THESIS

INCOMPATIBILITY IN SPRUCE HYBRIDIZATION

Submitted by

Susan V. Kossuth

In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

June, 1971

COLORADO STATE UNIVERSITY

May 17, 1971

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY Susan V. Kossuth

ENTITLED Incompatibility in Spruce Hybridization

BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF Master of Science

Committee on Graduate Work

Adviser

Head of Department

ABSTRACT OF THESIS

INCOMPATIBILITY IN SPRUCE HYBRIDIZATION

Strobili of reciprocally crossed Picea pungens Engelm. and Picea engelmannii Parry were periodically collected for microscopic study. The development of ovules from unpollinated blue spruce strobili was also studied.

Ovules from unpollinated blue spruce strobili began to break down nine days after the mid-period of strobilus receptivity. First, the female gametophyte became necrotic, usually protruding into the nucellus; then the nucellar cap, and finally the chalazal nucellus, broke down. Breakdown of the chalazal nucellus was usually preceded by a proliferation of the cells immediately surrounding the female gametophyte cavity. Blue spruce ovules, which did not receive any Engelmann spruce pollen though the strobili had been pollinated, followed this same sequence of breakdown, and although the female gametophyte became cellular, most cells contained no nuclei and little cytoplasm. Furthermore, the female gametophyte in these ovules did not usually protrude into the nucellus.

Some blue spruce ovules, containing germinated Engelmann spruce pollen, did not reach the egg stage. In others, an empty corrosion cavity formed, but in other ovules the female gametophyte showed no further development beyond the egg stage and degenerated. There seemed to be an inhibition of

Engelmann spruce pollen germination and/or growth of the pollen tube, in the blue spruce nucellus, which almost always prevented fertilization.

Most of the interspecifically crossed Engelmann spruce ovules also degenerated at an early stage of development. Two different conditions were observed in these ovules: 1) Some contained a female gametophyte consisting entirely of very large cells. 2) Others contained a greatly shrunken nucellar cap, but a developed female gametophyte. These two conditions were also observed in the crossed blue spruce ovules, but not simultaneously in the same ovule.

The average number of seeds per control-pollinated blue spruce cone was 278, and the average for open-pollinated cones was 302. This difference was not statistically significant according to a T-test. Control-pollinated blue spruce seed showed only a 0.48 percent germination, whereas seed from open-pollinated blue spruce showed 42.5 percent.

Susan V. Kossuth
Forest and Wood Science
Colorado State University
Fort Collins, Colorado 80521
June, 1971

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to Dr. Gilbert H. Fechner (major professor), Department of Forest and Wood Sciences, for the many hours he spent with me during my years at Colorado State University. During this time his passion to teach and mine to learn were matched in the work we performed and the research we conducted. His patience, encouragement and continuous interest in me and my research activities have strengthened my professional self-confidence and solidified the goals for which I will strive in my life.

Appreciation is also extended to my committee members, Dr. Robert V. Parke, Department of Botany and Plant Pathology, Dr. Jess Fults, Department of Botany and Plant Pathology, and Dr. Donald Wood, Department of Agronomy, for their assistance in the preparation of this thesis, and instruction in their respective courses.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

Chapter

TABLE OF CONTENTS CONTINUED

Chapter

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

CHAPTER I

INTRODUCTION - LITERATURE REVIEW

One of the basic tools of a tree improvement program can be the artificial hybridization between different varieties, species or genera. Hybridization offers a method of bringing together new gene combinations in new individuals. One goal of such combinations in tree species is to obtain heterosis in the F_1 generation, such that the progeny usually exceed either parent for a given trait. A second goal is the combination of desirable traits from two parents. In some agricultural crops, inbred lines may be developed before hybridization is attempted, but such a procedure is not practical in forest trees because of the relatively long time between generations. Thus hybridization of forest trees is closely coupled with selection of phenotypically desirable parent trees.

The first step in hybridization studies is to determine the species crossability pattern (Wright, 1962). In spruce, Picea A. Dietr.[1], crossability seems to depend on the morphological similarity between the two species in question and the closeness of their geographic range. Morphologically

---

[1]In this thesis, scientific binomials are in accord with those used by Little (1953) for trees native or naturalized in the United States, and with those used by Bailey (1963) and Bailey (1949) for species not listed by Little.

similar species, whose natural distributions overlap extensively, tend to be genetically isolated and therefore do not interbreed (Wright, 1955). One concludes that genetic differentiation has taken place in morphologically similar species if their ranges overlap but the two species do not interbreed. Artificial hybridization of species from adjacent areas of distribution is more likely to succeed if the differentiation between the two species in question is the result of geographic rather than genetic barriers.

Upon inspection of the natural distribution of Picea pungens Engelm. (blue spruce) (Sudworth, 1916) and Picea engelmannii Parry (Engelmann spruce) (Fowller, 1968), it would appear that these two species have similar distributions. However, blue spruce and Engelmann spruce are altitudinally or geographically isolated except for some slight altitudinal overlap. Therefore, one would expect crossing to occur readily, but attempts to artificially hybridize blue spruce and Engelmann spruce have not been very successful. Richens, in 1945, reported that the artificial cross Picea engelmannii Parry x Picea pungens Engelm. was successful, but he gave no indication of the degree of success. Fechner and Clark, in 1967, obtained one to two percent viable seed in their artificial cross Picea engelmannii Parry x Picea pungens Engelm. but no viable seed from the reciprocal cross. Johnson (personal communication with Wright, 1955) reported the occurrence of natural putative

hybrids between these two species. It was hoped that successful hybridization between blue spruce and Engelmann spruce would create a tree that combined the blue color and drought resistence of blue spruce with the fast growing timber qualities of the Engelmann spruce.

Objectives

The specific objectives of this study were:

1. To describe the ovular development of the reciprocal crosses between Picea pungens Engelm. and Picea engelmannii Parry and to describe the unpollinated ovule development of Picea pungens Engelm.

2. To compare the ovular development of the cross Picea pungens Engelm. x Picea engelmannii Parry and the unpollinated ovule development of Picea pungens Engelm. with the normal open pollinated Picea pungens Engelm. ovular development.

3. To observe other features of reporductive development of control pollinated and wind pollinated blue and Engelmann spruce seed and cones.

Angiosperm Incompatibility

Knowledge of past research in the area of incompatibility has been most helpful in interpretation of the results of this investigation. It may be that the principles and types of incompatibility observed in angiosperms have application in the behavioral patterns of incompatible gymnosperms. Thus a review of current and past research in both angiosperm and gymnosperm incompatibility seems appropriate.

Most work on incompatibility systems has been concentrated on the self-incompatibility mechanisms of the angiosperms. Bubar (1959) attempted to clarify terminology relating to systems whereby male and female gametes from the same plant did not produce viable seed. He explained that "self-sterile" should refer to plants which fail to set self seed when isolated from insects (voluntarily self-sterile). "Self-incompatibility" is a genetic outbreeding mechanism causing self-sterility by inhibiting the growth of self pollen tubes, such that fertilization never occurs. Somatoplastic sterility is another self-sterility mechanism wherein self fertilization occurs, but the embryo and ovule obort. Somatoplastic sterility results in inviable seed, while self-incompatibility systems, as pointed out by Brewbaker (1957), select against self pollen, thereby promoting outbreeding and the set of viable seed.

Most self-incompatibility systems in angiosperms are governed by the S-gene (self-sterility gene). The S-gene has many different alleles, and if identical alleles occur in both the male and female tissues, an incompatibility reaction inhibiting the development of the pollen will result. Thus foreign pollen (from another plant) could be just as incompatible as self pollen, if the foreign pollen and the female tissue contained the same S-alleles.

One of two types of incompatibility systems, gametophytic or sporophytic, may occur in conjunction with the self-sterility gene in angiosperms. These two systems differ only in

the time of the S-gene action during microsporogenesis. In the gametophytic system, the S-gene is determined by the genotype of the pollen. In this system, pollen is usually shed in the binucleate state and functions normally until the pollen tube has actively grown into the style or the ovary. Interaction with the female tissue inhibits the pollen tube at this time.

Dominance is important in the determination of the pollen phenotype or sensitivity in the sporophytic incompatibility system. The phenotype of the pollen grain is dependent upon the genotype of the plant from which it came. If, in the production of pollen from an S_2S_3 plant, S_2 is dominant to S_3, both the S_2 and S_3 pollen grains will behave as S_2 phenotypes. Thus a haploid pollen grain of genotype S_3 from this plant would be incompatible in the diploid female tissue of a plant with a genotype S_1S_2, but compatible in the female tissue of an S_1S_3 plant since it expresses an S_2 phenotype. Pollen grains of the sporophytic system of self-incompatibility, are usually shed in the trinucleate state and are inhibited on the stigma, frequently not even germinating (Brewbaker, 1957). The stigma, style and ovary are diploid tissue systems and may contain two kinds of alleles or two of the same allele.

Brewbaker (1957), felt that the action of the incompatibility alleles might be effected through the control of sucrose uptake and metabolism in the pollen grain. Trinucleate pollen, of the sporophytic incompatibility system, because they have undergone the final mitotic division, may

be so sucrose-or metabolite-deficient, that germination is inhibited. Binucleate pollen, of the gametophytic incompatibility system, not so depleted from the final mitotic division, may, under similar conditions, germinate and begin pollen tube growth without additional nutrition from the style. Nicotiana, Oenothera, and Trifolium show gametophytic incompatibility (Crowe, 1954, and Lundquist, 1954). Most Cruciferae show sporophytic incompatibility.

Time of the S-allele action

Linskens (1961) suggested four different times at which the incompatibility reaction may be manifest: 1) during germination on, and penetration of the pollen grain into the stigma; 2) during growth of the pollen tube through the conducting tissue of the style or ovary; 3) during the discharge of the pollen tube contents into the female sexual apparatus; 4) by abortion of the zygote or young embryo. The first manifestation is characteristic of sporophytically-determined incompatibility; the second, and probably the third, characterize gametophytic incompatibility; and the last is probably the inability of the two genomes to function together or, in self pollination, the expression of homozygous lethal recessives.

Function of the self-sterility gene

The S-alleles carried by the male and female cells are responsible for: 1) specificity of the incompatibility reaction; 2) induction of the reaction; and 3) energetic and

metabolic background of the incompatibility reaction (Linskens, 1963). Linskens (1963) has pointed out that in a self-incompatible plant, the same S-allele acts differently in the male and female cells. In the gametophytic system, the metabolic relationship between the stylar tissue and the pollen tubes takes one of three forms: 1) dissolution of the pectic material of the stigma by the pollen tube; 2) chemotropic guidance of the pollen tube; 3) absorption of metabolic substances by the pollen tube (Linskens and Tupý, 1966). Disruption of any of these relationships would inhibit the successful growth of the pollen tube.

Callose in the germinating pollen grain

According to Roggen and Stanley (1969), Currier reported that in pear pollen, a deposition of callose occurred in the intine, just below the pore from which the pollen tube would have protruded. Roggen and Stanley (1969) found that this deposition occurred after the pear pollen grain swelled, which took ten to twenty minutes in artificial media. If germination did not subsequently take place, the callose formation continued until the whole grain was filled with callose. In the germinated pollen grain, cell-wall-softening enzymes were released into the germination medium from the pollen grain. These same enzymes then entered the intine, presumably through the germ pores, and softened the intine wall, which facilitated pollen tube emergence from the pore area containing the callose. This callose then disappeared

from the tip, and pollen tube growth commenced. New callose, deposited just behind the pollen tube tip, served to strengthen the pollen tube wall, but if tube growth were halted, callose was deposited at the tip wall.

Nutrition of the pollen tube

Electron microscopic observations by Van der Pluijm and Linskens (1966), indicated that in some styles, pollen tubes grow within the matrix of the middle lamellae of the transmitting tissue by enzymatically dissolving a path in front of the pollen tube tip. According to Bhattacharyja and Linskens (1955), Vogel has presented photographs of pollen tube tips which ended in a mass of erect cellulose fibrils, which showed that the cytoplasm of the pollen tube was in direct contact with the intercellular fluid of the conducting tissue. Bhattacharyja and Linskens (1955) indicated Straub had found that pollen tubes can dissolve and probably assimilate the collenchyma-like cell wall thickenings of the stylar tissue. Other work by Linskens showed that the sugar content of the style was reduced by half (in Petunia) by the time the pollen tubes reached the embryo sac. Linskens and Esser (1959) applied radioactively labelled ^{14}C-glucose, ^{14}C-fructose and ^{14}C-alanine to the styles of Petunia at the time of anthesis. Later, pollen tubes that developed in the styles were found to be radioactive. These studies provided evidence that the stylar tissue supplies nutritional material for pollen tube growth.

Environment

Hagman (1963) working with self-incompatible Betula spp. found that at lower temperatures, the self-incompatibility reaction is slowed such that pollen tubes may effect self-fertilization. In 1967 he found that the self-incompatibility reaction and the pollen tube inhibition was more effective at higher temperatures. Thus the environment played a role in the timing and intensity of self-incompatible reactions. Hagman (1967) also found that radiation of the pollen grains may remove the self-incompatibility barrier.

Performed stylar incompatibility substance

The results of experiments conducted by Emerson (1940) and Lewis (1952) supported the idea that the stylar incompatibility substance is formed before the arrival of pollen on the stigma. Emerson grafted a compatible stigma and style onto an incompatible ovary and observed the inhibition of pollen tubes upon their contact with the tissue of the ovary. This was also an indication that the female tissue was passive, having supplied nutrition, but not stimulation, for growth of the pollen tubes. Sears (1936) suggested that the lack of any stimulation from the style could be argued since pollen tubes grown in vitro did not grow to the equivalent length of those in vivo, namely, the distance from stigma to ovule. Lewis (1952) self-pollinated Oenothera stigmas twice, at a four-hour interval, and found both sets of pollen tubes were inhibited at the same stage of penetration. Had the second

set of pollen tubes been less successful than the first, that is, penetrated a lesser distance into the style, one might conclude that the first set of pollen stimulated the production of the incompatibility substance in the style, but this was not observed. According to Tupý (1963) it seems logical to argue that the antigens of the pollen tube are not preformed in the gametophytic type incompatibilities, such as those above, since the reaction does not occur until the pollen tubes are into and actively growing in the stylar tissue.

Characteristics of the self-incompatibility reaction

The gametophytic self-incompatibility reaction affects the pollen tube in many ways. Linskens (1961) has found an increased respiration rate, and a disturbance in carbohydrate metabolism in germinated self-incompatible pollen tubes. These disturbances of the pollen tube were accompanied by an increased formation of cellulose cell wall thickenings, thick callose formation, branched tube tips and an increased number of callose plugs (Linskens, 1963).

It seems that the gametophytic self-incompatibility reaction may be the interaction of antigens of the pollen with antibodies of the female tissue, to form a reaction product which inhibits the pollen tube. Linskens (1961) has proposed three possible mechanisms for this action: 1) Incompatible pollen may stimulate or activate the formation of inhibitors in the conductive tissue of the style.

This would require a high genetic specificity such that special inhibitors would be formed for each different allele. 2) The growing pollen tube may form antigens that induce specific antibody production in the style. These two may interact, and produce reaction products which block enzyme systems leading to normal polymerization of carbohydrates and to normal nuclear metabolism of the pollen tube. 3) Antibodies may be preformed in the stylar conducting tissue and react with the preformed antigens of the pollen tube. The reaction products (glucoproteins) may be called "ward bodies" and serve as a block to a link in the reaction chain leading to fertilization. The "ward bodies" from an incompatible combination are believed to form a precipitate which inhibits pollen tube growth.

Serological analyses support the idea of an antigen-antibody reaction in self-incompatible pollinations. As a result of his work with Oenothera, Lewis (1952) believes that the antigens and antibodies are the end-products of S-allele action and that when they combine, pollen tube growth is inhibited. However, he also cautioned that the antigenic substances may only be enzymes controlling intermediate stages in the synthesis of incompatibility substances. Brewbaker (1957) also felt that the immediate S-allele products were of the antigen-antibody type and interacted to disrupt pollen tube growth. Bhattacharyja and Linskens (1955) reiterated Straub's "Consumption Theory." According to this theory, identical S-alleles lead to a build-up of

antagonistic substances in the style. These substances inactivated the pollen tube by preventing sufficient digestion of style conducting tissue for the continued growth of the pollen tube. Hagman (1963) found serologically detectable differences between the pollen of Betula verrocosa Ehrh. and Betula pubescens Ehrh. which may be connected to the interspecific incompatibility between these two species.

Chemical analysis

Bhattacharyja and Linskens (1955) referred to Linskens' earlier work of electrophoretic separation to determine the physio-chemical nature of the substance causing self-sterility. Extracts from selfed and outcrossed stylar-conducting tissue from different species, resulted in two new fractions of proteins from the self-pollinated styles and one new fraction from cross-pollinated conducting tissue, none of which occurred in the unpollinated styles. Linskens and Tupý (1966) found evidence for two different groups of amino acids in the style of Petunia in which pollen tubes were actively growing. One group of amino acids served as a storage pool and source of oxygen for respiration while the second and smaller group of amino acids provided amino acids for protein synthesis. Variation in these groups of free amino acids of the style was the physiological expression of pollen tube growth. After outbreeding in Petunia there was a striking increase in tryptophane which is a precursor of indole-acetic-acid. Linskens (1963) used radioactive tracer techniques and paper electrophoresis to show that a complex was formed

between pollen and stylar protein after an incompatibility reaction in the style. Vasil (1964) investigated the stimulatory effect of boron from stigmatic and stylar tissue on pollen grains inherently deficient in boron. He felt the stimulatory effect of boron may be due to 1) an increased absorption, translocation and metabolism of sugars through the sugar-borate complex, 2) increased oxygen uptake, or 3) the synthesis of pectic materials for the wall of the pollen tubes. He did not feel that boron was an activator for any enzyme system although it is an essential element for pollen tube growth.

Sporophytic self-incompatibility in Cruciferae

Linskens (1961) has reviewed the self-incompatibility mechanism among the Cruciferae. Members of this family (*Arabis* spp., *Brassica* spp. etc.) are sporophytically incompatible. Manifestation of the self-incompatibility reaction of selfed pollen grains in this family is on the stigma, where germination is prevented or penetration of the pollen tubes into the cutin of the stigma membrane does not occur. The Cruciferae pollen contain cutinase which belongs to a cutin-splitting enzyme system. This enzyme system in selfed pollen is probably not activated when the pollen lies on the stigma of the flower from which it came. Kroh (1966) working with *Arabis arenosa* Scop. and *Brassica nigra* Koch. found that if self-incompatible pollen were started on a compatible stigma and then transferred to an incompatible self stigma, they were able to penetrate the self stigma. This indicated that

there was an irreversible activation of the cutin-splitting enzyme system that did not occur in the self-incompatible species of the Cruciferae. Kroh (1966) reviewed Christ's work in which Christ was able to overcome the inhibition of germination of selfed Cruciferae pollen by increasing the humidity in the atmosphere. Even though germination occurred on the self stigma in the higher humidity, the pollen tubes remained incapable of penetrating the stigma. Kroh related that in an earlier study he observed normal fertilization when selfed Cruciferae pollen grains were brought into direct contact with the stylar tissue, having bypassed the stigma.

Interspecific and intergeneric incompatibility

Roggen and Linskens (1967) found different expressions of incompatibility in the reciprocal applications of Petunia and Salpiglossis pollen. Petunia pollen tubes in Salpiglossis styles were retarded in growth, but Salpiglossis pollen although able to produce pollen tubes, were unable to penetrate Petunia stigmas. Gardella (1950) found two kinds of barriers to interspecific hybridization within Datura. If the longer style of Datura inoxia were shortened by removing a center section of the style, the pollen tubes of D. ferox Linn. and D. quercifolia HBK., with short styles, were able to reach the ovules. Hagman (1963) found retarded pollen tube growth in both reciprocal crosses between Betula verrucosa Ehrh. and Betula pubescens Ehrh., with the incompatibility reaction more effective when Betula verrucosa Ehrh.

was used as the male parent. As mentioned earlier, serological differences between the pollen of these two birch species exists.

Gymnosperm Incompatibility

A system for the genetic explanation of incompatibility, such as the S-alleles in angiosperms, has not been found in gymnosperms. Consequently, much information has been obtained and many hypotheses proposed for explaining both self- and interspecific-incompatibility in this group. Studies in which set of viable seed from self- or interspecific-pollinations was not obtained indicated self or cross sterility or, self or interspecific incompatibility. Sterility or incompatibility was expressed in the gametes as gametic incompatibility (pollen germination or penetration of the nucellus or pollen tube growth is affected) or in the embryo as embryo inviability, both of which resulted in inviable seed. Wright (1955), Mikkola (1969), and Kriebel (1967) used the term "crossability" as a positive expression of the successful embryo development in interspecific crosses. The factors limiting crossability were the mechanisms of gametic incompatibility and embryo inviability. Orr-Ewing (1957a), working with Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir), refrained from designating the set of inviable seed after self fertilization as being the result of an incompatibility reaction until proof of an incompatibility system in gymnosperms has been presented.

Selective pollination

The first selective mechanisms to operate against selfing or cross-pollination are phenology and geographical distribution. Dispersion of pollen at a time when female strobili are not receptive excludes the pollen from the ovule with the same effectiveness as geographical isolation of the ovule from the pollen. Most gymnosperms are outbreeding or cross-fertilized for either or both of these two reasons, and as Mikkola (1969) indicated, internal barriers to hybridization of species have probably built up gradually in association with geographical isolation and general genetic differentiation.

The internal mechanisms that operate to prevent the formation of viable seed when self and cross pollen are artificially introduced to the gymnosperm ovule are effective, even though their action may not appear as quickly and conclusively as might be expected in a well-developed incompatibility mechanism. These are the incompatibility mechanisms involving biochemical reactions between pollen and ovule, which are ultimately controlled by the genetic constitution of the individuals and result in inviable seed.

Pollen germination

Stanley (1967) summarized primary and secondary environmental factors affecting *in vitro* pollen germination. The primary factors include: 1) temperature, 2) pH, 3) oxygen, 4) osmotic pressure, 5) moisture, 6) cations and anions, and 7) carbohydrates. All of these have narrow optimum ranges

for *in vitro* pollen germination. The secondary factors, which can have a wide range of variation, include 1) growth hormones, 2) carbon dioxide, 3) micronutrients and 4) irradiation. The most important factors determining *in vivo* pollen growth include 1) endogenous chemical levels in the pollen, 2) chemistry and structure of the female tissue through which the pollen tube grows, and 3) the numbers and kinds of pollen tubes growing together in the female tissue. *In vitro* pine pollen does not require boron or sugars in the medium in order to germinate, although some angiosperm pollen germination is enhanced by the addition of sugars or boron (Stanley, 1967). Fechner (1955) found the germination of fresh blue spruce pollen to be enhanced in a ten percent sucrose medium as compared to lower or higher percentages of sucrose.

It seems that the sugar and amino acids of the micropylar fluid of *Taxus barrata* L. (English yew) and *Pinus thunbergii* Parl. (Japanese black pine) stimulate pollen tube growth Stanley (1967). Anhaeusser (1953) too, found that the pollination drop of *Taxus* promoted pollen germination and tube growth. McWilliam (1960), working with *Pinus resinosa* Ait. (red pine) found that the micropylar fluid evaporated or was withdrawn at the time of pollen germination. He felt that, although the fluid was probably not a means of affecting an incompatibility reaction on foreign pollen, it may supply some initial stimulus for germination as it draws the pollen toward the nucellus. He also found that Austrian pine

(Pinus nigra Arnold) and red pine pollen failed to germinate in Austrian pine micropylar fluid, which led him to conclude that the micropylar fluid was not involved in the germination of pine pollen. McWilliam further found pine pollen was sensitive to metallic ions since it did not germinate in tap water.

Hagman and Mikkola (1963) working with Pinus peuce Griseb. (Macedonian pine), Pinus cembra L. (Swiss stone pine) and Pinus koraiensis Sieb. and Zucc. (Korean pine), concluded that there was no mechanism in Pinus to prevent the germination of pollen in selfed strobili. Observations of Barnes, Bingham and Squillace (1962) on Pinus monticola Dougl. (western white pine) supported this observation and Orr-Ewing (1957b) also found no inhibition of Douglas-fir pollen in selfed strobili.

Penetration of the nucellus

With the aid of electron microscopy, Willemse and Linskens (1969) studied the development of the pollen grain in Pinus sylvestris L. (Scotch pine) ovules. Both germinated and ungerminated pollen grains were found to secrete enzymes such as pectinase and cellulase which affected the nucellar cell wall. These enzymes were secreted just ahead of the germinated pollen tube and thus weakened the nucellar cells so that they could be pushed aside and later degenerated as the pollen tube penetrated the nucellus. McWilliam (1959) found differences in the concentration of amino acids in the

nucellar tissues of red pine and Austrian pine. These differences were believed to be the reason that red pine pollen did not germinate, or functioned ineffectively in the nucellus of the Austrian pine.

Pollen tube growth through the nucellus

The slowed development of Scotch pine pollen in the nucellus of Austrian pine appeared not to be synchronized with the more rapid development of the female gametophyte (Vidakovic and Jurkovic-Bevilacqua, 1970). Mikkola (1969) has suggested three physiological reasons for the inhibition or cessation of foreign pollen tube growth through the nucellus of different spruce species: 1) An antigen type protein in the pollen grain may react with an antibody of the nucellus to inhibit pollen tube growth. 2) The pollen tube may be incapable of clearing a path through the nucellus. 3) Normal metabolism between the pollen grain and the nucellus may not occur. Through his chromatographic analyses of purified extracts from red pine pollen, Michalski (1967) found an acidic stimulator, with properties similar to IAA, and a neutral inhibitor to be present. When Michalski simultaneously applied the acidic stimulator and the neutral inhibitor to an Avena coleoptile section, the growth response was greatly decreased, indicating the net effect of these two substances was inhibitory. Stanley (1967) proposed that the time required for inherent inhibitors in the pine pollen to diffuse out or to be broken down may account for the delay in pollen tube extension at the end of the first growing season. The

work of Chira and Berta, as reviewed by Stanley (1970) shows that pollen of taxonomically closely related Pinus species have similar sugars, but those not so closely related have different sugars. This Stanley felt may be the cause of interspecific incompatibility between certain pines. McWilliam (1959) found that the pollen tubes of Austrian pine and Pinus rigida Mill. (pitch pine) growing in Pinus elliottii Engelm. (slash pine) nucellar tissue were of smaller than normal size.

Fertilization

Mikkola (1969) found in his interspecific crosses with spruce that fertilization did not necessarily occur even though the pollen tube had penetrated the nucellus. The pollen tube may not reach the neck cells of the archegonium or it may not penetrate the neck cells if it did grow directly to them. Also, if the pollen tube did penetrate the neck cells, fertilization still may not have been effected if the sperm nuclei did not move down the tube sufficiently to be discharged into the archegonium.

Embryo inviability

Hagman and Mikkola (1963) found hybrid proembryos were formed in the cross Macedonian pine x Swiss pine and Macedonian pine x Korean pine, but the proembryos aborted at an early stage. Abortion seemed to be correlated with the stage when cell walls were formed around the first four nuclei of the embryo. It appeared to the authors as if the proembryo were no longer capable of communicating with the surrounding

cytoplasm. In self-pollinated Douglas-fir embryos (Orr-Ewing, 1957), the abortion of the proembryos was attirubted to recessive lethal genes brought together in a homozygous condition. The fact that the individual trees studied differed in the number of viable seed produced supported this supposition since some probably had more recessive lethals present in the heterozygous condition than others.

Development of unpollinated gymnosperm ovules

Mikkola (1969) found that during early development of the spruce ovule a "primary threshold" had to be overcome, or else the ovule did not develop. If germinated pollen was not present in the ovule, the threshold was usually not passed by the ovule. Degeneration of the unpollinated spruce ovules originated in the free nuclear female gametophyte and proceeded into the nucellar cap; then the remainder of the nucellus degenerated. By the time fertilization should have occurred, the nucellus had died and shrunk. The integument too, slowed or stopped growing and these unpollinated ovules were flatter but not shorter than normal ones. This was the condition of the small, empty seeds. Mikkola also found that some unpollinated ovules continued development, and archegonia formed in the female gametophyte. Fifty to 60 days after pollination the corrosion cavity was formed, and the female gametophyte began to shrink. This kind of development in unpollinated ovules gave rise to large, empty seeds.

Mikkola's mature-unpollinated spruce seeds could not be distinguished macroscopically as being small or large as they

could in the earlier stages of development. In a poorly-pollinated strobilus in which less than half the ovules received pollen, many of the unpollinated ovules developed into the large-type empty seeds but in well-pollinated strobili or strobili completely devoid of pollen, the individual, unpollinated ovules developed into the small-type, empty seeds.

Hagman and Mikkola (1963) left Macedonian pine strobili unpollinated and observed that by June of the second year, all of the unpollinated strobili had abscised or dried. Unpollinated strobili of Austrian pine remained receptive longer than pollinated strobili, and the micropyle closed later in the unpollinated form (McWilliam, 1959). Three weeks after the time of pollination, the Austrian pine megaspore (in unpollinated ovules) degenerated. This was followed by the proliferation of the spongy tissue and its subsequent degeneration. The seeds resulting from unpollinated ovules contained a large centrally located, necrotic region in the ovule. Within two months, all of the Austrian pine ovules in the unpollinated strobilus had collapsed and the strobilus then abscised (McWilliam, 1959). McWilliam (1959) attributed the cause of the breakdown of the female gametophyte to a lack of some growth factor which the pollen tube growing in the nucellus, usually supplied. In no instance was parthenocarpy observed when strobili were isolated from all pollen.

Development of self-pollinated gymnosperm ovules

Orr-Ewing (1957a) found the effects of inbreeding Douglas-fir to vary considerably depending upon the individual tree, although all inbred progeny were weaker and less vigorous than cross-pollinated progeny from the same tree. Some Douglas-fir trees yielded considerably fewer viable inbred seed than others, and this was attributed to the number of lethal recessives which varied from tree to tree. The increased homozygosity of these recessive, deleterious genes was considered to be the cause of the observed embryo collapse in selfed ovules. Orr-Ewing (1957b) found no inhibition of fertilization or early embryo development in selfed Douglas-fir. Degeneration progressed from the terminal embryo to the female gametophyte. Douglas-fir ovules, in which the self pollen did not fertilize the egg, continued development of a corrosion cavity in which a residue was deposited as the archegonia degenerated. The fertilized ovules contained no such residue but did contain a stainable material in the cells of the female gametophyte which the unfertilized female gametophyte cells did not contain. Orr-Ewing felt this stainable material was probably the food supply for the developing embryo.

To test the ability of self pollen to compete with wind pollen, Squillace and Bingham (1958) applied equal mixtures of self and cross pollen to strobili of western white pine, a naturally outbreeding gymnosperm. In the highly self-fertile tree, either selfing or crossing was favored depending

on the fertility and ability of the cross pollen to compete with the self pollen. However, crossing exceeded selfing in moderately self-fertile trees and in the self-sterile trees.

Interspecific incompatibility in gymnosperms

McWilliam (1959) investigated interspecific incompatibility among several species of pine. In the cross slash pine x Austrian pine, the Austrian pine pollen did not penetrate the slash pine nucellus. In the crosses red pine x pitch pine, Austrian pine x pitch pine, and red pine x Austrian pine, pollen germinated and penetrated the foreign nucellar tissues, but in every instance the pollen appeared not to function normally in the nucellar tissue of a foreign species such that no fertilization occurred. The Austrian pine x red pine cross was more closely studied than the others mentioned. Germination and penetration of the red pine pollen into the Austrian pine nucellus occurred normally, but after two to four months, the ovule began to break down, and further deterioration occurred the following spring. The first sign of breakdown occurred in the magaspore followed by the disintegration of the surrounding cells and finally isolation and collapse of the nucellar tissue. Finally, McWilliam observed that the entire ovule appeared necrotic and shrunken. No successful fertilization occurred in the Austrian x red pine cross, although some seed was full-sized, indicating that ovular development continued until the time of fertilization when the seed coat had reached its mature size. This

was the same as that in the unpollinated Austrian pine ovule except for the timing. In the unpollinated strobili, all the ovules were completely deteriorated after two months and the strobilus abscised in the fall of the first season. McWilliam believes that chemical differences must have existed between the red pine pollen and the Austrian pine ovule. The differences apparently not only limited the pollen tube growth, but also prevented the pollen from conveying the necessary stimulus for normal development of the female gametophyte.

Kriebel (1967, 1970) has attempted to establish some crossability pattems within the different taxonomic groups of the pines. McWilliam (1959) established that the incompatibility among "hard" pines seemed to be due to the abnormal functioning of the pollen in a foreign nucellar tissue. This he found to be true in the crosses slash pine x Austrian pine, red pine x pitch pine, Austrian pine x pitch pine, red pine x Austrian pine and Austrian pine x red pine. Kriebel found that among the "white" pines, Pinus strobus L. (eastern white pine) hybridized successfully with other members of its subsection Strobi but not with members of the white pine subsection Cembrae. This, one could interpret as supporting evidence for the present classification and close relationship of members of the subsection Strobi. One exception however, was found in that some fertilization occurred in the cross eastern white pine x Swiss stone pine. In Kriebel's hybridizations, fertilization followed by proembryo breakdown was the general pattern in the cross P. strobus L. x P. flexilis

James (limber pine). Eastern white pine x western white pine was a successful hybridization yielding viable seed (Kriebel, 1967). The general incompatibility pattern in the white pines seems to be for normal fertilization to occur but for embryo inviability to be the cause of inviable seed, however in hard pines incompatibility is manifest prior to fertilization (Kriebel, 1970).

Hagman and Mikkola (1963) also conducted hybridization studies within the white pine group between members of the two subsections Strobi and Cembrae. When Macedonian pine (subsection Strobi), as the female parent, was crossed with Swiss stone pine and Korean pine (both of the subsection Cembrae), development proceeded normally up to the four nucleate proembryo stage but no cell walls formed around the nuclei. The corrosion cavity formed, although it remained empty, and the female gametophyte developed almost to its normal, full size. Apparently, in these crosses, fertilization and the formation of the proembryo are sufficient to stimulate the development of the female gametophyte. No completely empty seed resulted from these two interspecific crosses (Hagman and Mikkola, 1963).

Mikkola (1969) observed degrees of interspecific sterility (inability to produce offspring) in spruce species, ranging from partial embryo mortality to inhibition of pollen grain germination. According to Mikkola, spruce ovules probably pass two thresholds in development to maturity. The presence of some kind of pollen is apparently necessary for the ovule

to pass the primary threshold and develop up to the fertilization stage. The second threshold is passed when an embryo is present in the corrosion cavity, which allows final development of the seed. Mikkola observed some ovules to pass the primary threshold, which is much lower than the second threshold, without the presence of pollen. In Mikkola's work, large empty seed from interspecific crosses degenerated internally, shortly after fertilization, or just before the second threshold. Mikkola believed this was probably due to one of four reasons:

1. The ovule lacked pollen grains.
2. The ovule contained only ungerminated pollen grains.
3. The pollen grains germinated but did not effect fertilization.
4. Fertilization occurred, but the embryo died at an early stage.

Mikkola (1969) was able to categorize all incompatibility that occurred in his interspecific spruce crosses into four types:

1. Pollen did not germinate normally and any tubes formed were short and of irregular shape /*Picea abies* Karst. (Norway spruce) x *P. jezoensis* Carr. (Yeddo spruce)/.
2. Pollen germinated but no tubes penetrated the nucellus (*Picea abies* Karst. x *Pinus sylvestris* L.).
3. Pollen germinated and some tubes penetrated the nucellus. The most advanced tubes grew only half way through the nucellus /*Picea abies* Karst. x *P. mariana* Mill. B.S.P. (black spruce) and the reciprocal cross, *P. abies* Karst. x *P. omorika* Purkyne (Serbian spruce) and the reciprocal cross, *P. jezoensis* Carr. x *P. abies* Karst., *P. mariana* Mill. B.S.P. x *P. glauca* (Moench) Voss (white spruce), *P. mariana* Mill B.S.P. x *P. asperata* Mast. and *P. mariana* Mill. B.S.P. x *P. jezoensis* Carr./.

4. Many of the pollen tubes penetrated the nucellus but most usually stopped growing half-way through the nucellus /*Picea abies* Karst. x *P. glauca* (Moench) Voss and the reciprocal cross, *P. abies* Karst. x *P. asperata* Mast. and the reciprocal cross, *P. abies* Karst. x *P. koyamai* Shiras., *P. abies* Karst. x *P. obovata* Ledeb. (Siberian spruce), *P. mariana* Mill. B.S.P. x *P. omorika* Purkyne, *P. sitchensis* (Bong.) Carr. x *P. abies* Karst., *P. sitchensis* (Bong.) Carr. x *P. jezoensis* Carr., and *P. jezoensis* Carr. x *P. omorika* Purkyne/.

Mikkola believes there is some significant interaction of pollen tube tips and the tissue about half way through the nucellus because in both his intraspecific control crosses and his interspecific crosses, pollen tubes stopped growing when the tube tip reached this half-way point into the nucellus.

Not all of Mikkola's (1969) reciprocal crosses were similar, i.e., they did not always involve the same trees. The pollen of Norway spruce was more effective in penetration of the nucellus of white spruce, black spruce and Serbian spruce, than the reciprocal pollen on Norway spruce nucellus. Furthermore, when white spruce pollen of different provenances was applied to Norway spruce ovules, the depth of penetration of the pollen tube varied, depending upon the provenance of the pollen.

Mikkola (1969) observed fertilization followed by embryo degeneration in the following spruce crosses: Norway spruce x white spruce and the reciprocal cross, Norway spruce x *P. asperata*, Norway spruce x *P. koyamai*, Norway spruce x Siberian spruce, black spruce x Serbian spruce, and Yeddo spruce x

Serbian spruce. Some structurally-normal and filled seed resulted from the following crosses: Norway spruce x P. asperata, Norway spruce x P. koyamai, Norway spruce x Siberian spruce, and black spruce x Serbian spruce.

Klaehn and Wheeler (1961) X-rayed mature seeds of Norway spruce following open, cross, intraspecific, and interspecific pollinations and classified them according to the presence or absence of embryos and the condition of the female gametophyte. Neither selfed nor inter-crossed seed were found to be completely empty, but 95 percent of the seed from unpollinated cones X-rayed as empty. Mikkola (1969) also X-rayed mature seed from some of his interspecific spruce crosses and found that he could classify seeds according to whether the female gametophyte degenerated at the free nuclear stage or shortly after fertilization. Klaehn and Wheeler (1961) believed the X-ray technique to be good for determining if fertilization occurred and the stage of development at which the embryo aborted.

Because pollen seems to provide a necessary stimulus for the development of the female gametophyte, it is logical to suspect growth substances or extracts from germinated pollen might provide the same stimulus artificially. Hagman and Mikkola (1963) sprayed receptive Macedonian pine cones with idole-3 acetic acid, gibberellic acid, and a water solution in which pollen of the same species had germinated. The cones treated with IAA and the pollen solution abscised, but those treated with gibberellic acid continued elongation even though

all the ovules aborted. Four of the twenty-six gibberellin-sprayed strobili did remain on the twig and grew somewhat during the second season. McWilliam (1959) treated receptive strobili of Pinus with auxins, ether-and-alcohol extracts of fresh pollen, and heat-killed pollen, in an effort to induce parthenocarpy. The only response observed was among the ovules treated with the heat-killed pollen in which a one-month delay in the breakdown of the ovules occurred.

An attempt to stimulate inhibited or slow pollen tube growth in interspecific crosses, irradiation of both pollen and ovules has been made. Kriebel (1967) found that irradiation of limber pine pollen had no effect on pollen tube growth or on initial ovule development. Vidakovic and Jurkovic-Bevilacqua (1970) irradiated Scotch pine pollen with gamma-rays of different doses and then artificially pollinated Austrian pine ovules with it. Ordinarily the Scotch pine pollen grew too fast or too slow in relationship to the development of the Austrian pine female gametophyte. At low X-ray dosages of the Scotch pine pollen, this growth rate was corrected and hybrids were obtained from the cross Austrian pine x Scotch pine.

CHAPTER II

PROCEDURES

In the spring of 1969, a single blue spruce (Picea pungens Engelm.)(Figure 1) and a single Engelmann spruce (Picea engelmannii Parry)(Figure 2) were selected on the campus of Colorado State University, Fort Collins, Colorado, for study of ovule development following interspecific pollination. In the spring of 1970, two additional blue spruce trees in Fort Collins were selected; one tree for artificial pollination with Engelmann spruce pollen and the other to study development of unpollinated ovules.[2] (Figure 3)

Strobili Isolation

On April 4, 1969, 105 branchlets with one or more elongating female strobili were isolated on the blue spruce tree with Central States "Slip Ezy" pollination bags. Twenty branchlets, each with more than one elongating female strobilus per branchlet, were isolated with the same kind of pollination bag on the Engelmann spruce tree, on April 30, 1969. The bud scales covering the female strobili of both trees were tightly closed at the time of bagging. Cotton was wrapped around each branchlet near the mouth of the isolation bag which was

---

[2]A description of the blue spruce and Engelmann spruce tree used in the hybridization part of this study (1969) and a description of the blue spruce tree used in the 1970 unpollinated ovule study are found in the Appendix.

Figure 1 - Blue spruce female parent used in the control-pollination study.

Figure 2 - Engelmann spruce mother tree and pollen source used in the control-pollination study.

Figure 3 - Blue spruce mother tree used in the study of unpollinated ovules.

closed with a "twist 'em". All male strobili near the bag were removed before the isolation bags were attached. No pollen of other tree species was observed to be shedding prior to the time of isolation, so it is believed that no contaminating pollen was enclosed within the bags. The pollination bags were removed from both the blue spruce and Engelmann spruce mother trees on June 30, 1969, and the corresponding branchlets were identified with numbered tags attached just below the pollinated stobili. All artificially-pollinated cones, which had not been collected by September, were enclosed in net bags at that time for later counts of seed per cone and germination tests.

Pollination

On May 7, 1969 and again on May 8, 1969, a mix of blue spruce pollen from two different trees (Pi pun 11 and Pi pun 40) was applied to receptive Engelmann spruce strobili. This pollen had been collected in the spring of 1968 and stored in sealed glass jars in a refrigerator. No petri dish germination tests were conducted on this pollen mix. On May 12, and 15, 1969, fresh pollen from the Engelmann spruce parent (Pi eng 9), was applied to receptive blue spruce strobili. This fresh pollen was extracted from the stobili two days after the male strobili began shedding naturally on May 3, 1969. The first date of artificial pollination for each tree was estimated to be approximately mid way through the receptive period of the female strobili, and the second application of

pollen was made to insure that an adequate amount of pollen was available for each ovule.

Disposable syringes, equipped with a rubber bulb and a twenty-gauge needle were used to introduce the pollen into the bag. Following pollination a piece of masking tape was used to seal the hole made in the bag by the needle. Precautions were taken to insure that only the desired pollen was introduced into each pollination bag. Motion of the pollination bags in the wind should have facilitated individual ovule pollination after initial pollen dispersion from the needle.

Collections

Beginning on May 12, 1969, open-pollinated and artificially-pollinated blue spruce strobili were collected every three days for the first seven weeks, after which weekly collections were made until the cones opened and shed seed September 15, 1969. Usually a single strobilus or developing cone of each type was removed for each collection. A total of 30 wind-pollinated and 30 artificially-pollinated blue spruce cones collected during 1969 were used for the study. Additional open-pollinated and artificially-pollinated cones were collected and frozen or dried, but none of these cones was processed further. Three nearby blue spruce trees supplied abundant pollen for the open-pollinated cones.

A total of seventeen collections were made of open-pollinated and artificially-pollinated Engelmann spruce cones. The seventeen open-pollinated cones were not processed

further after killing, because there were no other Engelmann spruce trees in the vicinity and pollination of these cones was uncertain. Only the first three of the artificially-pollinated Engelmann spruce cones were processed further than the killing fluid stage.

In addition to external morphological observations of the tree and of development of the unbagged cones, the length, width, color, and orientation of the collected cones were recorded. The Munsell Color Chart was used as the color reference. Length and width measurements were made to the nearest millimeter. A sample data sheet is presented in the Appendix.

1970 Isolations

In the spring of 1970 very few blue spruce strobili were produced in northern Colorado. Only two blue spruce trees were found bearing strobili. Approximately 35 strobili on one of these were isolated, pollinated with 1969 Engelmann spruce pollen, collected and fixed in killing solutions. Abnormal cone development and poor results obtained in a pollen germination test resulted in discarding all these samples. Twelve of the 16 total strobili on the other blue spruce tree were isolated and left unpollinated. One week after the estimated mid period of female strobilus receptivity, alternate day collections were begun of the cones until all twelve cones had been collected and killed.

Preparation of microscope slides

Upon collection, each cone was cut longitudinally, further subdivided into smaller pieces and then placed immediately into Craf III killing fluid, and aspirated (see Appendix). As the seed coats became progressively hardened, beginning about the end of June, ovules from the cones of the 1969 collections were dissected out of the seed coats or the seed coats were slit to allow penetration of the killing fluid. The processing schedules are found in the Appendix. Ovules, at first attached to the scale, and later individually, were dehydrated in alcohol, embedded in paraffin, and sectioned on a rotary microtome. Longitudinal sections through the micropyle were made of each ovule. Most of the ovules were cut parallel to the axis of the cone, but some were cut perpendicular to the cone axis. Most ovules were sectioned at ten microns, but a few were cut at different thicknesses between ten and 22 microns. Ovules with hardened seed coats were soaked in Molliflex, a softening agent, (see Appendix), for three to eight days, or they were soaked in a 1:1 mixture of alcohol-glycerine prior to cutting.

Initially, a safranin-fast green stain series was used, but material processed later was stained with Conant's quadruple stain. An iron-hematoxylin stain series was also used successfully. Conant's quadruple stain gave superior results in the writer's opinion (see Appendix).

Photography

A Nikon, photomic-F, single lens reflex camera with a Micro-Nikkor lens and a Honeywell Pentax camera with close-up lenses were used to photograph both trees and cones. Using Panatomic-X black and white film, approximately 1000 photomicrographs of sections of developing ovules were made. Color and black-and-white photographs were made of the collected cones from both blue spruce trees used in the 1970 collections.

An eyepiece micrometer was used to measure a portion of each section photographed. The corresponding portion was then measured on the print to determine the relative magnification.

Mature 1969 Cones

Thirty-eight blue spruce x Engelmann spruce cones were not collected and remained enclosed in net bags after the study period. These cones were collected on September 15, 1969. All of the seeds from these cones were extracted, counted, and germination tests were subsequently run. The cones and extracted seeds were kept at room temperature until the germination test was conducted in January, 1970. One hundred seeds from each cone, or 200 seeds if two cones were included in a net bag, were placed on blotter paper enclosed in plastic boxes in a germination room of the National Seed Storage Laboratory on the Colorado State University campus. The humidity was 100 percent in the germination room with

the daytime temperature maintained at 30°C., and night temperature at 20°C. Five more of the open-pollinated blue spruce cones than the excess control-pollinated cones were also bagged and collected. The number of seeds per cone was determined and tested for germination in the January test.

CHAPTER III

RESULTS

Development of Unpollinated Blue Spruce Ovules

A total of 108 unpollinated blue spruce ovules from 11 collections (12 unpollinated cones collected in 1970) were studied microscopically as shown in Table 1.

Unpollinated ovules from the first collection (May 26, seven days after pollination)[3] appeared to contain normal cellular female gametophytes. In these ovules archegonia would probably have been initiated in approximately one week. One ovule contained an empty, triangular-shaped female gametophyte cavity, the periphery of which stained darkly (Figure 4).[4] Some of the nucellar cells adjacent to the chalazal end of the empty female gametophyte cavity were slightly enlarged, suggestive of later observed stages. These cells, in other ovules, proliferated (abnormal enlargement and/or multiplication of cells) in advanced stages of ovule degeneration. Also observed in this ovule was a zone of separation where the nucellar tissue seemed to have been pulled apart.

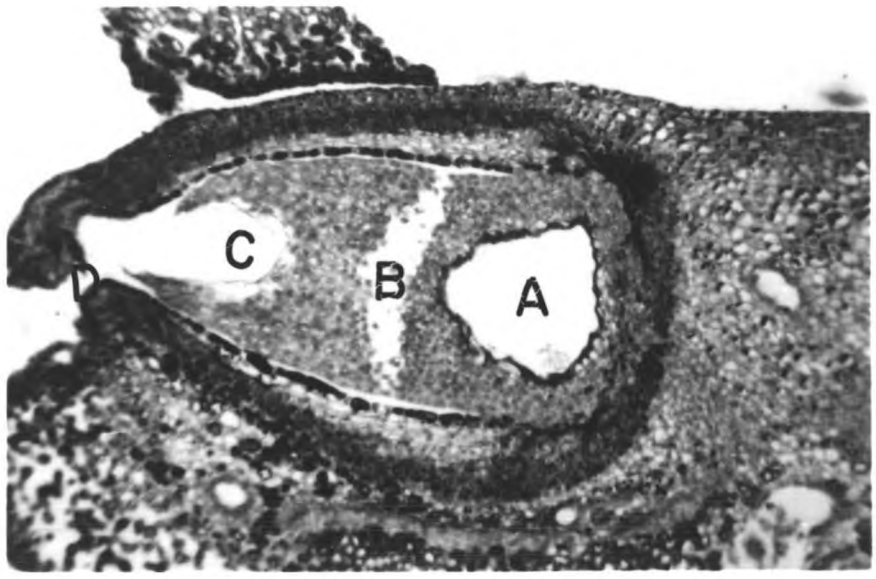
---

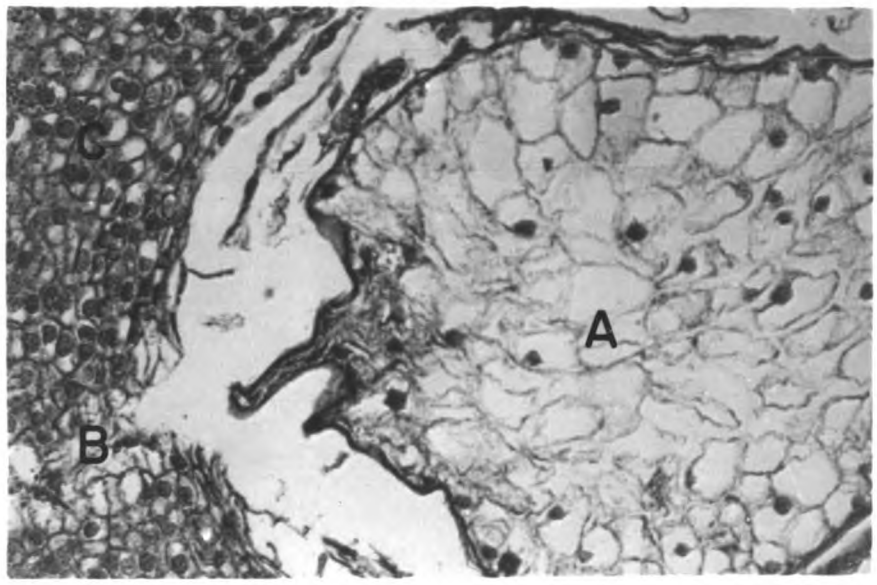
[3]"Pollination day" was assumed to be the day mid-way through the period of receptivity of the female strobili.

[4]All photomicrographs are oriented with the direction of the micropyle to the left of the picture.

Figure 4 - Ovule from unpollinated blue spruce strobilus 7 days after pollination date (x70) showing:
A - female gametophyte cavity, necrotic at the periphery
B - tear through nucellar zone of division
C - nucellar cup
D - micropyle

Figure 5 - Female gametophyte from unpollinated blue spruce strobilus 9 days after pollination date (x225) showing:
A - cellular female gametophyte
B - breakdown in nucellus
C - nucellus


C
B
A


C
A
B

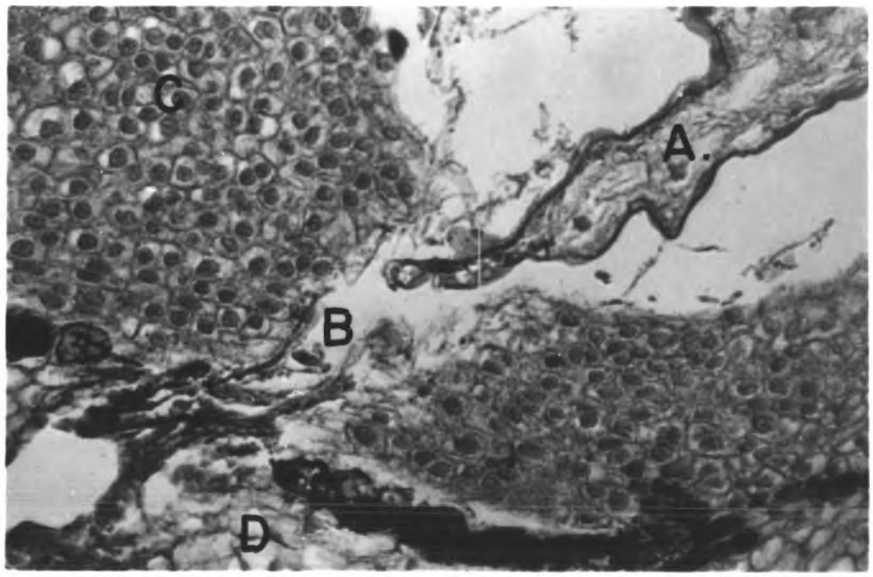
The separation occurred in the nucellar zone of division where the nucellar cells divide and increase the volume of this tissue.

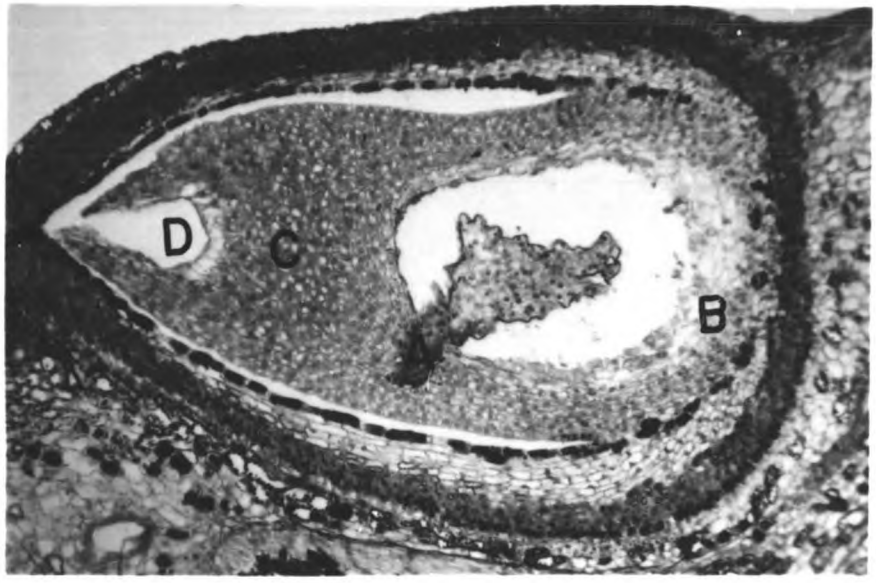
Ovules from the second collection, (May 28, nine days after pollination) contained large oval-shaped, cellular female gametophytes, which nearly filled the cavity. The cells were not always contiguous even though cell walls had formed around the individual nuclei. In all of the ovules from this collection one or two areas of tissue breakdown in the nucellus were observed adjacent to one or both ends (chalazal or micropylar) of the female gametophyte cavity (Figure 5). The initial breakdown of the female gametophyte was characterized first by large cell size and sparseness of cytoplasm. This was followed later by a deterioration of the cell walls and condensation of all the cells into a darkly staining amorphous mass. Often breakdown of the nucellus involved the integument as well (Figure 6). Usually the cells of the female gametophyte were somewhat condensed (probably due to the breakdown of cell walls) on the side toward the nucellar breakdown areas. The conglomerations formed by the female gametophyte produced small projections oriented toward the nucellar areas of breakdown.

The third collection (May 30, 11 days after pollination) contained two types of ovules. The first type exhibited more degeneration of the nucellar tissue and clumping of the female gametophyte tissue than was observed earlier. However, the cellular female gametophyte was no longer oval. Rather,

Figure 6 - Female gametophyte and nucellus from unpollinated blue spruce strobilus 9 days after pollination date (x210) showing:
A - clumped female gametophyte
B - breakdown in nucellus extending through integument
C - nucellus
D - integument

Figure 7 - Ovule from unpollinated blue spruce strobilus 11 days after pollination date (x70) showing:
A - clumped and protruded female gametophyte
B - slightly enlarged nucellar cells
C - nucellus
D - nucellar cup


C
A.
B
D

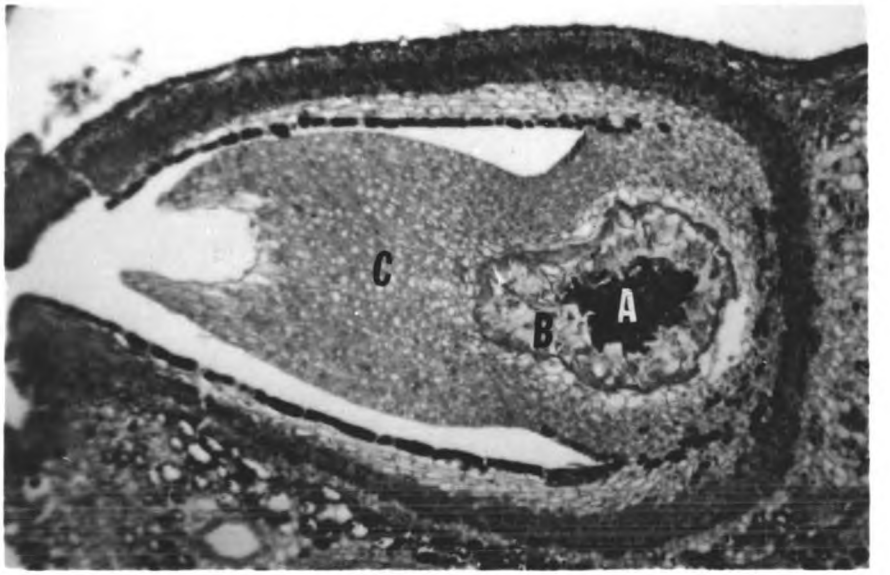

D
C
B

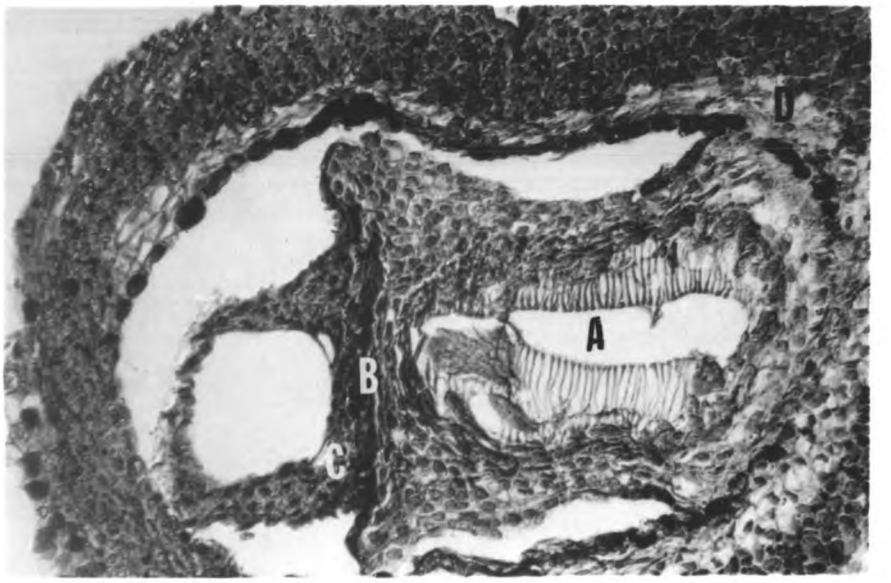
it had condensed to form a dense, tube-like mass projecting into the nucellus from the female gametophyte cavity, along the line of breakdown observed earlier in the nucellus. Protrusions were observed in the micropylar end of the nucellus either parallel to the longitudinal axis of the ovule or off to one side (Figure 7). Some ovules contained two protrusions, one extending toward each side of the ovule. In the second type of ovule observed, the female gametophyte cavity was of an irregular, oval shape, with the center completely filled with a darkly-staining, necrotic mass. This necrotic mass was surrounded by what appeared to be cytoplasmic constituents of an incompletely-formed, cellular female gametophyte (Figure 8).

The fourth unpollinated blue spruce collection (June 2, 13 days after pollination) contained ovules undergoing a different kind of degeneration from that observed in the first three collections. All the ovules were noticeably smaller than those of the preceeding or succeeding collections, and the nucellar cap (the nucellar tissue from the micropylar end of the female gametophyte cavity to the nucellar cup where the pollen grains germinate) had apparently shrunk and died. The dead nucellar cells were characterized by small size, apparent lack of cell walls and dark staining. Shrinkage had occurred in the nucellar zone of division, where a separation in the nucellar tissue had been observed in earlier ovules. The remaining portion of the nucellus up to and surrounding the female gametophyte appeared to be slightly shrunken. The

Figure 8 - Ovule from unpollinated blue spruce strobilus 11 days after pollination date (x75) showing:
A - necrotic female gametophyte
B - female gametophyte cavity filled with broken cellular material
C - nucellus

Figure 9 - Ovule from unpollinated blue spruce strobilus 13 days after pollination date (x175) showing:
A - female gametophyte cavity with cytoplasmic strands
B - nucellar zone of division
C - nucellar cap, dead and shrunken
D - integument


C
B
A


D
A
B
C

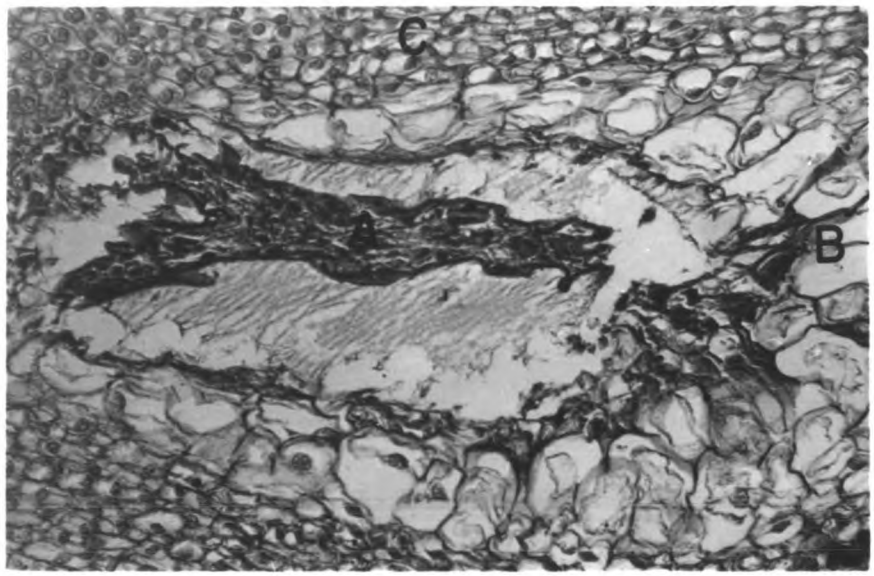
nucellar tissue surrounding the female gametophyte cavity normally degenerates in advance of the enlarging female gametophyte cavity and some of this normal degeneration was evident. A necrotic female gametophyte was usually observed to be centrally located with cytoplasmic strands extending in a regular pattern to the periphery of the female gametophyte cavity (Figure 9).

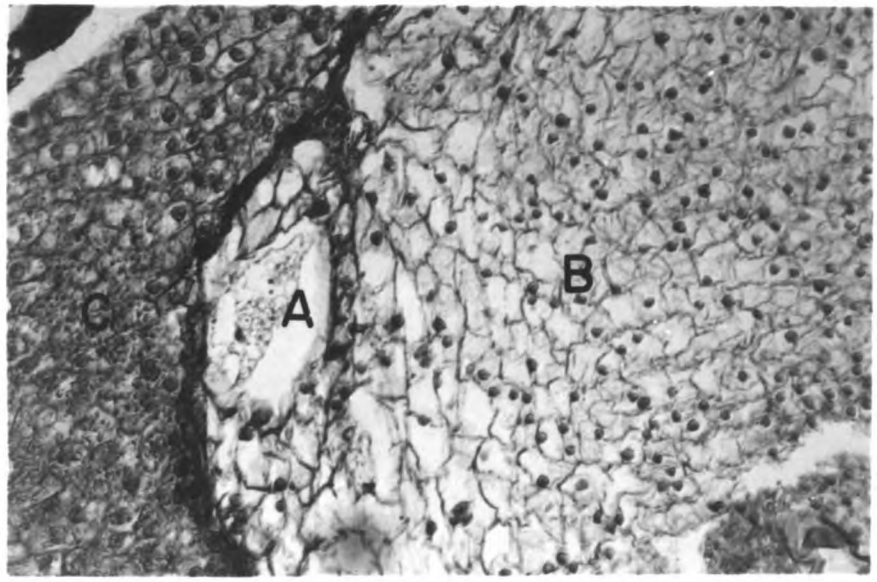
The fifth collection of unpollinated blue spruce cones (June 3, 14 days after pollination) contained ovules in which the degeneration pattern set in the first three collections was continued. In most instances the female gametophyte had protruded into the nucellus along one or two lines of nucellar tissue breakdown, similar to the situation observed in earlier ovules. The breakdown of the nucellus in these areas of protrusion by the female gametophyte was very localized, and adjacent nucellar tissue was unaffected. All female gametophytes in all ovules observed were necrotic, having become aggregated into dense masses. This aggregation was not due to processing. The nucellar cap remained normal, but around the female gametophyte, and for several cell layers outward into the nucellus at the chalazal end, the nucellar cells were noticeably enlarged and proliferated.

Unpollinated blue spruce ovules from the sixth collection (June 5, 16 days after pollination) were very similar to those from the fifth collection, but they showed more marked proliferation of the nucellar tissue surrounding the female gametophyte cavity at the chalazal end and more clumping of the female gametophyte tissue itself (Figure 10).

Figure 10 - Ovule from unpollinated blue spruce strobilus 16 days after pollination date (x180) showing:
A - necrotic female gametophyte surrounded by cytoplasmic strands
B - proliferated nucellar cells at the chalazal end of the ovule
C - nucellus

Figure 11 - Female gametophyte and archegonium from unpollinated blue spruce strobilus 18 days after pollination date (x190) showing:
A - mis-shapen archegonium
B - female gametophyte
C - nucellus


C
B

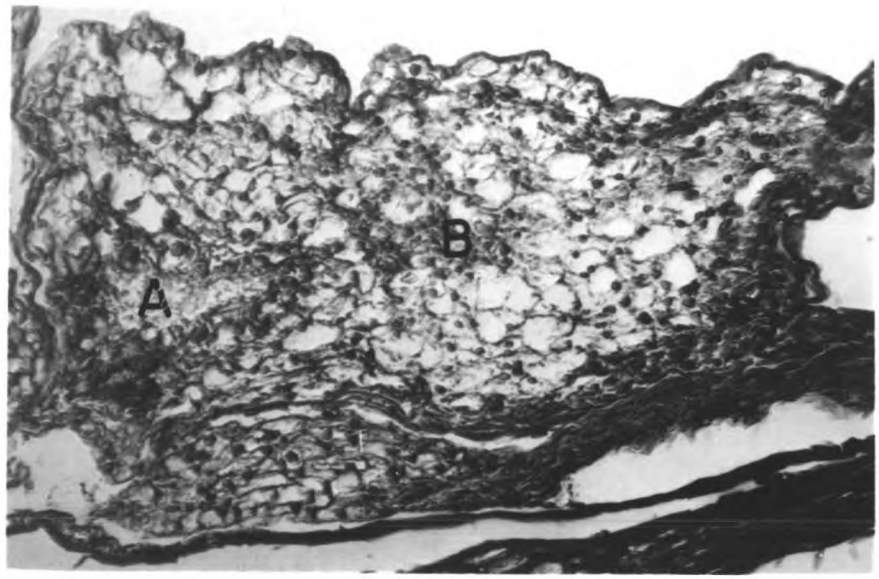

C
A
B

Ovules of the seventh collection (June 7, 18 days after pollination) had elongated considerably, but deterioration of the female gametophyte had been delayed, and some even contained poorly-formed and often ill-defined archegonia (Figure 11 and 12). Even though the nucellus appeared normal, the female gametophyte was not always properly developed. In one ovule, the area in the female gametophyte where the archegonia should have formed, had proliferated. There was also some necrosis at the point of contact between the female gametophyte and the nucellus (Figure 13).

A collection 7-B (B = broken) was also made (June 7) of a blue spruce cone detached from the twig and believed to have been broken during manipulations of equipment during the previous collection, two days earlier. Most of the unpollinated ovules in this cone were elongated and curved inward along the abaxial surface of the ovule, which gave them a flattened or dented appearance macroscopically (Figure 14). Again, the female gametophyte was necrotic and protruded into the nucellus. The nucellus was normal except in the area surrounding the female gametophyte cavity, where it was usually proliferated. Excessive cytoplasmic constituents or torn cellular material occupied much of the female gametophyte cavity in some ovules. One of these contained archegonial initials centrally located in the female gametophyte (Figure 15). This female gametophyte was necrotic at the point of contact with the nucellus at the micropylar end and contained enlarged and proliferated cells just beyond its contact with the nucellus.

Figure 12 - Female gametophyte and archegonium from unpollinated blue spruce strobilus 18 days after pollination date (x190) showing:
A - poorly developed archegonium
B - female gametophyte tissue

Figure 13 - Ovule from unpollinated blue spruce strobilus 18 days after pollination date (x65) showing:
A - proliferated female gametophyte tissue
B - necrotic female gametophyte tissue
C - normal female gametophyte tissue
D - nucellus


A
B

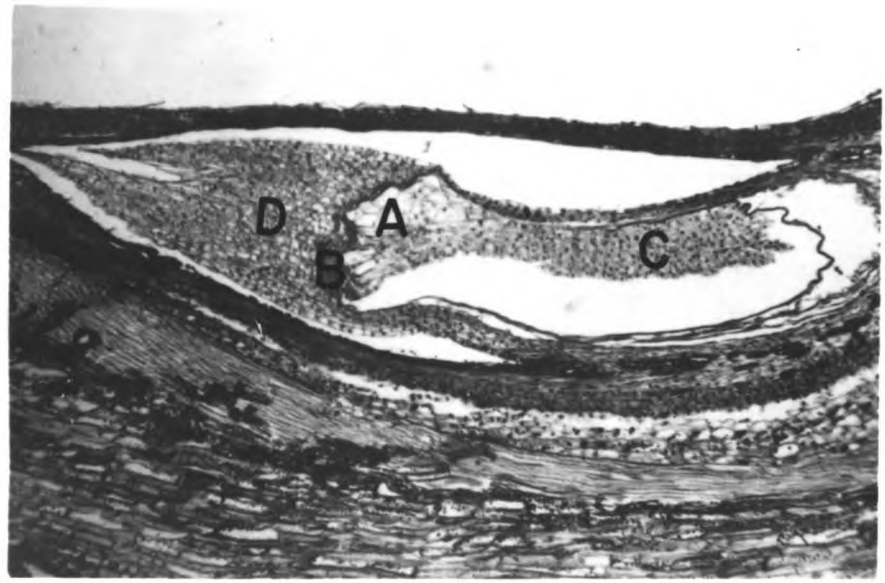
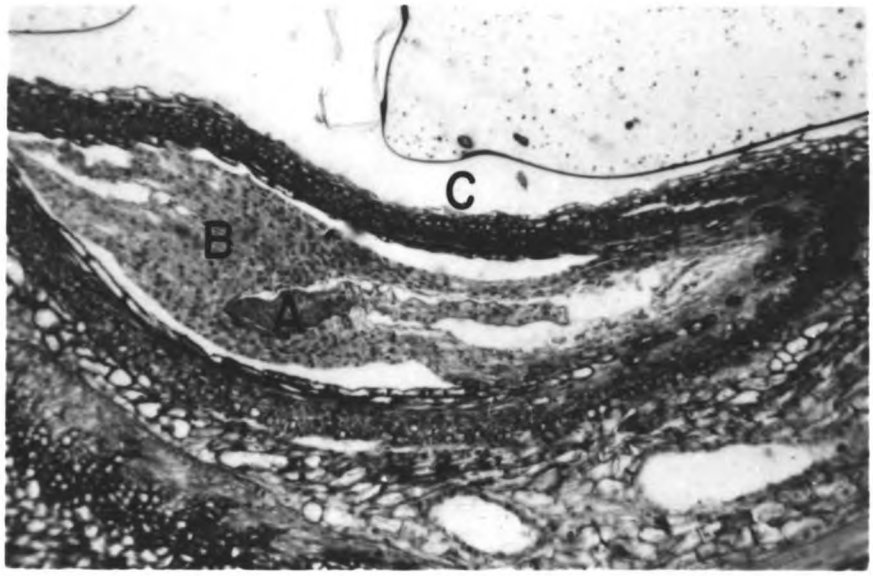
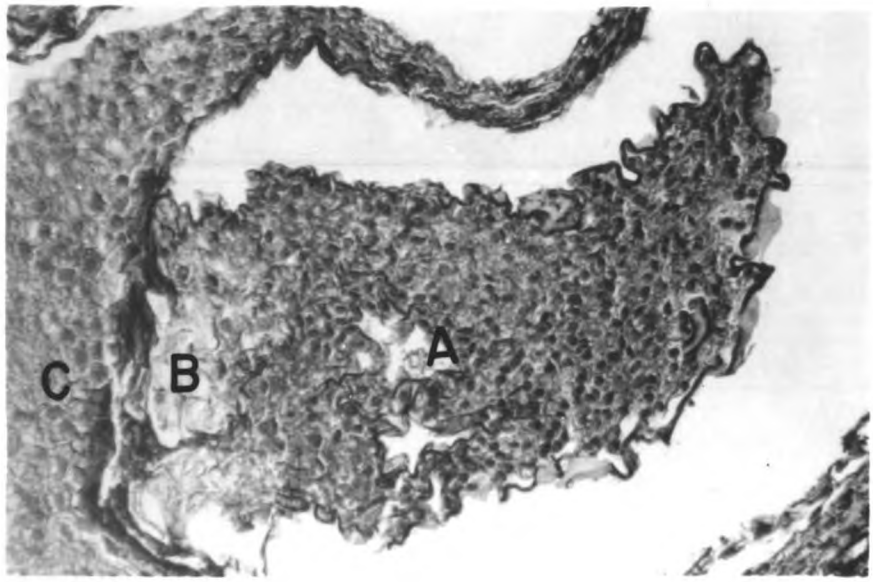

D
A
B
C

Figure 14 - Ovule from unpollinated blue spruce strobilus 18 days after pollination date (x70) showing:
A - necrotic and protruded female gametophyte
B - nucellus
C - indented surface of ovule

Figure 15 - Female gametophyte from unpollinated blue spruce strobilus 18 days after pollination date (x190) showing:
A - enlarged archegonial initials
B - proliferated and necrotic female gametophyte
C - nucellus


C
B
A

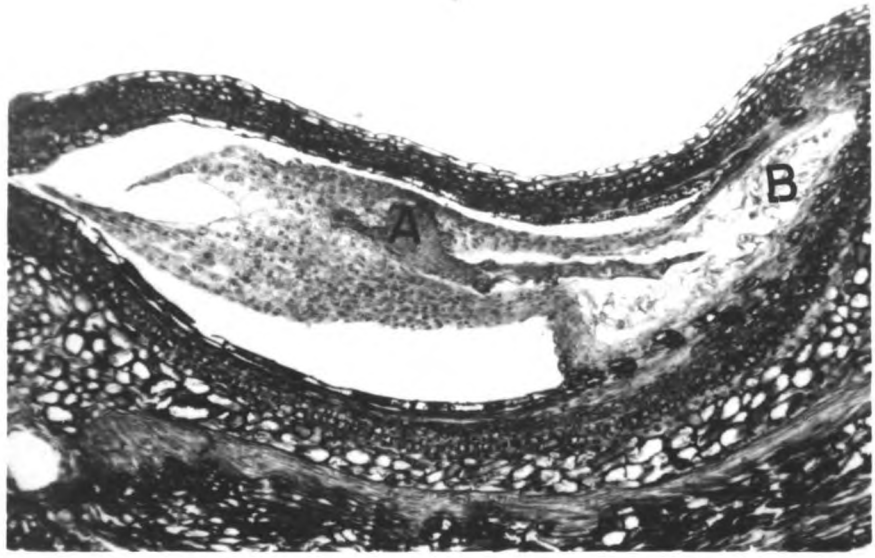

C
B
A

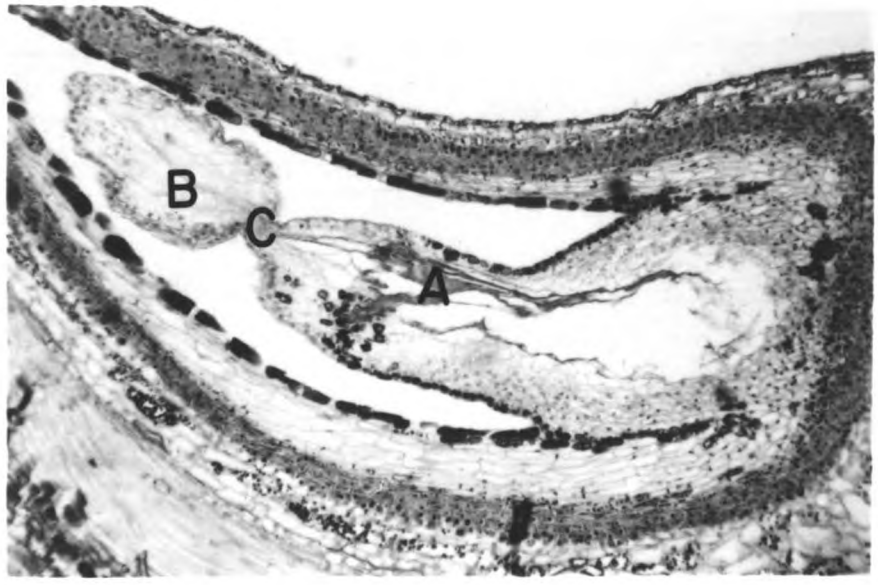
The degenerated state of the ovules from the unpollinated blue spruce cones in the last four collections were all similar. Most ovules had elongated, and the female gametophyte cavity, too, had enlarged and elongated. The female gametophyte usually consisted of one necrotic mass protruding into the upper part of the nucellus (Figure 15). The nucellar tissue had died, as evidenced by the lightly stained cells, and were apparently devoid of cytoplasm. In ovules where the female gametophyte and protruded, there often occurred a sharp constriction in the nucellus at the furthest point of protrusion. This again, was the zone of division of the nucellus, and the constriction occurred here even in ovules in which the necrotic female gametophyte remained within the organized female gametophyte cavity and did not protrude (Figure 17). All of the unpollinated ovules from these last collections were either dented or at least flattened on the abaxial surface.

One unpollinated ovule from the ninth collection, June 11, 22 days after pollination, contained a mass of disorganized female gametophyte nuclei surrounded by a normal nucellus. Another ovule, from the tenth collection, June 13, 24 days after pollination, also had a normal nucellus, but the female gametophyte contained only large proliferated cells clumped at the micropylar end of the tissue. In this ovule the female gametophyte cavity was dumbell-shaped, the enlarged ends being oriented parallel to the axis of the ovule.

Figure 16 - Indented ovule from unpollinated blue spruce strobilus 18 days after pollination date (x70) showing:
A - necrotic female gametophyte protruded into upper nucellus
B - excess, unknown, cellular material

Figure 17 - Ovule from unpollinated blue spruce strobilus 26 days after pollination date (x65) showing:
A - necrotic female gametophyte
B - dead nucellar cap
C - constriction in the nucellar zone of division


A
B


B
C
A

Blue Spruce X Engelmann Spruce Hybrid Embryogeny

A variable number of ovules from each cone were observed microscopically. No attempt was made to count the number of seeds per collection cone, or to classify the individual ovules as large or small at the time of collection. In ovules from cones collected beginning in about June, so many ovules were small and degenerated internally that an effort was then made to process only larger ovules which may have developed somewhat more, internally. Those collections containing ovules of special interest were more extensively investigated by processing more of the ovules from them. A total of 783 blue spruce ovules were microscopically examined from cones that had received Engelmann spruce pollen (Table 2).

Fechner, (1964) has described the reproductive cycle of blue spruce. Observations on the embryogeny of normal wind pollinated trees were made and compared with observations of the control-pollinated ovules from the same tree in the same year. All observations by the present author on the open-pollinated ovules corroborated the observations of Fechner (1964). The number of ovules examined microscopically from open-pollinated cones was not tallied. "Pollination day," (May 12, 1969) for both open- and control-pollinated ovules, was the day the Engelmann spruce pollen was first applied to the isolated blue spruce strobili. Comparison of the developmental stages of the open-pollinated ovules to that of the control-pollinated ovules was made for different dates following pollination day.

0 to 6 days after pollination

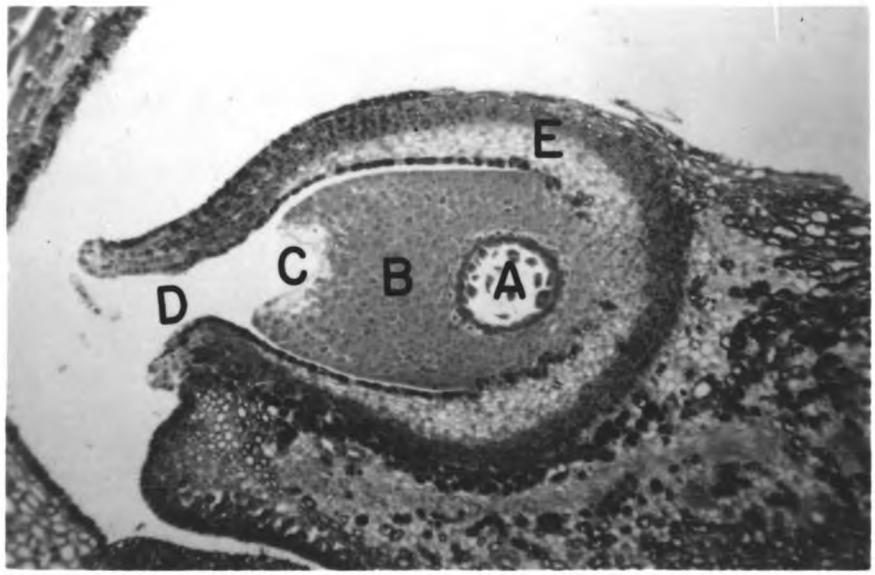
Ovules from open- and control-pollinated strobili collected during the first six days after pollination were in the free nuclear to early cellular female gametophyte stages (Figures 18 and 19). The ovules were still oval in shape and normal breakdown of the nucellar cells immediately surrounding the female gametophyte cavity was evident.

9 days after pollination

Although the control-pollinated strobilus was still erect, the scales had begun to close, whereas the scales of the open-pollinated strobili were still open. The wind-pollinated ovules were elongated and the first developmental stages of the cellular female gametophyte were evident. The first abnormalities in the control-pollinated ovules were observed nine days after the Engelmann spruce pollen was introduced. Two kinds of development occurred: 1) Either the female gametophyte aborted before becoming cellular (Figure 20), or 2) the cellular female gametophyte protruded into the nucellus (Figure 21), in a similar manner to that observed in unpollinated ovules. Those ovules classified as having aborted before the female gametophyte became cellular, did produce female gametophyte cell walls, which filled the entire female gametophyte cavity, but they enclosed only a few nuclei and very little cytoplasm. In one hybrid ovule (interspecifically pollinated ovule) cytoplasmic and cell wall fragments were scattered around the periphery of the female gametophyte cavity. In another ovule, the female

Figure 18 - Normal ovule from open-pollinated blue spruce strobilus on pollination day (x65) showing:
A - free nuclear female gametophyte
B - nucellus
C - nucellar cup
D - micropyle

Figure 19 - Normal ovule from open-pollinated blue spruce strobilus 6 days after pollination date (x180) showing:
A - cell walls formed around some female gametophyte nuclei
B - normal degeneration of nucellar cells surrounding female gametophyte due to growth of the latter
C - nucellus
D - deteriorated cells of the nucellar cup


E
C
B
A
D

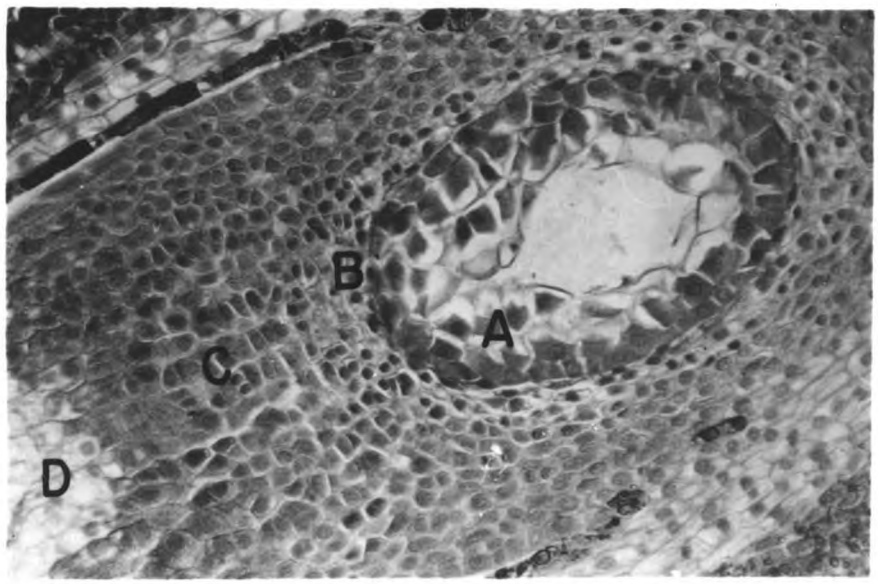
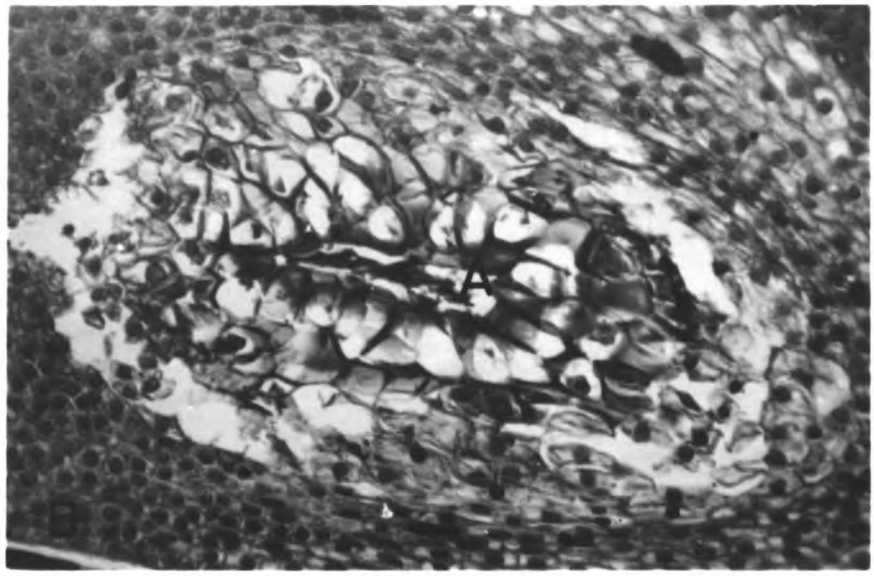
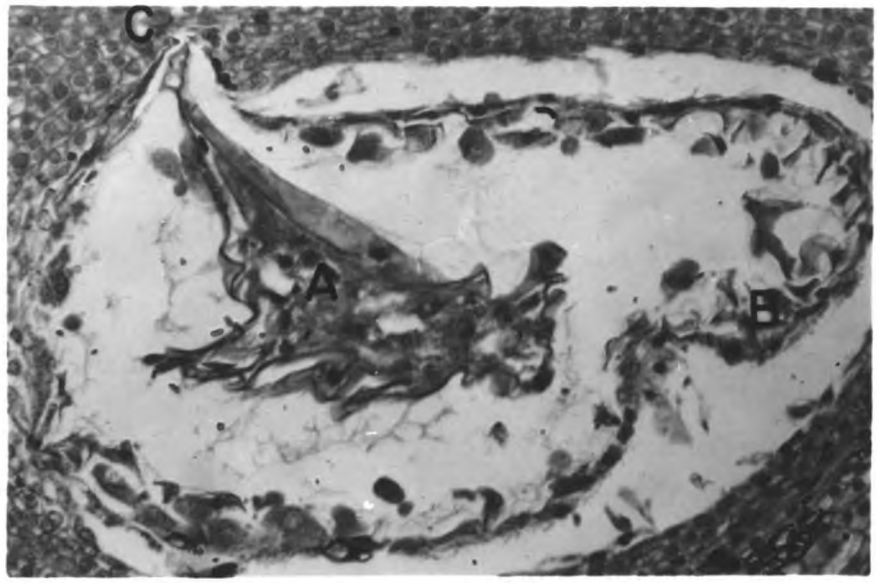

B
A
C
D

Figure 20 - Female gametophyte of blue spruce ovule 12 days after strobilus received Engelmann spruce pollen (x185) showing:
A - cellular female gametophyte enclosing few nuclei and little cytoplasm
B - nucellus

Figure 21 - Female gametophyte of blue spruce ovule 9 days after strobilus received Engelmann spruce pollen (x190) showing:
A - clumped and protruded female gametophyte
B - female gametophyte; free nuclear
C - nucellus


A
B


C
A
B

gametophyte was clumped into a T-shaped structure. Here, dense masses of cytoplasm filled the remaining, empty space within the female gametophyte cavity. No pollen was observed in these ovules and no normal ovule elongation occurred.

12 days after pollination

Both the open- and control-pollinated strobili were erect, but the scales of the control-pollinated strobilus had started to close, and the strobilus was beginning to curve, forming an open U shape. The open-pollinated ovules were partially elongated and the female gametophytes had become cellular. The ovules from the control-pollinated strobilus contained female gametophytes of three different types: 1) those that had aborted before becoming cellular, 2) those that had protruded into the nucellus or 3) those with normal cellular or free nuclear female gametophytes (Figure 22). Most of the hybrid ovules examined microscopically had begun to elongate.

15 days after pollination

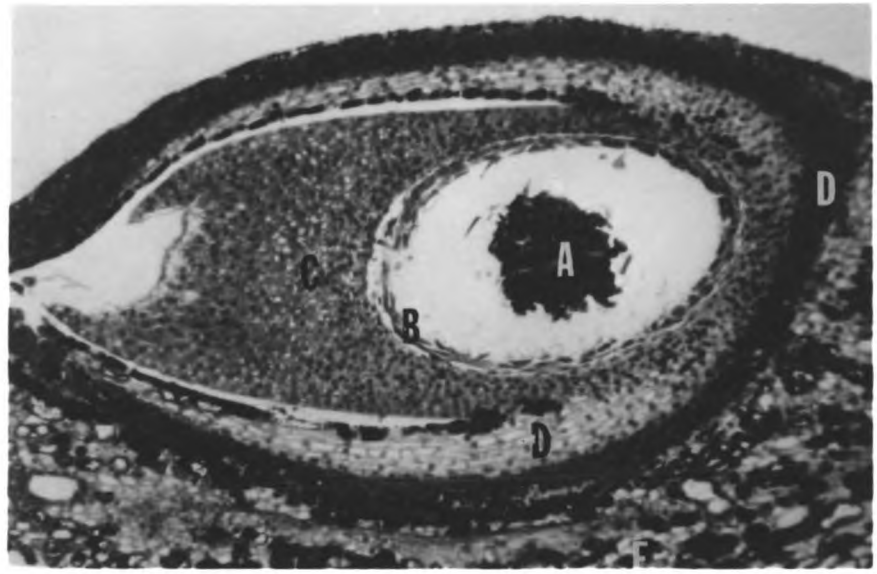
Both conelets collected had begun to turn downward and were approaching a horizontal position on the branches. The scales of the control-pollinated conelet were more tightly closed than those of the open-pollinated one. Ovules from the open-pollinated conelet contained well-developed female gametophytes just prior to archegonial initiation (Figure 23). Cellular female gametophytes were found in interspecifically crossed ovules with vigorously growing pollen tubes and in

Figure 22 - Normal ovule from open-pollinated blue spruce strobilus 12 days after pollination date (x55) showing:

A - normal cellular female gametophyte
B - female gametophyte; free nucleate
C - nucellus
D - integument

Figure 23 - Normal ovule from open-pollinated blue spruce strobilus 15 days after pollination (x65) just prior to archegonial initiation, showing:

A - cellular female gametophyte
B - ungerminated pollen grains in micropylar area
C - germinated pollen grains having penetrated the nucellus


D
A
C
B
D

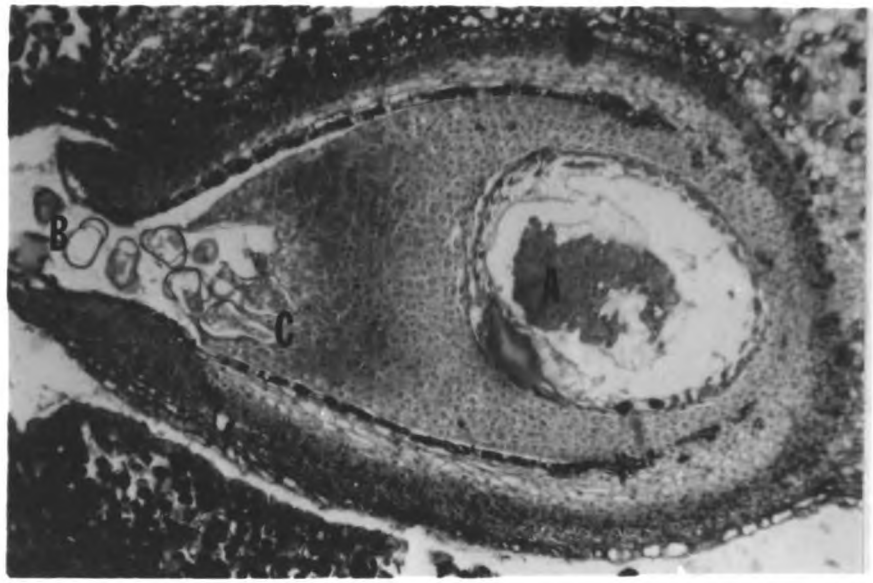
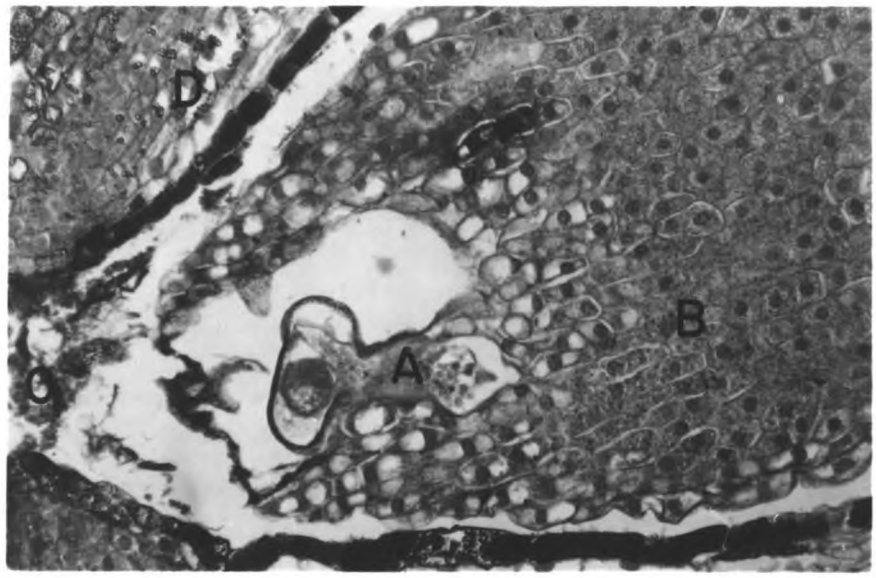

B
A
C

Figure 24 - Engelmann spruce pollen grain having germinated and penetrated the blue spruce nucellar tissue 15 days after pollination date (x180). Swollen end may be indicative of a stage prior to pollen tip burst.

A - pollen tube
B - nucellus
C - micropyle
D - integument

Figure 25 - Ovule from blue spruce strobilus 15 days after receiving Engelmann spruce pollen (x65) showing:

A - necrotic female gametophyte
B - dead, necrotic and greatly shrunken nucellus
C - proliferated nucellar tissue
D - micropyle


D
A
B
C

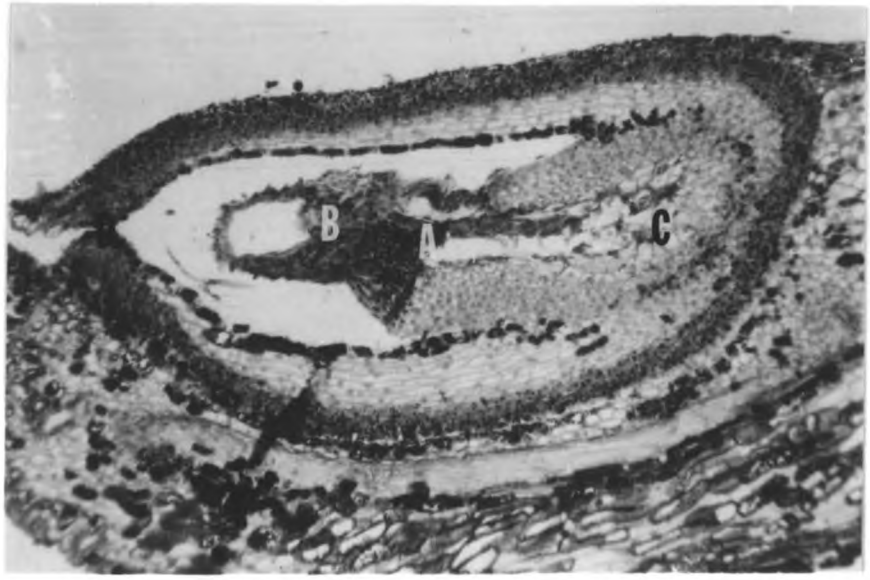
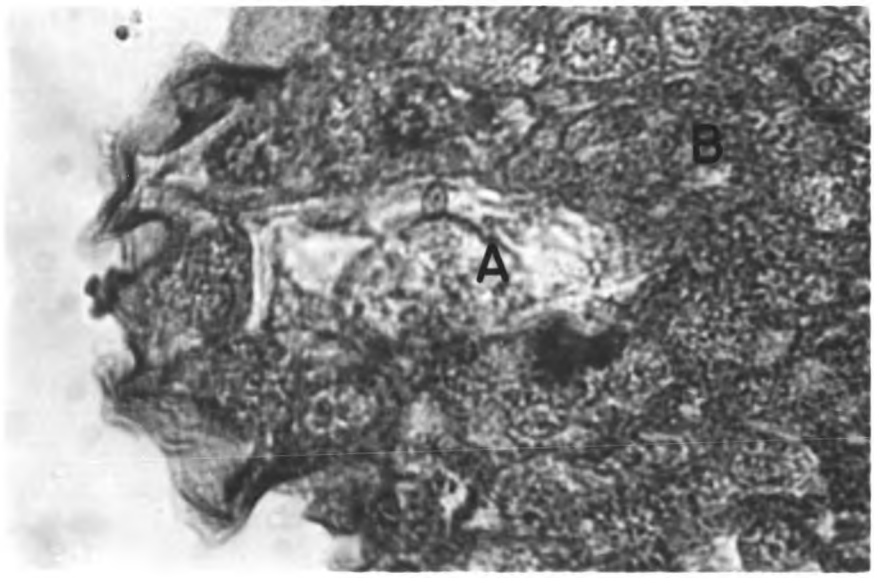
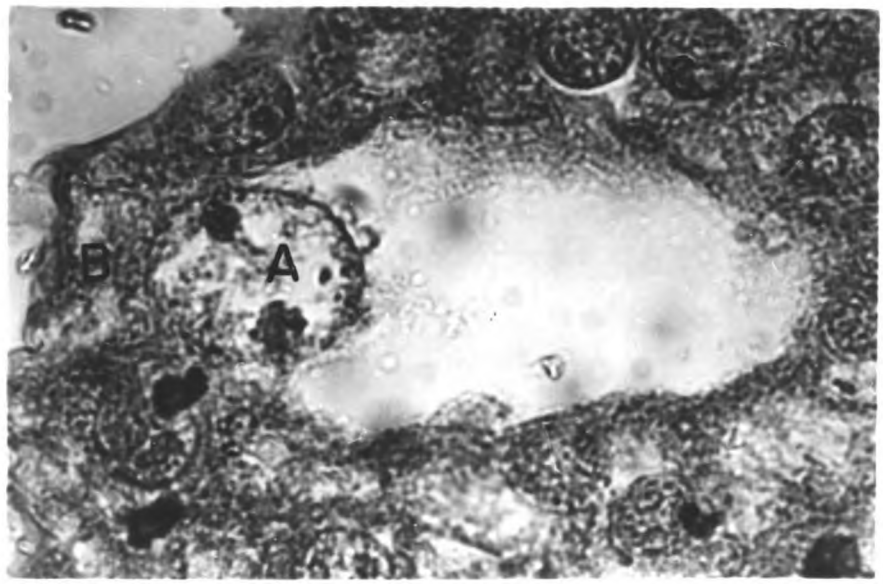

B
A
C

Figure 26 - Normal archegonial initial (A) at micropylar end of the female gametophyte (B) of ovule from open-pollinated blue spruce cone collected by G. H. Fechner, June 30, 1964 in Roosevelt National Forest, Larimer County, Colorado located R 73W, T 8N, S 13 (x800).

Figure 27 - Normal vacuolate archegonium of ovule from open-pollinated blue spruce cone collected by G. H. Fechner, June 30, 1964 in Roosevelt National Forest, Larimer County, Colorado R 73W, T 8N, S 13 (x870) showing:

A - central cell nucleus
B - two neck cells
C - archegonial jacket


B
A


B
A

ovules in which no pollen could be found (Figure 24). Hybrid ovules, both with and without pollen, had aborted before the female gametophyte became cellular. Some of these ovules contained female gametophytes that had protruded into the nucellus.

A different kind of abnormality was found in this material in which the nucellar tip, extending from the nucellar cup nearly to the female gametophyte cavity, appeared dead and greatly shrunken (Figure 25). In these ovules the female gametophyte appeared to be necrotic and protruded into the dead nucellar tip. The dead and shrunken nucellar tissue ended abruptly at the zone of nucellar division where, in the unpollinated blue spruce ovules, a similar condition had been observed in ovules from the fourth collection. From this point to the chalazal end of the ovule, the nucellar tissue appeared normal, except where the cells had proliferated immediately around the female gametophyte cavity.

18 days after pollination

Both collection conelets were horizontally oriented on the tree, with the cone scales partly closed. Archegonia had been initiated in ovules from the open-pollinated conelets and in some ovules from the control-pollinated conelets (Figure 26). Some of these had attained an early vacuolate stage of development (Figure 27). In the hybrid ovules, some were found in which the female gametophyte had aborted before becoming cellular. Others were found in which the female gametophyte had protruded into the nucellus. In some of these

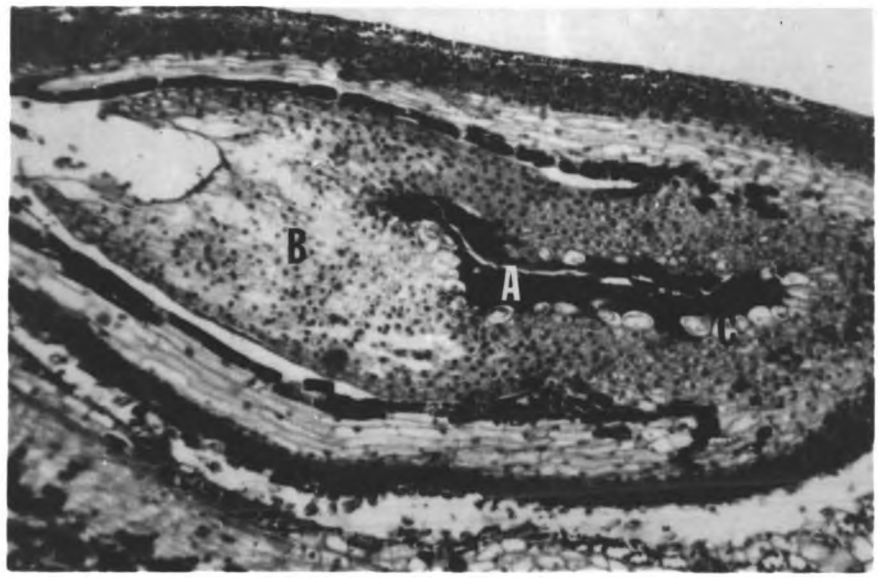
ovules the nucellus appeared dead as was evidenced by the lack of cytoplasm in many of the cells (Figure 28). Protruded female gametophyteswere also found in ovules in which the nucellar tip appeared dead and extremely shrunken (Figure 29). A third type of hybrid ovule with a protruded female gametophyte was observed. In this ovule the protrusion apparently continued completely through the nucellar tissue, which remained normal and alive outside the protrusion path, to the nucellar cup (Figure 30). This latter condition resulted in one long continuous cavity from the female gametophyte cavity to the nucellar cup.

In two of the hybrid ovules the female gametophytes appeared to be of normal size, but at the micropylar end the cells were proliferated. These appeared the same as the unpollinated ovule in Fugure 15. These female gametophytes showed no evidence of protrusion into the normal nucellar tissue. One ovule was observed to have a female gametophyte similar to the two described above, except that at the micropylar end there was some clumped material that protruded into the nucellar tissue.

Two ovules from control-pollinated conelets contained female gametophytes with archegonia. The archegonia of one ovule were empty, apparently containing no cytoplasm. The enlarged archegonial jacket cells were either empty or necrotic, although their structural continuity could be identified (Figure 31). The archegonium in the other ovule contained a central cell that was located at a central position in

Figure 28 - Blue spruce ovule 18 days after the strobilus received Engelmann spruce pollen (x75) showing:
A - necrotic, protruded female gametophyte
B - dead nucellus
C - proliferated nucellar cells

Figure 29 - Blue spruce ovule 18 days after the strobilus received Engelmann spruce pollen (x70) showing:
A - necrotic female gametophyte
B - dead and shrunken nucellar cap
C - normal nucellus
D - integument


B
A

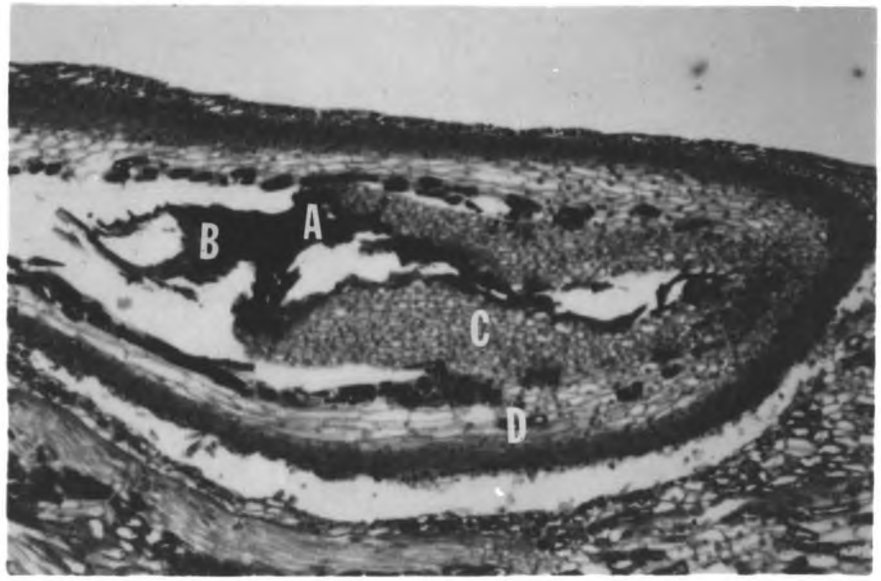
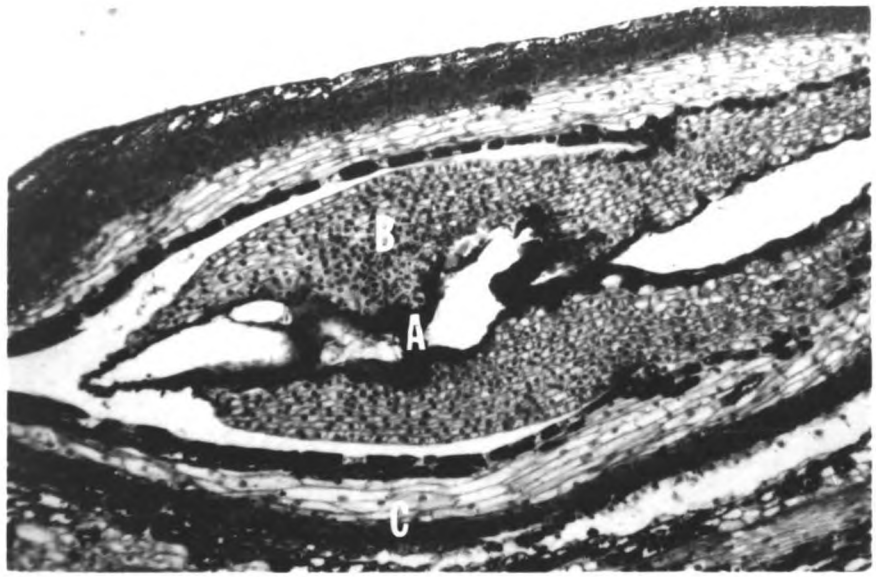
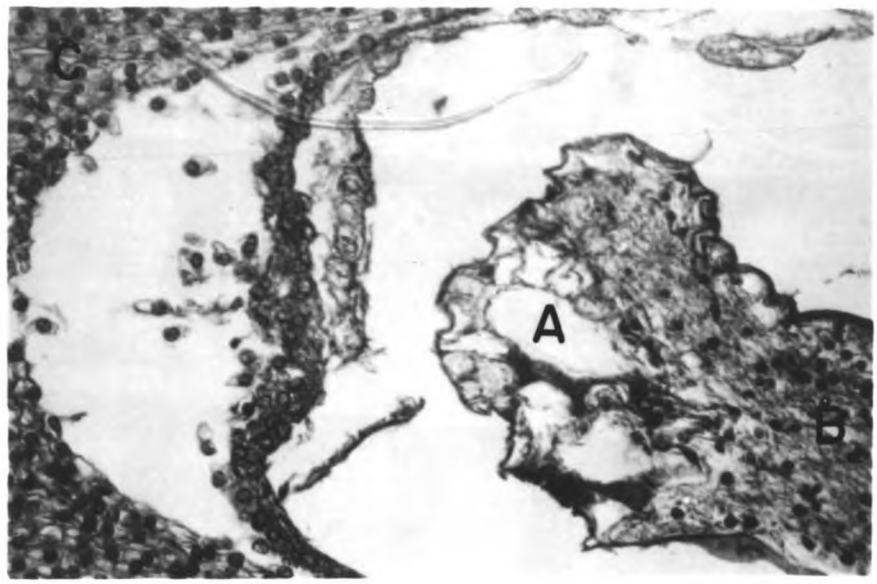

B
A
C
D

Figure 30 - Blue spruce ovule 18 days after strobilus received Engelmann spruce pollen (x70) showing:
A - necrotic female gametophyte protruded through nucellus to nucellar cup
B - normal nucellar tissue
C - integument

Figure 31 - Blue spruce female gametophyte 18 days after strobilus received Engelmann spruce pollen (x190) showing:
A - archegonium with dead jacket cells
B - female gametophyte tissue
C - nucellus


B
A
C


C
A
B

the archegonium rather than at the tip of the archegonium near the neck cells, as would ordinarily be expected (Figure 32).

21 days after pollination

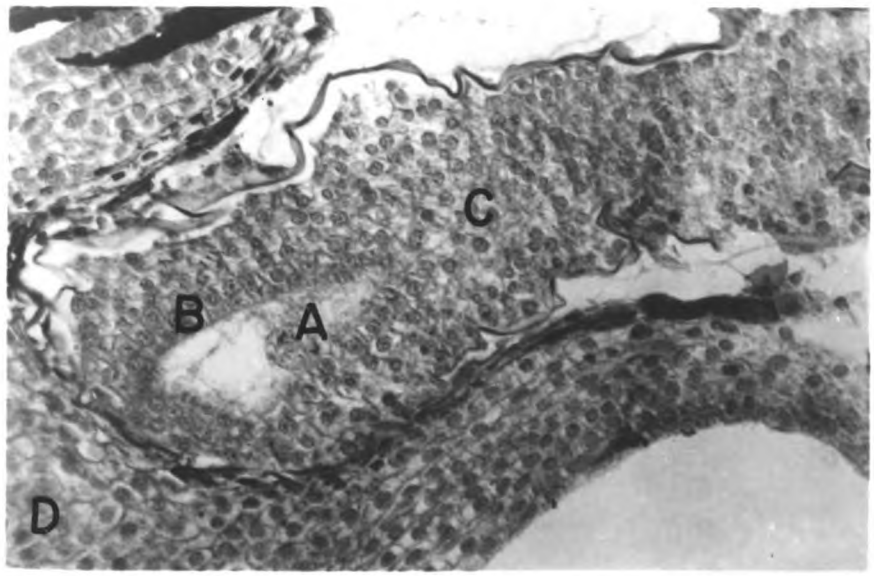
The control-pollinated cone was almost pendent, but the open-pollinated cone was horizontal and the scales were not completely closed. The ovules from the open-pollinated cones contained well-developed, vacuolate archegonia (Figure 33), but none of the artificially pollinated ovules examined microscopically contained any sign of a cellular female gametophyte. Most of the hybrid ovules had apparently aborted before the female gametophyte became cellular. The nucellus, however, was not shrunken in these ovules and some ovules even contained pollen which had weakly penetrated the nucellus. Ten of the hybrid ovules observed contained female gametophytes which had not developed beyond the free nuclear or early cellular stages. In these ovules, the free nuclei seemed to have formed a ring with interconnecting strands of cytoplasm (Figure 34).

24 days after pollination

Most of the unbagged blue spruce cones were pendent except for the uppermost 10 percent of the cones on the tree which were still horizontal. The ovules from open-pollinated cones and some of the ovules from the control-pollinated cones contained archegonia filled with cytoplasm and were at the stage of division of the central cell (Figure 35). Many

Figure 32 - Blue spruce female gametophyte 18 days after strobilus received Engelmann spruce pollen (x175) showing:
A - mis-placed central cell
B - archegonial jacket
C - female gametophyte
D - nucellus

Figure 33 - Normal female gametophyte from open-pollinated blue spruce strobilus 21 days after pollination (x180) showing:
A - vacuolate archegonium
B - archegonial jacket
C - female gametophyte


C
B
A
D

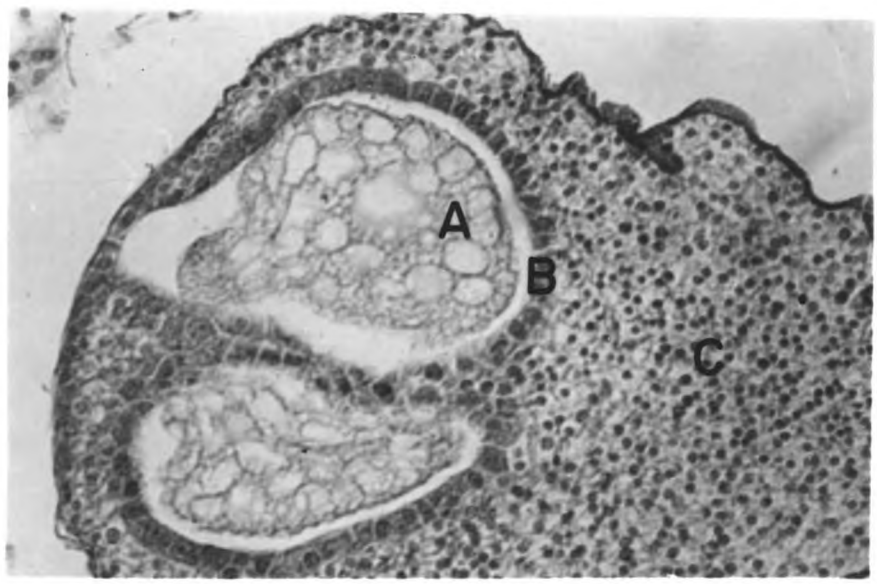
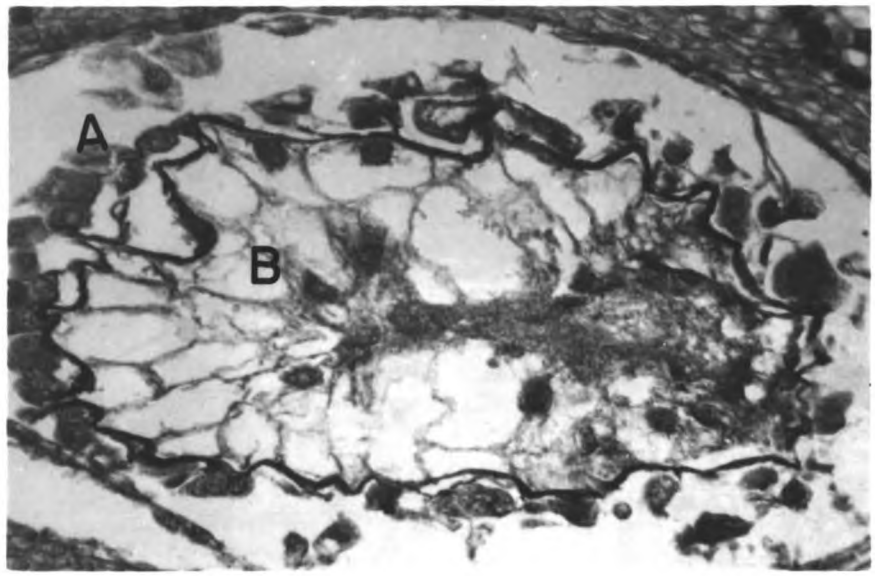
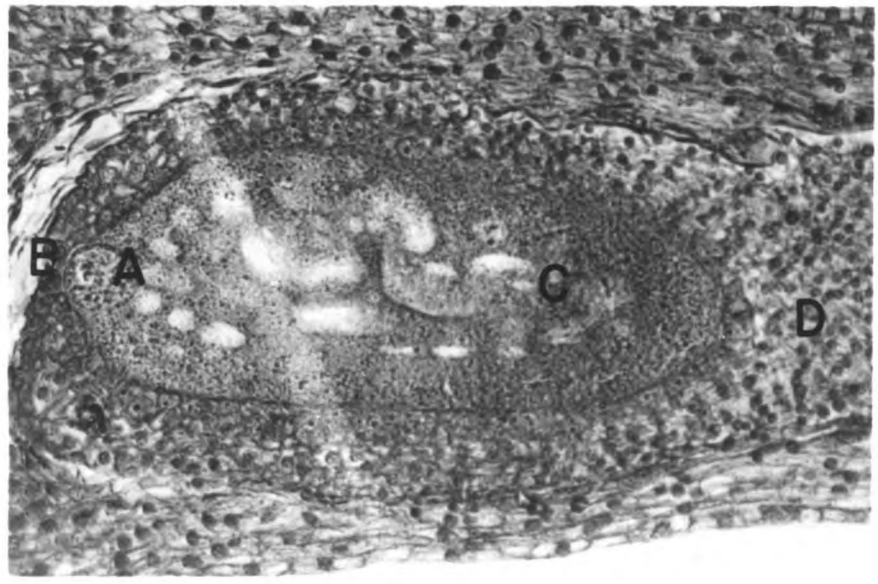

A
B
C

Figure 34 - Female gametophyte of blue spruce ovule 21 days after strobilus received Engelmann spruce pollen (x200) showing:
A - free nuclei
B - interconnecting strands of cytoplasm
C - nucellus

Figure 35 - Normal archegonium with very few vacuoles remaining of an ovule from an open-pollinated blue spruce cone collected by G. H. Fechner, June 30, 1964 in Roosevelt National Forest, Larimer County, Colorado located R 73W, T 8N, S 13 (x185) showing:
A - central cell
B - archegonial neck
C - cytoplasm
D - female gametophyte


A
B


A
C
D

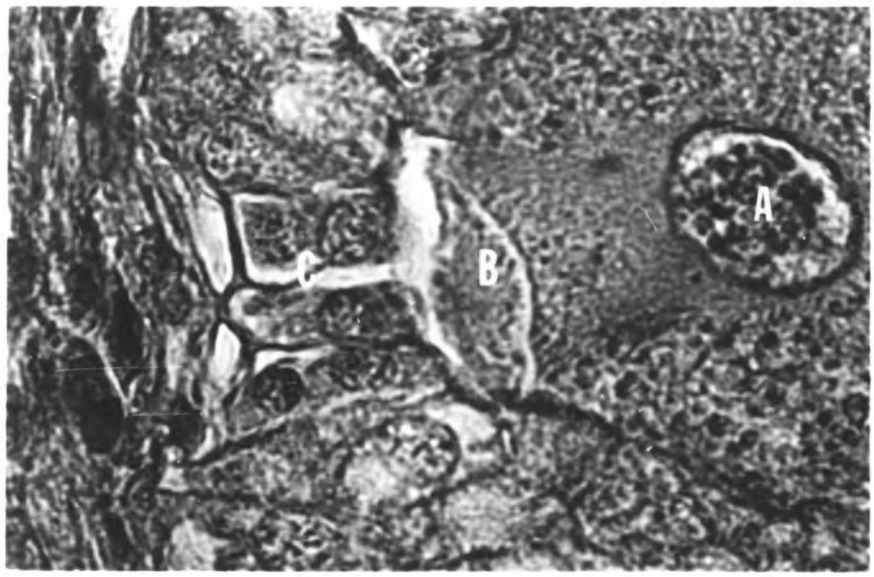
hybrid ovules showing completion of the division of the central cell were observed and apparently were developing normally prior to collection (Figure 36). One such ovule clearly showed aberrent direction of growth of a pollen tube off toward the side of the ovule. At least one artificially pollinated blue spruce ovule was normal at this date. It contained a mature egg and a pollen tube that had penetrated the archegonial neck cells (Figure 37). Two normal hybrid ovules contained vacuolate archegonia.

All the earlier observed abnormalities plus several new ones were found in the ovules of the control-pollinated cone. The entire nucellar tip appeared to be in the process of degeneration in those ovules in which the female gametophyte aborted before becoming cellular (Figure 38). Often the nucellar tissue proliferated at the chalazal end of the female gametophyte cavity. Hybrid ovules in which the female gametophyte became necrotic and protruded into the nucellus included ovules in which the nucellar tip was shrunken and necrotic (Figure 29) as well as those in which the cells of the nucellar tip appeared dead, and empty, but not shrunken (Figure 28). As was the usual situation in these kinds of ovules, the nucellus was proliferated at the chalazal end of the ovule.

In four of the ovules from the control-pollinated cone the archegonia had begun to fuse with the female gametophyte tissue. That is, the archegonial jacket was indistinguishable from the rest of the female gametophyte. Three conditions

Figure 36 - Recently divided normal central cell of blue spruce ovule 24 days after strobilus received Engelmann spruce pollen (x840) showing:
A - egg
B - ventral canal cell
C - archegonial neck
D - nucellus

Figure 37 - Normal archegonium of blue spruce 24 days after the strobilus received Engelmann spruce pollen (x190) showing:
A - receptive egg
B - egg cytoplasm
C - pollen tube having penetrated the archegonium
D - female gametophyte


A
B
C

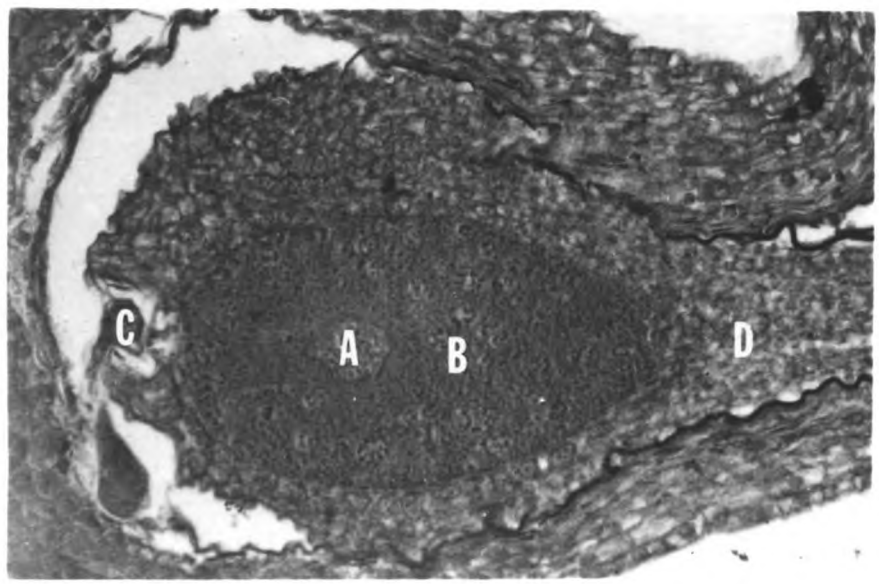
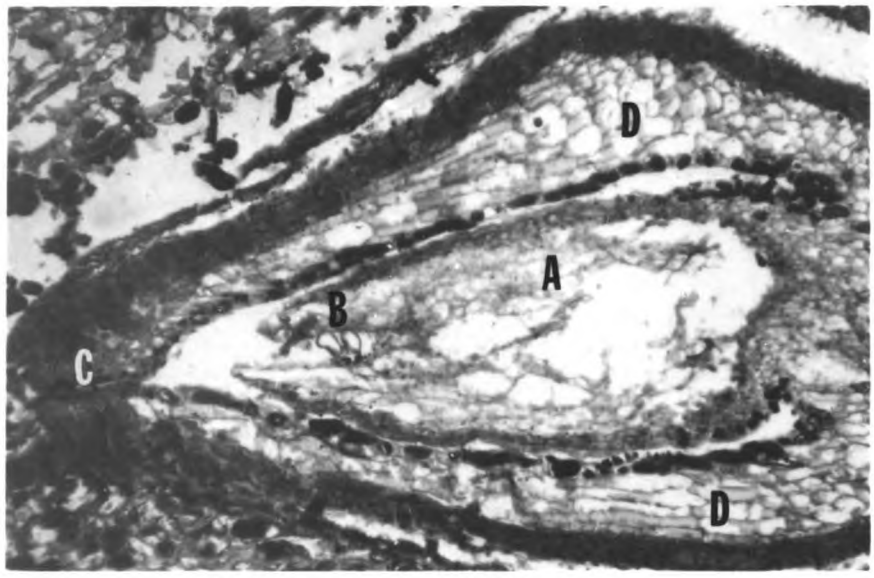
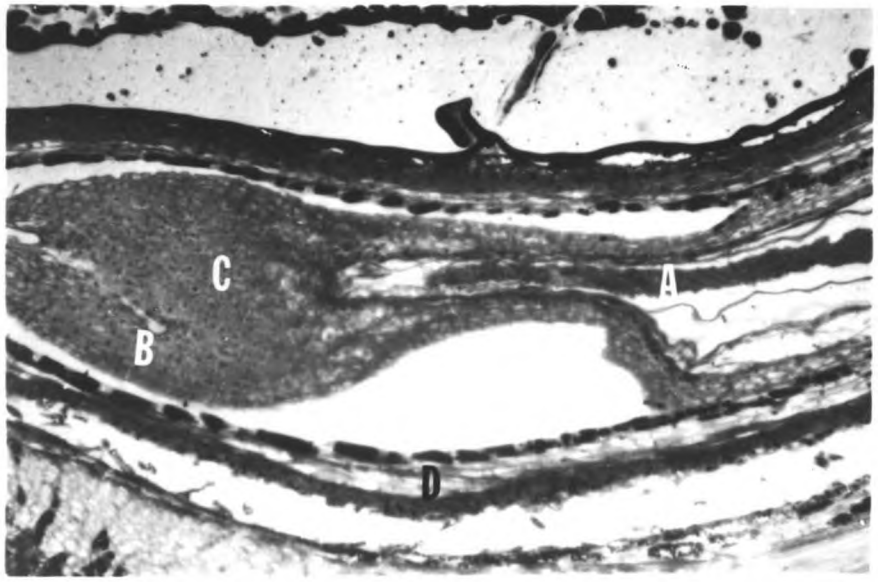

C
A
B
D

Figure 38 - Blue spruce ovule 30 days after strobilus received Engelmann spruce pollen (x65) showing:
A - degenerated nucellus
B - ungerminated Engelmann spruce pollen grain
C - micropyle
D - integument

Figure 39 - Blue spruce ovule 24 days after strobilus received Engelmann spruce pollen (x70) showing:
A - narrow female gametophyte
B - aberrant growth of pollen tube
C - nucellus
D - integument


D
A
B
C
D


C
A
B
D

were observed in these ovules: 1) an egg cell was present or 2) an undivided central cell was present or 3) no nuclei were found. Fusion of the ovular tissues may have constituted a stage prior to that in which the archegonial jackets were indistinct and in which the upper portion of the female gametophyte tissue including the archegonia, was segmented. Even though the female gametophyte may have been segmented with archegonia that had fused with the surrounding tissue, some ovules were found in which the egg or undivided central cell could be recognized.

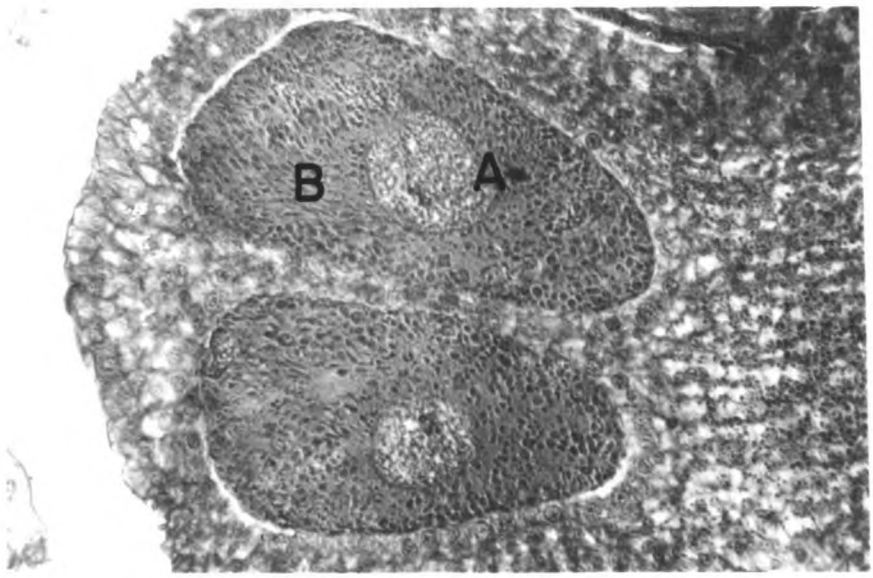
At least one flattened ovule, from the control-pollinated cone collected 24 days after pollination, was observed in which the pollen tube had grown to one side of the nucellar tissue. In this ovule the female gametophyte was very narrow. Apparently the whole ovule did not increase in diameter and no archegonium developed in the limited female gametophyte cavity (Figure 39). Some of the ovules from the control-pollinated cone however, developed full-sized female gametophytes with normal nucellar tissue and full-sized archegonia. These archegonia, surrounded by well-defined archegonial jackets, contained what appeared to be gigantic protein bodies. In one such ovule a remnant central cell was observed (Figure 40). Another archegonium with these large protein-like bodies contained a small egg, which, having lost its nuclear envelope appeared to be in the process of fusing with the surrounding cytoplasm.

Figure 41 - Normal blue spruce archegonia from open-pollinated strobilus 27 days after pollination date (x190) showing:

A - receptive egg
B - protein bodies
C - female gametophyte

Figure 40 - Blue spruce archegonia 24 days after strobilus received Engelmann spruce pollen (x205) showing:

A - apparent protein bodies
B - remnants of central cell
C - archegonial jacket
D - female gametophyte


B
A

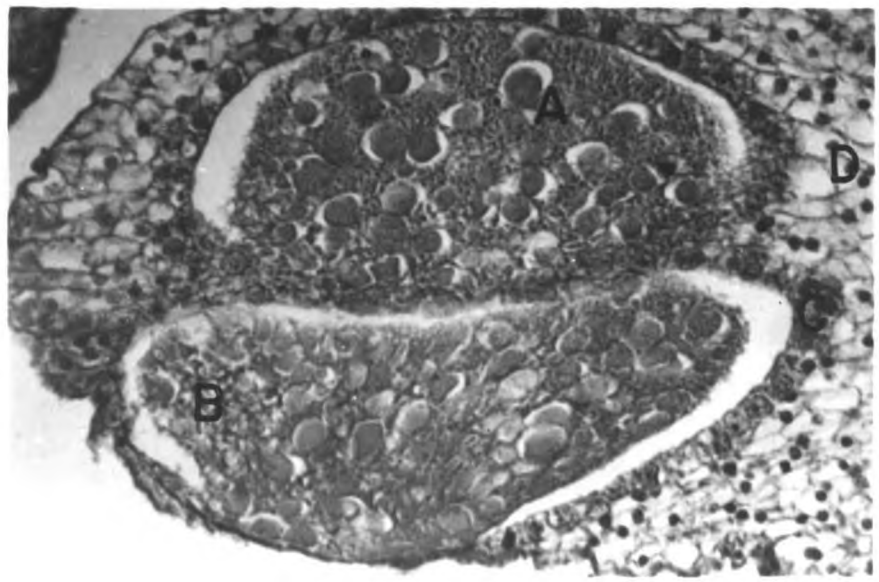
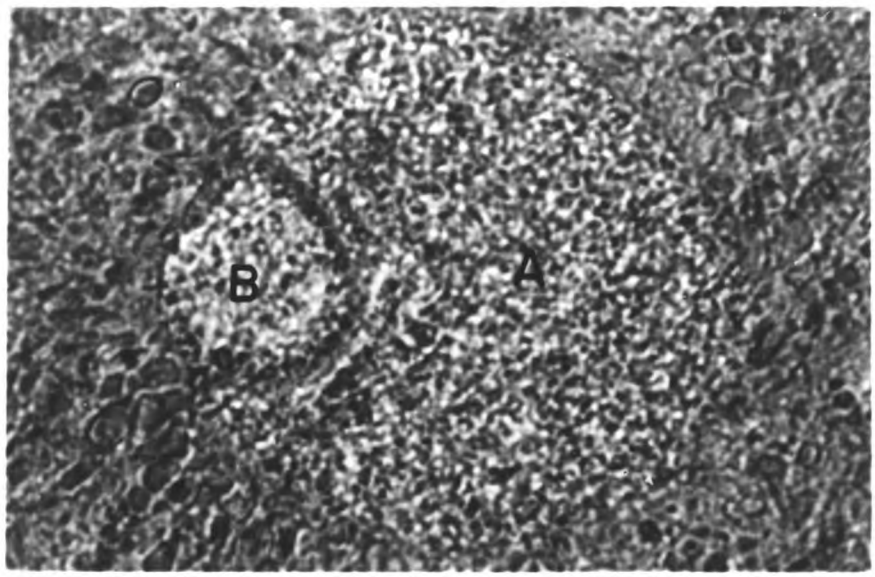

A
D
C
B

Figure 42 - Syngamy in normal blue spruce ovule 30 days after open-pollination (x805) showing:
A - egg
B - sperm nucleus

Figure 43 - Normal archegonium of ovule from open-pollinated blue spruce strobilus 33 days after pollination (x200) showing:
A - two nucleate proembryo
B - egg cytoplasm
C - degenerating pollen tube in archegonial neck
D - female gametophyte
E - nucellus


B
A

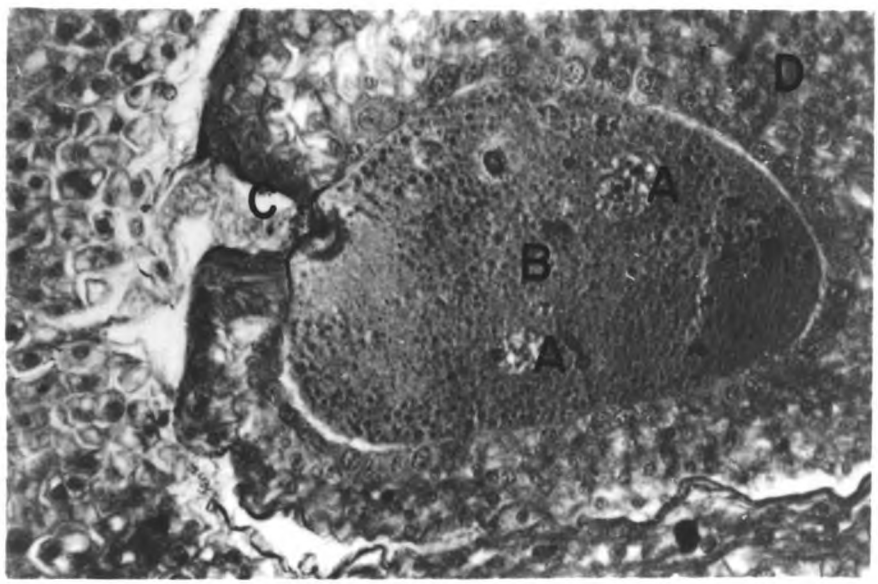
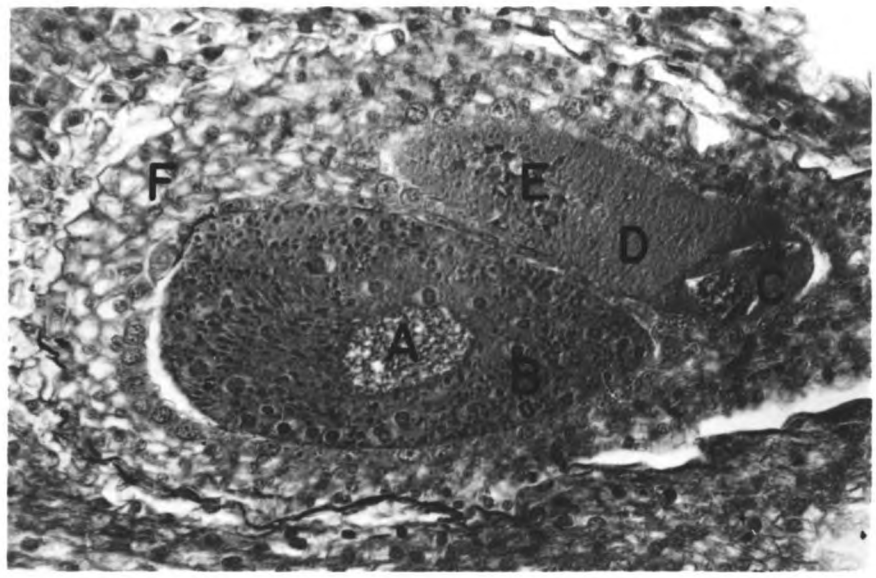

D
C
A
B
A

Figure 44 - Normal proembryo and unfertilized egg in blue spruce ovule from open-pollinated strobilus 33 days after pollination date (x145) showing:

A - receptive egg
B - granular cytoplasm typical of unfertilized archegonia
C - two-nucleate proembryo
D - fine cytoplasm typical of fertilized
E - suspected degenerating pollen tube nuclei
F - female gametophyte

Figure 45 - Normal second proembryo division in blue spruce ovule 30 days after open-pollination date (x880).


F
E
D
C
A
B

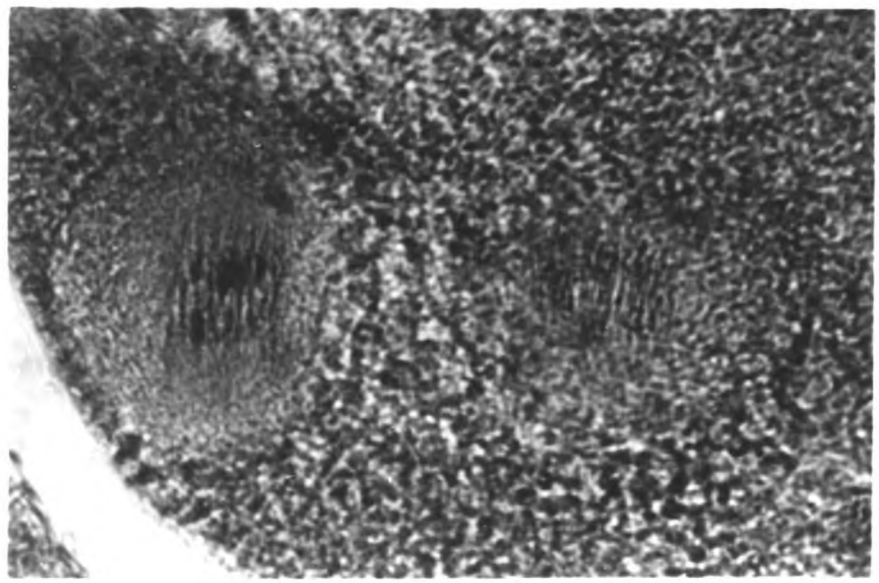
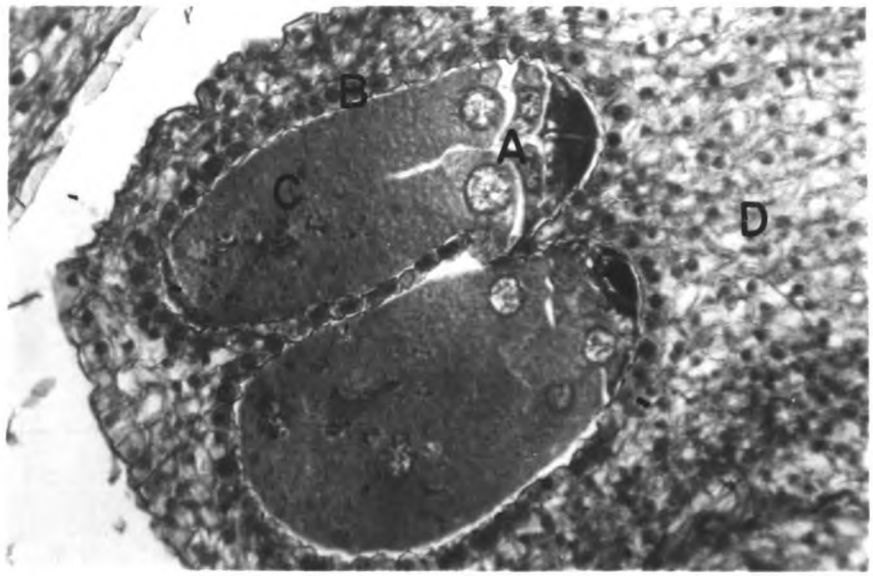
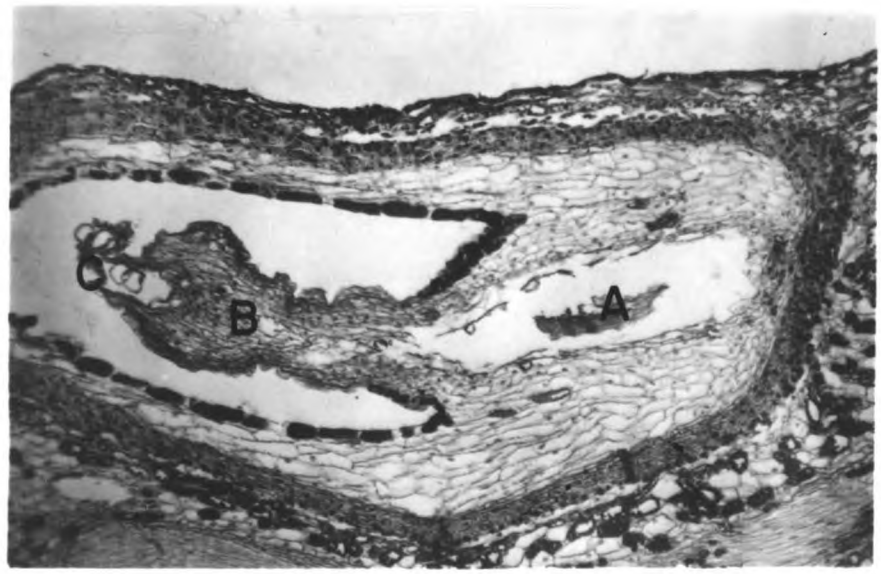


Figure 46 - Normal blue spruce proembryos from open-pollinated strobilus 24 days after pollination date (x165) showing:
A - proembryo
B - archegonial jacket
C - egg cytoplasm
D - female gametophyte

Figure 47 - Blue spruce ovule 30 days after the strobilus received Engelmann spruce pollen (x65) showing:
A - necrotic unprotruded female gametophyte
B - dead nucellus
C - ungerminated Engelmann spruce pollen grains


B
A
C
D


C
B
A

One ovule from this cone contained an archegonium filled with cytoplasm, in which the central cell displayed a large indentation. Still another abnormal hybrid ovule from this cone contained an archegonium with the neck cells offset to one side and with the central cell undivided.

27 days after pollination

Several developmental stages ranging from the receptive egg to the proembryo were observed in ovules from the open-pollinated cone (Figure 41 and 46). A germination test of the seeds included in the control-pollination bag of this collection indicated that contamination of the Engelmann spruce pollen had probably occurred and that some blue spruce pollen may have been included. The high germination percentage (36 percent) of the putative hybrid seed suggests contamination. Therefore, all the control-pollinated ovules from this collection were discarded.

30 days after pollination

The collection cone that had been pollinated with Engelmann spruce pollen was in a horizontal position, and the open-pollinated cone was pendent. Syngamy and proembryo stages were observed in the open-pollinated ovules (Figures 42 and 45). All the hybrid ovules examined had apparently aborted before the female gametophyte became cellular (Figure 47). In the aborted ovules, the nucellus remained intact, but clearly showed deterioration as evidenced by a lack of

cytoplasm in most of the cells. Occasionally, an ungerminated pollen grain was observed in these ovules indicating that they had received pollen.

33 days after pollination

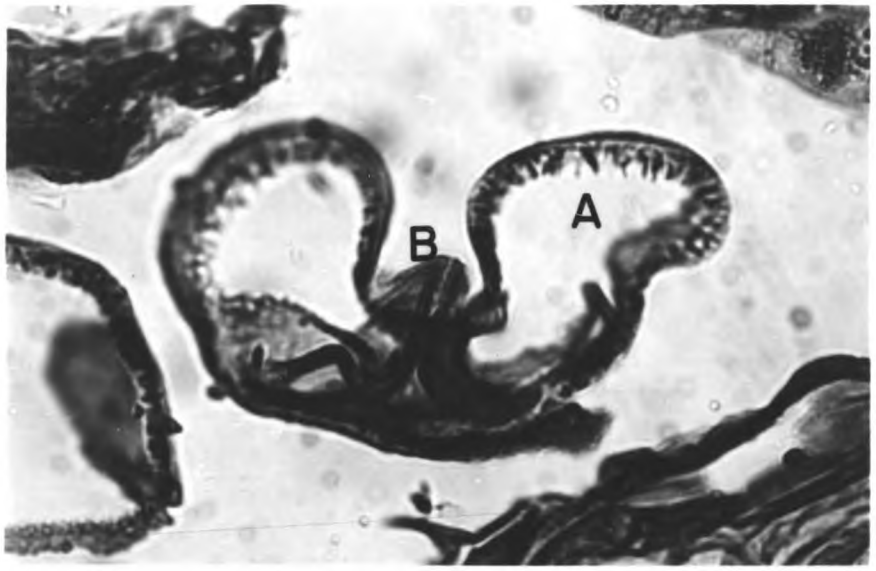
The control-pollinated cone had started to turn downward and all unbagged cones were pendent. The control-pollinated cone was considerably smaller than the open-pollinated cone collected on this date, and its green scales were bluish tinted. The open-pollinated ovules contained proembryos (Figures 43 and 44). All but one of the ovules from the control-pollinated cone had aborted before the female gametophyte became cellular. Over half of the control-pollinated ovules contained ungerminated, or poorly-germinated, pollen in the nucellar cup (Figure 48), but only one ovule had pollen that had penetrated into the nucellus. The hybrid ovule that developed was just slightly segmented through the archegonium, and no archegonial jacket was evident. One of the archegonia contained a central cell and two neck cells. The ovule was about the size of those collected 18 days after pollination and the frequently observed constriction of the nucellus, just distal to the archegonial area had not yet occurred.

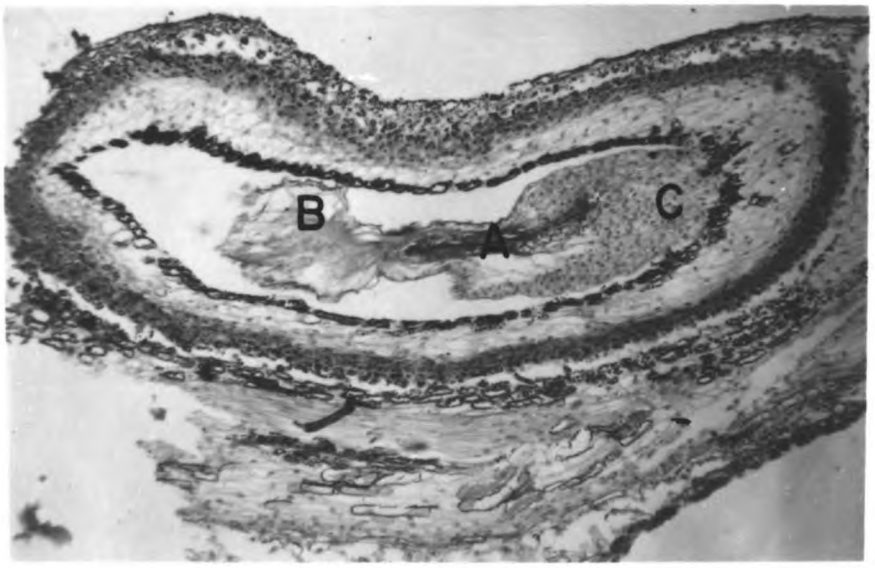
36 days after pollination

All of the cones on the blue spruce study tree were pendent. The control-pollinated cone collected was only 60 percent as long as the open-pollinated cones, and somewhat

Figure 48 - Poorly-germinated Engelmann spruce pollen in blue spruce ovule 33 days after the strobilus received pollen (x790) showing:
A - pollen air sacs
B - poorly developed pollen tube

Figure 49 - Blue spruce ovule 36 days after the strobilus received Engelmann spruce pollen (x60) showing:
A - necrotic female gametophyte
B - somewhat shrunken and dead nucellus
C - normal nucellus


A
B


B
A
C

narrower. The scales of the control-pollinated cone were not tightly closed, although elongation was probably complete.[5] Many abnormalities were observed in the ovule from this cone that were not found in other collections. The open-pollinated ovules contained proembryos or embryos which were growing out of the archegonia.

Those ovules from the control-pollinated cone in which the female gametophyte was necrotic and the nucellus deteriorated could be placed in three groups:

1) In ovules in which the necrotic female gametophyte showed no proliferation but protruded into the nucellus, the nucellar cells were often proliferated at the chalazal end of the female gametophyte cavity.

2) Proliferation of cells was observed in unprotruded female gametophyte tissue or in nucellar tissue, but never in both tissues simultaneously.

3) The necrotic female gametophyte and deteriorated nucellus was found in both shrunken and non-shrunken ovules (Figure 49). The nucellus in shrunken ovules proliferated either at the chalazal end or at the nucellar cup, but never at both places (Figure 50).

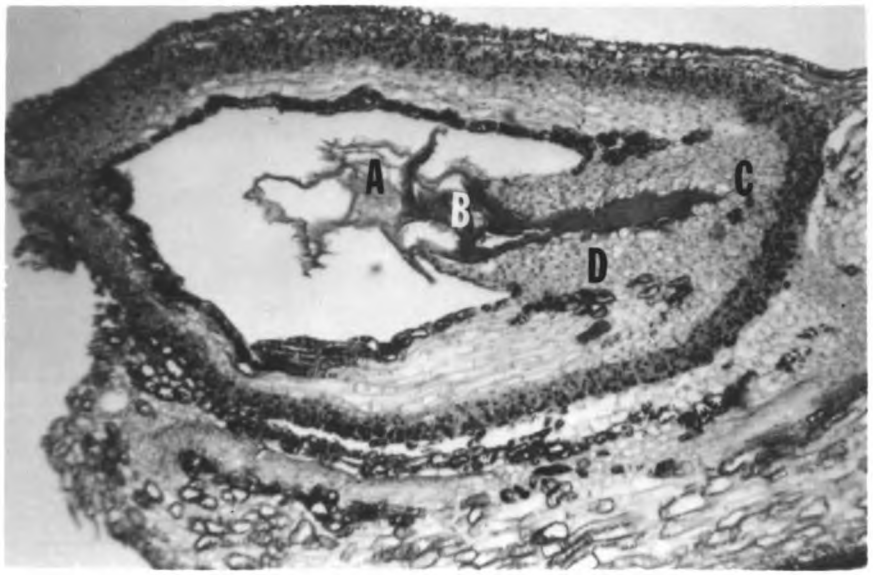
Ovules from this control-pollinated cone that were segmented through both the archegonium and the surrounding female gametophyte tissue also contained normal nucellar tissue. A typical female gametophyte in this type of ovule was expanded in the archegonial region, but narrow and undeveloped toward

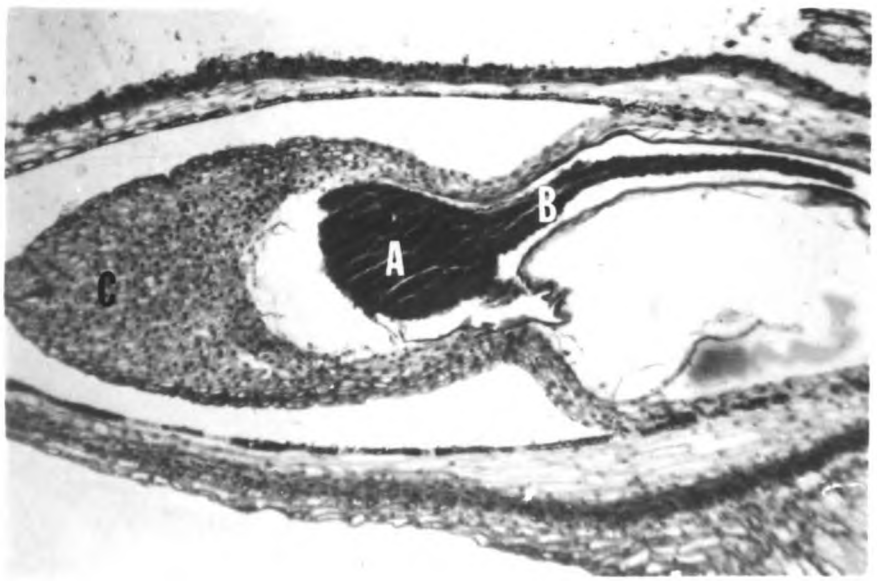
---

[5]This phenemenon of loosely closed cone scales at the cessation of cone elongation was very common in the cones of this hybridization study in 1970. These 1970 cones were eventually discarded and not processed due to the frequency of the irregularity.

Figure 50 - Shrunken blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x70) showing:
A - dead and shrunken nucellus
B - necrotic and protruded female gametophyte
C - proliferated nucellar cells
D - normal nucellus

Figure 51 - Female gametophyte of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x65) showing:
A - segmented archegonia and female gametophyte
B - narrow undeveloped female gametophyte where corrosion cavity should be
C - nucellus


A
B
C
D


A
B
C

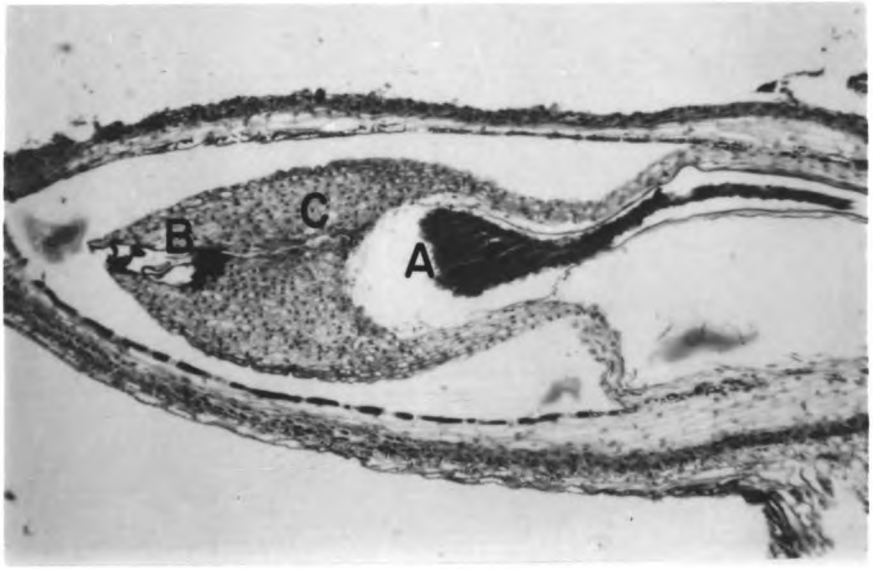
the chalazal end (Figure 51). Some of these segmented ovules contained pollen that had penetrated into the nucellus. They were found to contain either degenerating egg nuclei or degenerating central cells (Figures 52 and 53).

Several of the ovules from the control-pollinated cone had archegonia with an additional amount of scattered chromatin-like material. Archegonia of this type contained: 1) a central cell, 2) a receptive egg (or enlarged egg in an early stage of degeneration) or 3) several nuclei of egg size or larger (Figures 54 and 55). Ovules with these three different kinds of nuclei were found with pollen having penetrated the nucellus, without pollen, or with ungerminated pollen present.

Some developmental abnormalities were observed only once. One ovule appeared normal except that the archegonia were completely empty. Two ovules contained archegonia at an early vacuolate stage of development. One of these showed preliminary signs of proliferation of the female gametophyte tissue around the archegonium (Figure 56). The ovules with the vacuolate archegonia were considerably smaller than those collected 18 days after pollination. Another ovule appeared to have normal, receptive eggs present in the archegonia, but only ungerminated pollen could be found in the ovule. The most advanced ovule of this collection contained an embryo which was growing out of the archegonium. The second archegonium of this ovule contained an oblong-shaped egg, apparently not yet fertilized (Figure 57).

Figure 52 - Blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x65) showing:
A - segmented female gametophyte, narrow at the chalazal end
B - dead pollen tubes
C - pollen tube path through nucellus

Figure 53 - Female gametophyte of blue spruce ovule 36 days after receiving Engelmann spruce pollen (x75) showing:
A - degenerating egg
B - degenerating archegonium
C - nucellus


B
C
A

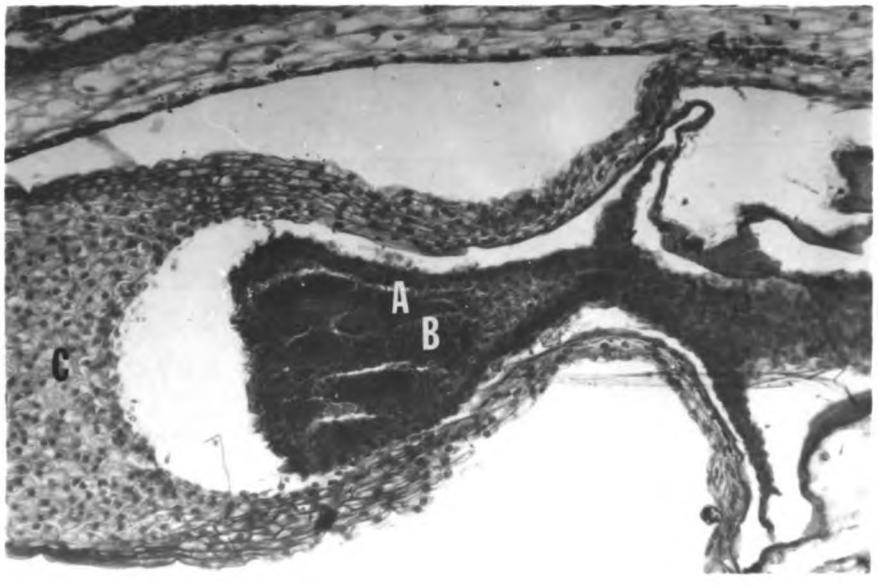
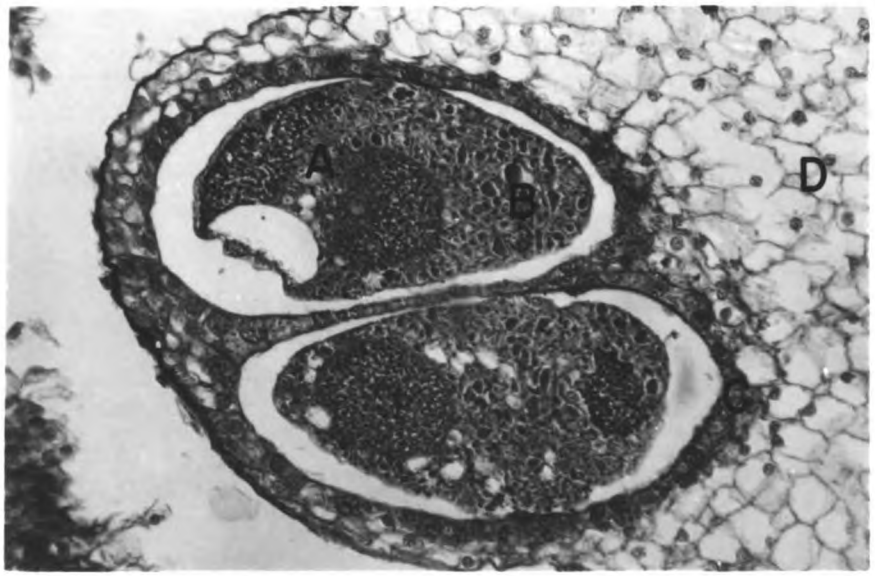

A
B
C

Figure 54 - Archegonia of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x185) showing:
A - nucleus or chromatic material
B - granular cytoplasm indicative of unfertilized condition
C - archegonial jacket
D - female gametophyte

Figure 55 - Archegonium of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x165) showing:
A - unfertilized egg nucleus
B - scattered chromatin
C - corrosion cavity area


A
B
D

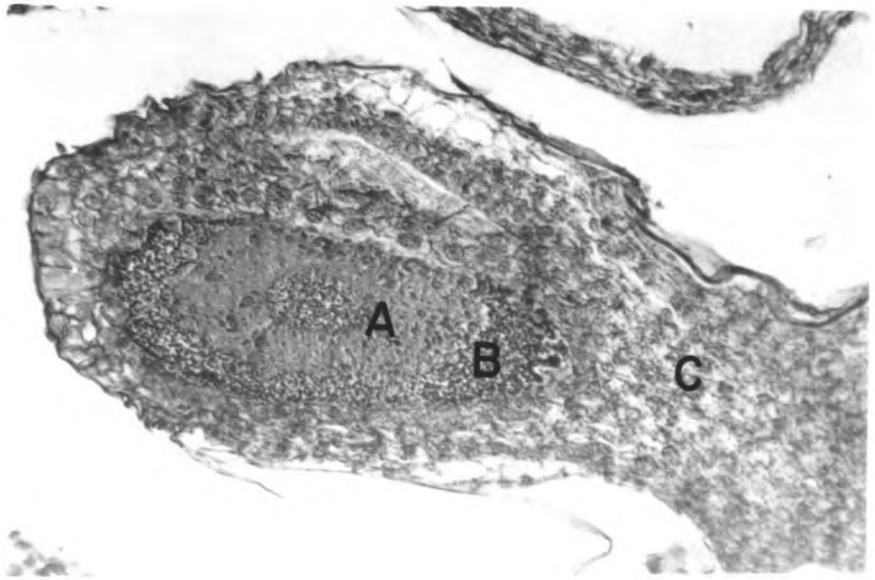
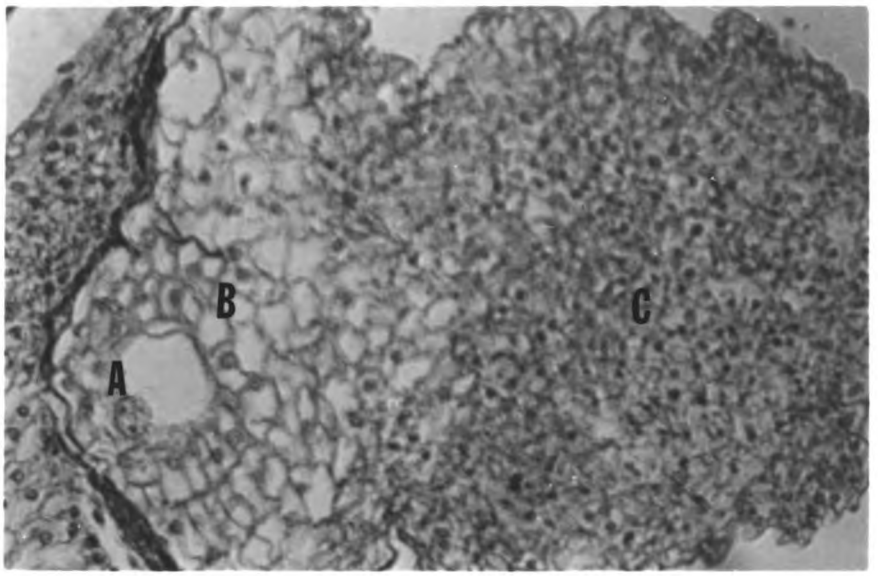
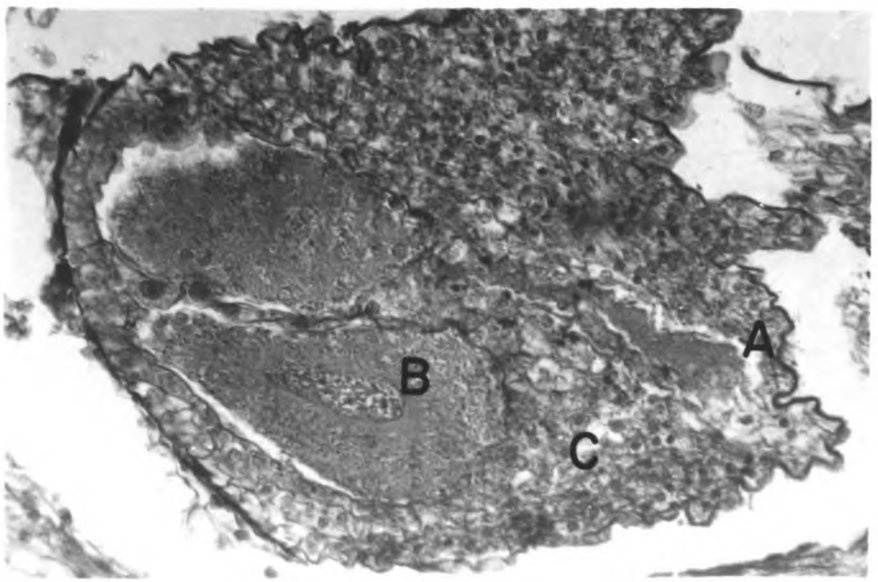

A
B
C

Figure 56 - Female gametophyte and archegonium of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x200) showing:
A - central cell in partially developed archegonium
B - early proliferation in female gametophyte tissue
C - normal female gametophyte tissue

Figure 57 - Normal embryo and unfertilized egg of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x185) showing:
A - embryo grown out of the archegonium
B - elongated degenerated egg
C - female gametophyte


A
B
C


A
B
C

A few ovlues contained female gametophytes which had proliferated in the archegonial region. One such proliferated female gametophyte was also clumped near the micropylar end (Figure 58). Another showed extensive proliferation throughout the tissue without clumping (Figure 59).

39 days after pollination

Both collection cones were pendent, and the control-pollinated cone was a lighter green color than the open-pollinated cone. The open-pollinated ovules contained embryos growing out of the archegonia, but all of the hybrid ovules had aborted before the female gametophytes became cellular. Some of the contents of the ovules from the control-pollinated cones were shrunken, and some were not. Some ovules contained proliferated nucellar tissue while others did not. In all the hybrid ovules the nucellus was dead and often had disappeared.

42 days after pollination

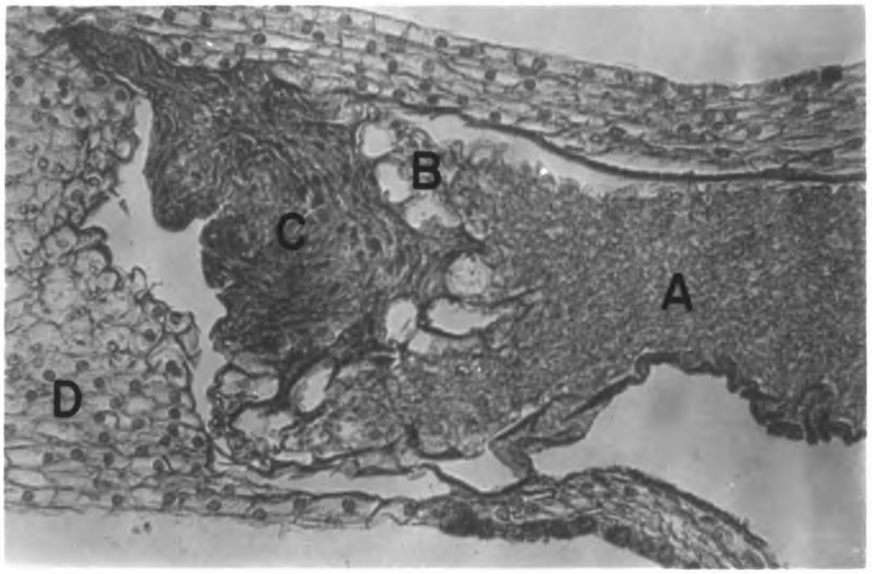
The control-pollinated cone was only 55 percent as long as the open-pollinated cone. Both cones were somewhat woody. The ovules from the open-pollinated cone contained embryos growing out of the archegonia and initial formation of the corrosion cavity was recognizable. Several of the hybrid ovules examined microscopically contained necrotic egg cytoplasm enlarged aborting eggs, or empty archegonia (Figure 60). Associated with deterioration of the archegonial contents was the breakdown of the archegonial jacket cells, especially those between adjacent archegonia (Figure 61). Pollen was

Figure 58 - Proliferated, necrotic and normal female gametophyte tissue of blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x185) showing:

A - normal female gametophyte tissue
B - proliferated cells of female gametophyte
C - necrotic and protruded tissue of female gametophyte
D - nucellus

Figure 59 - Blue spruce ovule 36 days after strobilus received Engelmann spruce pollen (x65) showing:

A - proliferated female gametophyte tissue
B - nucellus
C - seed coat


B
C
A
D

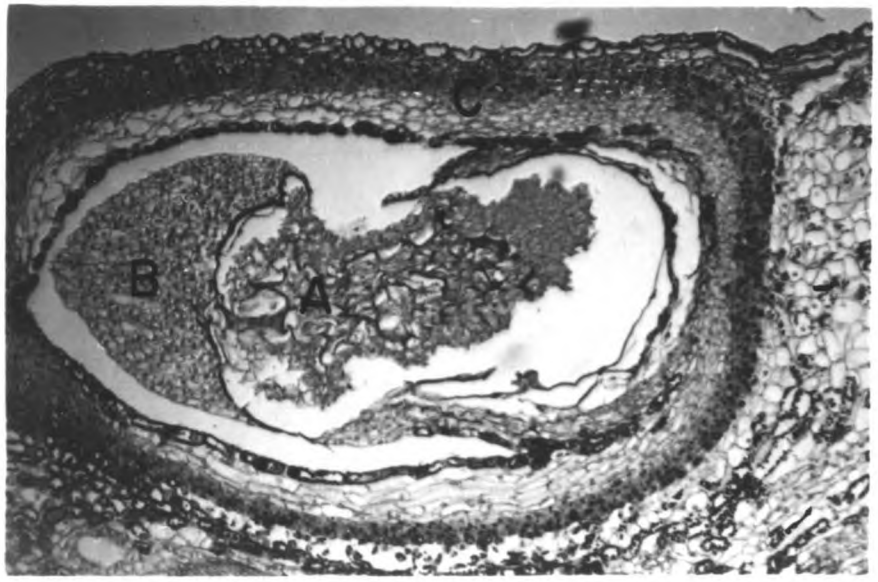
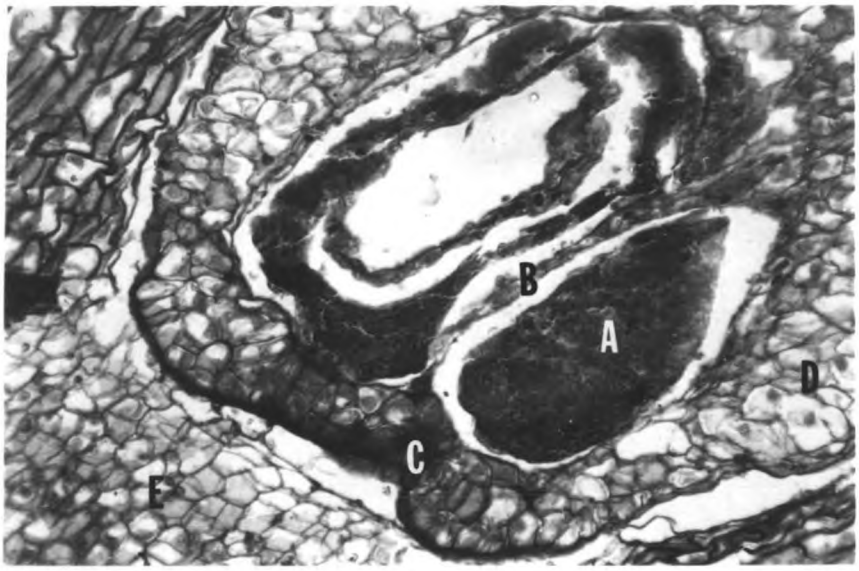
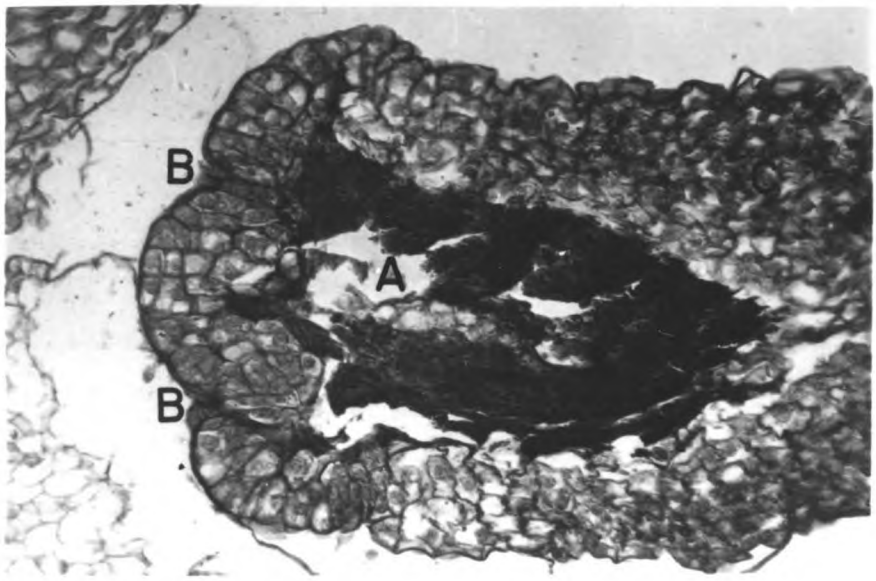

C
B
A

Figure 60 - Archegonia of blue spruce ovule 42 days after strobilus received Engelmann spruce pollen (x175) showing:
A - necrotic egg cytoplasm
B - deteriorated archegonial jackets of adjacent archegonia
C - archegonial neck
D - female gametophyte
E - nucellus

Figure 61 - Female gametophyte and fused necrotic archegonia of blue spruce ovule 42 days after strobilus received Engelmann spruce pollen (x180) showing:
A - necrotic contents of two archegonia
B - archegonial necks
C - female gametophyte


B
A
D
C
F


B
A
B

observed to have penetrated into the nucellus of many of these ovules (Figure 62).

Other hybrid ovules contained necrotic female gametophytes with shrunken ovular tissue. The female gametophytes of some of these ovules protruded into the nucellus, and the nucellar cells were proliferated at the chalazal end. Some ovules of this collection showed no protrusion of the female gametophyte and no proliferation of the nucellar cells.

Other abnormalities included one ovule in which the female gametophyte contained no archegonia and one in which the female gametophyte was necrotic at its micropylar tip and had proliferated cells adjacent to the necrotic portion (Figure 63). Still another ovule contained an embryo that had apparently emerged recently from the archegonium but had then died. All that remained of the embryo was a small dense necrotic mass (Figure 64).

It appears that when archegonia abort and begin to deteriorate, the extreme micropylar tip of the female gametophyte often responds in a hypertrophy of these cells to form what might be called the female gametophyte cap (Figure 65).

45 days after pollination

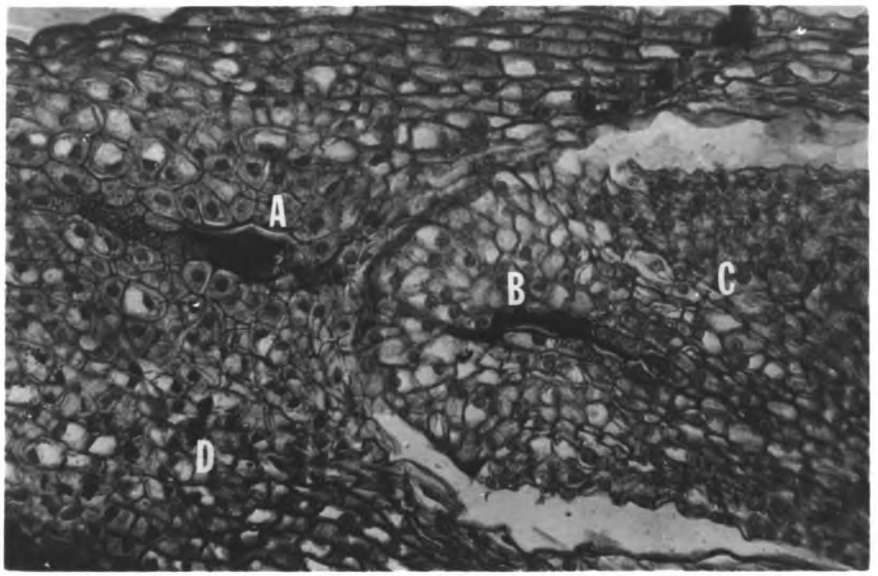
Open-pollinated ovules contained young embryos within young corrosion cavities. All the investigated ovules from control-pollinated cones contained female gametophytes that had aborted prior to becoming cellular. All of these ovules were small, but in none had the nucellus died and shrunk at

Figure 62 - Pollen tube penetration and edge of necrotic archegonium of blue spruce ovule 42 days after strobilus received Engelmann spruce pollen (x180) showing:

A - pollen tube penetration to female gametophyte
B - edge of necrotic archegonium
C - female gametophyte
D - nucellus

Figure 63 - Female gametophyte of blue spruce ovule 42 days after strobilus received Engelmann spruce pollen (x185) showing:

A - necrotic female gametophyte tip
B - proliferated female gametophyte tissue
C - nucellus


A
B
C
D

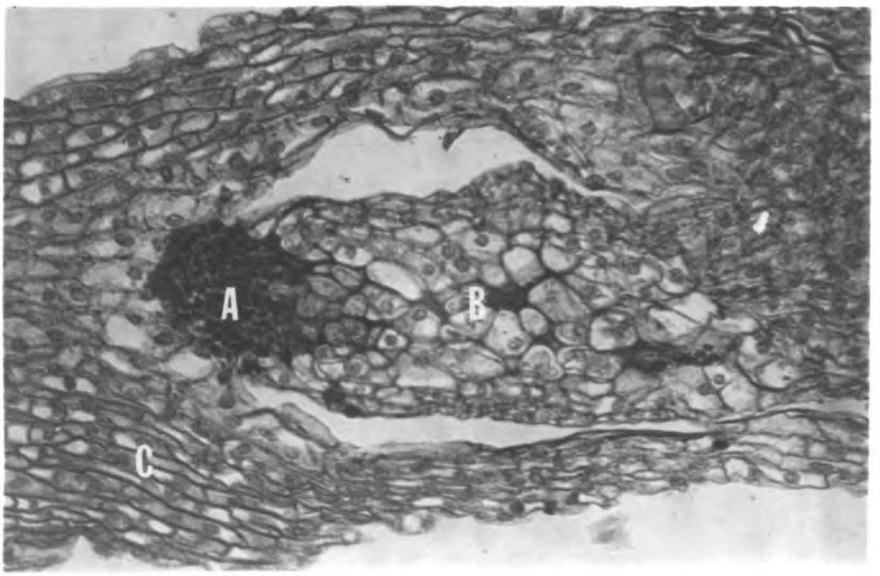
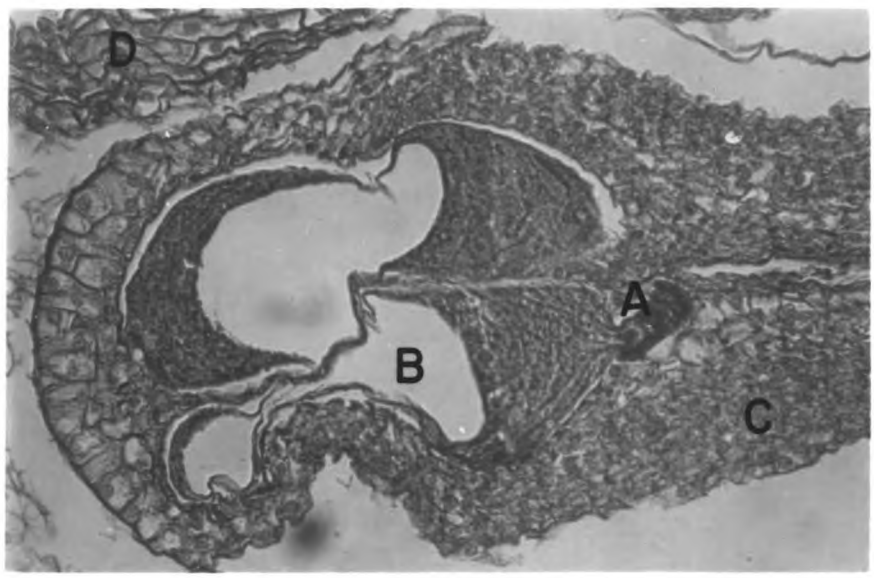
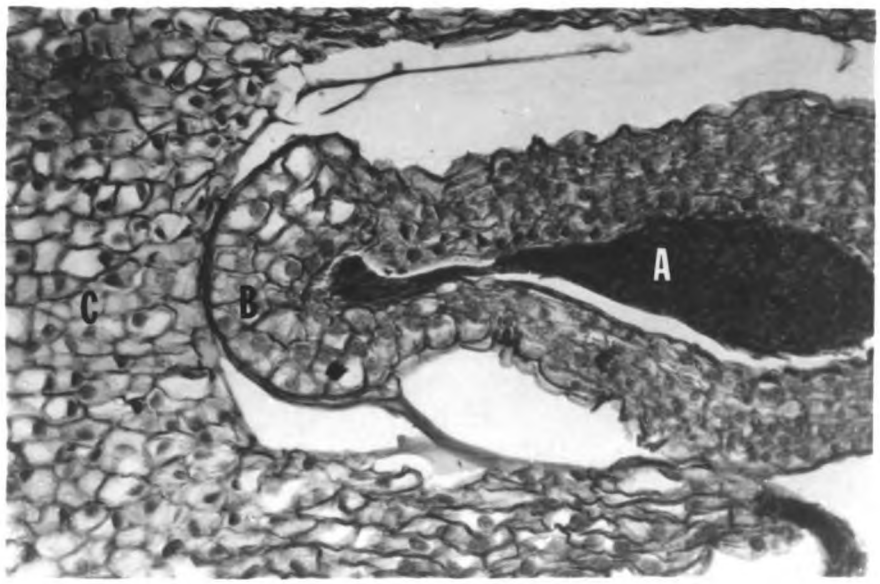

A
B
C

Figure 64 - Necrotic embryo just emerged from chalazal end of an archegonium 42 days after strobilus received Engelmann spruce pollen (x185) showing:
A - necrotic embryo
B - collapsed archegonium
C - female gametophyte tissue
D - nucellus

Figure 65 - Female gametophyte of blue spruce ovule 42 days after strobilus received Engelmann spruce pollen (x195) showing:
A - long archegonium, broader at the chalazal end filled with necrotic egg cytoplasm
B - hypertrophied female gametophyte cap
C - nucellus


D
A
B
C


A
C
B

an early age; rather, deterioration had led to progressive shrinkage of the nucellus as indicated by the samll volume of this tissue.

49 days after pollination

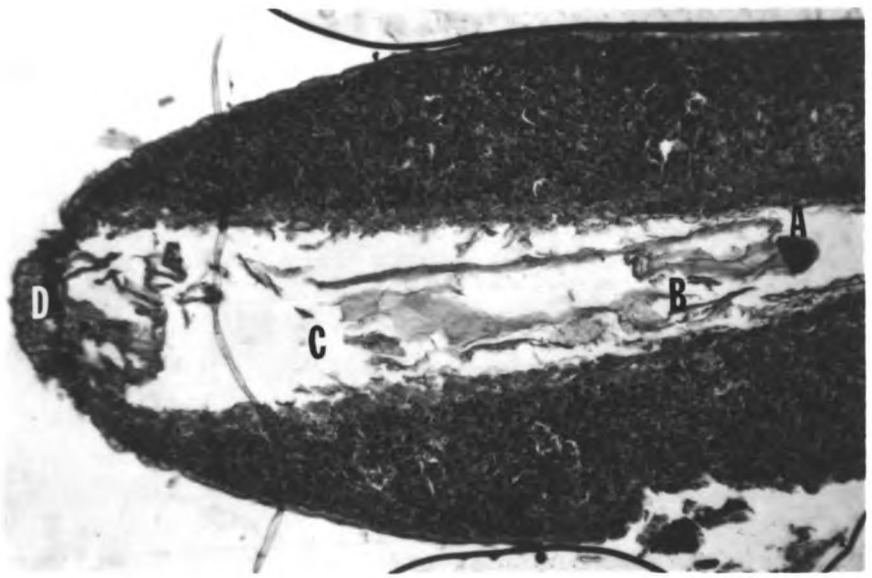
The control-pollinated cone was not straight but curved and smaller than the open-pollinated cones. Ovules from the open-pollinated cone contained young embryos suspended in the center of well-developed corrosion cavities (Figure 66). Ovules from the control-pollinated cone contained well-developed female gametophytes with corrosion cavities; however, the nucellus had been almost completely used. A few of these ovules had large, empty spaces where the archegonia should have been located, above which the hypertrophied female gametophyte cap was identifiable. In one of these ovules, the female gametophyte cells had proliferated at the periphery of the tissue, but normal cells occupied most of the interior of the female gametophyte tissue. Only one ovule contained an embryo, and this one was rather small, being located exactly where the archegonia had been, rather than in the center of the corrosion cavity (Figure 67).

53 days after pollination

The scales of both cones showed initial signs of color change from green to brown. More embryos of somewhat larger size than those of the previous collection were found in ovules from the open-pollinated cone (Figure 68). Ovules from the control-pollinated cones were similar to those in

Figure 66 - Ovule from open-pollinated blue spruce strobilus 49 days after pollination (x70) showing:
A - embryo
B - remnants of suspensor cells
C - corrosion cavity
D - female gametophyte cap

Figure 67 - Hybrid embryo in empty archegonial cavity of blue spruce ovule 49 days after pollination (x200) showing:
A - small embryo
B - female gametophyte tissue
C - female gametophyte cap
D - archegonial cavity
E - remnants of archegonial jackets


A
B
C
D

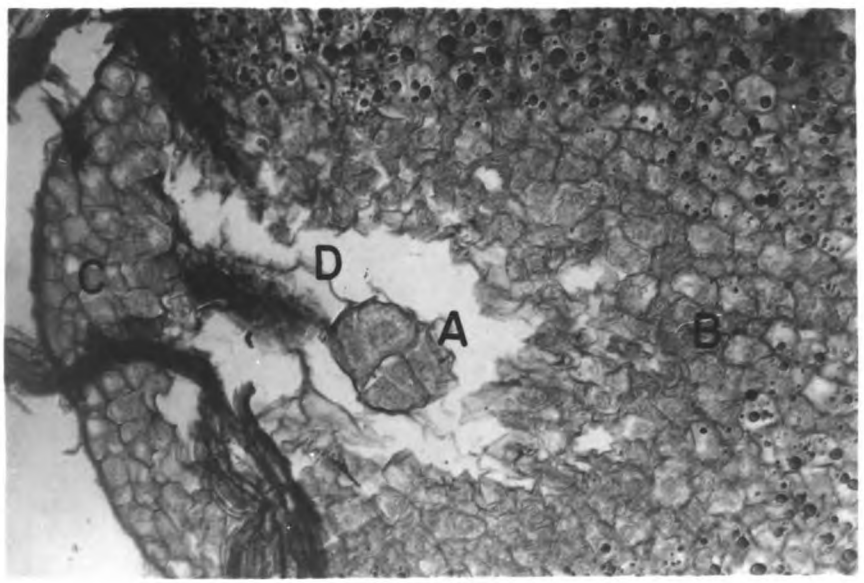
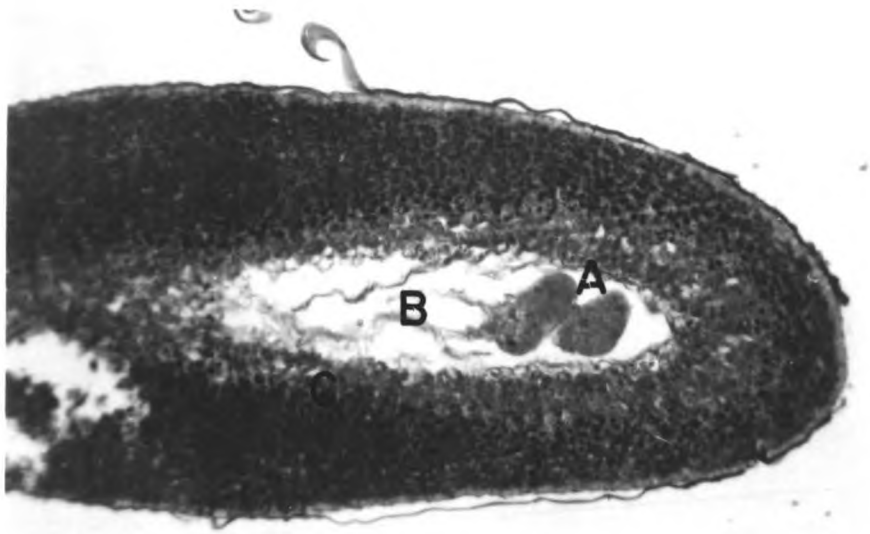
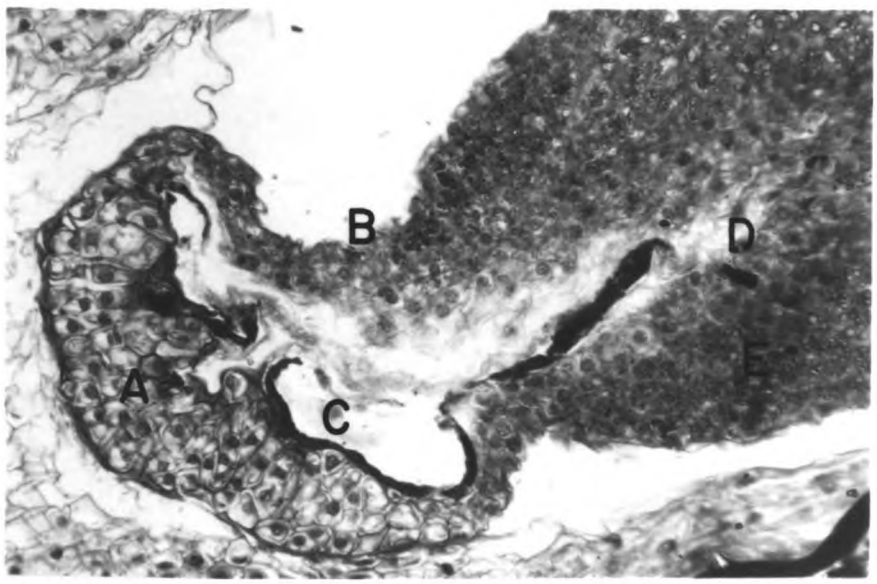

C
D
A
B

Figure 68 - Ovule from open-pollinated blue spruce strobilus 53 days after pollination date (x65) showing:
A - normal embryos
B - corrosion cavity
C - female gametophyte

Figure 69 - Female gametophyte of blue spruce ovule 53 days after strobilus received Engelmann spruce pollen (x180) showing:
A - hypertrophied female gametophyte cap
B - constriction in female gametophyte
C - archegonial cavities
D - corrosion cavity
E - female gametophyte tissue


A
B
C


B
D
A
C

the previous collection in that the female gametophyte was well developed, the corrosion cavity had begun to form, but no embryos were present and only remnants of the archegonia remained. Two ovules were found in which the nucellar tip was necrotic and the contents of the ovule had shrunk to fill only one-half of the female gametophyte cavity.

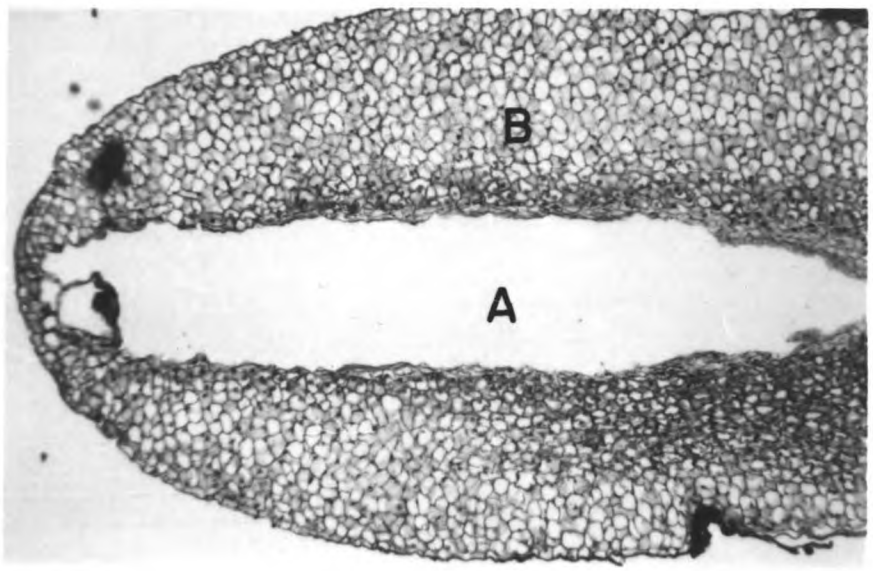
Those female gametophytes in which the corrosion cavity never fully developed often were constricted at the area where the archegonia had probably been and then apparently developed the hypertrophied cap (Figure 69).

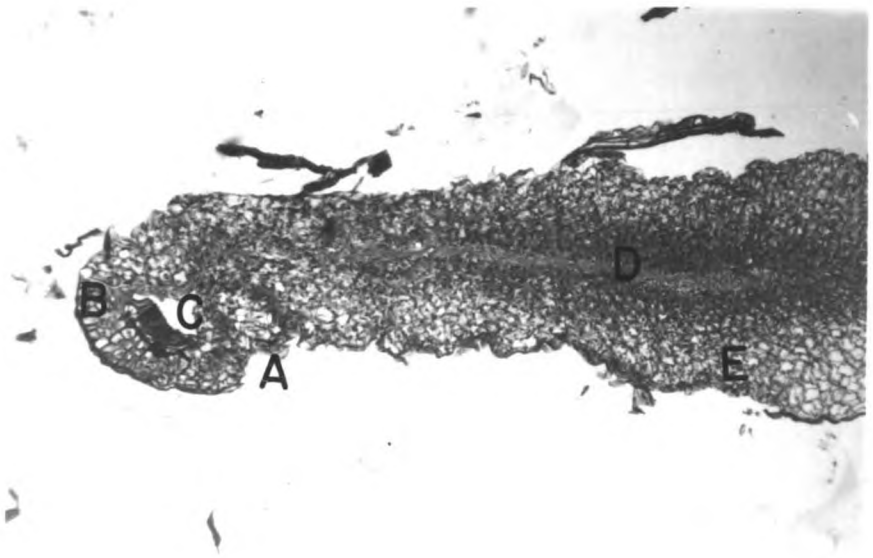
56 days after pollination

The control-pollinated cone was curved, and somewhat "J" shaped. The filled ovules from this cone, appeared, macroscopically, to be a grayish color while similar ovules from the open-pollinated cone were a milky white. Although of similar size, the grayish ovules contained no embryos. Many of the ovules from the control-pollinated cone contained these well-developed female gametophytes with empty corrosion cavities (Figure 70). One contained a narrow but full length female gametophyte with an undeveloped corrosion cavity (Figure 71). Several of these also displayed a hypertrophy of the female gametophyte cap. Another ovule from the control-pollinated cone contained only the shrunken remnants of the nucellus, and a similar ovule was nearly empty except for slight outlines of the tissues. Two other ovules were flattened, and contained a necrotic female gametophyte and a

Figure 70 - Female gametophyte of blue spruce ovule 56 days after strobilus received Engelmann spruce pollen (x65) showing:
A - empty corrosion cavity
B - female gametophyte tissue

Figure 71 - Female gametophyte of blue spruce ovule 56 days after strobilus received Engelmann spruce pollen (x80) showing:
A - constriction just above archegonium
B - female gametophyte cap
C - necrotic remnants of archegonium
D - undeveloped corrosion cavity
E - female gametophyte tissue


B
A


D
B
C
A
E

degenerated nucellus which had proliferated around the chalazal end of the female gametophyte cavity.

60 days after pollination

The control-pollinated cone was curved into a J-shape. All the seeds contained in it were similar in size, so it was difficult to macroscopically separate the aborted seed from the filled seed. The shoot apical meristem and cotyledon primordia were evident in the embryos of ovules from the open-pollinated cone, (Figure 72). Some of the ovules from the control-pollinated cone appeared to be almost completely empty except for a hypertrophied female gametophyte cap (Figure 73), while others contained only remnants of unidentifiable tissues. In one of these ovules a narrow corrosion cavity had developed, which contained a small, aborted embryo located about in the center of the corrosion cavity (Figure 74).

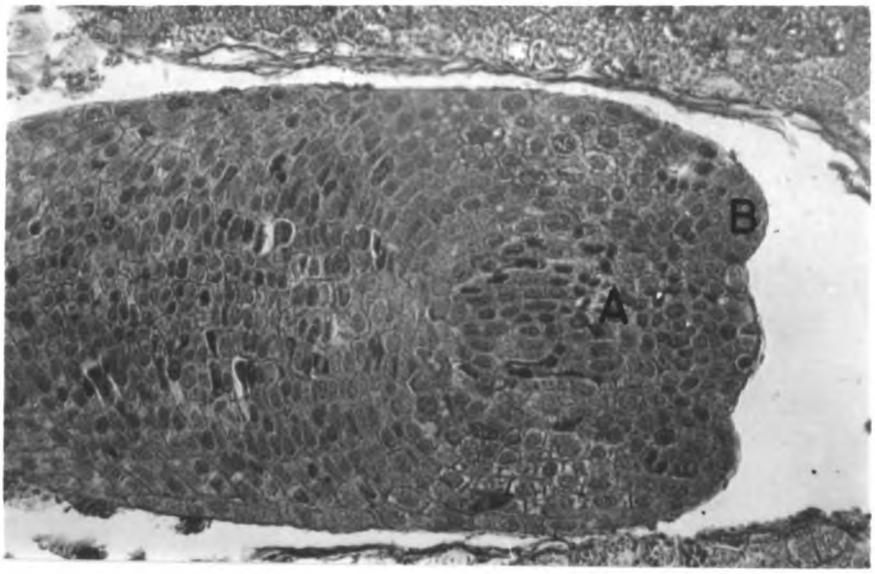
63 to 119 days after pollination

The ovules from open-pollinated cones, 63 days after pollination were almost completely developed and the embryos 77 days after pollination were very well developed (Figure 75). Seed was not shed until September 8, 1969, approximately 119 days after pollination.

The ovules from the control-pollinated cones microscopically examined from collections 60 to 119 days after pollination were all aborted, with only fragments of any tissues remaining in the ovule. In all instances the seed coats matured and hardened, but often the ovules were of much smaller than normal size.

Figure 72 - Embryo of ovule from open-pollinated blue spruce strobilus 60 days after pollination date (x60) showing:
A - shoot apical meristem
B - cotyledon primordia

Figure 73 - Blue spruce ovule 60 days after strobilus received Engelmann spruce pollen (x65) showing:
A - hypertrophied female gametophyte cap
B - seed coat


B
A

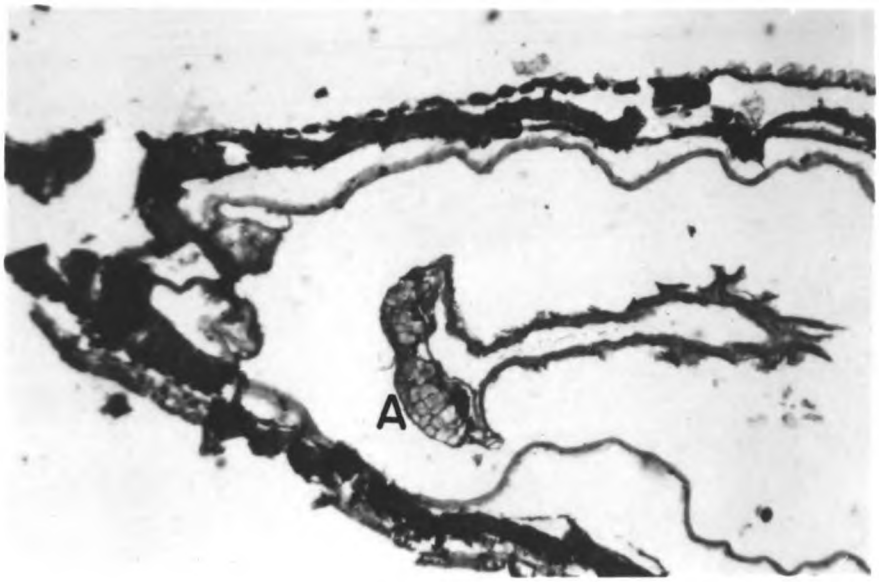
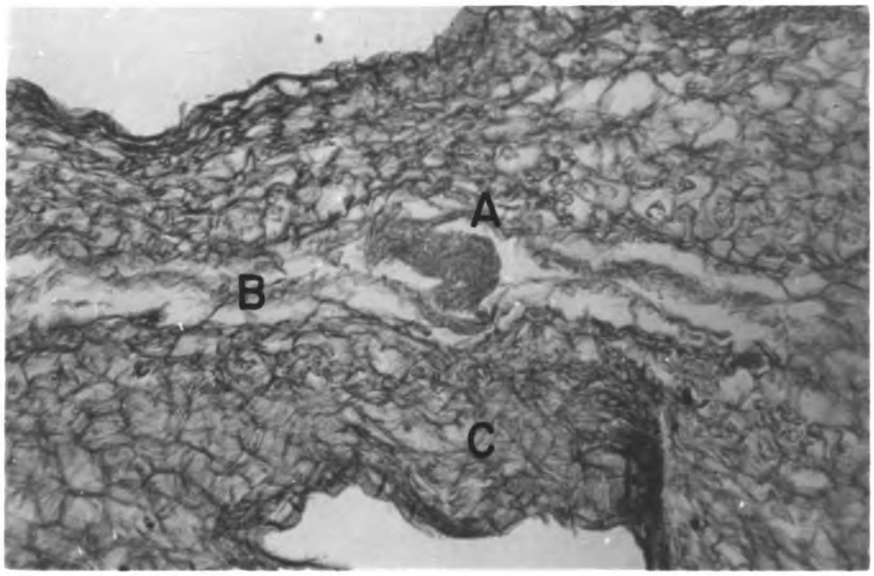
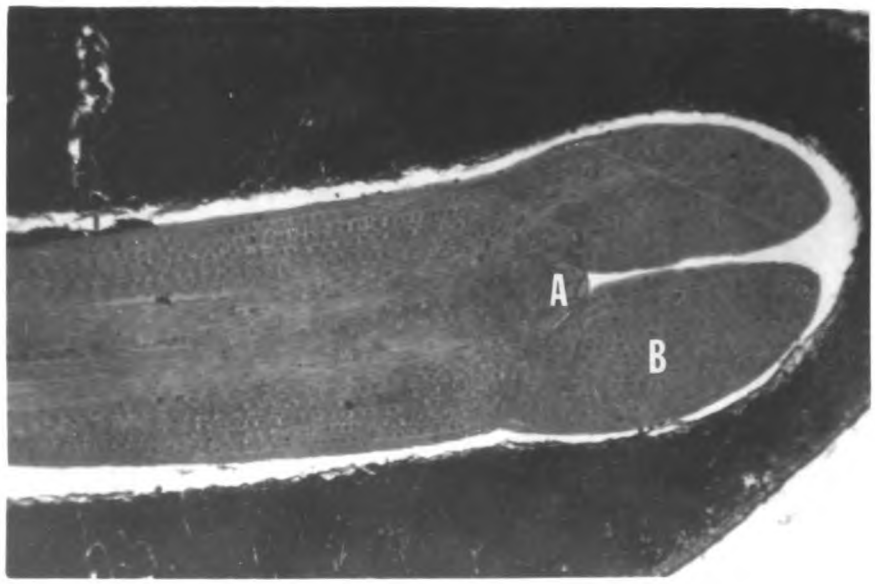

A

Figure 74 - Dead embryo in corrosion cavity of blue spruce ovule 60 days after receiving Engelmann spruce pollen (x175) showing:
A - embryo
B - corrosion cavity
C - female gametophyte tissue

Figure 75 - Ovule from open-pollinated blue spruce strobilus 77 days after pollination date (x70) showing:
A - shoot apical meristem
B - cotyledon primordia


A
B
C


A
B

Additional control-pollinated cones collected 42 days after pollination

Two additional cones were collected 42 days after pollination because they had stopped development, became dried out and broke from the branchlet when touched. Some of the ovules from these cones were also microscopically examined.

The first control-pollinated cone, from bag number 134, contained ovules without pollen or with ungerminated pollen grains in the nucellar cup of the ovules examined. In all the ovules examined from this cone, the nucellar cap had died and had greatly shrunk but the rest of the nucellar tissue toward the chalazal end of the ovule seemed to have completely disintegrated (Figure 76). In these ovules the female gametophyte cavity was completely empty, and the entire ovules were quite small.

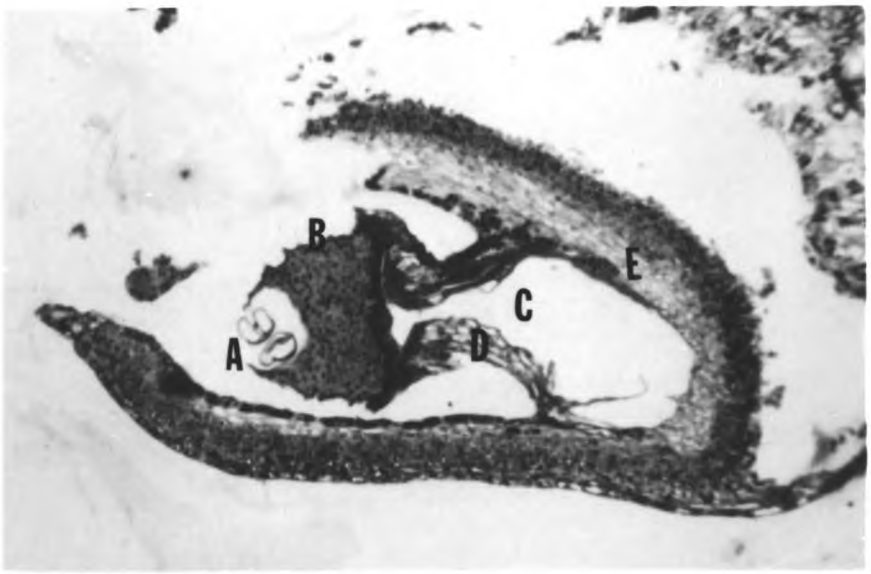
Ovules of the second cone, from bag number 156, showed several abnormalities. In some ovules the female gametophyte had developed a corrosion cavity, and the remnants of empty or necrotic archegonial cavities were evident (Figure 77). In another hybrid ovule the female gametophyte was oval-shaped with enlarged cells that surrounded a dense centrally-located necrotic mass. Another of these ovules contained an abnormal-sized female gametophyte that had proliferated at the micropylar end around an archegonium. The central cell of this archegonium was dislodged from its position by the neck cells and was in the center of the archegonium. The archegonial jacket was also somewhat indistinct. Still another ovule

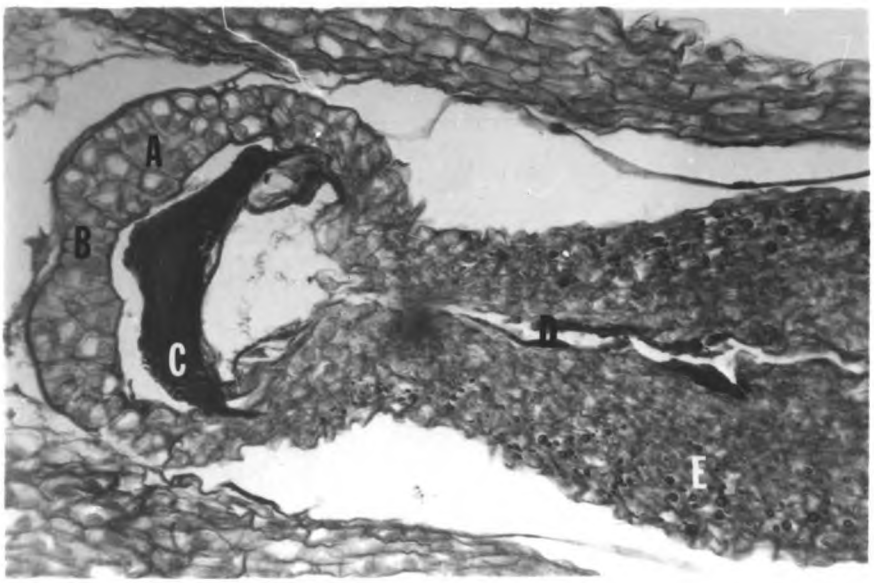
Figure 76 - Blue spruce ovule from cone that stopped developing, collected 42 days after strobilus received Engelmann spruce pollen (x75) showing:

A - ungerminated pollen grains
B - dead and shrunken nucellus
C - empty female gametophyte cavity
D - nucellus
E - integument

Figure 77 - Female gametophyte of blue spruce ovule from cone that stopped developing, collected 42 days after strobilus received Engelmann spruce pollen (x185) showing:

A - hypertrophied female gametophyte cap
B - archegonial neck cells
C - necrotic remnants of archegonia
D - corrosion cavity
E - female gametophyte


A
B
C
D
E


A
B
C
E

from this cone showed evidence of pollen tube penetration deep into the nucellus toward two archegonia which contained masses of scattered chromatin, similar to that found in earlier collections.

Anomalies In Open-Pollinated Blue Spruce Ovules

Not all of the ovules from open-pollinated cones that were microscopically examined were developing. Most of the abnormalities observed could be attributed to a lack of observed pollen, but a few were unexplainable.

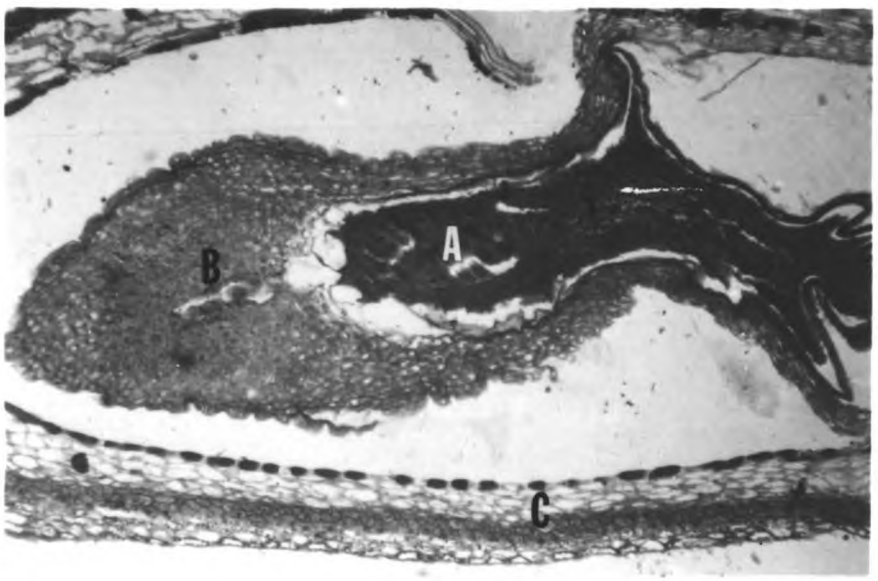
Many of the ovules that did not receive pollen via wind appeared to have degenerated before the female gametophyte became cellular. The nucellus of these ovules underwent progressive deterioration. Very early developmental stages in these unpollinated ovules were not observed, but the later stages were very similar to suspected unpollinated ovules from the control-pollinated cones.

One ovule collected 21 days after pollination, contained pollen growing in the nucellus, but the female gametophyte was somewhat clumped and protruded into one side of the nucellus (Figure 78). This degeneration of the female gametophyte was similar to that observed in unpollinated ovules. In another ovule collected 33 days after pollination, the female gametophyte seemed to have developed normally and at least one pollen tube was observed to have approached the archegonium, but the archegonium appeared segmented and no nuclei could be found (Figure 79). In another female gametophyte, from an ovule collected 39 days after pollination, three archegonia

Figure 78 - Ovule from open-pollinated blue spruce strobilus 21 days after pollination date (x65) showing:
A - clumped, protruded female gametophyte
B - germinated pollen tubes

Figure 79 - Ovule from open-pollinated blue spruce strobilus 33 days after pollination date (x65) showing:
A - segmented archegonium
B - path of pollen tube penetration
C - seed coat


A
B


B
A
C

were found in unusual relationships to each other. One of these archegonia was directly above another (i.e., at the micropylar end of the female gametophyte), and the third archegonium was distal to these two archegonia, located where the corrosion cavity first begins to form and where the embryos would ordinarily have been developing (Figure 80). Pollen was observed to have penetrated as far as the female gametophyte in this ovule, and the cytoplasm of all three archegonia was finely textured as it becomes after the egg has been fertilized. However, no nuclei were observed in any of these three archegonia.

The Reciprocal Cross - Picea engelmannii (Parry) x Picea pungens (Engelm.)

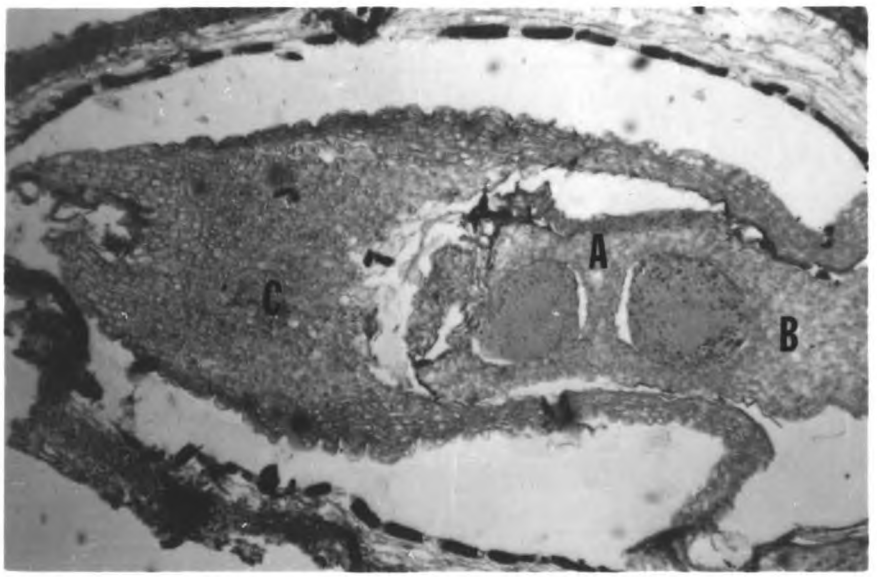
Because of collections of the reciprocally crossed cones, Picea engelmannii (Parry) X Picea pungens (Engelm.) were irregular and widely spaced, ovules from only the second to fourth collections (12, 24 and 30 days after pollination) were examined microscopically. None of the ovules from the open-pollinated Engelmann spruce cones was processed because the absence of other Engelmann spruce trees in the vicinity precluded normal development. A total of 33 ovules from the three control-pollinated cones were examined microscopically (Table 3).

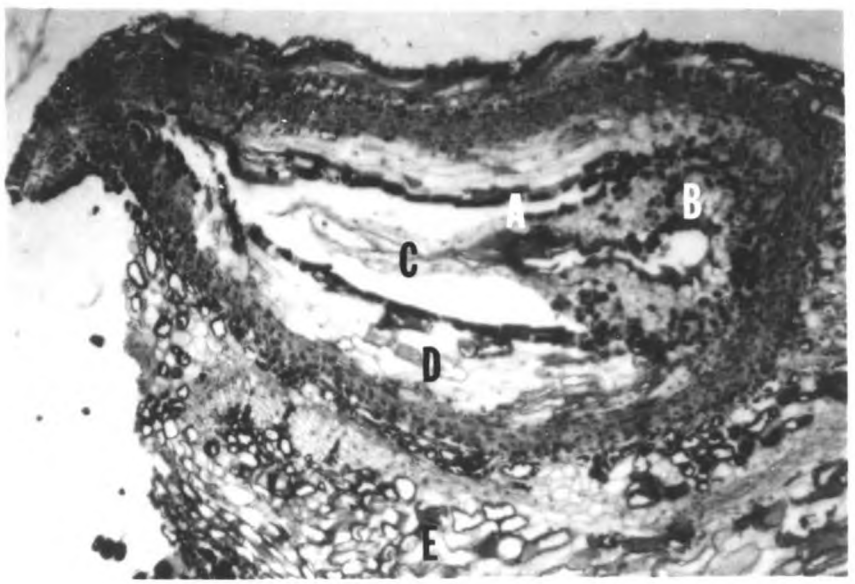
12 days after pollination

Most of the ovules from the control-pollinated cones that were examined, were shrunken with dead nucellar caps and

Figure 80 - Ovule from open-pollinated blue spruce strobilus 39 days after pollination date (x65) showing:
A - two archegonia, one distal of the other
B - female gametophyte
C - nucellus

Figure 81 - Engelmann spruce ovule 12 days after the strobilus received blue spruce pollen (x70) showing:
A - necrotic female gametophyte tissue
B - somewhat normal nucellar tissue with some proliferated cells
C - degenerated nucellus
D - integument


A
B
C


A
B
C
D
E

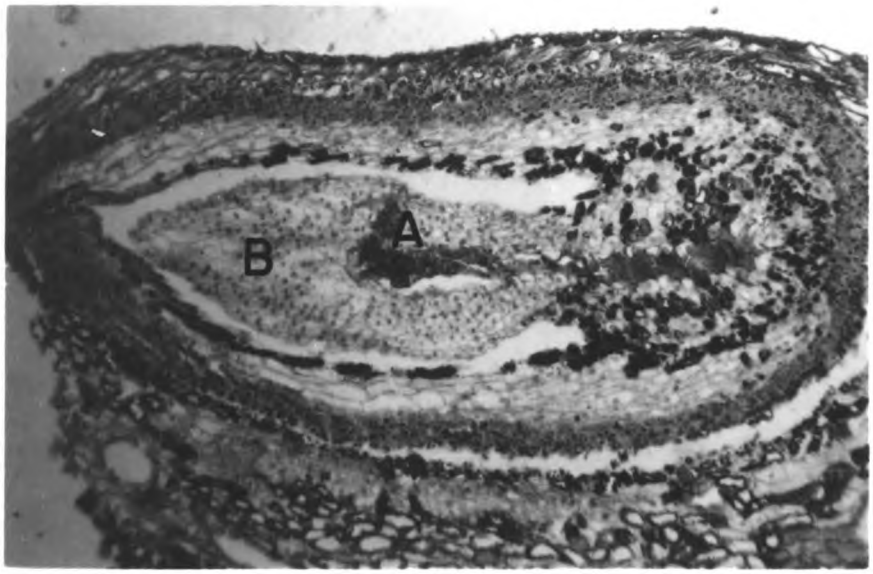
empty or necrotic female gametophyte cavities. These were surrounded by proliferated nucellar tissue at the chalazal end of the ovule (Figure 81). One ovule was observed to have a full-sized, but dead nucellar cap, and a necrotic female gametophyte which had protruded into the nucellus (Figure 82). All of the ovules examined were aborted. The ovules from the reciprocal cross collected on this date (discussed in the previous section) differed from these ovules in that in most ovules from the reciprocal cross the nucellus was normal, no proliferations were evident, and if the female gametophyte had aborted, it was still at the free nuclear stage.

24 days after pollination

All of the Engelmann spruce ovules from the control-pollinated cone collected on this date were aborted. Several ovules contained female gametophytes made up of greatly enlarged and proliferated cells (Figure 83). Several other ovules contained necrotic female gametophytes surrounded by proliferated nucellar tissue at the chalazal end of the ovule (Figure 84). Still another ovule had a dead and shrunken nucellar tip, but the rest of the nucellus and the female gametophyte also, seemed to be in an early stage of condensation and necrosis (Figure 85). Another ovule with a developed female gametophyte appeared to be somewhat normal except that the cells toward the micropylar end, where the archegonia should have been, were proliferated.

Figure 82 - Engelmann spruce ovule 12 days after the strobilus received blue spruce pollen (x65) showing:
A - necrotic and protruded female gametophyte tissue
B - dead but unshrunken nucellus

Figure 83 - Female gametophyte of Engelmann spruce ovule 24 days after strobilus received blue spruce pollen (x195) showing:
A - proliferated female gametophyte tissue
B - nucellus


B
A

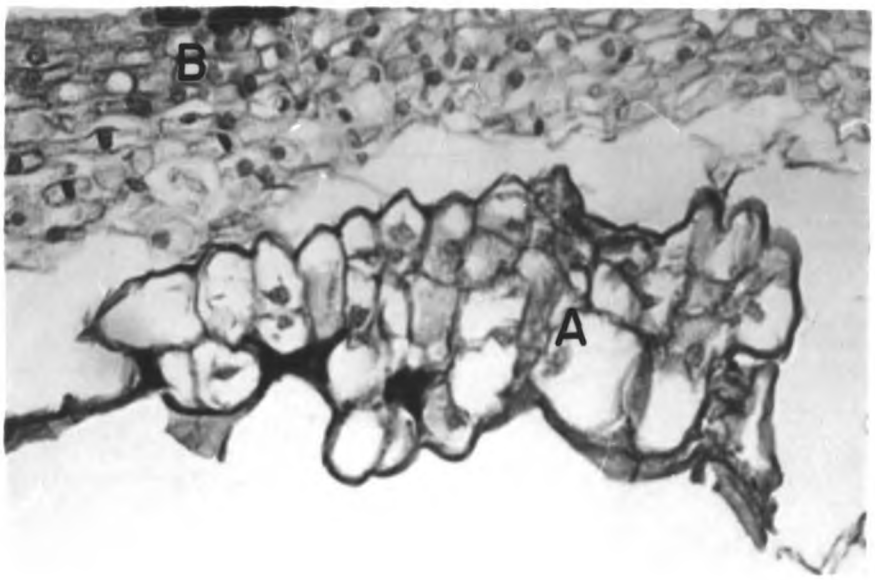
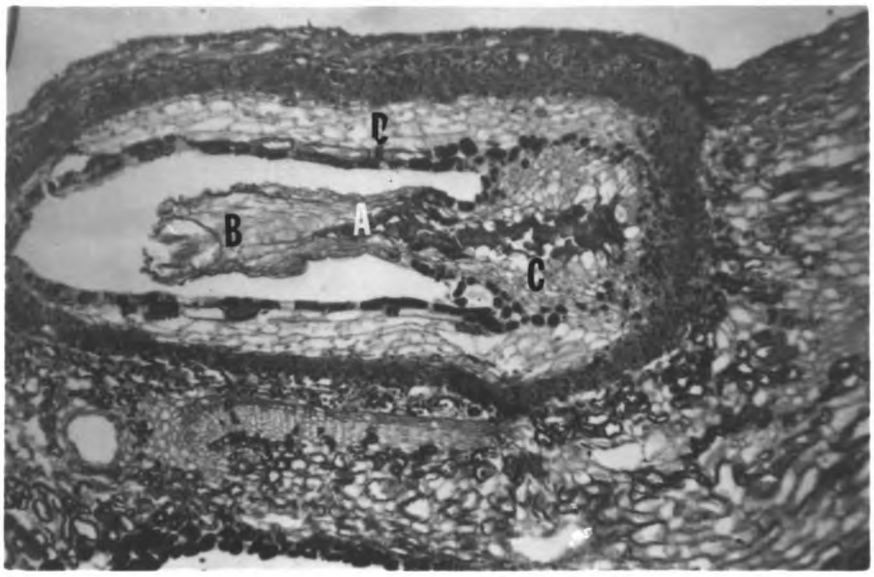
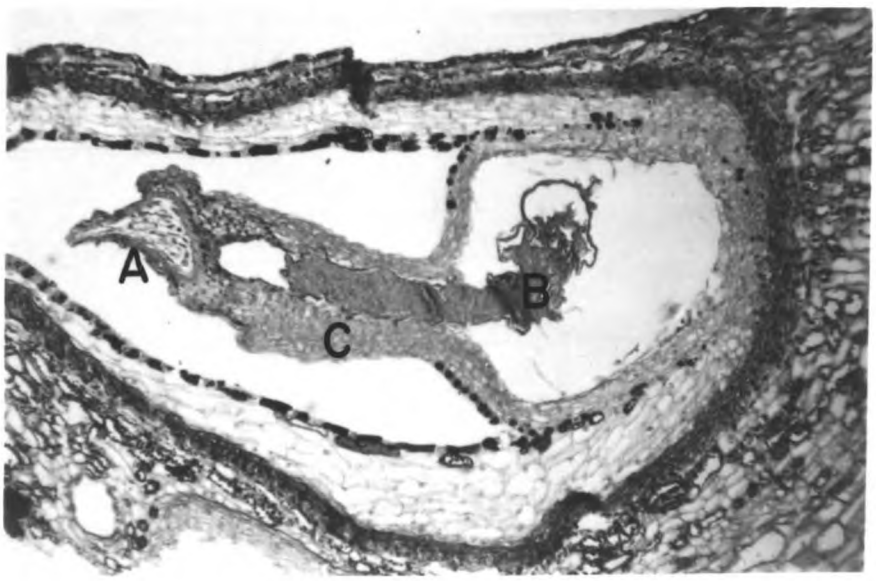

B
A

Figure 84 - Engelmann spruce ovule 24 days after the strobilus received blue spruce pollen (x75) showing:
A - necrotic and somewhat protruded female gametophyte
B - degenerated nucellus
C - proliferated nucellar cells
D - integument

Figure 85 - Engelmann spruce ovule 24 days after the strobilus received blue spruce pollen (x65) showing:
A - necrotic and shrunken nucellar cap
B - female gametophyte
C - nucellus


D
B
A
C


A
B
C

Ovules from control-pollinated blue spruce cones of the reciprocal cross collected on this date were generally a little more developed. In the normal blue spruce ovules, the central cell had divided to form the egg. In the abnormal blue spruce ovules the archegonial jackets were fused with the female gametophyte tissue and the archegonia were breaking up into segments. Other abnormal hybrid blue spruce ovules contained archegonia with gigantic protein bodies. Others were very narrow with small female gametophytes and seemed to contain inadequate tissue for archegonia to be initiated.

30 days after pollination

By this time, the Engelmann spruce ovules, from cones pollinated with blue spruce pollen, had developed far enough to contain archegonia, but those archegonia observed appeared to be deteriorated. Some archegonia were found to contain the scattered chromatin which was common in ovules of the reciprocal cross collected 36 days after pollination.

Engelmann spruce ovules were observed from all three of these collections in which the female gametophyte was composed entirely of large cells (Figure 83).

Length And Width Of Open And Control-Pollinated Blue Spruce Cones

Measurements of the length and width of the control-pollinated and open-pollinated collection cones (See Appendix) show that the control-pollinated cones were generally longer than the open-pollinated cones.

Seed Yield And Germination Of Picea pungens (Engelm.) Cones

The average number of seeds per control-pollinated cone was 278.54 and the average number of seeds per open-pollinated cone was 302.50. By a T-test, with unequal sample variances, this difference was not significant at the .05 level. Based on 33 samples of 100 seeds each, from different cones, seeds from the control-pollinated cones showed a .48 percent average germination. Twenty-six of the 100 seed samples had no germinations and seven samples had germinations from 1 to 5 percent. If the tip of the radicle emerged from the seed, it was considered to have germinated. Based on 37 samples of 100 seeds each, from different cones, seeds from the open-pollinated cones showed an average of 42.41 percent germination.

CHAPTER IV

DISCUSSION

To this date, no one has proposed a genetic mechanism to explain interspecific incompatibility in gymnosperms, and very few microscopic studies of ovule development following interspecific pollination have been made. Only one such study, which was quite comprehensive for the genus, has been conducted in spruce, and this study did not include blue spruce or Engelmann spruce (Mikkola, 1969). In the present study blue spruce ovules receiving Engelmann spruce pollen were usually not fertilized. Very often the female gametophyte developed to the central cell or egg stage, but the pollen seemed not to grow rapidly to the archegonium and the archegonium degenerated.

Unpollinated Ovules

The deterioration of unpollinated ovules occurring usually before the female gametophyte became cellularly organized, is indicative of the necessity of pollen for the development of the female gametophyte. Mikkola (1969) found that the presence of pollen, even ungerminated, was sufficient to stimulate development of most female gametophytes to the egg stage. In contrast, many of the blue spruce female gametophytes in this study deteriorated before becoming cellular even though pollen grains were observed growing in the nucellus. Perhaps

the receptivity of the strobilus (erect position with scales open) is longer than receptivity of some of the ovules. If this were the case, those ovules that degenerated at an early stage, even with pollen present in their nucellar cup, may simply have passed their individual receptivity period and any stimulus received from the pollen was then too late and thus ineffective in stimulating female gametophyte development. It is doubtful that the early degeneration of the female gametophyte was due to an improper stimulus by the Engelmann spruce pollen or a stimulus different from that which a blue spruce pollen grain may have given, because many blue spruce ovules with Engelmann spruce pollen did develope normally up to the egg stage.

Deterioration of the female gametophyte in ovules of the 1969 study, that apparently did not receive Engelmann spruce pollen (Figure 20), was somewhat different from those in which the entire cone was void of pollen (Figures 5, 6 and 7), as in the 1970 study. Thus one is led to believe that the presence of pollen in some ovules in the blue spruce cone may have an effect on other ovules of that cone that did not receive pollen. This is the case in blue spruce where the early cellular female gametophyte in ovules from a completely unpollinated cone may become condensed, protrude into the nucellus and become necrotic (Figures 6, 7 and 8). Those ovules in control-pollinated cones that did not catch pollen before the cellular stage was organized, became necrotic, but rarely protruded into the nucellus (Figures 20 and 47). Presumably one

effect of the presence of pollen in the cone generally, might be the continued development of unpollinated ovules. This may or may not have occurred in this study because it was not always determined if pollen had penetrated the nucellus or been present in ovules that had been developing at the time of fertilization. Mikkola (1969) recognized three conditions for unpollinated ovules: 1) If the strobilus were unpollinated, very few of the ovules enlarged. 2) If half or fewer ovules of a strobilus received pollen, many of the unpollinated ovules enlarged. If many of the ovules of a strobilus received pollen, then very few of the unpollinated ovules of that strobilus enlarged and continued development.

McWilliam (1959) observed that unpollinated Austrian pine ovules deteriorated at the early free nuclear stage, accompanied by proliferation of the surrounding spongy tissue, as early as three weeks after pollination. Proliferation of the nucellar tissue surrounding the female gametophyte cavity was also a very common phenemenon in the unpollinated blue spruce ovules used in this study (Figure 10), and in the pollinated ovules showing early degeneration of the female gametophyte (Figures 25, 28 and 50).

Mikkola's (1969) paper included photomicrographs of degenerated spruce ovules that received no pollen. He indicated that an unpollinated ovule would progress from an enlarging empty female gametophyte cavity with a full sized nucellus to one in which the nucellus and female gametophyte cavity were greatly shrunken at the chalazal end of the ovule. The

unpollinated blue spruce ovules did not follow this kind of pattern, but rather, followed two patterns for the two kinds of ovules Mikkola (1969) described. Unpollinated blue spruce ovules similar to early stage unpollinated ovules in Mikkola's work (1969) showed progressive dying of the nucellus and emptying of the cells (Figures 28, 38, 41 and 49). Eventually a narrow layer of collapsed nucellar cells represented the nucellar tissue. Little shrinkage toward the chalazal end occurred. Some unpollinated blue spruce ovules probably started out, within a week after pollination, with dead and greatly shrunken nucellar caps (Figure 25 and 29). This condition Mikkola (1969) described in unpollinated ovules of later developmental stages. Shrinkage occurred up to the nucellar zone of division and ended abruptly. From this stage the ovular contents of blue spruce ovules continued to shrink until they occupied only a small portion of the ovule at the chalazal end.

Mikkola (1969) stated, in an uncited reference, that the tip and basal scales of the cone usually contain sterile or unfilled seed. This sterility is apparently predetermined, thus it is expected that the presence of pollen would not stimulate ovule development in those locations. One should not assume that the sterility of the tip and basal scale ovules is due to a lack of pollen because there is no apparent cause for discrimination of pollen dispersion to them. Because of the earliness of the necrotic, shrunken condition of the nucellus of some of the blue spruce ovules used in this study (Figure 25,

15 days after pollination), it would seem that their inviability was predetermined. No environmental influence had been operating on these ovules for a long enough period of time to promote such a completely degenerated condition. One cannot help drawing a parallel between these ovules and those of the tip and basal scales of a cone, both of which seem to show early predetermined sterility.

Picea pungens (Engelm.) X Picea engelmannii (Parry)

The development of Engelmann spruce pollen tubes in the blue spruce ovules seemed to follow the general pattern of incompatibility that Mikkola (1969) observed in his spruce hybridizations where fertilization usually did not occur. The pattern in this study was for the Engelmann spruce pollen tubes to grow slowly and often die before reaching the archegonium (Figures 52 and 62). Death was evident since a dark staining mass was formed at the furthest point of pollen tube penetration in the nucellus.

No one has previously reported the unusual segmentation (in interspecifically crossed ovules) of the female gametophyte that occurred in the blue spruce ovules after the archegonia had been formed (Figures 51 and 52). Often, if this segmantation or early phases of it were present, the portion of the female gametophyte distal from the archegonia was small and narrow. This condition probably precluded corrosion cavity formation. Segmentation was observed in ovules from more than one control-pollinated cone and is probably one of several patterns of breakdown that can occur from incompatible crosses.

Another pattern of breakdown in interspecifically crossed ovules in which the female gametophyte developes occurs after the eggs have been receptive but are not fertilized. While the egg is degenerating the archegonial jacket begins to breakdown and the archegonia and egg fuse with the surrounding female gametophyte tissue (Figure 52). Later the archegonial tissue becomes necrotic and the walls of adjacent archegonia breakdown (Figure 60). Soon all that remains is a necrotic mass located in the former position of the archegonia (Figure 61). Often the female gametophyte tissue proximal to and including the neck cells becomes hypertrophied (Figure 65). Where this occurs the corrosion cavity often forms and eventually a large female gametophyte with a corrosion cavity and often with a hypertropied female gametophyte cap is present in the ovule (Figure 70). This condition was noted as late as 56 days after pollination, so it is not known if the female gametophyte then shrinks and degenerates, or maintains this condition up until seed is shed. Mikkola (1969) reported that in the interspecifically crossed spruce ovules used in his study in which the female gametophyte continued to develope and formed a corrosion cavity the tissue eventually died and shrunk before the seeds were mature in the fall.

A third developmental pattern occurred in some ovules in which the female gametophyte developed to a large size, but archegonia were not initiated. In these ovules the female gametophyte tissue proliferated and often became necrotic in the area where the archegonia would normally have occurred

(Figures 58, 59 and 63). Presumably corrosion cavities did not form in these female gametophytes. It is believed that the presence of archegonia is required before a corrosion cavity can be developed.

Within interspecifically crossed ovules in which the female gametophyte and archegonia developed, several interesting abnormalities occurred. Hitherto no one has reported the occurrence of what appeared to be chromatin or nucleic material scattered throughout the cytoplasm of unfertilized eggs (Figure 55). Mikkola (1969) explained that as an unfertilized egg degenerates, it first expands (Figures 53 and 57). Often in these spruce ovules, the egg was still intact, so its degeneration or scattering could not account for the source of the scattered chromatin. This condition was observed in at least nine ovules from control-pollinated cones.

A few interspecifically crossed ovules contained archegonia with several nuclei (Figure 54). Many of these were the size of the egg or larger and their occurrence is unexplained.

In a very few interspecifically crossed ovules the archegonia seemed to be filled with gigantic protein bodies (Figure 40). At this time there seems to be no explanation for this condition.

It is interesting to note that when McWilliam (1959) crossed Austrian pine and red pine, 70 percent of the pollen did not germinate or germinated poorly and the ovules broke down by the end of the first season while still in a free

nuclear condition. This breakdown in pine was similar and occurred proportionately earlier in the developmental period than that in spruce where the female gametophyte became cellular at the initial time of breakdown. Whether or not any evolutionary significance should be attached to this generic difference is not clear.

The zone of division and growth of the nucellus is found about 2/3 to 3/4 of the distance through the nucellus. This particular area of the ovule was involved in many of the morphological changes that occurred in the degenerating blue spruce ovules. In ovules from unpollinated cones, a break occurred in this zone in what appeared to be a normal healthy nucellus (Figure 4). Perhaps this is a zone of weakness and the break occurred during processing. In ovules from both control-pollinated and unpollinated cones the nucellar cap died and became greatly shrunken at an early stage of development. Shrinkage of the nucellar cap did not occur all the way up to the female gametophyte cavity, but rather stopped abruptly at or in the nucellar zone of division (Figures 9, 25 and 29). In ovules from unpollinated cones, three weeks after pollination should have occurred, a constriction appeared in the nucellus at this point (Figure 17). When the female gametophyte protruded into the nucellus it protruded only as far as the nucellar zone of division and stopped (Figures 14, 16 and 28). There are some important characteristics of this area of the nucellus, no doubt biochemical in nature, which probably cause the above morphological abnormalities to develop in degenerating ovules.

Many of the interspecifically crossed blue spruce ovules contained female gametophytes that became necrotic and protruded into the nucellus before growing very large (Figures 21 and 18). Occasionally a few enlarged female gametophyte cells would appear in the necrotic mass of protruding tissue. Initially the nucellus in these ovules was normal but then underwent progressive degeneration (Figures 21 and 28). Sometimes an ovule of this type was found in which the nucellar cap died and shrunk up to the nucellar zone of division (Figure 29).

Other ovules observed 21 days after being control-pollinated had simply stopped developing and appeared to be at the late free nuclear stage of development (Figure 34). This is the stage that they had probably reached at pollination time.

Picea engelmannii (Parry) X Picea pungens (Engelm.)

Development of Engelmann spruce ovules from control-pollinated cones was similar to that of the blue spruce from control-pollinated cones in that most of them produced a necrotic female gametophyte that protruded into the nucellar tissue (Figures 82, 84 and 81). Some of the Engelmann spruce ovules did produce female gametophytes consisting entirely of gigantic cells, some of which appeared to have two or more nuclei (Figure 83). This condition was not observed in blue spruce ovules. In some Engelmann spruce ovules the nucellar cap had died and shrunk as was the case in some ovules from the control-pollinated blue spruce cones. In other ovules, underdeveloped cellular female gametophytes were produced in ovules in which the nucellar cap had died and was shrunken.

This combination of a dead and shrunken nucellar cap and a cellular female gametophyte was not observed in any blue spruce ovules (Figure 85).

This study substantiates the work of Fechner and Clark (1968) in which they stated that low seed set in the reciprocal crosses between blue and Engelmann spruce was due to a definite barrier to crossing. Surely the control of this incompatibility barrier lies in a genetic scheme, that is manifest in biochemical reactions resulting in the observed morphological abnormalities.

The present morphological study has established the approximate time of incompatibility reactions between these two species. Hopefully, a biochemical study of the tissues involved could add more information to the understanding of this situation.

If spruce hybridization is to be more successful, experiments attempting to overcome the incompatibility barrier should be investigated. These could include such methods as application of growth substances to the strobili or X-ray treatment of pollen.

The author believes that further investigations of factors affecting crossability between blue spruce and Engelmann spruce should yield basic information concerning the mechanisms of interspecific incompatibility in spruces particularly and gymnosperms in general. It seems doubtful that an effective and economic method of overcoming the existing barrier to crossing between these two species of spruce will be found in the near future.

CHAPTER V

SUMMARY AND CONCLUSION

Summary

In the spring of 1969, reciprocal crosses were made between blue spruce and Engelmann spruce. Collections from the Engelmann spruce mother tree were made at irregular dates. One open-pollinated and one control-pollinated cone were collected every third day beginning May 12, 1969, from the blue spruce mother tree until July. After July 14, 1969, weekly collections were made. In 1970, 12 blue spruce strobili were isolated, and one unpollinated strobilus was collected every two days. After killing, individual ovules were sectioned on a rotary microtome for microscopic examination. Thirty-three control-pollinated blue spruce cones which had not been collected during the summer and 38 open-pollinated blue spruce cones were used for seed set and germination studies.

Unpollinated blue spruce strobili

Ovules from unpollinated strobili began to breakdown approximately nine days after the mid-period of receptivity of the strobilus. Breakdown usually began in the early cellular female gametophyte as a clumping and protrusion of this tissue into the nucellus. Following this protrusion, the nucellar cap gradually died. The nucellar tissue, at the

chalazal end, characteristically became proliferated around the female gametophyte cavity, but nucellar cells outside this immediate area were the last to degenerate.

Blue spruce X Engelmann spruce

Sections of 783 interspecifically crossed ovules were examined in this part of the study. It was found that the presence of germinated Engelmann spruce pollen in a blue spruce ovule did not necessarily guarantee full development of the female gametophyte. All levels of pollen viability were observed, but the main cause of inviable seed from this cross seemed to be due to the early death of germinated pollen, or to slowed pollen tube growth, such that fertilization never occurred. Many interspecifically crossed ovules developed to the egg stage. Beyond this stage in development, if the egg was not fertilized, two paths of ovule breakdown were common: 1) The archegonia and surrounding female gametophyte became segmented, then necrotic and the tissues deteriorated. In these ovules the female gametophyte usually did not form a corrosion cavity. 2) The archegonia became necrotic and the jacket cells deteriorated. A large empty space or a necrotic mass formed where the archegonia had been. The female gametophyte, however, continued to develop, forming a large volume of nutritive tissue with a well-developed, but empty, corrosion cavity, and often a hypertrophied female gametophyte cap. Abnormalities within the archegonia before degeneration included the presence of apparently gigantic protein bodies,

the presence of additional nuclei, and the presence of additional scattered chromatin-like material.

Frequently, interspecifically crossed ovules developed female gametophytes, but no archegonia. The ovules of this type usually became necrotic or the cells proliferated in the area where the archegonia should have been located.

Ovules from control-pollinated blue spruce strobili that apparently did not receive pollen usually aborted at about the same time as the blue spruce ovules from unpollinated strobili, nine days after pollination. The sequence of breakdown of tissues was similar in both kinds of unpollinated ovules except that the female gametophyte looked somewhat differnet in initial breakdown stages and often did not protrude into the nucellus. Rather, the female gametophyte became a small necrotic mass in the center of the female gametophyte cavity and remained as such for several weeks while the nucellus progressively deteriorated.

Engelmann spruce X blue spruce

Only the first three control-pollinated cones, 12, 24 and 30 days after pollination were processed from this cross. Three progressive stages of nucellar breakdown were observed in ovules in which the female gametophyte was necrotic and often protruded. In these stages the chalazal nucellar cells remained normal except for proliferation of those cells immediately surrounding the female gametophyte cavity. Two conditions were observed in Engelmann spruce ovules from cones that had received blue spruce pollen that were not found in the

reciprocal cross: 1) In some ovules from both reciprocal crosses a condition whereby the nucellar cap died and was greatly shrunken was observed. This condition in the interspecifically crossed Engelmann spruce ovules was also found in conjunction with a well-developed female gametophyte. In the blue spruce ovules with this condition, the female gametophyte was always necrotic and often protruded. 2) Some interspecifically crossed Engelmann spruce ovules contained only a few very large cells representing the female gametophyte.

Conclusion

There is a mechanism, probably genetic in nature and ultimately biochemical, which inhibits development of reciprocal pollen of blue spruce and Engelmann spruce on or in the foreign nucellus. This influence on the pollen may interfere with the normal development of the ovule up to the egg stage, and the lack of fertilization, in most ovules, precludes the development of viable seed. The fact that ovules from blue spruce strobili without pollen began to degenerate as early as nine days after the mid-period of strobilus receptivity attests to the necessity of pollen for normal ovule development.

LITERATURE CITED

Anhaeusser, H. 1953. Keimung und Schlauechwachstum des Gymnospermenpollens unter besonderer Berucksichtigung des Wuchastoffproblems. Beitr. Biol. Pflanzen 29(3) 297-338. In "Biological Abstracts" 28, Part 1, Abstract No. 4391.

Bailey, L. H. 1949. Manual of cultivated plants. The MacMillan Co. New York. 1116 pp.

Bailey, L. H. 1963. The standard cyclopedia of horticulture The MacMillan Co. New York. Volumes I, II and III. 3639 pp.

Barnes, Burton V., R.T. Bingham, and A. E. Squillace. 1962. Selective fertilization in Pinus monticola Dougl. II. Results of Additional Tests. Silvae Genet. 11: 103-111.

Bhattacharyja, S. S. and H. F. Linskens. 1955. Recent advances in the physiology of self-sterility in plants. Science and Culture 20: 370-373.

Brewbaker, James L. 1957. Pollen cytology and self-incompatibility systems in plants. J. Heredity 48: 271-277.

Bubar, J. S. 1959. Differences between self-incompatibility and self-sterility. Nature (Lond.) 183: 411-412.

Crowe, Leslie K. 1954. Incompatibility in Cosmos bipinnatus. Heredity 8: 1-11.

Emerson, Sterling. 1940. Growth of incompatible pollen tubes in Oenothera organensis. Bot. Gaz. 101: 890-911.

Fechner, Gilbert H. 1955. Preliminary study of the viability and vitality of certain Rocky Mountain tree species. Master's Thesis, Colorado State University, Fort Collins. 85 pp.

_____. 1964. The reproductive cycle of Picea pungens Engelmann. PhD Thesis, University of Minnesota, St. Paul. 199 pp.

_____. and Roger W. Clark. August 8-10, 1968. Preliminary observations on hybridization of Rocky Mountain spruces. Proc. Meeting, Comm. on Forest Tree Breeding in Canada. 11: 237-247.

Fowells, H. A. 1965. Silvics of forest trees of the United States. U.S.D.A. Agr. Handbook No. 271. 762 pp.

Gardella, Catherine. 1950. Overcoming barriers to crossability due to style length. Amer. J. Bot. 37: 219-224.

Hagman, Max and Lauri Mikkola. 1963. Observations on cross-, self-, and interspecific pollinations in Pinus peuce Griseb. Silvae Genetica 12(3): 73-79.

Hagman, Max. 1963. Incompatibility in Betula verrucosa Ehrh. and Betula pubescens Ehrh. Genetics Today. S. J. Geerts, editor. Vol. 1 Abstracts p. 211.

Hagman, Max. 1967. Serological studies of pollen, and the incompatibility in forest trees. Proc. XIV IUFRO Kongress, München III Section 22. pp. 60-71.

Klaehn, Friedrich Ulrich and William P. Wheeler. 1961. X-Ray study of artificial crosses in Picea abies (L.) Karst. and Picea glauca (Moench.) Voss. Silvae Genet. 10: 71-77.

Kriebel, Howard B. 1967. The timing of the incompatibility reaction in interspecific crosses of Pinus strobus L. Proc., XIV IUFRO Kongress, München VIII Section 22. pp. 77-87.

Kriebel, H. B. 1970. Embryo development and hybridity barriers in the white pines (Section strobus). To be published in Silvae Genet.; published in condensed form in the Proc. of the Meeting of the IUFRO Section 22, Working group on sexual reproduction of forest trees, Varparanta, Finland 1970.

Kroh, M. 1966. Reaction of pollen after transfer from one stigma to another (Contribution to the character of the incompatibility mechanism in Cruciferae). Züchter/ Genet. Breed. Res. 36(4): 185-189.

Lewis, D. 1952. Serological reactions of pollen incompatibility substances. Proc. Roy. Soc. Series B. 140: 127-135.

Linskens, H. F. and K. Esser. 1959. Stoffaufnahme der pollen schläuche aus dem leitgewebe des griffels. Proc. Dutch Academy of Science. Series C. 62(2): 150-154. (English Summary).

Linskens, H. F. 1961. Biochemical aspects of incompatibility. Recent Advances in Botany. 2: 1500-1503.

Linskens, H. F. 1963. Biochemistry of incompatibility. Proc., XI International Congress of Genetics. 3: 629-636.

Linskens, H. F. and J. Tupy. 1966. The amino acids pool in the style of self-incompatible strains of Petunia and self- and cross-pollination. Der Züchter 36(4): 151-158.

Little, E. L. 1953. Check List of Native and Naturalized Trees of the United States. U.S.D.A. Handbook No. 41. 472 pp.

Lundquist, A. 1954. Studies on self-sterility in rye, Secale cereale L. Hereditas 40: 278-294.

McWilliam J. R. 1959. Interspecific incompatibility in Pinus. Amer. J. Bot. 46 (6): 425-433.

McWilliam, J. R. 1960. Pollen germination of Pinus as affected by the environment. For. Sci. 6: 27-39.

Michalski, L. 1967. Growth regulators in the pollen of pine (Pinus silvestris L.). Acta Scietatis Botanicorum Poloniae 36 (3): 475-481.

Mikkola, Lauri. 1969. Observations on interspecific sterility in Picea. Ann. Bot. Fennici 6: 285-339.

Orr-Ewing, A. L. 1957a. Further inbreeding studies with Douglas-fir. Forest. Chron. 33: 318-332.

_____. 1957b. A ctyological study of the effects of self-pollination on Pseudotsuga menziesii (Mirb.) Franco. Silvae Genet. 6: 179-185.

Richens, R. H. 1945. Forest Tree Breeding and Genetics. Imperial Agr. Bureau Joint Publication No. 8 Imperial Bureau of Plant Breeding and Genetics. Cambridge, England. 79 pp.

Roggen, H. P. J. R. and L. F. Linskens. 1967. Pollen tube growth and respiration in incompatible intergeneric crosses. Sonderuck aus der Zeitschrift die Naturwissenschaften 20: 524-543. Springer-Verlag, New York.

Roggen, H. P. J. R. and R. G. Stanley. 1969. Cell-wall-hydrolysing enzymes in wall formation as measured by pollen-tube extension. Planta (Berl.). 84: 295-303.

Sears, E. R. 1936. Cytological phenomena connected with self-sterility in the flowering plants. Genetics 22: 130-181.

Stanley, Robert G. 1967. Factors affecting germination of the pollen grain. Proc. XIV IUFRO Kongress, München, III Section 22. pp. 38-59.

Stanley, Robert G. 1970. Biochemical approaches to forest genetics. International Review of Forestry Research 3: 253-300.

Squillace, A. E. and R. T. Bingham. 1958. Selective fertilization in Pinus monticola Dougl. I Preliminary Results. Silvae Genet. 7: 188-196.

Sudworth, George B. Feb. 19,1916. The spruce and balsam fir trees of the Rocky Mountain Region. U.S.D.A. Bull. No. 327, 43 pp.

Tupý, Jaroslav. 1963. Changes in pollen proteins during pollen tube growth from the incompatibility point of view. Genetics Today. S.J. Geerts, editor. Vol. 1 Abstracts p. 212.

Van Der Pluijm, J. and H. F. Linskens. 1966. Fine structure of pollen tubes in the style of Petunia. Züchter/Genet. Breed. Res. 36 (5): 220-224.

Vasil, I. K. 1964. Effect of boron on pollen germination and pollen tube growth. In "Pollen Physiology and Fertilization." H. F. Linskens, editor. pp. 108-119. North-Holland Publ., Amsterdam.

Vidakovic, M. and B. Jurkovic-Bevilacqua. 1970. Observations on the ovule development following cross pollination between Austrian and Scots pines using irradiated and non-irradiated pollen. Proc. of the Meeting of the IUFRO Section 22, Working group on sexual reproduction of forest trees, Varparanta, Finland 1970.

Willemse, M. Th. M. and H. F. Linskens. 1969. Development du microgametophyte chez le Pinus sylvestris entre la meiose et la fecondation. Rev. Cytol. et Biol. veg., 32: 121-128. (English summary).

Wright, Jonathan W. 1962. Genetics of forest tree improvement. Food and Agriculture Organization of the United Nations. FAO Forestry and Forest Products Studies No. 16. Rome. 399 pp.

Wright, J. W. 1955. Species crossability in spruce in relation to distribution and taxonomy. Forest Science. 1(4): 319-349.

APPENDIX

TREE DESCRIPTIONS

The blue spruce mother tree used for the control- and open-pollinated collections in this study is located south of the Forestry Building on the Colorado State University campus. On March 30, 1971, it was 37 feet tall, 9.6 inches DBH (4.5 feet above ground level) and contained 31 growth rings at breast height. The maximum crown width was 14 feet.

The Engelmann spruce tree used in this study is located directly north of the Music Building on the Colorado State University campus. On March 30, 1971, it was 45 feet tall, 16 inches DBH and contained 47 growth rings at breast height. The maximum crown width was 22 feet.

The blue spruce mother tree used for the study of unpollinated ovules is located south of the front door to Dunn Elementary School on Washington Street in Fort Collins, Colorado. On March 30, 1971, this tree was 29 feet tall, 11 inches DBH and contained 19 growth rings at breast height. The maximum crown width was 16 feet.

INITIAL COLLECTION DATA

on Picea pungens Engelm. #50 female cones

artificially and wind pollinated

Bag No.	Date of collection	Collection Number p or w[1]	Length in centimeters	Width in centimeters[2]	Color of cone[3]	Position on tree	Remarks

[1] p = pollinated May 12, 1969 and May 15,1969 with Pi eng #9 pollen. w = wind pollinated and unbagged.

[2] midpoint diameter.

[3] Munsell Color Charts for Plant Tissues.

Killing and Fixing Fluids

Craf III killing fluid was used on all material collected for this study. Most commonly the material was dehydrated to 70 percent ethyl alcohol and stored in the refrigerator.

Craf III

A - 300 cc 1% chromic acid
200 cc 10% acetic acid

B - 100 cc formalin (40% formaldehyde)
400 cc distilled water

Dehydration, Infiltration, and Embedding Schedule

All material was handled according to the following schedule. Different equipment was used in the embedding process.

Medium	Length of time
1. Dehydration	
a. 5% ethanol	15 minutes
b. 10% ethanol	20 minutes
c. 16% ethanol; 2% n-butanol	30 minutes
d. 23% ethanol; 7% n-butanol	45 minutes
e. 28% ethanol; 15% n-butanol	1 hour
f. 30% ethanol; 27% n-butanol	2 hours
g. 30% ethanol; 40% n-butanol	Overnight
h. 27% ethanol; 55% n-butanol	2½ hours
i. 21% ethanol; 70% n-butanol	3 hours
j. 12% ethanol; 85% n-butanol	3 hours
k. 100% n-butanol	2 hours
l. 100% n-butanol	Overnight
2. Infiltration	
a. 1 part parowax : 1 part 100% n-butanol	Until sinking
b. parowax	1 to 2 hours
c. parowax	1 to 2 hours
d. hard paraffin (56° to 58°C melting)	Overnight
3. Embedding	
a. cast in aluminum boats	
b. cast in Tissue Tek embedding rings	

MOLLIFLEX - SOFTENING AGENT

Molliflex was purchases from:

Gallard Scheisinger Chemical Manufacturing Company
584 Minneola Avenue
Carle Place
New York 11514

The catalog number for Molliflex is 3273. In February, 1970, the cost of Molliflex was $3.00 per 500 ml.

Staining Schedules

Fluid	Length of time
Safranin O - fast green	
1. Xylene	5 minutes
2. Xylene	5 minutes
3. 95% ethanol	5 minutes
4. 1% safranin O in 95% ethanol	1 minute
5. 95% ethanol	Several dips
6. 95% ethanol	Several dips
7. 1% fast green in 95% ethanol	30 seconds
8. 95% ethanol	Several dips
9. Absolute ethanol	Several dips
10. 1 absolute ethanol : 1 xylene	10 minutes
11. Xylene	5 minutes
12. Xylene	5 minutes
13. Mount in Euparal	
Heidenhain's Iron Hematoxylin	
1. Xylene	5 minutes
2. Xylene	5 minutes
3. 1 Xylene : 1 absolute ethanol	5 minutes
4. Absolute ethanol	3 to 4 minutes
5. 95% ethanol	2 minutes
6. 90% ethanol	2 minutes
7. 80% ethanol	2 minutes
8. 70% ethanol	2 minutes
9. 50% ethanol	2 minutes

Heidenhain's Iron Hematoxylin, continued

10. 35% ethanol	2 minutes
11. 20% ethanol	2 minutes
12. 10% ethanol	2 minutes
13. Distilled water	3 minutes
14. 4% aqueous ferric ammonium sulfate	10 to 30 minutes
15. Running tap water	10 to 15 minutes
16. Distilled water	3 minutes
17. 0.5% aqueous hematoxylin	5 to 30 minutes
18. Running tap water	10 minutes
19. Saturated picric acid	20 to 30 minutes
20. Running tap water	20 to 30 minutes
21. 20% ethanol	3 to 5 minutes
22. 50% ethanol	3 to 5 minutes
23. 70% ethanol	3 to 5 minutes
24. 95% ethanol	3 to 5 minutes
25. 1% fast green in 95% ethanol	30 seconds
26. 95% ethanol	3 to 5 minutes
27. Absolute ethanol	3 to 5 minutes
28. 1 clove oil : 1 absolute ethanol	30 seconds
29. 1 absolute ethanol : 1 xylene	5 minutes
30. Xylene	5 minutes
31. Xylene	5 minutes
32. Mount in Euparal	

Conants Quadruple stain

1. Xylene	5 minutes
2. Xylene	5 minutes
3. 1 Xylene : 1 absolute ethanol	5 minutes
4. Absolute ethanol	2 minutes
5. 95% ethanol	2 minutes
6. 70% ethanol	2 minutes
7. 50% ethanol	2 minutes
8. 1% safranin 0 in 95% ethanol	24 hours
9. 2 drops HCl/100 ml. 50% ethanol	Several dips
10. Tap water	Several dips
11. Tap water	Several dips
12. .25% crystal violet in water	5 to 10 seconds
13. 50% ethanol	2 minutes
14. 70% ethanol	2 minutes
15. 95% ethanol	2 minutes
16. Absolute ethanol	2 minutes
17. Orange II + Fast green	1 to 5 minutes
18. Clove oil	5 minutes
19. 75% Clove oil : 25% Xylene	5 minutes
20. 25% Clove oil : 75% Xylene	5 minutes
21. Xylene	5 minutes
22. Xylene	5 minutes
23. Mount in Euparal	

Table 1.--Number of ovules microscopically examined from each unpollinated blue spruce cone collected, 1970.

Date of Collection	Days after Pollination	Ovules Examined
	Number	Number
May 26	7	12
May 28	9	9
May 30	11	9
June 2	13	7
June 3	14	11
June 5	16	6
June 7 (broken)	18	13
June 7	18	11
June 9	20	7
June 11	22	8
June 13	24	7
June 15	26	8
Total		108

Table 2.--Number of ovules microscopically examined from each Picea pungens x Picea engelmannii cone collected, 1969.

Date of Collection	Days after Pollination	Ovules Examined
	Number	Number
May 15	3	45
May 18	6	15
May 21	9	37
May 24	12	25
May 27	15	46
May 30	18	48
June 2	21	76
June 5	24	75
June 11	30	70
June 14	33	41
June 17	36	71
June 20	39	33
June 23	42	22
June 23; Bag 156	42	9
June 23; Bag 134	42	5
June 26	45	38
June 30	49	7
July 4	53	22
July 7	56	21
July 11	60	14
July 21	63	3
August 4	84	6
August 11	91	15
August 18	98	8
September 1	112	10
September 8	119	7
Total		783

Table 3.--Number of ovules microscopically examined from each Picea engelmanni x Picea pungens cone collected, 1969.

Date of Collection	Days after Pollination	Ovules Examined
	Number	Number
May 24	12	9
June 5	24	17
June 11	30	7
Total		33

Table 4.--Slide and negative sleeve references for photographs of interspecifically crossed Picea pungens and Picea engelmannii ovules, and unpollinated Picea pungens ovules

Figure Number	Slide Number	Section Number	Negative Sleeve	Collection Date	Remarks
1			151		Pi pun mother tree
2			151		Pi eng mother tree
3			151		Unpoll mother tree
4	591	15	43	5-26-1970	Unpollinated blue
5	597	26	45	5-28-1970	Unpollinated blue
6	597	5	44	5-28-1970	Unpollinated blue
7	609	14	49	5-30-1970	Unpollinated blue
8	608	21	49	5-30-1970	Unpollinated blue
9	612	13	50	6-02-1970	Unpollinated blue
10	628	32	58	6-05-1970	Unpollinated blue
11	643	8(Row 4)	61	6-07-1970	Unpollinated blue
12	645	39	62	6-07-1970	Unpollinated blue
13	639	20	149	6-07-1970	Unpollinated blue
14	630	11	58	6-07-1970	Unpollinated blue
15	632	9	59	6-07-1970	Unpollinated blue
16	635	28	60	6-07-1970	Unpollinated-broken
17	663	5(Row 2)	91	6-15-1970	Unpollinated blue
18	146	3	*	5-12-1969	Wind-blue spruce
19	231	2		5-18-1969	Wind-blue spruce
20	398	19	1	5-24-1969	Blue X Engelmann

Table 4.--Continued

Figure Number	Slide Number	Section Number	Negative Sleeve	Collection Date	Remarks
21	199	4		5-21-1969	Blue X Engelmann
22	397	12	1	5-24-1969	Blue X Engelmann
23	186	5		5-27-1969	Wind-blue spruce
24	174	8		5-27-1969	Blue X Engelmann
25	679	11(Row 2)	95	5-27-1969	Blue X Engelmann
26	609	21	2	6-30-1964	Pi pun 4
27	622	35	13	7-2-1964	Pi pun 4
28	413	25	4	5-30-1969	Blue X Engelmann
29	412	5(Row 2)	67	5-30-1969	Blue X Engelmann
30	410	12	3	5-30-1969	Blue X Engelmann
31	415	10	4	5-30-1969	Blue X Engelmann
32	191	9		5-30-1969	Blue X Engelmann
33	27	9		6-02-1969	Wind-blue spruce
34	422	11	5	6-02-1969	Blue X Engelmann
35	650	8	38	7-11-1964	Pi pun 4
36	699	6(Row 2)	100	6-05-1969	Blue X Engelmann
37	429	5(Row 2)	72	6-05-1969	Blue X Engelmann
38	100	1	75	6-11-1969	Blue X Engelmann
39	257	4	69	6-05-1969	Blue X Engelmann
40	532	39	33	6-05-1969	Blue X Engelmann
41	287	20		6-08-1969	Wind-blue spruce
42	481	12	19	6-11-1969	Wind-blue spruce

Table 4.--Continued

Figure Number	Slide Number	Section Number	Negative Sleeve	Collection Date	Remarks
43	279	30		6-14-1969	Wind-blue spruce
44	284	27		6-11-1969	Wind-blue spruce
45	473	1	18	6-11-1969	Wind-blue spruce
46	367	12		6-08-1969	Wind-blue spruce
47	714	2(Row 4)	19	6-11-1969	Blue X Engelmann
48	443	17	10	6-14-1969	Blue X Engelmann
49	334	14		6-17-1969	Blue X Engelmann
50	731	9(Row 7)	149	6-11-1969	Blue X Engelmann
51	272	4		6-17-1969	Blue X Engelmann
52	271	1(Row 3)	79	6-17-1969	Blue X Engelmann
53	265	3		6-17-1969	Blue X Engelmann
54	453	22	14	6-17-1969	Blue X Engelmann
55	452	17	12	6-17-1969	Blue X Engelmann
56	726	6(Row 5)	107	6-17-1969	Blue X Engelmann
57	451	6	12	6-17-1969	Blue X Engelmann
58	728	5(Row 3)	110	6-17-1969	Blue X Engelmann
59	728	3(Row 2)	149	6-17-1969	Blue X Engelmann
60	348	3		6-23-1969	Blue X Engelmann
61	458	last	15	6-23-1969	Blue X Engelmann
62	455	13	14	6-23-1969	Blue X Engelmann
63	456	2	149	6-23-1969	Blue X Engelmann
64	742	9(Row 4)	129	6-23-1969	Blue X Engelmann
65	540	14	35	6-23-1969	Blue X Engelmann

Table 4.--Continued

Figure Number	Slide Number	Section Number	Negative Sleeve	Collection Date	Remarks
66	513	22	26	6-30-1969	Wind-blue spruce
67	554	38	37	6-30-1969	Blue X Engelmann
68	514	6	27	7-04-1969	Wind-blue spruce
69	384	34		6-27-1969	Blue X Engelmann
70	568	14	40	7-07-1969	Blue X Engelmann
71	567	11	149	7-07-1969	Blue X Engelmann
72	516	21	28	7-11-1969	Wind-blue spruce
73	575	4	40	7-11-1969	Blue X Engelmann
74	580	9	42	7-11-1969	Blue X Engelmann
75	519	15	29	7-28-1969	Wind-blue spruce
76	468	8	17	6-23-1969	B X E Bag 134
77	467	18	17	6-23-1969	B X E Bag 134
78	353	21		6-02-1969	Wind-blue spruce
79	275	8		6-14-1969	Wind-blue spruce
80	510	35	150	6-29-1969	Wind-blue spruce
81	760	9	133	5-24-1969	Engelmann X blue
82	761	11(Row 2)	133	5-24-1969	Engelmann X blue
83	763	13(Row 2)	135	6-05-1969	Engelmann X blue
84	764	3(Row 3)	150	6-05-1969	Engelmann X blue
85	762	3(Row 4)	150	6-05-1969	Engelmann X blue

*Some negatives were cut separately and filed in envelopes by collection number.

Table 5.--Length and width of control- and wind pollinated blue spruce cones

Coll. Date 1969	Days after Poll.	Length in cm.		Width in cm.	
		Art. Poll.	Wind Poll.	Art. Poll.	Wind Poll.
May 12	0	3.95	3.73	2.04	1.75
May 15	3	4.04		1.64	
May 18	6	4.00	3.96	1.92	1.87
May 21	9	5.00	3.97	1.80	2.8
May 24	12	4.90	4.43	1.65	1.73
May 27	15	5.36	4.53	1.82	1.70
May 30	18	5.91	5.44	1.95	1.96
June 2	21	5.05	6.17	1.56	2.00
June 5	24	6.30	8.03	2.77	2.58
June 8	27	7.32	8.96	1.93	2.25
June 11	30	7.63	8.73	1.98	2.14
June 14	33	6.40	8.70	1.74	2.07
June 17	36	4.81	8.16	1.82	2.15
June 20	39	8.10	9.29	2.00	2.27
June 23	42	5.30	9.68	1.60	2.30
June 27	46	6.50	8.49	1.70	2.13
June 30	49	7.72	9.02	2.15	2.20
July 4	53	9.55	8.18	1.95	2.00
July 7	56	8.42	9.0	2.10	2.15
July 11	60	8.10	8.87	1.96	2.00

Table 5.--Continued

Coll. Date 1969	Days after Poll.	Length in cm.		Width in cm.	
		Art. Poll.	Wind Poll.	Art. Poll.	Wind Poll.
July 14	63	9.39	9.90	2.15	2.24
July 21	70	8.20	9.14	2.15	2.05
July 28	77	7.71	10.13	1.89	2.28
August 4	84	9.33	9.35	2.15	2.20
August 11	91	7,80	9.71	1.98	2.20
August 18	98	8.57	8.30	2.05	2.13
August 25	105	8.33	8.49	2.03	2.18
September 1	112	8.14	9.45	2.25	2.24
September 8	119	7.93	9.05	2.30	2.28

SAMPLE CALCULATIONS OF MAGNIFICATIONS

To determine the magnification of each print, the distance between two points was measured under the microscope using an eyepiece micrometer. The same distance was measured on the print to the nearest one-half millimeter. The eyepiece micrometer was calibrated, with the aid of a stage micrometer, at the three magnifications used for the photographs. All measurements and photographs were with a 10x eyepiece lens. All magnifications were rounded to the nearest five.

1. 3.5x objective

1 eyepiece micrometer space = .01428571 mm.

Distance on slide = 65 eyepiece micrometer spaces
Distance on print = 61 mm.

Magnification: 61 mm./ 65 spaces x .01428571 mm./space

= 61 mm./ .02857115 mm.

= 65.6923

= x65

2. 10x objective

1 eyepiece micrometer space = .00506329 mm.

Distance on slide = 80 micrometer spaces
Distance on print = 74 mm.

Magnification: 74 mm./ 80 spaces x .00506329 mm./space

= 74 mm./ .4050632 mm.

= 182.6875

= x185

SAMPLE CALCULATIONS OF MAGNIFICATIONS CONT'D.

3. 45x objective

1 eyepiece micrometer space = .00111111 mm.

Distance on slide = 30 micrometer spaces
Distance on print = 28 mm.

Magnification: 28 mm./ 30 spaces x .00111111 mm.

= 28 mm./ .0333333 mm.

= 840.0008

= x840