wind	168		7509	2935	39.1
H	Self	56	60.7	3400	445	13.1
	Cross	37		2246	1078	48.0
	Wind	99		6010	1886	31.4
J	Self	63	48.6	3061	454	14.8
	Cross	41		1992	1051	52.8
	Wind	56		2721	668	24.5

Sowing

The different seed lots were first soaked in water for twenty-four hours and then stratified for a period of five weeks by the method recommended for Pseudotsuga by Allen and Bientjes (1954). The seed was then ready for sowing. A randomised block, split plot design was used in order that the environmental effects on the seed lots would be equalised as far as possible. Each of the eighteen seed lots was lightly dusted with a fungicide and divided

into equal numbers of seed in order that they would be all represented in each of the four standard seed beds at the nursery. Each of these four seed beds was divided up into six plots and each plot was divided into three sub plots. The six parent trees were assigned at random to each of the six plots in each seed bed, the three types of pollination of each parent tree were then assigned at random to the three sub plots within the parent tree plot. One of the four seed beds is illustrated in figure 1, plate XII. The method of seeding was modified from that reported by Liddicoet and Kimbrough (1950). The seed was individually sown in spots which were six inches apart and seven to a row; the spots at the end of each row were four inches from the sides of the seed bed and the rows were six inches apart. The location of each spot within a row was marked mechanically by large headed nails, set at the appropriate intervals on the underside of a flat board which could be laid across the seed bed. Pressure was applied to this board, then it was removed, and the position of each seed spot was visible on the soil surface.

The seed was sown in early May. The number per spot varied from one to three depending upon the amount of seed available; if more than one was sown in the same spot, half an inch was left between each seed. The seeds were then covered by a thin layer of soil and their location marked with a tooth pick. This method of sowing had many advantages since records could be kept of each seed spot and the apparent germination readily obtained. The seed beds

were covered as a protection against birds and animals by fine meshed netting and poison bait was used as an added precaution against any possible rodent damage. The seed beds were shaded throughout the summer months and watered when necessary.

Germination

The seed spots were checked for germination at intervals of three days. The first germination took place sixteen days after sowing. Some delayed germination occurred owing to unusually cold weather but the time of germination of seeds from different pollinations did not differ markedly. At the end of June, all seeds which had not germinated were uncovered and examined to see whether or not they contained embryos; they were then recorded as either filled or empty. Germination percentage was generally high in all of the beds which indicated that the seed had been thoroughly cleaned. It was found that the precautions taken to equalise the environmental effects on the different seed lots in the four seed beds were justified. An analysis of variance showed that there were significant differences between beds, between trees and between pollinations. None of the interactions was significant and for this reason, the germination of each seed lot has been summarised in table 26.

It was of some interest to find that isolation of the female cones must have interfered with the life cycle of Megastigmus spermotrophus, the Douglas fir seed chalcid which can cause considerable damage. According to a recently revised publication

(Anonymous 1954), attack takes place in the spring when the female forces her long ovipositor through the bracts and scales of the cone and lays her eggs in the ovule. The eggs later hatch and the larvae feed on the contents of the seed, finally emerging the next spring as adults through a small hole. The results from table 26, however, indicate that only the seeds from wind-pollinated cones contained larvae of this seed chalcid.

TABLE 26 SUMMARY OF GERMINATION RESULTS. 1955

Tree	Type of pollin.	Seeds sown	Seeds germin.	Appar. germin. per cent	Ungerminated seeds			
					Filled	Empty	Not found	With larvae
A	Self	90	16	17.8	-	62	12	-
	Cross	420	330	78.6	7	61	22	-
	Wind	420	166	39.5	131	59	51	13
C	Self	56	23	41.1	30	3	-	-
	Cross	420	394	93.8	6	10	10	-
	Wind	420	222	52.9	29	121	28	20
D	Self	6	4	66.7	1	1	-	-
	Cross	217	208	95.8	2	5	2	-
	Wind	420	381	90.7	1	23	15	-
F	Self	959	820	85.5	35	92	12	-
	Cross	420	400	95.2	4	13	3	-
	Wind	420	358	85.2	10	47	4	2
H	Self	445	430	96.6	10	4	1	-
	Cross	420	406	96.7	8	5	1	-
	Wind	420	395	94.1	12	7	4	2
J	Self	454	363	79.9	21	63	7	-
	Cross	420	397	94.5	7	13	3	-
	Wind	420	352	83.8	14	30	9	15

Seedling Mortality

Records were kept of the mortality that occurred during 1955, almost all of which was caused by damping-off fungi. Mortality in the four seed beds is summarised in table 27; the data indicates an apparent relationship between first-year survival and the type of pollination. Mortality of inbred seedlings was consistently higher than that of the crossbred seedlings, and losses of the seedlings from wind-pollination were intermediate. The higher mortality rate of the inbred seedlings was not unexpected since many obviously were far less vigorous than the crossbred seedlings. This is clearly illustrated in figure 2, plate XII, which shows an inbred and a crossbred seedling from the same parent and of almost identical ages. The mortality of the inbreds and of the crossbreds was compared by the Chi square method and the difference was found to be highly significant for those from trees A, D and H.

TABLE 27 SUMMARY OF SEEDLING MORTALITY. 1955.

Tree	<u>Self-pollination</u>			<u>Cross-pollination</u>			<u>Wind-pollination</u>		
	Total	Dead	Percent	Total	Dead	Percent	Total	Dead	Percent
A	16	4	25.0	330	32	9.7	166	28	16.9
C	23	3	13.0	394	46	11.7	222	23	10.4
D	4	3	75.0	208	9	4.3	380	35	9.2
F	820	83	10.1	400	28	7.0	358	29	8.1
H	430	75	17.4	406	47	11.6	395	36	9.1
J	363	27	7.4	397	21	5.3	351	27	7.7
Total	1656	195	11.7	2135	183	8.6	1872	178	9.5

Comparative Height Development

Thirty seedlings from each pollination type, for each of the six trees, were selected at random in each of the four seed beds

and their heights measured from ground level to the tip of the terminal bud. In cases where there was only a few seedlings representing a particular pollination, they were all measured. It was again found that the precautions taken to equalise the environmental effects on the different seed lots in the seed beds were justified. An analysis of variance showed that there were significant differences between beds, between trees and between pollinations. None of the interactions were significant, however, so the heights from each pollination type have been summarised. The frequency distributions and coefficients of variation for the progeny of all six trees are shown in table 28. Selfing has again produced seedlings which are smaller than those from controlled cross-pollination while those from wind-pollination are intermediate between them. The coefficients of variation for any one inbred line differed considerably, those for trees A and F, for example, being very close to those of the seedlings from controlled cross-pollination. In the case of tree J, however, there is a much greater difference between the two values. This variation is probably related to the undetermined genotype of the parent tree. Figures 2 and 3, plate XII, illustrate the variation in any one inbred line, the seedling in figure 2 is inherently weak and unlikely to survive whereas the seedling in figure 3 is vigorous. This seedling already has a tendency to branch and should be compared with the seedling in figure 4 which has resulted from controlled cross-pollination of the same parent tree.

It is of some considerable interest that in five of the trees,

controlled cross-pollination has resulted in a higher proportion of large seedlings than were obtained from wind-pollination although no attempt was made to select particularly vigorous pollen parents. The comparative results of controlled cross-pollination on different trees are also of interest. The most vigorous crosses appeared to be those in which both parent trees came from different localities. The crosses A x F and J x C were both made with Victoria and Lake Cowichan trees, the superiority of their progeny at the present time over those from wind-pollination of the same parent trees is quite marked. It moreover can not be matched by any other cross between parents from the same locality.

It remains to be seen whether such differences persist as it is fully realised that hasty conclusions should not be made at this early stage of development. Several investigators such as Righter (1945) have effectively demonstrated that seed weight in the Pinaceae is of considerable importance in initial growth, and that the difference in inherent growth may be concealed by the effects of initial seed weight. The effects of seed weight are only temporary but they should not be overlooked at this stage of development.

TABLE 28 FREQUENCY DISTRIBUTIONS AND COEFFICIENTS OF VARIATION
BY HEIGHT CLASSES

Tree	Pollen parent	Height classes in millimeters at end of first year							Total no.	Mean ht.	C.V.
		10-29	30-49	50-69	70-89	90-109	110-129	130-149			
A	Self			2	4	4	2		12	88.8	18.1
	X F		1	5	22	45	41	6	120	101.2	18.6
	Wind	3	9	13	37	45	11	2	120	83.9	28.0
C	Self	1	3	9	3	2	1		19	63.7	34.5
	X B*	2	7	24	44	27	16		120	80.7	28.2
	Wind		4	20	47	38	9	2	120	83.2	23.8
D	Self			1					1		
	X C		2	7	29	51	29	2	120	96.1	19.1
	Wind			17	40	50	12	1	120	86.7	19.3
F	Self	2	21	57	31	9			120	62.2	25.9
	X H	1	5	32	41	37	4		120	77.8	24.7
	Wind	2	24	46	21	24	3		120	66.8	29.7
H	Self	3	61	39	15	1	1		120	50.8	31.4
	X J		6	40	44	22	8		120	75.9	24.8
	Wind	5	23	48	27	16	1		120	63.1	31.8
J	Self	21	9	46	32	12			120	59.3	40.5
	X C	1	6	12	37	41	19	4	120	88.9	25.2
	Wind	7	10	42	46	13	2		120	67.5	31.4

* Tree B was also included in the investigation but was damaged later by fire.

PLATE X

Figure 1. A dwarf form of inbred seedling from tree 2.
October, 1955.

Figure 2. A bushy form of inbred seedling from tree 2.
October, 1955.

Figure 3. An inbred seedling from tree 2, comparable in
form to those from cross-pollination.
October, 1955.

Figure 4. A vigorous inbred seedling from tree 2 with
a very branching habit, it is twenty-six inches
in height. October, 1955.

PLATE X

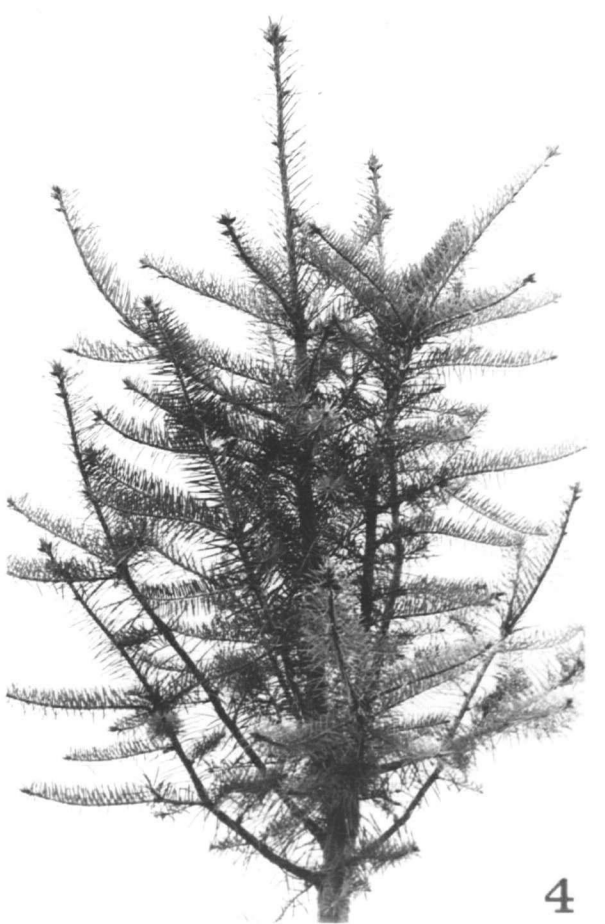
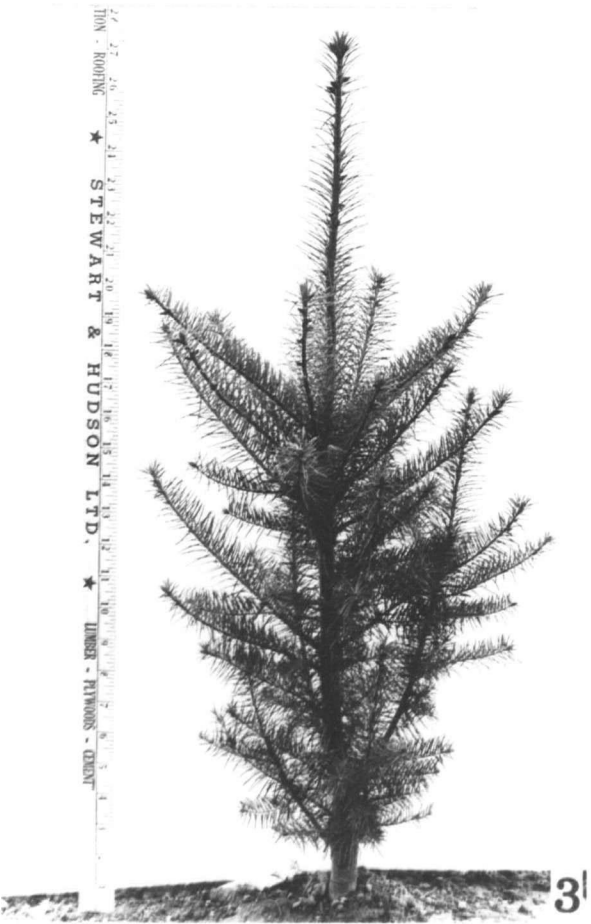
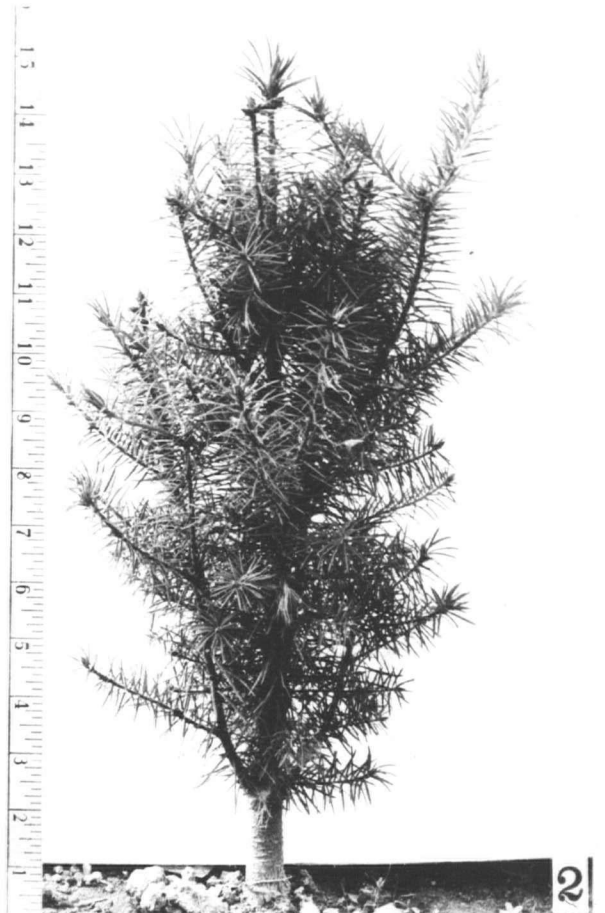


PLATE XI

Figures 1 and 2. Two seedlings from tree 2, believed to be apomictic; note the similarity in height and form. October, 1955.

Figure 3. A seedling from the wind-pollination of tree 2; compare with figure 4. October, 1955.

Figure 4. A vigorous seedling from wind-pollination of tree 2; it is thirty-four inches in height. October, 1955.

PLATE XI

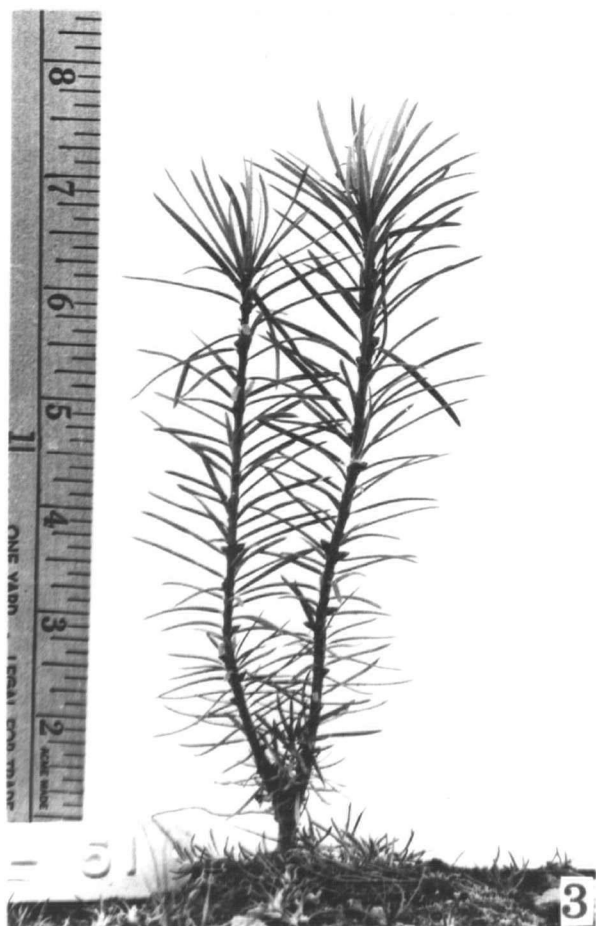


PLATE XII

- Figure 1. One of the four seed beds used for the 1955 progeny test, the laths separate the progeny of one parent tree from another. October, 1955.
- Figure 2. Variation in vigour between an inbred and a seedling from controlled cross-pollination of the same parent tree, both are the same age and from tree C. October, 1955.
- Figure 3. A vigorous inbred from tree C with a pronounced branching habit. October, 1955.
- Figure 4. A seedling from controlled cross-pollination of tree C, note the improved form compared to figure 3. October, 1955.
- Figure 5. Twin terminal buds, possibly representing a recessive character revealed by inbreeding tree 2. October, 1953.
- Figure 6. The same seedling, one year later, October, 1954.
- Figure 7. The same seedling two years later, note the persistence of the two leaders. October, 1955.

PLATE XII

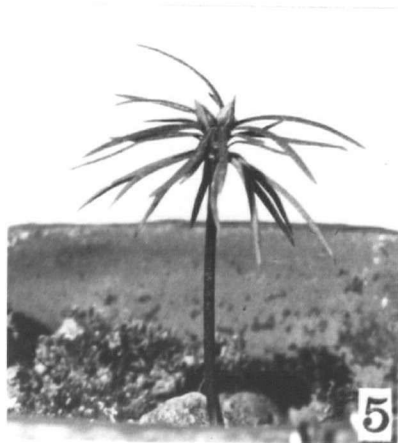
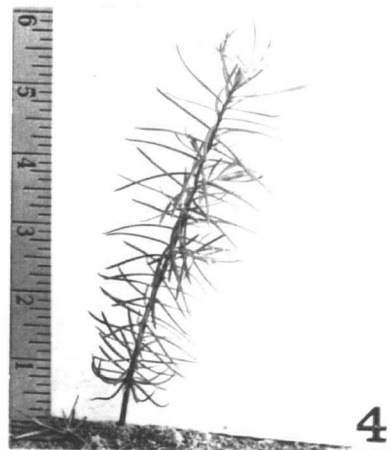
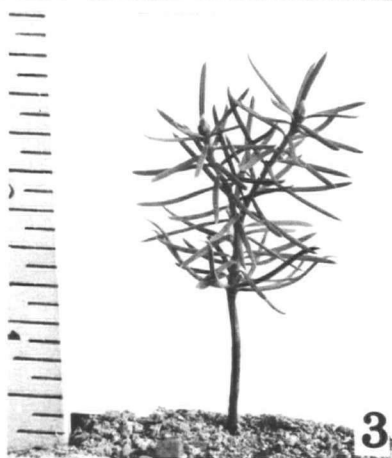
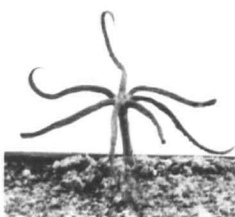
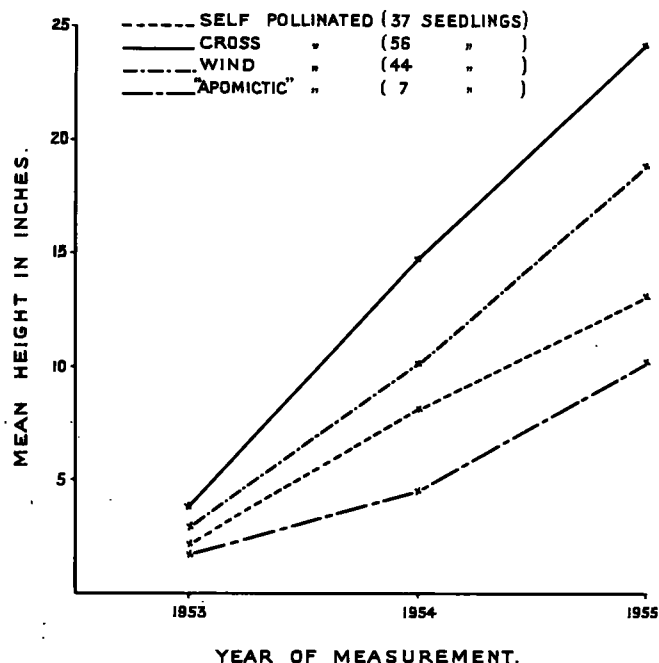


PLATE XIII

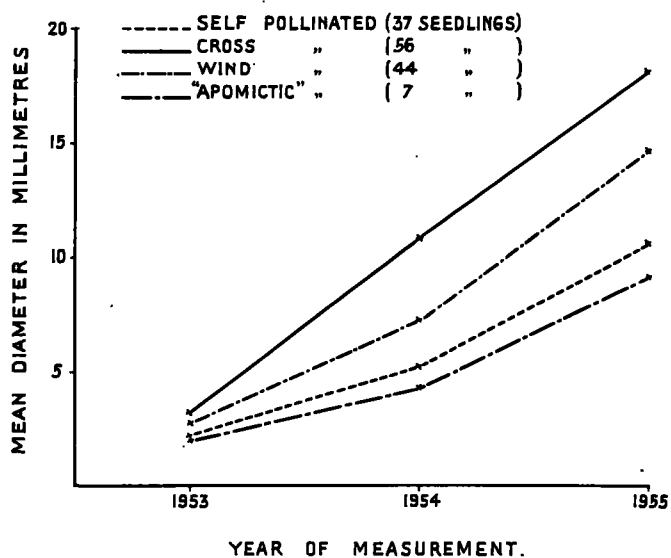
The comparative heights and diameters of the
progeny of tree 2 from 1953 to 1955.

PLATE XIII

COMPARATIVE HEIGHTS OF SELFED, CROSSED, WIND
POLLINATED AND "APOMICTIC" PROGENY FROM TREE 2.



COMPARATIVE DIAMETERS OF SELFED, CROSSED, WIND
POLLINATED AND "APOMICTIC" PROGENY FROM TREE 2.



III SOME PRACTICAL ASPECTS OF SELF-POLLINATION

Natural Regeneration

The fact that Pseudotsuga can be self-pollinated and produce seedlings which are generally inferior to those resulting from cross-pollination is of significance in planning for natural regeneration of cutover areas. Johnson (1945) previously pointed out the possibility that self-pollination will occur if a few widely spaced individual trees are left to reseed a cutover area. He added that the silviculturist should, among other things, leave sufficient seed trees to provide for cross-pollination among individuals of the same species. It is possible that a loose form of inbreeding could take place on cutover areas in the Douglas fir region where such areas have regenerated from single or a small number of seed trees. This important aspect of regeneration has been given scant attention and no studies have yet been conducted on the crucial matter of distance of pollen flight in Pseudotsuga.

Several misconceptions, moreover, have arisen in regard to self-pollination in Pseudotsuga. It was formerly considered that viable seed could not be obtained from selfing and, indeed, that self-pollination itself was almost impossible, owing to the different times of maturation of the male and female cones. Syrach Larson (1937, page 67) believed that Pseudotsuga is markedly metandric and that self-pollination can occur to only a limited extent. This statement was corrected by Allen (1942), who pointed out that many trees showed no evidence of dichogamy and that there was much variation from tree to tree. Some years later, however,

Isaac (1949, page 49) wrote:

Since pistillate flowers of Douglas fir receive their pollen from staminate flowers of another Douglas fir tree, there is certain to be cross-fertilization with other good trees in the plantation. Consequently, the line degeneration with attendant loss of vigor, which usually accompanies self-fertilization is automatically and naturally avoided.

This statement is, of course, both incorrect and misleading and can not be substantiated by experimental evidence. On the contrary, this study has shown that there are definite opportunities for natural self-pollination in some trees. Particular attention was given to the development of both the male and female cones and, in several trees, pollination was in progress at the time when the female cones had reached the peak of the receptive stage. In addition, the appearance of some of the seedlings produced by wind-pollination was very similar to that of the inbreds and in all probability they were the result of self-pollination. It can be definitely concluded that self-pollination does occur under natural conditions in Pseudotsuga, that self-pollination does result in variable amounts of viable seed, and that the resulting seedlings will usually be less vigorous than those produced by cross-pollination.

These proven facts should be of some significance in the selection of silvicultural methods for regenerating cutover land.

Artificial Regeneration

The knowledge that Pseudotsuga can produce viable seed after self-pollination should have obvious implications in regard to

cone collections. The necessity of not collecting cones from isolated trees and for ensuring that there has been adequate cross-pollination in young stands producing cones needs no further elaboration.

The deleterious results of self-pollination could also be a matter of some significance in the establishment of seed orchards. There has been considerable emphasis in recent years on the necessity for improving the inherent quality of seed for reforestation purposes and seed orchards have been recommended for this purpose. These would consist of a number of clones taken from selected trees thought to be inherently superior in growth and form to the remaining trees in a stand. It is hoped that the clones from these selected trees will cross-pollinate freely and produce abundant superior seed. Various suggestions for the formation of such orchards have been proposed by Isaac (1949), Duffield (1950), and Meyer (1951), but these were all based on the improbability of viable seed being produced after self-pollination. It is clear, however, that much careful planning of these orchards will be necessary if inbreeding is to be reduced as much as possible. This danger in seed orchards has already been appreciated in Europe. Langner (1953), for example, has recently made a detailed study of the most desirable designs for planting out ten clones from each of ten selected trees in order to reduce self-pollination. There has in addition been some controversy in regard to the number of clones that should be represented in a seed orchard. In Sweden, Gustaffson (1950) has stated that the clones

should not be too few in number in order to prevent a depauperization of genes as well as future risks of homozygotization and inbreeding depression of variable intensity. He has suggested that there should be at least twenty to thirty clones from as many parent trees to represent each provenance zone. Jensen (1954, page 63), on the other hand, has pointed out that he has been repeatedly criticized for selecting only nine parent trees to represent each provenance zone in Sweden. It was felt that this small number could lead to inbreeding deterioration. In his opinion there was no theoretical proof that inbreeding appeared as a result of the crossing of a limited number of parent individuals in a natural population. Such controversies show that the formation of a seed orchard is no easy matter.

In conclusion it should be pointed out that inbreeding could also be of value to future forestry practice. Ehrenberg et al (1955) have recently pointed out that a seed orchard utilizing the phenomenon of hybrid vigour could be established on the basis of inbred lines. The intercrossing of such lines, moreover, might in some cases effect a higher heterosis than in the other types of heterotic orchards. Wright (1953) again has concluded that recent developments such as the induction of precocious reproduction, indicate that an inbreeding program started now could lead to very useful results within the next half century. He also pointed out that several tree breeders at the present time are maintaining selfed lines for the use of their successors. Such long term planning presents a challenge to all concerned with programs in forest genetics.

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