POLLINATION AND POLLEN AND SEED DEVELOPMENT IN WESTERN HEMLOCK

Anna M. Colangeli
B.Sc. University of British Columbia, 1978
M.Sc. University of British Columbia, 1982

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Biology

ACCEPTED
FACULTY OF GRADUATE STUDIES
DEAN
DATE 25 April 1989

We accept this dissertation as conforming
to the required standard

Dr. J.N. Owens

Dr. E.D. Styles

Dr. R.D. Burke

Dr. T.M. Fyles

Dr. E.E. Ishiguro

Dr. J.E. Webber

Dr. M.D. Meagher

UNIVERSITY OF VICTORIA

1989

Supervisor: Dr. J.N. Owens

ABSTRACT

Pollen development, pollen forcing, the pollination mechanism, the optimal time for pollination, and factors affecting ovule and seed development after self, cross, wind or no pollination were studied in field-grown and container-grown western hemlock clones (Tsuga heterophylla).

Pollen-cone development under ambient temperatures was compared to pollen cytology. Phenology proved to be an accurate indicator of cytology, independent of collection dates and rate of development. Some practical implications of relating pollen-cone phenology to cytology are discussed.

Two trials were conducted to determine the effect of forcing on pollen development and quality; (1) on cut branches at room temperature and (2) on container-grown trees at two different temperatures in growth chambers. Forcing pollen on cut branches at early phenological stages aborted pollen cones, decreased pollen quantity, increased pollen abnormalities and reduced the fertilizing potential (pollen quality). Forcing after the pollen cones were at least 50% emerged through the bud scales did not decrease yield or fertilizing potential. Forcing pollen on container-grown ramets in growth chambers at 18°C accelerated development three-fold relative to ambient temperatures, altered the relationship between pollen phenology and cytology and, in one clone, resulted in abnormal development. Pollen forced at early stages and stored for one or two years had a lower fertilizing potential than fresh pollen or pollen forced at later stages of development. The feasibility of pollen forcing to ensure adequate supplies of pollen for controlled crosses or supplemental mass pollination is discussed.

Controlled pollinations at various stages following bud burst were used to determine the pollination mechanism and the optimal time for pollination. Western hemlock has a non-micropylar type of pollination mechanism, where the pollen is not deposited near or in the micropyles of the ovules; instead, the mechanism involves an interaction between the roughly sculptured pollen grains and the long epicuticular waxes on the bracts. Maximal seed-cone receptivity occurred when the cones were completely emerged through the bud scales. Receptivity was maintained until cone closure. Several weeks after pollination, pollen germinated on the bracts and formed long pollen tubes which grew towards and into the micropyles.

Selective sampling between pollination and seed maturity after self, cross, wind and no pollination revealed several stages where a potential reduction in seed yield occurred. The stages were divided into two major classes, pre-fertilization and post-fertilization. Pre-fertilization losses included pre- and post-pollination ovule abortion, inadequate pollination, pollen inviability and low pollen vigor. Post-fertilization losses included embryo degeneration and megagametophyte degeneration. Embryo degeneration was subdivided into degeneration at cleavage and during early embryo development. All stages where a reduction in potential seed yield could occur were found in self-, and cross- and wind-pollinated ovules. The lower seed efficiency observed in the selfed cones was due to greater losses at some of these stages, particularilу from embryo abortion. Factors affecting seed development are discussed in terms of maternal effects, pollen vigor and viability, self-incompatibility, self-inviability and embryonic lethals.

Examiners:

Dr. J.N. Owens

Dr. E.D. Styles

Dr. R.D. Burke

Dr. T.M. Fyles

Dr. E.E. Ishiguro

Dr. J.E. Webber

Dr. M.D. Meagher

CONTENTS

TABLES

FIGURES

ACKNOWLEDGMENTS

This research was supported by a Science Council of B.C. Graduate Research, Engineering and Technology Award (G.R.E.A.T) and by a Natural Sciences and Engineering Research Council Operating Grant (A1982).

I wish to express my appreciation to Dr. John N. Owens, under whose supervision these studies were undertaken, for his valuable advice and constructive criticism during the research, and for his guidance in writing this manuscript. I am grateful to Dr. John Barker, Roy Collins and the staff at Western Forest Products, Lost Lake Seed Orchard for the use of the orchard and container-grown ramets; to Frank Portlock and Doug Taylor, of Pacific Forestry Center for use of container-grown material and the X-ray machine; to Sheila Morris (Simpson) for technical assistance over the years; to Diane Mothersill for photographic assistance; to Mike Decker for graphic assistance and to Diane Gray for typing parts of the manuscript.

Chapter I
INTRODUCTION

Western hemlock [*Tsuga heterophylla* (Raf.) Sarg.] is a commercially important conifer in British Columbia for which approximately 23.5 hectares of soil-based seed orchards have been established (Hanson 1985). As well as conventional seed orchards, the British Columbia Ministry of Forests and MacMillan Bloedel Ltd. have been actively involved with the development of container-grown western hemlock seed orchards (Bower *et al.* 1986; Ross *et al.* 1986). Western hemlock is a prime candidate for containerised seed orchards because of the relative ease with which it roots (Brix and Barker 1975; Foster *et al.* 1984) and its positive response to pollen- and seed-cone induction by gibberellin ($GA_{4/7}$) application (Ross *et al.* 1981; Brix and Portlock 1982; Pollard and Portlock 1984). To maximize seed-set efficiency and to ensure effective artificial and supplemental pollinations, all aspects of the reproductive cycle should be understood fully. These include pollen development, pollen forcing, seed and cone development, pollination mechanism, optimal time of pollination and factors affecting seed and cone development under various pollination regimes.

Successful pollination, fertilization and seed yield depend on adequate supplies of viable pollen. Seasonal variation in pollen production, as well as individual tree and clonal variation in the timing of pollen release in seed orchards, implies that some combinations among individuals or clones will not occur naturally (Frampton *et al.* 1982; El-Kassaby *et al.* 1984). Supplemental mass pollination of seed orchards has been suggested as a means of increasing the amount of pollen available in poor pollen years (Bridgwater and Trew 1981; Bridgwater and Bramlett 1982) or as a means of increasing random mating of more clones and higher seed sets if pollen from early and late pollen shedding trees were collected and applied (Daniels 1978).

With increased interest in pollen management and container-grown seed and breeding orchards, knowledge of the relationship between pollen-cone phenology and pollen cytology can be an valuable tool. This is especially true in the area of containerised seed orchards, since it is now possible to control photoperiod, temperature, moisture or humidity in environmental growth chambers or greenhouses. In seed orchards it would be possible to determine when mature pollen is present, hence safe to collect, or predict pollen shed by the rate of pollen-cone development.

Induced pollen cones can be forced to shed pollen early. However, before pollen cones are subjected to forcing treatments, or any other conditions that may affect development, pollen-cone and pollen-grain development under ambient conditions should be fully understood. Pollen development has been studied in many species (Singh 1978; Owens and Blake 1985), including western hemlock (Ho and Owens 1974b) but few studies have described pollen-cone development and fewer still have related pollen-cone phenology to cytological development.

The advantages of pollen forcing are that large amounts of pollen from specific clones or clones that consistently shed pollen late can be collected well before seed-cone receptivity to ensure an adequate supply of pollen for specific crosses or supplemental mass pollinations. A disadvantage to pollen forcing is that not enough is known about the effect of elevated temperatures on pollen development and quality. Several studies have indicated that certain stages of microsporogenesis, especially meiosis, are heat sensitive (Chira 1965; Andersson 1965; Eriksson *et al.* 1970a; Sarvas 1972; Jonsson 1974; Luomajoki 1977). Collection of unripe pollen cones resulted in low pollen yields and pollen of low viability (Snyder and Clausen 1974). Before temperature is used to accelerate pollen development, the effect of elevated temperatures on pollen cytology and pollen quality should be determined. The effect of forcing on pollen development has not been described for western hemlock.

To ensure successful pollination in both field- and container-grown seed orchards, a detailed understanding of the pollination mechanism and the optimal time of pollination would help ensure maximal returns in seed yield. This would be especially advantageous where supplemental pollinations are to be conducted in order to maintain the diversity of the gene pool and increase seed production. Stanlake and Owens (1974) observed megagametophyte, ovule and embryo development in western hemlock but did not determine the causes of empty seed.

Selfing and inbreeding have been cited as the principal reason for empty seed. It has been noted in many studies (reviewed by Owens and Blake, 1985) that, even under controlled pollinations, empty seed are produced. The developmental stages where a reduction in potential seed yield after self, cross, wind or no pollination occur are not known for western hemlock. In order to maximize seed yield, a better understanding of the factors that result in empty seed would be beneficial.

The objectives of this study include:

(1) To document pollen-grain development under ambient conditions and determine the relationship between pollen-cone phenology and cytological development in western hemlock.

(2) To determine the earliest stage that pollen on cut branches could be forced successfully to shed without adversely affecting pollen quality and quantity.

(3) To determine any differences in cytology of pollen development and pollen quality caused by elevated temperatures.

(4) To describe and relate postdormancy seed-cone development to the structure and function of the pollination mechanism and condcut tests to determine the optimal time of pollination for maximal seed efficiency.

(5) To determine the developmental stages where a reduction in potential seed yield occurs after self, cross, wind or no pollination in both field- and container-grown clones.

Chapter II

LITERATURE REVIEW

2.1 Reproductive Cycles

Three general types of reproductive cycles occur in north temperate conifers. In each cycle cone-bud induction and differentiation occur the year before pollination. The most-common reproductive cycle, the 2-year cycle, is found in *Abies* (Owens and Molder 1977d; Singh and Owens 1981b, 1982), *Larix* (Owens and Molder 1979b), *Picea* (Owens and Molder 1979a, 1980a; Singh and Owens 1981a), *Pseudotsuga* (Allen and Owens 1972), *Thuja* (Owens and Molder 1980b; Colangeli and Owens 1989), *Tsuga* (Stanlake and Owens 1974; Owens and Molder 1975b) and some species of *Chamaecyparis* and *Juniperus* (Singh 1978), where pollination, fertilization and complete embryo and seed development occur in the second year. In most members of *Pinus* and some *Juniperus* species, pollination and pollen-tube formation occur in one growing season, while fertilization and seed development are completed in the following year, resulting in a 3-year cycle (Lill 1976; Owens and Molder 1977b; Singh 1978; Owens *et al.* 1982). A variation on the 3-year cycle is found in some species of *Chamaecyparis* and *Juniperus* where pollination, fertilization and early embryo development occur in one growing season, but late-embryo development and seed development are completed the following year (Owens and Molder 1975a; Singh 1978).

2.2 Cone Differentiation

2.2.1 Pollen-Cone Differentiation

Pollen cones differentiate during the late spring or summer of the year prior to pollen shed. Several patterns and times of pollen-cone differentiation have been observed.

Recent studies have demonstrated that pollen-cone differentiation in the Pinaceae correlates with the end of bud-scale initiation and rapid shoot elongation.

In *Pseudotsuga* (Owens 1969; Allen and Owens 1972) and *Abies* (Powell 1974; Owens and Molder 1977a; Owens and Singh 1982; Singh and Owens 1982) pollen cones differentiate from newly initiated axillary apices. In *Picea* (Owens and Molder 1976, 1977e; Harrison and Owens 1983) and *Tsuga mertensiana* (Bong.) Carr. (Owens 1984a) pollen cones differentiate from either newly initiated axillary or terminal apices. In *T. heterophylla* (Owens and Molder 1974a), pollen cones differentiate from newly initiated axillary apicies, terminal apices or the previous years' latent buds. In *Larix* (Owens and Molder 1979c) pollen cones differentiate from dwarf-shoot apices on less-vigorous branches, while in *Pinus* (Owens and Molder 1977b; Owens *et al.* 1981a) pollen cones differentiate from newly initiated axillary apicies within the long shoot terminal bud. In the Cupressaceae, which lack bud scales, pollen-cone differentiation occurs by the transition of vegetative apices into pollen cones in late June (Owens and Pharis 1967, 1971; Owens and Molder 1974b).

2.2.2 Seed-Cone Differentiation

In all the conifers studied to date, with the exception of the soft pines (Haploxylon), seed-cone differentiation occurs in the spring, summer or fall preceding pollination.

As was observed for pollen cones, seed-cone differentiation in the Pinaceae correlates well with the end of bud-scale initiation and rapid lateral shoot elongation. Seed cones differentiate from newly initiated, undetermined, axillary apicies in *Pseudotsuga* (Owens and Smith 1964; Owens 1969; Allen and Owens 1972) and *Abies* (Powell 1974; Owens and Molder 1977a; Owens and Singh 1982; Owens 1984b). In *Tsuga* (Owens and Molder 1974a; Owens 1984a), seed cones differentiate from terminal apices on lateral shoots of moderate vigor, while in *Picea* (Owens and Molder 1976, 1977e; Harrison and Owens 1983) either axillary or terminal apices can differentiate into seed cones. In *Larix* (Owens and Molder 1979c), seed cones differentiate from distal, younger dwarf shoot buds on vigorous branches. Two patterns of seed-cone differentiation are observed in *Pinus*. In

P. contorta Dougl. (Owens *et al.* 1981a) and other hard pines, seed cones differentiate from newly initiated axillary apicies in the fall. In *P. monticola* Dougl. (Owens and Molder 1977c) and other soft pines, seed cones do not differentiate until just before pollination the following spring. In *Chamaecyparis* and *Thuja* of the Cupressaceae, seed cones differentiate from vegetative apices in July (Owens and Pharis 1971; Owens and Molder 1974b Owens and Molder 1977f).

2.2.3 Cone Induction in Western Hemlock

In recent years there has been much emphasis placed on enhancing cone crops for commercially important conifers within the Pinaceae (Pharis and Ross 1984). There has been considerable success through the use of plant growth regulators, namely the less-polar gibberellins (GA_4, GA_5, GA_7 and GA_9). Western hemlock has proven to be an ideal subject for $GA_{4/7}$ treatments and has given excellent and consistent cone crops under a variety of experimental conditions and tree ages. Cones have been induced on one- to five-year-old seedlings, and seven- to eight-year-old rooted ramets grown in the field. Treatments of container-grown trees were most effective where foliar sprays of $GA_{4/7}$ at 200 mgL^{-1} were coupled with water stress treatments and applied at weekly intervals for six weeks in May, June and early July before the natural time for anatomical differentiation of cone buds (Pollard and Portlock 1984; Brix and Portlock 1982; Ross *et al.* 1981; Rottink 1986).

2.3 Pollen Development

Pollen development in gymnosperms has been studied since the middle of the last century. This body of work was reviewed recently by Singh (1978) and Moitra and Bhatnagar (1982). With a few exceptions the emphasis of this review will be placed on the native genera of British Columbia.

2.3.1 Predormancy Development

After bud differentiation, microsporophyll initiation occurs until the late summer. Pollen cones are preformed before winter dormancy in that they possess all microsporophylls and microsporangia but variation occurs between genera in the stage of sporogenous tissue development reached before winter dormancy (Eriksson 1968; Andersson *et al.* 1969; Owens 1980). Pollen cones may become dormant: (1) before sporogenous tissue development, as in the soft pines (Konar 1960; Owston 1969; Owens and Molder 1977b); (2) after the sporogenous tissue development, as in the hard pines (Konar 1960; Mergen *et al.* 1963; Runquist 1968; Willemse 1971a,b,c; Ekberg *et al.* 1972; Ho and Owens 1974a; Kupila-Ahvenniemi *et al.* 1978, 1980; Owens *et al.* 1981a); (3) after pollen mother cells (PMC) differentiate from the sporogenous tissue, as in *Abies* (Mergen and Lester 1961; Owens and Molder 1977a; Singh and Owens 1981b, 1982) and *Picea* (Eriksson *et al.* 1970a; Moir and Fox 1975; Owens and Molder 1979a, 1980a; Singh and Owens 1981a); (4) during meiotic prophase of the PMC, as in *Larix* (Chandler and Mavrodineanu 1965; Ekberg and Eriksson 1967; Ekberg *et al.* 1968; Eriksson 1968; Eriksson *et al.* 1970b; Hall and Brown 1976; Owens and Molder 1979b; Hall 1982), *Pseudotsuga* (Owens and Smith 1964; Owens and Molder 1971; Allen and Owens 1972), *Thuja* (Owens and Molder 1980b) and *Tsuga* (Ho and Owens 1974b; Owens 1984a); or, (5) after the pollen matures, as in *Chamaecyparis nootkatensis* (D. Don.) Spach and some species of *Juniperus* (Owens and Molder 1974b; Singh 1978).

A number of studies have shown that the dormant sporogenous cells in *Pinus sylvestris* L. Lamb. and *P. banksiana* (Kupila-Ahvenniemi *et al.* 1978; Hohtola *et al.* 1984) and the PMC in *Pseudotsuga menziesii* (Mirb.) Franco (Singh *et al.* 1983; Cecich 1984) are metabolically active and undergo many ultrastructural changes during winter dormancy. In the nucleoli the nuclear membrane becomes less distinct and euchromatin and many nuclei appear, while in the cytoplasm starch content decreases and abundant concentric cisternae form, apparently from the endoplasmic reticulum (Singh *et al.* 1983; Hohtola *et al.* 1982), implying that these cells are not dormant, but rather in a state of reduced metabolic activity.

2.3.2 Postdormancy Development

Two patterns of cell division and pollen characteristics occur during pollen development. In *Chamaecyparis*, *Juniperus*, *Taxus* and *Thuja*, pollen is small, lacks sacci, is sculptured with orbicules, storage products are oil droplets, and the pollen is shed at the 1- or 2-cell stage (Owens and Molder 1974b, 1980b; Singh 1978). In *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga* pollen is large, sacci are present in some genera, storage products are in the form of starch and pollen is shed at the 4- or 5-cell stage (Ho and Owens 1974b; Owens and Molder 1971, 1975b, 1977d, 1979a, b, 1980a; Owens *et al.* 1981a; Singh and Owens 1981a, b).

In *C. nootkatensis*, as well as in some species of *Juniperus*, where the pollen matures before dormancy, no changes are observed during the postdormancy stage which lasts only one to two weeks (Owens and Molder 1975a).

Pollen development in other conifers has five postdormancy stages. The duration of each stage varies between genera and between species (Owens 1982). The first stage is the initiation or resumption of meiosis, depending on the degree of development before dormancy. This stage can last for as little as one week in *Pseudotsuga* and *Larix* (Owens and Molder 1971, 1979b) to four weeks in *Pinus monticola* (Owens and Molder 1977c). The second stage is meiotic division resulting in microspores. This stage is invariably short, lasting no more than one week (Owens 1982). A relationship between low temperature, meiotic irregularities and sterile pollen has been observed in *Larix* (Andersson *et al.* 1969; Ekberg and Eriksson 1967; Eriksson 1968, 1970), *Abies* (Mergen and Lester 1961; Andersson 1980) and *Picea* (Jonsson 1974). High temperatures also have been implicated as the cause of meiotic irregularities. Eriksson *et al.* (1970a) reported that high temperatures induced meiotic irregularities in *Picea abies* (L.) Karst. Chira (1965) claimed that long-term temperatures above 15°C caused plasmolysis of the pollen mother cells in *P. abies*. The third stage is microspore development. During this stage microspores within the tetrad enlarge, separate, the exine, followed soon after by the intine, thickens, starch or oil droplets accumulate and sacci may form. This stage varies from one week in *Pinus* and *Thuja* (Owens and Molder 1977c, 1980b) to six weeks in

Tsuga mertensiana (Owens and Molder 1975b). The fourth stage is the period of cell division. In *Thuja plicata* Donn. only one cell division occurs and this stage is completed in one week (Owens and Molder 1980b). In the Pinaceae, three to four cell divisions occur and this stage lasts up to three weeks (Owens 1982). In the fifth stage, elongation of the cone axis causes the microsporophylls to separate and the pollen sacs (microsporangia) to open, releasing the pollen (Owens 1982).

2.4 Pre-Pollination Seed-Cone Development

2.4.1 Predormancy Development

Several patterns of predormancy development have been observed in native genera. *Pinus contorta* (Owens *et al.* 1981a) initiates about two-thirds of its bract primordia but no axillary ovuliferous scale primordia before dormancy. *Tsuga mertensiana* (Owens 1984a) initiates all bracts and ovuliferous scales, but no ovule primordia, before dormancy. *Abies grandis* (Dougl.) Lindl. (Owens 1984b) initiates bracts, ovuliferous scales and ovule primordia but no megaspore mother cells (MMC), before dormancy. The most common pattern occurs in *Tsuga heterophylla* (Owens and Molder 1974a), *Larix* (Owens and Molder 1979c), *Pseudotsuga* (Owens and Smith 1964; Allen and Owens 1972), *Picea* (Owens and Molder 1976, 1977e; Harrison and Owens 1983), *Abies lasiocarpa* (Hook.) Nutt. (Owens and Singh 1982) and *A. amabilis* (Dougl.) Forbes (Owens and Molder 1977a) where bracts, ovuliferous scales, ovule primordia and premeiotic MMCs develop before dormancy.

In *Thuja* (Owens and Pharis 1971; Owens and Molder 1980b) and *Chamaecyparis* (Owens and Molder 1974b) the ovules develop before dormancy. A ring of meristematic tissue develops at the distal end of each ovule and elongates to form the integument tip with a micropyle in the center (Owens *et al.* 1980). The MMC of *Chamaecyparis* overwinters at a premeiotic stage (Owens and Molder 1974b) while the MMC of *Thuja* (Owens and Molder 1980b) commences meiosis in the fall and overwinters at pachytene.

2.4.2 Postdormancy Development

Seed-cone development resumes in February or March. The amount of development varies, from very little change in *Thuja* (Owens and Pharis 1971; Owens and Molder 1980b) to complete seed-cone development in *Pinus monticola* (Owens and Molder 1977c), where the seed cones must first differentiate and then initiate bracts, ovuliferous scales and ovules. For species that overwintered at the ovule primordia stage, the distal portion of the ovules initiate a ring of meristematic tissue which elongates to form the integument tip and micropyle. The structure of the integument tip greatly influences the pollination mechanism of the species.

2.5 Pollination Mechanisms

Pollination mechanism was defined by Lill and Sweet (1977) as the process by which male gametophytes are transported to the proximity of the megagametophyte. Owens and Blake (1984) described pollination mechanisms as the structure of the ovule tip and the process by which the pollen is taken into the micropyle. There are three major types of pollination mechanisms found in gymnosperms. These pollination mechanisms were first described by Doyle (1945) and have been reviewed by Dogra (1964), Konar and Oberoi (1969), Singh (1978) and Owens and Blake (1985).

The most common type of pollination mechanism involves the formation of a pollination drop. This mechanism is found in the Taxodiaceae, Podocarpaceae, Cupressaceae, Taxaceae, Cephalotaxaceae and some members of the Pinaceae. The second mechanism exhibited by a few members of the Pinaceae involves a stigmatic integument tip and no pollination drop. In the third mechanism, the pollen grains land away from the micropyle on the bract and develop long pollen tubes that grow towards and into the micropyle. This occurs in the Araucariaceae and one member each in the Podocarpaceae and Pinaceae (*Tsuga canadensis*).

2.5.1 Pollination Drop Mechanism

Of the native genera, pollination drops have been observed in *Pinus* (Doyle and O'Leary 1935a; Sarvas 1962; Lill 1976; Lill and Sweet 1977; Owens and Molder 1977c; and Owens *et al.* 1981a, 1982) and *Picea* (Doyle and Kane 1943; Owens and Molder 1980a; Singh and Owens 1981a; Owens and Blake 1984; Owens *et al.* 1987) of the Pinaceae and *Chamaecyparis* (Owens *et al.* 1980) and *Thuja* (Owens and Molder 1980b) of the Cupressaceae. The pollination drop, produced by the breakdown of nucellar cells within the ovule, is exuded through the micropyle (Owens *et al.* 1987).

In all the families exhibiting a pollination drop, except the Pinaceae, the integument develops into a simple funnel-shaped structure surrounding the nucellus (Doyle 1945). In *Pinus* and *Picea* the integument tip develops two prongs or arms. The epidermal cells of the integument arms secrete tiny droplets to which pollen adheres (Owens *et al.* 1982; Owens and Blake 1984; Owens *et al.* 1987). This pollen is picked up when the drop is exuded, or the pollen may land directly into the drop. The pollination drop retracts soon after the pollen grains sink into it, carrying pollen to the nucellus.

2.5.2 Stigmatic Integument Tip

This mechanism has been found in several members of the Pinaceae. Two variations have been found, one having two stigmatic lobes and the second a stigmatic funnel.

The stigmatic-lobe mechanism has been observed in *Pseudotsuga* and *Larix* (Lawson 1909; Doyle 1926; Doyle and O'Leary 1935b; Barner and Christiansen 1960, 1962; Allen 1963;

Allen and Owens 1972; Owens and Molder 1979b; Ho 1980; Owens *et al.* 1981b; Owens and Simpson 1982; Villar *et al.* 1984). The integument tip develops into two unequal lobes, a short abaxial and a large adaxial lobe, on which develop numerous long epidermal hairs. A long slit-like micropyle can be found between the two lobes. Pollen grains become entangled in the stigmatic hairs. No secretions were found on the hairs (Owens and Molder 1979b). The two lobes grow into the micropyle, engulfing the entangled pollen. Engulfment by the stigmatic tip is due to differential elongation of the cells of the integument tip and occurs in the presence or absence of pollen.

The stigmatic funnel mechanism occurs in *Abies* (Doyle 1945; Owens and Molder 1977d; Singh and Owens 1981b, 1982), *Cedrus* (Doyle and O'Leary 1935b; Doyle 1945; Chowdhury 1961) and *Tsuga mertensiana* (Doyle 1945; Owens and Molder 1975b; Owens and Blake 1983).

In *Abies* and *Cedrus* the integument tip is flared out into a wide, funnel-shaped stigmatic surface. In *T. mertensiana*, the integument tip consists of two large micropylar flaps. In all cases, the epidermal cells of the integument tip secrete tiny droplets to which incoming pollen grains adhere. The funnels and flaps crimp in entrapping the pollen grains within the micropylar canal. Pollen germinates inside the canal.

2.5.3 Germination Outside the Micropyle

This mechanism occurs in *Agathis* and *Araucaria* of the Araucariaceae (Haines *et al.* 1984), *Saxegotheae* of the Podocarpaceae and in *Tsuga canadensis* of the Pinaceae (Doyle and O'Leary 1935b; Doyle and Kane 1943; Doyle 1945). In these taxa, pollen is received on the bracts, scales or fused bract/scales. After cone closure pollen grains germinate and pollen tubes grow towards the micropyle and nucellus. In *Saxegotheae* the nucellus is flared out of the micropyle (Doyle 1945), in *Araucaria* and *Agathis* the nucellus projects beyond the micropyle (Haines *et al.* 1984) and in *Tsuga canadensis* the nucellus is flush with or just inside the micropyle (Doyle and O'Leary 1935b).

2.6 Megagametophyte and Embryo Development

Since the turn of the century, many papers have appeared on the morphology, anatomy, embryology and cytology of conifers. One of the first comprehensive reviews of conifer reproduction was Chamberlain's book (1935) entitled "Gymnosperms, Structure and Evolution". Since then megagametophytic and embryonic development in gymnosperms have been reviewed by Chowdhury

(1962), Doyle (1963), Maheshwari and Sanwal (1963), Dogra (1964, 1967), Maheshwari and Singh (1967) Konar and Oberoi (1969), Chesnoy and Thomas (1971), Singh and Johri (1972), Mehra and Dogra (1975, 1977) and Singh (1978). The following will emphasize the general patterns observed in the native genera.

2.6.1 Megagametophyte Development

Soon after dormancy ends, the MMC enlarges and begins meiosis. Species that did not overwinter at the MMC stage quickly form MMCs and in both cases meiosis commonly occurs about the time of pollination. Meiosis usually produces four megaspores, of which three normally degenerate. The functional megaspore undergoes several weeks of free nuclear division, during which time several hundred free nuclei form. Cell wall formation occurs between all nuclei, forming a multicellular megagametophyte. The cells divide periclinally and then elongate, filling in the central vacuole.

Several cells at the micropylar end do not divide, but enlarge to form the archegonial initials. These divide unequally, giving rise to a small primary neck cell at the micropylar end and a larger central cell. The neck cell divides to form one or more tiers of cells. The central cell enlarges and divides unequally, producing a ventral canal cell next to the neck cells and a larger egg cell. The egg cell accumulates lipids and proteins in large and small inclusions. The egg nucleus is surrounded by a perinuclear zone rich in mitochondria.

The egg cell is surrounded by a single layer of cells, the archegonial jacket. The average number of archegonia in the Pinaceae is three to five (Willson and Burley 1983). In the Cupressaceae and Taxodiaceae no sterile cells form between adjacent archegonia, instead a single layer archegonial jacket encloses all archegonia forming an archegonial complex. The number of archegonia per archegonial complex may vary from five to 100 (Singh 1978; Willson and Burley 1983).

2.7 Fertilization

Pollen grains germinate within 1 to 3 weeks of pollination and pollen tubes grow through the nucellus to the archegonia in all conifers except *Pinus*. In *Pinus* pollen germination and pollen tube growth into the nucellus occurs before dormancy. The pollen tubes remain dormant within the nucellus for several months (overwinter) before development and fertilization occur the next spring. The *body* cell divides to form the two male gametes before the pollen tube penetrates the archegonium. Male gametes may be two equal-sized cells, as in the Cupressaceae, Taxodiaceae and Araucariaceae, two unequal-sized male cells, as in some Taxaceae and Podocarpaceae or two equal-sized nuclei as in the Pinaceae and Cephalotaxaceae (Singh 1978).

In most conifers, the pollen tube penetrates through the neck cells, the ventral canal cell and releases both gametes inside an archegonium. Never has more than one pollen tube been observed penetrating an archegonium. A receptive vacuole forms in the egg cytoplasm at the site of pollen-tube penetration. The pollen tube discharges its contents which, as well as male gametes, may include the stalk cell and the tube nucleus (Singh 1978). One male gamete migrates to the center of the cell and fuses with the egg nucleus producing a diploid zygote.

2.7.1 Proembryo Development

The zygote undergoes two mitotic divisions, resulting in four free nuclei enclosed by dense neocytoplasm. The neocytoplasm results from the aggregation of perinuclear zone and paternal cytoplasm from the pollen tube. The four nuclei and the neocytoplasm migrate to the chalazal end of the archegonium, resulting in one tier of four nuclei. The four nuclei divide once quickly followed by cell wall formation, producing two tiers of eight cells. This is followed by one or two cell divisions depending on the genus, resulting in a 12- or 16-cell proembryo. The 12-cell proembryo consists of embryo, suspensor and open tiers of four cells each. This has been found in *Pseudotsuga* (Owens and Smith 1965; Allen and Owens 1972) and *Thuja* (Owens and Molder 1980b). The 16-celled proembryo has an embryo, suspensor, rosette or disfunctional suspensor (Singh 1978) and open tier. This structure has been observed in *Abies* (Singh and Owens 1981b, 1982), *Larix* (Owens and Molder 1979b; Kozinski 1987), *Tsuga* (Stanlake and Owens 1974; Owens and Molder 1975b), *Picea* (Owens and Molder 1979a, 1980a; Singh and Owens 1981a), *Pinus* (Doyle 1963, Lill 1976; Owens and Molder 1977c; Owens *et al.* 1982) and *Chamaecyparis* (Owens and Molder 1975a). The rosette, or disfunctional suspensor cells, may divide a few times as in *Pinus*, forming what have been termed rosette embryos, which serve no function and soon degenerate. The proembryo stage ends when the primary suspensor cells elongate and the embryo tier is pushed through the base of the archegonia into the megagametophytic tissue.

2.7.2 Embryo Development

The suspensor tier elongates, pushing the embryo deep within the megagametophyte. Since more than one archegonium may be fertilized, the megagametophyte may contain several developing embryos. This is referred to as simple polyembryony and occurs in most gymnosperms (Singh 1978; Willson and Burley 1983). In some genera, a single zygote forms multiple embryos which arise by the cleavage or splitting of the embryonal tier into four embryonal units. This is referred to as cleavage polyembryony (Singh 1978). Among the native genera both simple and cleavage polyembryony are found in *Tsuga* (Stanlake and Owens 1974; Owens and Molder 1975b), *Pinus* (Lill 1976; Owens and Molder 1977c; Owens *et al.* 1982) and *Chamaecyparis* (Owens and Molder 1975a). *Abies* (Singh and Owens 1981b, 1982), *Larix* (Owens and Molder 1979b; Kozinski 1987), *Picea* (Owens and Molder 1979a, 1980a; Singh and Owens 1981a), *Pseudotsuga* (Owens and Smith 1965; Allen and Owens 1972) and *Thuja* (Owens and Molder 1980b) exhibit only simple polyembryony.

While many embryos may be found during early development, one usually dominates and the rest degenerate. The cell walls in the megagametophyte adjacent to the developing embryo break down, forming a corrosion cavity. The embryo continues to divide in all planes, resulting in an embryonal mass (Singh 1978) or club-shaped embryo (Allen 1942). The developing embryo elongates and begins to form distinct meristematic regions. The apical region becomes separated from the suspensor system by a long rib meristem. Cells at the base of the apical region form the root apex. The shoot apical meristem forms in the distal region. Cells adjacent to the shoot apex divide, forming the cotyledons. Cotyledons, unlike true leaves, arise independently of the shoot apex.

Many changes occur in the megagametophyte during embryo development. At the mature-archegonial stage, the cells of the gametophyte are devoid of storage products. During embryo development, deposition of lipoprotein bodies occurs in the gametophytic cells. These continue to accumulate, so that by seed maturity the gametophytic cells are packed with what have been identified as lipid droplets and/or lipoproteins (Favre-Duchartre 1956; Hakansson 1956; Takao 1960; Simola 1974; Butler *et al.* 1979; Singh and Owens 1981a, b, 1982; Owens *et al.* 1982).

Seed maturation in gymnosperms has been reviewed by Singh (1978). The mature seed consists of a three-layered seed coat (testa) surrounding a nucellus (megasporangium) and megaspore cell wall. Within these structures is the mature embryo enclosed within the megagametophyte.

2.8 Factors Affecting Seed Development

Controlling seed losses is a major concern in forestry, especially in tree-breeding programs. Many causes for seed losses have been found. Several of the key areas where reduction in potential seed yield occurs will be discussed.

2.8.1 Ovule Abortion

Ovule abortion occurs in *Pinus* and, to a lesser extent, *Picea* (McWilliam 1958; Sarvas 1962, 1968; Mikkola 1969; Kossuth and Fechner 1973; Sweet 1973; Plym-Forshell 1974; Cecich 1979; Fechner 1979; Owens *et al.* 1981a; Owens and Blake 1984) because pollen is essential for normal ovule and megagametophytic development. Ovule abortion and/or arrestment of megagametophytic development prior to pollination also has been reported in the Pinaceae and Cupressaceae, resulting in small, flattened, empty seed. While the actual causes are uncertain, several theories have been postulated. These include: competition for nutrients (Lyons 1956; Burdon and Low 1973), drought (Simak and Gustafsson 1954; Sarvas 1962; Dogra 1967) and low temperatures (Dogra 1967; Sweet and Bollmann 1972; Owens and Molder 1980b) at the time of megagametophytic development.

Very few studies have actually measured the percent lost due to ovule abortion. Sweet and Bollmann (1972) estimated ovule abortion from 13 to 52% in *Pseudotsuga* while in *Picea abies*, Sarvas (1968) found that about 7% of the pollinated ovules aborted.

2.8.2 Insufficient Pollen

Insufficient pollen has been cited as a principal reason for low seed yields (Sarvas 1962; Bramlett 1974; Hall and Brown 1976, 1977; Daniels 1978; Kozinski 1987). For *Larix*, it has been calculated that even under conditions of supplemental pollinations, about 24% of the ovules contain no pollen in the pollen chambers (Hall and Brown 1976). This is a serious problem in young seed orchards where for the first few years of the trees' reproductive lives, pollen cone production is extremely low compared to seed cone production (Daniels 1978).

2.8.3 Embryo Abortion

Bramlett and Popham (1971), Hadders and Koski (1975), O'Reilly *et al.* (1983), McKinley and Cunningham (1983) and El-Kassaby *et al.* (1984) reported in different coniferous species that a primary cause of empty seeds, other than insufficient pollen, is embryo abortion caused by homozygous lethal alleles, usually as a product of inbreeding. Reduced seed yields after selfing and inbreeding have been reported for *Abies* (Sorensen *et al.* 1976), *Pseudotsuga* (Orr-Ewing 1954, 1957, 1965; Sorensen 1969, 1971, 1973; Sorensen and Miles 1974; El-Kassaby *et al.* 1981; Shaw and Allard 1982), *Larix* (Park and Fowler 1982), *Picea* (Mergen *et al.* 1965; Sarvas 1968; Koski 1971; Coles and Fowler 1976; Singh and Owens 1981a; Fowler and Park 1983; Cram 1984; Park and Fowler 1984) and *Pinus* (Bingham and

Squillace 1955; Sarvas 1962; Hagman and Mikkola 1963; Hagman 1964; Fowler 1965; Koski 1971; Franklin 1970, 1971; Bramlett and Pepper 1974; Plym-Forshell 1974; Squillace and Goddard 1982).

Cytological studies in *Picea* (Mergen *et al.* 1965), *Pinus* (Hagman and Mikkola 1963; Hagman 1964 Plym-Forshell 1974;) and *Pseudotsuga* (Orr-Ewing 1957) have shown that neither pollen germination, growth of pollen tube, nor fertilization capability were reduced as a result of self-pollination. There is general agreement among these authors and others (Ehrenberg *et al.* 1955; Sarvas 1962; Kraus and Squillace 1964; Fowler 1964, 1965) that embryo abortion between fertilization and seed maturation accounts for reduced yields of filled seed after selfing (reviewed by Franklin 1970). Orr-Ewing (1954, 1957), Hagman and Mikkola (1963) and Mergen *et al.* (1965) postulated that self-incompatibility may be the result of physiological incompatibility between early embryos and the megagametophyte.

Embryo abortion occurs under cross-pollinations which cannot be attributed to inbreeding or selfing. Several suggestions have been made as to why post-fertilization abortion may occur. These include competition for nutrients (Lyons 1956; Allen and Trousdell 1961; Burdon and Low 1973) and environmental factors such as drought (Simak and Gustafsson 1954; Sarvas 1962; Dogra 1967).

2.8.4 Maternal Effect

Brown (1971, 1973) observed that in *Pinus* the degree of loss of developing cones is associated positively with the amount of pollen applied but that the strength of this association is modified by inherent factors, implying that certain clones have a greater inherent tendency to retain cones than others. Bramlett (1974) found that differences between trees for seed and ovule characteristics from a sample of 118 clones were significant statistically, which may imply an inherent ability of each tree to produce filled seed.

2.9 Tsuga

Tsuga has a 2-year reproductive cycle, where pollination, fertilization and complete embryo and seed development occur in the second year (Stanlake and Owens 1974; Owens and Molder 1975b). In *T. heterophylla*, pollen cones differentiate from newly initiated axillary apices, terminal apices or previous years' latent buds (Owens and Molder 1974a), whereas in *T. mertensiana* pollen cones differentiate from either newly initiated axillary or terminal apices (Owens 1984a). Seed cones differentiate from terminal apices on lateral shoots in all species of *Tsuga*. Pollen development in *T. heterophylla* was observed by Ho and Owens (1974), but the effects of forcing on pollen development, quality and quantity in this species has not been studied.

The pollination mechanism in *T. heterophylla* was described by Stanlake and Owens (1974) and Owens and Blake (1983) but this information was not used to determine the optimal time for pollination. The pollination mechanism in *T. canadensis* was described by Doyle and O'Leary (1935b) and in *T. mertensiana* by Doyle (1945), Owens and Molder 1975b and Owens and Blake (1983).

Megagametophyte, ovule and embryo development has been described for *T. heterophylla* (Stanlake and Owens 1974), *T. mertensiana* (Owens and Molder 1975b) and to a lesser extent, in *T. canadensis* (Murrill 1900; Sterling 1948) and *T. caroliniana* (Buchholz 1931). The effects of selfing or no pollination on megagametophyte. ovule and embryo development in *Tsuga* has not been described. The stages where a potential reduction in seed yield results in empty seed has not been determined for *Tsuga*.

Chapter III

Materials and Methods

3.1 Fixation Techniques for Light Microscopy

All material that was to be used for light microscopy was fixed immediately upon return to the lab. Pollen-cone buds were fixed whole after the buds scales were removed. Bud scales were removed from seed-cone buds and the cones sliced along both sides before fixing. After bud burst, separate ovuliferous scales were fixed. Later, once the cones had started to elongate and the ovules increased in size, individual ovules were removed from the scales, sliced along one side and fixed. All material was fixed in Navashin's chromic acid - acetic acid -formalin (CRAF) fixative (Berlyn and Miksche 1977). Fixed specimens were dehydrated in a tertiary butyl alcohol series (Johansen 1940) and embedded in Tissue-Prep. Serial longitudinal sections were cut at 6μm and stained with safranin and iron hematoxylin for microscopic examination. The description of the developmental stages for pollen- and seed-cone development was summarized from many observations of each stage.

3.2 Fixation Techniques for Scanning Electron Microscopy

Three methods were used to prepare material for scanning electron microscopy (SEM) observations. Fresh seed cones were dissected, ovuliferous scales, ovules and bracts were mounted fresh on a stub using silver conductive paint and observed immediately with a JEOL-35U SEM, operating at 10Kv. This was suitable for observations at low magnifications. At higher magnifications and after pollen was present on bracts and scales, fresh material was gold coated using a Technics Hummer Sputter Coater before scanning electron micrographs were made. As well as reducing the charging of the pollen grains, the gold particles enhanced the epicuticular waxes on the surface of the bracts and scales. Partially dissected cones and ovuliferous scales with and without pollen and pollen tubes were fixed in Zircle-Erliki fluid (Conn *et al.* 1960), dehydrated through an ethanol series to absolute ethanol followed by dry-amyl acetate. The specimens were critical point dried and kept under vacuum in a dessicator. The specimens were mounted on stubs, gold coated and scanning electron micrographs were made.

3.3 Pollination Technique

In all trials in which controlled crosses were conducted, the seed-cone buds were isolated using windowed, paper pollination bags before or during bud swell. With the exception of the two studies used to determine the optimal time of pollination, all controlled crosses were conducted after the strobili within a bag had completely emerged through the bud scales. A rubber bulb with a one-way valve, attached to a 20-cc syringe with a 13-gauge needle was used to spray approximately one ml of pollen at the cones in each bag. The needle was inserted through the paper; and the plastic window in the underside of the bags allowed a good view of the cones. The puncture holes were immediately sealed with tape. The bags were shaken to ensure an even distribution of the pollen to all the cones, judged by visual inspection of cones through the plastic window.

The bags were left on the branches for several weeks until the cones closed and began to elongate. At this time, the branches were tagged and the pollination bags replaced with mesh insect bags. The cones were allowed to mature within the insect bags. Cone survival within the bags was near 100%.

3.4 Cone Analysis

Mature cones were collected in mid-September after they had turned brown and showed signs of drying. For each controlled pollination, twenty cones per trial were placed in individual envelopes to finish drying and flexing. For each cone the number of sterile distal scales, fertile scales, sterile basal scales, round seeds, flat seeds and total seeds were determined. All round seeds were dewinged and then radiographed on Industrex Instant 600 paper using a Faxitron X-ray unit and an exposure factor of 360mA at 12.5 KV to determine the number of filled seed per cone (containing a full-size embryo). Seed potential (SP) and seed efficiency (SEF) were calculated per cone. SP, which is the maximum number of seeds a cone is capable of producing, was calculated by multiplying the number of fertile scales by two because each fertile scale bears two ovules each capable of producing a seed. SP is determined the year before pollination by the number of bracts, ovuliferous scales and ovule primordia initiated. SEF which is the ratio of filled seed (FILL) to potential seed (SP), expresses the productivity of a cone in relation to its biological capacity (Bramlett *et al.* 1977).

Since SEF is expressed as a percentage, the angular transformation (arcsine transformation) was performed on the ratio FILL/SP (ARCSEF) whenever statistical analyses were used to compare SEFs. A modification of the Freeman and Tukey (1950) transformation was used because many of the SEF values were zero or close to zero (Zar 1984).

$$\mathrm{ARCSEF} = 0.5(\arcsin(\mathrm{FILL}/(\mathrm{SP}+1))^{1/2} + \arcsin((\mathrm{FILL}+1)/(\mathrm{SP}+1))^{1/2})$$

3.5 Pollen Cone Development

Twigs, 3 to 5 cm in length, bearing pollen-cone buds, and later pollen cones were collected in 1983 from eight western hemlock clones located at the Canadian Forest Service's Cobble Hill Clone Bank, 55 km north of Victoria, B.C. Specimens were collected weekly from January 10 to March 3, then twice weekly until April 3, when pollen shed commenced. Twigs were placed in vials of water and immediately transported to the University of Victoria where the cones were fixed, embedded, stained and sectioned for microscopic examination.

Pollen-cone development was divided arbitrarily into nine phenological stages (Table 1). The stages of development for each of the eight clones were monitored throughout the collection period so that pollen-cone phenology could be compared to pollen cytology.

Pollen phenology was monitored for an additional 52 clones at two- to three-day intervals from March 19 to April 26. The period of pollen shed was noted for each clone.

TABLE 1: Classification of western hemlock pollen cones.

STAGE	DESCRIPTION
1	Quiescent bud
2	Swollen bud
3	Bud burst, Cone less than one-quarter emerged[1]
4	Cone one-quarter to one-half emerged
5	Cone one-half to Less than fully emerged
6	Cone fully emerged
7	Elongating stalk
8	Pollen shed
9	Empty cone

[1] Beyond the bud scales.

3.6 Effect of Forcing on Pollen Development

Two methods were used to study the effect of forcing (above ambient temperatures) on pollen development. The first method involved forcing pollen on cut branches with bases placed in flasks of water at room temperature. The second method consisted of forcing small containerized trees with abundant pollen cones in growth chambers.

3.6.1 Cut-Branch Method

Small branches (40-60 cm), with pollen-cone buds and, later, pollen cones, were collected on six occasions between February 9 and March 30, 1983 from two clones at the Cobble Hill Clone Bank. The six collections were labelled pollen forcing trials, PFT1 to PFT6 (Table 2). The buds and cones were classified according to the phenological stages described in Table 1. The sixth collection was obtained two days before the pollen shed in the field. The branches were transported to the University and the bases placed in flasks of water on a laboratory bench. The temperature in the room ranged from 18 to 21°C. At one- to two-day intervals from the time of collection, four to six pollen cones were sampled from the branches, bud scales removed, and the cones fixed, embedded, sectioned and stained for microscopic examination. The number of abnormal or aborted pollen cones that did not shed pollen as well as the number of cones that shed pollen were tallied for each collection. From this information the proportion of pollen cones (%) that shed pollen was determined. The volume of pollen collected was recorded. The percent pollen cones shed and the total pollen produced in each trial were compared using multiple comparisons for proportions (Zar 1984) after the data were transformed using the same formula as for the SEFs. The final length of 20 pollen cones at dehiscence was measured for each collection.

TABLE 2: Collection dates and phenological stages (STAGE) for western hemlock pollen cones in the cut-branch pollen forcing-trial (PFT), 1983.

TRIAL	DATE COLLECTED	STAGE
PFT1	February 9	1
PFT2	February 23	2
PFT3	March 6	3
PFT4	March 13	4
PFT5	March 19	5
PFT6	March 30	7

The fertilizing potential of the pollen produced by each trial was tested in early April, 1983 by controlled pollinations of two clones at the Cobble Hill Clone Bank. The pollen produced by PFT1 was still insufficient for controlled crosses, so only pollen from PFT2 to PFT6 were used. Due to the small amount of pollen produced in PFT2 and PFT3 it was applied to each cone using a small #2 camel hair paint brush. The rubber bulb-syringe method was used to apply the pollen produced in PFT4, PFT5 and PFT6. Pollen was retested in the spring of 1985 after two years in storage at 4°C by controlled pollinations of containerized ramets using camel hair paint brushes.

Twenty to 25 seed-cone buds on five branches per clone were isolated in mid-March, 1985. Controlled pollinations were conducted in early April. The cones were collected in early September of 1983 and 1985 just before seed shed. For each clone, 20 cones per trial were analyzed. The ARCSEF values were compared between trials for the two clones in 1983 and the single clone in 1985 using the Kruskal-Wallis test. Whenever a significant Kruskal-Wallis test ($p<.05$) was found, pair-wise comparisons were made using the Wilcoxon 2-sample test.

Since all pollen cones used in the six pollen-forcing trials were collected from the same two clones, the only difference between the pollen produced in PFT1 to PFT6 was the degree of pollen forcing. All pollinations were conducted within a clone so the maternal effect on seed development was constant, therefore SEF should give an indication of the fertilizing potential of the forced pollen.

3.6.2 Whole-Tree Method

In 1983, pollen-cone buds were induced on eight-year-old western hemlock rooted cuttings by a combination of water stress and $GA_{4/7}$. The cones were induced by spraying the trees weekly with a 100 mgL^{-1} solution of $GA_{4/7}$ for six weeks starting the week of May 12, when 50% of the buds were at vegetative bud burst (Ross *et al.* 1981; Brix and Portlock 1982; Pollard and Portlock 1984).

Three clones with abundant pollen cones, represented by five to seven ramets each, were used in the whole-tree pollen forcing trial. In early 1984, the ramets were placed into growth chambers on three different dates (Table 3). Each date is designated by a different pollen forcing trial number (PFT) as well as the the temperature at which the trees were forced. On January 16 (PFT1-18), 22 (PFT2-18) and February 5 (PFT3-18), one ramet of each clone was placed into a growth chamber with a day and night temperature of 18°C and a 16-hour photoperiod. Also on February 5, one ramet of each clone was placed into a fourth growth chamber set at 10°C day and night with a 16-hour photoperiod (PFT3-10). One ramet of each clone remained outdoors in a lath house under ambient conditions to serve as controls (PFT3-C). One clone contained an extra 2 ramets; one of each was added to PFT3-10 and PFT3-C.

TABLE 3: Starting dates, growth chamber temperatures and pollen cone phenology (STAGE) for the western hemlock whole-tree pollen forcing trial (PFT), 1984.

TRIAL	DATE	TEMPERATURE	STAGE
PFT1-18	January 16	18°C	1
PFT2-18	January 22	18°C	1
PFT3-18	February 5	18°C	1
PFT3-10	February 5	10°C	1
PFT3-C	February 5	ambient	1

The data were analyzed as two separate studies. The first study included the three ramets placed into 18°C growth chambers on three different dates (PFT1-18, PFT2-18 and PFT3-18). The second study included the three pollen-forcing trials started on February 5 (PFT3-18, PFT3-10 and PFT3-C), and maintained at different temperatures.

Pollen-cone phenology was monitored for each trial from the time the trees were placed in the growth chambers until pollen shed, using the same nine phenological stages described in Table 1. At two- to three-day intervals, four to six cones per trial were collected, fixed, embedded, sectioned and stained for microscopic examination. Cytological development was correlated to phenological development. All pollen collected in each trial was pooled.

The fertilizing potential of the pollen produced in each trial was tested by controlled pollinations. Seed-cone buds on three ramets of one clone were isolated in late March and the cones were pollinated in early April. Unfortunately, all cones were lost due to root weevil infestation of the ramets. The pollinations were repeated on two ramets of one clone, in the spring of 1985. The cones were collected and analyzed in October, 1985. The ARCSEF of the different trials were compared using Kruskal-Wallis test. When a significant Kruskal-Wallis test ($p<.05$) was found pair-wise comparisons were conducted using Wilcoxon's 2-sample test.

3.7 Pollination Mechanism

Seed-cone development was divided into nine distinct phenological stages ranging from the quiescent bud to cone closure (Table 4) so that seed-cone phenology could be compared to ovule development. Seed-cone buds and young cones from a minimum of 3 clones per date were collected from the Cobble Hill Clone Bank and Western Forest Products', Lost Lake Seed Orchard in 1983 and 1984, respectively, at different stages of development, before, during and after pollination. Fresh and fixed material was observed using a SEM.

3.8 Optimal Time of Pollination Study

To determine the optimal time of pollination in western hemlock a preliminary study was conducted in 1983 at the Cobble Hill Clone Bank and a more intensive study was conducted in 1984 at Western Forest Products' Lost Lake Seed Orchard.

3.8.1 Cobble Hill - 1983

Two clones (7-10 and 9-3) from the Cobble Hill Clone Bank were selected in 1983. In mid-March, before bud burst, seven branches with at least 25 seed-cone buds were isolated per clone. Stage 6 and 7 pollen cones were collected from 50 clones between March 19 and March 26. The cones were allowed to dry at room temperature and shed pollen. The pollen was extracted, filtered to remove debris and bulked for use in the pollination trials. On March 30, all cones in one bag per clone were pollinated as described previously. The cones on clone 9-3 were at stage 4, and stage 3-4 on clone 7-10 (Table 4). The bags were shaken to ensure an even distribution of pollen to the cones. After 30 minutes, when the pollen had settled, the bags were removed, excess pollen shaken off the foliage and a new bag placed on the branch to ensure no pollen contamination during subsequent development.

TABLE 4: Classification of western hemlock seed cones.

STAGE	DESCRIPTION
1	Quiescent bud
2	Swollen bud
3	Bud burst, Cone less than one-quarter emerged[1]
4	Cone one-quarter to one-half emerged
5	Cone one-half to Less than fully emerged
6	Cone fully emerged
7	Elongating ovuliferous scales Less than one-half bract covered
8	Elongating ovuliferous scales Greater than one-half bract covered
9	Closed cone

[1] Beyond the bud scales.

One bag per clone was pollinated every two days regardless of cone stage from March 30 to April 9 and final bags pollinated April 14 (Table 5). The phenology of the seed-cones in the bags was recorded at the time of pollination. The pollination bags were replaced with insect bags on May 14, 2 to 3 weeks after all the cones had closed (stage 9). The cones were left in the insect bags until maturity. They were collected in mid-September after they showed signs of drying and processed as described previously.

TABLE 5: Pollination dates and seed-cone phenology for two western hemlock clones at Cobble Hill in the optimal time of pollination study, 1983.

	CLONE 7-10	CLONE 9-3
DATE	STAGE	STAGE
March 30	3-4	4
April 1	3-4	4-5
April 3	4	5
April 5	4	6
April 7	4-5	6
April 9	5-6	6
April 14	6-7	7

For each clone, 20 cones per pollination date were placed in individual envelopes and allowed to dry and flex. For each cone, the number of sterile distal scales, fertile scales, sterile basal scales, round seed, flat seed and total seed were determined. Filled seed, SP and SEF were calculated. The SEFs for each clone were plotted versus date and stage.

3.8.2 Lost Lake - 1984

Four clones with abundant seed-cone buds were selected in early March, 1984 for the second optimal-time-of-pollination study. Stage six and seven pollen cones were collected from 15 clones located at the Pacific Forestry Center (PFC) in Victoria. The cones were air dried at room temperature and the pollen extracted and filtered to remove debris. Six to seven branches per clone were bagged when the seed-cone buds were at stage 2. Unlike the Cobble Hill trial, pollinations were conducted by stage rather than by date (Table 6). Due to a shortage of cone buds, pollinations could not be carried out at each stage for each clone. Except for stage

2, at least three clones were pollinated for any one stage (Table 6). Before pollen was sprayed into each bag, any cones that were delayed or advanced were removed. One ml of pollen was sprayed into each bag using the rubber bulb-syringe method. The paper pollination bags were replaced with mesh insect bags on April 9 after all the cones had closed (stage 9). The cones were left in the insect bags until mid-September after they showed signs of drying and were processed as previously described.

3.9 Seed Development in Field-Grown Clones

Separate experiments were conducted to determine the effect of wind, cross, self or no pollination on seed development in field-grown western hemlock clones at the Cobble Hill Clone Bank. Seed cone and ovule development were monitored from early January, before the end of dormancy, until seed maturity and seed shed in late September. Seed development and seed yield were compared among the different types of pollinations.

TABLE 6: Seed-cone phenology (STAGE) and pollination dates for four western hemlock clones at the Lost Lake Seed Orchard in the optimal-time-of-pollination study, 1984.

STAGE	CLONE 39	CLONE 142	CLONE 143	CLONE 190
2	March 14	---[1]	---[1]	March 14
3	March 16	March 14	March 14	March 17
4	---[1]	March 16	March 16	March 21[2]
5	March 21	March 18	March 18[2]	---[1]
6	---[1]	March 21	March 21	March 24
7	March 26	March 24	March 24	March 31
8	March 31	March 26	March 31	April 4[2]
9	April 4[2]	March 31	April 4	---[1]

[1] No pollinations
[2] Lost in wind storm

3.9.1 Wind Pollination

Twigs bearing seed-cone buds or seed cones were collected in 1983 at the Cobble Hill Clone Bank from the same eight clones used in the pollen-development study. Specimens were sampled weekly (January 10 to March 3) until bud burst, twice weekly from bud burst to cone closure (March 3 to May 1) and weekly until mature seeds were found (May 1 to July 10). Twigs, 2 to 5 cm long, with cones were placed in vials of water and transported immediately to the University. The cones and ovules were fixed, embedded, sectioned and stained for microscopic examination. Seed-cone phenology was observed for 60 clones at 2- to 3-day intervals from March 19 to April 26 including the periods before, during and after pollination, so that seed-cone phenology could be compared to peak periods of pollen shed.

In September, when the cones started to turn brown and dry, 20 seed cones were collected from the mid-crown region of 60 clones. The cones were bulked by clone and allowed to open. Seeds were extracted, dewinged and filled seeds were determined by radiography. The number of sterile distal scales, fertile scales and sterile basal scales were determined for 20 cones per clone and the average SP calculated for each clone. SEF was calculated from the average number of filled seed per cone and the average SP.

3.9.2 Cross Pollination

One to four branches with 20 to 30 swollen seed-cone buds were isolated on 16 clones in late March. On April 3 and 5, when most of the seed cones were at stage 6, one ml of pollen was sprayed into the pollination bags as described previously with the same pollen polymix used in the optimal-time-of-pollination study (section 3.8.1).

Seed cones were collected from 4 of the crossed clones chosen at random, for subsequent microscopic examination using the same collection and treatment schedules as for the wind-pollinated clones. The remaining cones were left until mid-September, when the cones were collected, dried and the seeds extracted. SP and SEF were calculated for 20 cones per clone. The SEFs resulting from cross pollinations were compared to SEFs produced by the same wind-pollinated clones.

3.9.3 No Pollination versus Cross pollination

Four branches with a minimum of 30 swollen seed-cone buds (stage 2) were isolated on one clone in late March. Two of the bags were pollinated with one ml of pollen using the rubber-bulb-syringe method on April 2, when the seed-cone buds were stages 5 and 6. Cones in the other two bags remained unpollinated. One cone per bag was sampled weekly from the 2 pollinated and 2 unpollinated bags starting on pollination day (April 2) until June 9, then every 10 days until July 10. The cones were dissected and four to six ovules per cone were fixed, embedded, sectioned and stained for microscopic examination. The remaining cones were left until maturity. In mid-September the cones plus unpollinated cones from 3 more clones were collected from the 4 clones, dried and the seeds extracted. SP and SEF were calculated for individual cones.

3.9.4 Self Pollination

Ten clones from the Cobble Hill Clone Bank were selected to determine the effect of selfing on seed development. One branch per clone, with a minimum of 30 seed-cone buds at stage 2, was isolated in late March. Small branches with pollen cones were collected between March 19 and 24 from all 10 clones. The pollen cones had emerged completely through the bud scales (stage six) and in some cases the stalk had started to elongate (stage seven). The branches were transported to the University where the cut ends of the branches were placed in flasks of water. The pollen was collected as it was shed. On April 3 when the cones were completely beyond the bud scales (stage 6), one ml of self-pollen was sprayed into the pollination bags using the rubber bulb-syringe method.

Two seed cones were collected per clone weekly from May 1 to June 9, then every 10 days until July 10. The material was fixed, embedded, sectioned and stained for microscopic examination. The remaining cones were left until maturity.

In mid-September they were collected and dried. SP, SEF and percent filled seed (PCFILL) were calculated for individual cones. PCFILL was calculated by dividing the number of filled seed per cone (FILL) by the round seed (RND) and multiplying by 100. The SEFs resulting from selfing were compared to SEFs produced by wind pollination in the same clones.

3.10 Seed Development in Container-grown Clones.

The 1983 seed-development study identified several key stages where a reduction in potential seed yield occurred in the selfed and out-crossed cones. These included pre- and post-pollination ovule abortion, no fertilization, megagametophyte degeneration and embryo degeneration. The 1983 sampling was too infrequent and the sample size too small to determine the effect of these stages on the final seed yield. Based on the trends observed in 1983, a detailed study of the factors influencing seed development after self- and cross-pollination was undertaken in 1984 at the Pacific Forestry Centre (PFC) in Victoria. Four containerized clones with a basal stem age of 4-5 years and approximately 1 m tall, bearing both seed- and pollen-cone buds were selected. Each clone was represented by two ramets. Approximately 50 to 60 seed-cone buds per clone were isolated in early March using several small, windowed, pollination bags per clone. Stage six pollen cones were collected from each clone, from which the pollen was dried, extracted and filtered. Pollen for the cross-pollinations were collected and bulked from four clones at the Lost Lake Seed Orchard as previously described.

On March 23, when the seed cones had emerged completely beyond the bud scales, one ramet per clone was selfed while the other ramet was cross-pollinated. Two cones per ramet were collected randomly at one-week intervals to determine when pollen germination began. Once germination started, one or two cones from the selfed and crossed bags per clone were collected weekly from May 8 to July 16. Eight to 10 ovules per cone were dissected and fixed in Navashin's fixative, embedded, sectioned and stained for microscopic examination. The stages at which pollen tubes, the megagametophyte or embryos aborted were determined.

Chapter IV
RESULTS

4.1 Pollen Development in situ

Western hemlock pollen-cone buds were found on proximal branches in the lower regions of the crown. They were borne terminally or in axils of spirally arranged leaves, appearing as a cluster just below the terminal vegetative or pollen-cone bud. Axillary pollen-cone buds were found also in higher regions of the crown on proximal branches of low vigor. Quiescent or stage 1 pollen-cone buds averaged 1.8 (±.2) mm in length (Fig. 1). Each bud consisted of spirally arranged microsporophylls and was enclosed by bud scales (Fig. 2). On the abaxial surface of each microsporophyll were two microsporangia, each containing loosely-packed, thin-walled pollen mother cells (PMC) with densely-stained cytoplasm and a large nucleus (Fig. 3). Within each nucleus were several densely-stained nucleoli and lightly-stained chromatin strands. The PMCs were arrested at pachytene of meiosis. Surrounding the PMCs was a uninucleate, single-layered tapetum (Fig. 3).

Cytological development resumed during the first week in February. There was a ten-day difference between dates when the first and last tree ended pollen cone-bud dormancy. The nucleus of a few of the tapetal cells divided (karyokinesis) without subsequent cell wall formation, resulting in binucleate cells (Fig. 4). Karyokinesis of all tapetal cells occurred over the next two to three weeks. A few random divisions were observed in the cells of the microsporangial wall and cone-bud axis. Meiosis of the PMCs resumed approximately one week later, between February 12 and 20. The chromatin condensed and passed rapidly through pachytene, diplotene and diakinesis. The nucleoli dispersed at diakinesis. Following bivalent formation, the subsequent stages of meiosis from metaphase I to telophase II were completed in 3 to 5 days. The nuclear membrane disappeared just prior to metaphase I (Fig. 4). Telophase II resulted in four microspores enclosed within the PMC wall (Fig. 5). After telophase II the chromosomes uncoiled, the nucleoli reappeared and the nuclear membrane reformed. Meiosis was completed in six to 10 days. By the end of meiosis, all tapetal cells had undergone karyokinesis, resulting in a continuous layer of binucleate cells. Random cell divisions in the microsporangial wall caused an increase in the size of the microsporangia. The tapetal cells increased in size due to the enlargement of the microsporangia. The tapetal cell walls appeared to pull away from the cell membrane (Fig. 5). The tapetal cell walls began to degenerate, starting with the radial walls (Fig. 5).

Meiosis resumed and was completed while phenotypically the buds appeared quiescent (stage 1). The pollen cones started to increase in size during meiosis (stage 1), the increase was not noticeable externally until the end of meiosis. By the time the buds had visibly enlarged enough to be considered swollen (stage 2), meiosis was complete. After meiosis, the buds enlarged rapidly within the bud scales due to numerous cell divisions along the central axis and in the microsporophylls, resulting in round, swollen, stage 2, buds (Fig. 6).

The tetrads were enclosed within the PMC wall and floated within a lightly staining liquid, the thecal fluid (Fig. 7). About five to seven days after tetrad formation, the PMC walls degenerated and tetrads separated, releasing the 1-cell microspores. The tapetal cell walls were almost completely degenerated during separation of the microspores. The tapetal cytoplasm became very vacuolate and irregular in outline (Fig. 8).

The pollen cones reached stage 3 (bud burst) about one week after tetrad separation (Fig. 9). The increase in cone size was due to continued elongation and some cell division in the cone axis. Baculae were visible as small projections on the microspore walls five to seven days after tetrad separation. Granular Ubisch bodies formed on the surface of the tapetal cytoplasm, were released into the thecal fluid and incorporated into the pollen wall (Fig. 10). The baculae increased in size and fused forming a tectum about two weeks after tetrad separation. The exine thickened, becoming 3 and 6 μm (Fig. 10). There was little increase in microspore size during this time.

Figs. 1-5. Stage 1 pollen cones.

Fig. 1. Spirally arranged axillary pollen-cone buds. x6.

Fig. 2. Longitudinal section of a quiescent pollen-cone bud showing microsporophylls (msp) and bud scales (bs). x27.

Fig. 3. Longitudinal section of a microsporangium (ms) within a microsporophyll from a quiescent pollen cone showing loosely-packed, thin-walled pollen mother cells (pmc) arrested at pachytene of meiosis. Surrounding the pollen mother cells is a uninucleate, single-layered tapetum (tp). x120.

Fig. 4. Resumption of meiosis; bivalents are lined up during metaphase I. The tapetal nuclei divide to form binucleate cells (arrow). The thecal fluid (tf) has started to accumulate within the microsporangia. x240.

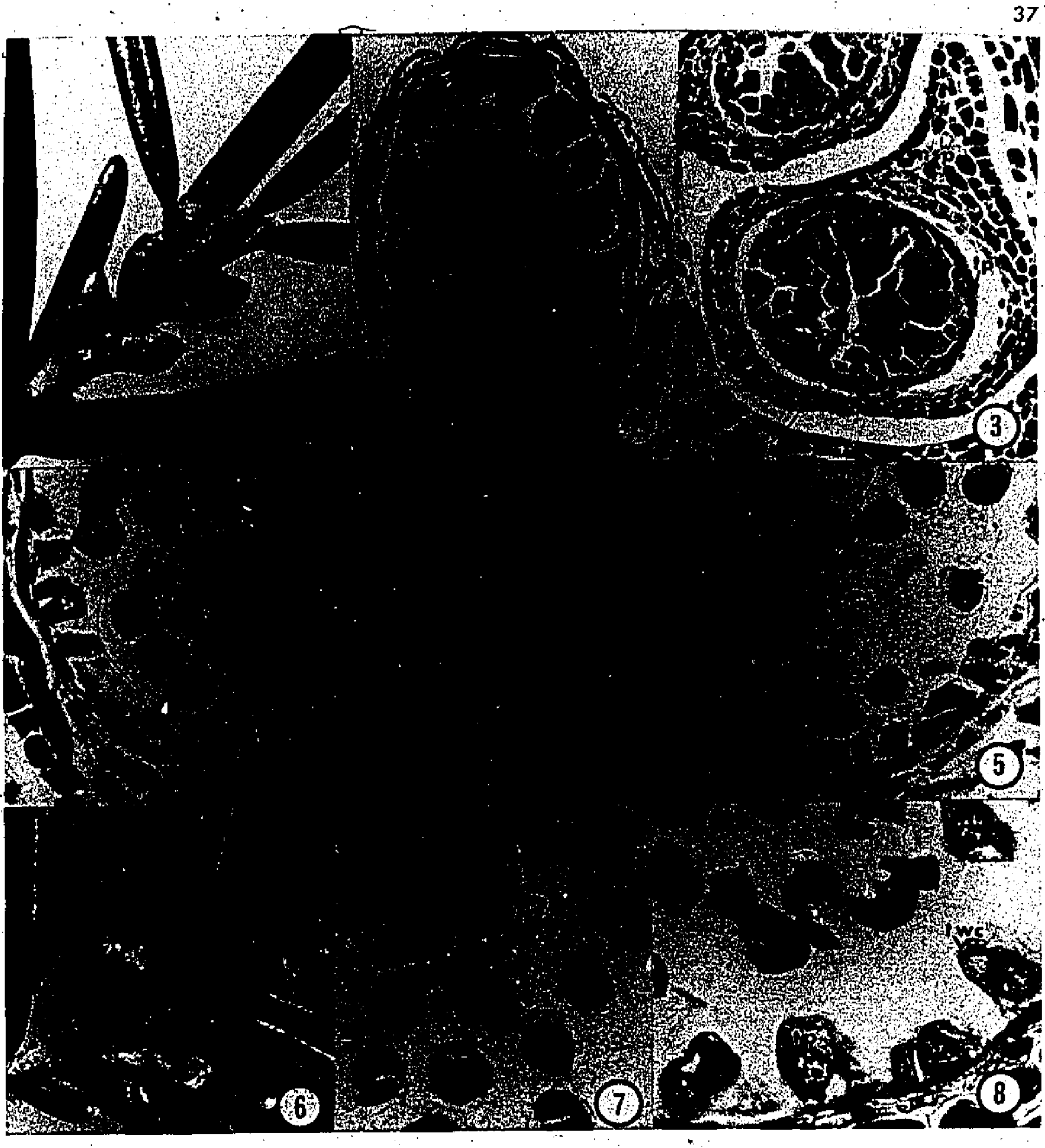
Fig. 5. Telophase II; the thecal fluid continues to accumulate in the microsporangia. The tapetal walls (twc) have pulled away from the cell membrane and have started to break down. x240.

Figs. 6-8. Stage 2 pollen cones.

Fig. 6. Swollen pollen-cone buds. x6.

Fig. 7. Tetrads of microspores (tm) enclosed by the pollen mother cell wall (mcw) floating within the thecal fluid. x400.

Fig. 8. Breakdown of the pollen mother cell wall releases the one-celled microspores (m). The tapetal cell wall has degenerated almost completely and the tapetal cytoplasm is irregular in shape. x440.


P
P
3
5
wc
6
7
8

By stage 4, about 10 to 12 days after tetrad separation, the pollen cones were one-quarter to one-half emerged (Fig. 11). The microspores started to expand rapidly, accumulated reserves and the tapetum was severely depleted (Fig. 12). An intine was observed shortly after microspore expansion. The exine pulled away from the inner layers of the pollen wall along the margin of the microspores, forming vestigial wings (Fig. 12). These became more pronounced as the pollen increased in size and were visible throughout pollen development (Figs. 12, 14, 16, 17).

Stage 5 began in mid-March, about 14 to 16 days after tetrad separation at which time the cones were greater than one-half but less than fully emerged beyond the bud scales (Fig. 13). During stage 5 cell division began. The microspore nucleus divided to produce a small, lens-shaped primary prothallial cell and a larger embryonal cell (Fig. 14). A thin intine formed around the prothallial cell. The 2-cell pollen grains were almost full sized and the tapetal cells were almost completely degenerated.

Four to seven days later, the cones emerged completely beyond the bud scales to reach stage 6 (Fig. 15). Two cell divisions occurred during stage 6. The embryonal cell divided to form a second small prothallial cell and an anthridial initial (Fig. 16). A thin layer of intine formed around the second prothallial cell. The prothallial cells were pushed against the pollen-grain wall making the two cells difficult to distinguish during later stages of development. Remnants of the tapetal cells were still visible. During late stage 6, just before stalk elongation and two to four days after the second cell division, the third cell division produced a generative cell and tube cell (Fig. 17). The generative cell formed next to the two prothallial cells while the tube nucleus remained near the middle of the pollen grain. A thin layer of intine formed around the generative cell. By this time, the pollen grains were full sized and the tapetum was no longer visible.

During stage 7, the stalk of the emerged pollen cone elongated (Fig. 18). This began two to four days after the third division. During stage 7, the fourth and final cell division of the generative cell resulted in mature, 5-cell pollen consisting of two prothallial cells, stalk cell, body cell and a large tube cell (Fig. 19).

Figs. 9-10. Stage 3 pollen cones.

Fig. 9. Less than one-quarter of the pollen cone has emerged through the bud scales (bs). x5.

Fig. 10. Ubisch bodies (ub) released from the surface of the degenerating tapetal cells are incorporated into the exine (e) of the one-celled microspores, resulting in a thick pollen wall. x725.

Figs. 11-12. Stage 4 pollen cones.

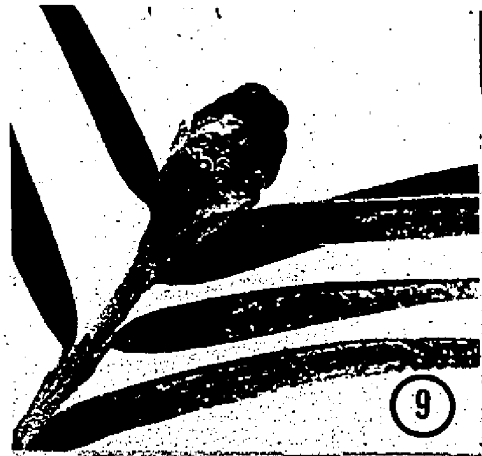
Fig. 11. Between one-quarter and one-half of the pollen cone has emerged through the bud scales. x6.

Fig. 12. The one-celled microspores are starting to expand. Small vestigial wings (vw) develop on the microspores and remain throughout development. The tapetum is severely degenerated. x75.

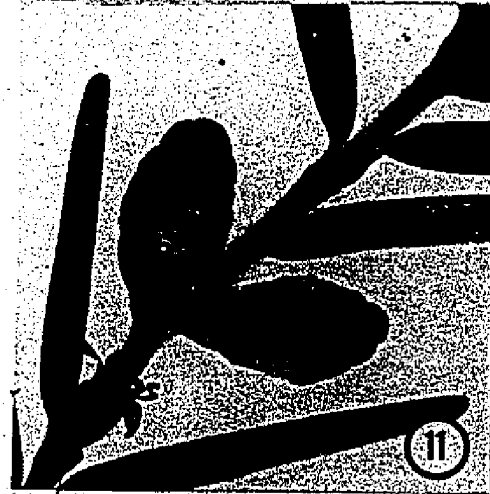
Figs. 13-14. Stage 5 pollen cones.

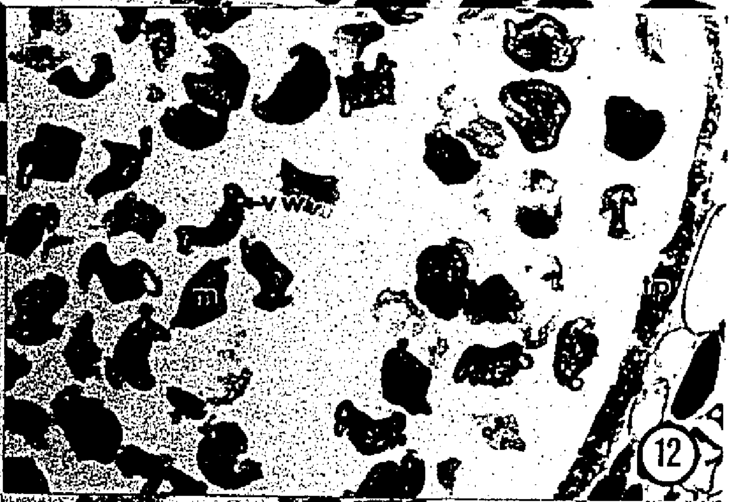
Fig. 13. Approximately three-quarters of the pollen-cone has emerged through the bud scales. x5.

Fig. 14. The first cell division produces two-celled pollen consisting of the small first prothallial cell (p_1) and a larger embryonal cell (ec). The pollen wall consists of an outer exine (e), an inner intine (i) and two small vestigial wings. x530.

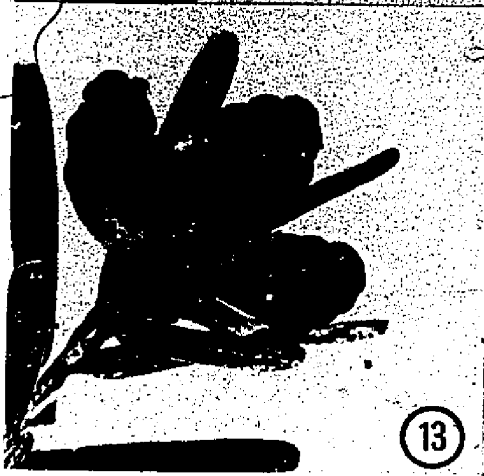

9

10


11



12


13

14

Figs. 15-17. Stage 6 pollen cones.

Fig. 15. The pollen cones have emerged completely from the bud scales. x6.

Fig. 16. The second cell division produces three-celled pollen which includes a small second prothallial cell (p_2) and an antheridial initial (ai). x530.

Fig. 17. The third division, resulting in four-celled pollen, produces a generative cell (gc) and a large tube cell (tc). The tube nucleus (tn) remains near the center of the cell. x530.

Figs. 18-19. Stage 7 pollen cones.

Fig. 18. The elongating stalk (s) pushed the microsporangia away from the stem. x8.

Fig. 19. The fourth and final division produces mature five-celled pollen consisting of two prothallial cells, stalk cell (sc), body cell (bc) and tube cell. x530.

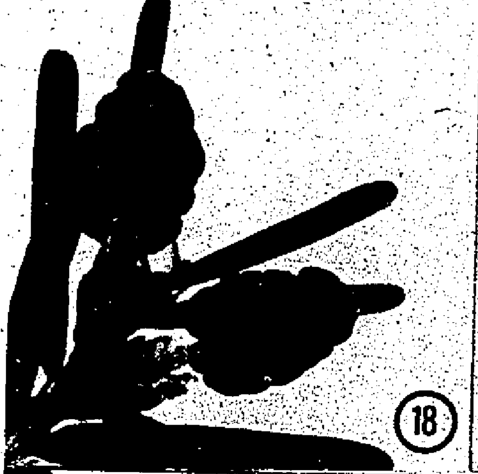
Fig. 20. Stage 8, pollen shedding. x7.

Fig. 21. Stage 9, the empty pollen cone. x8.

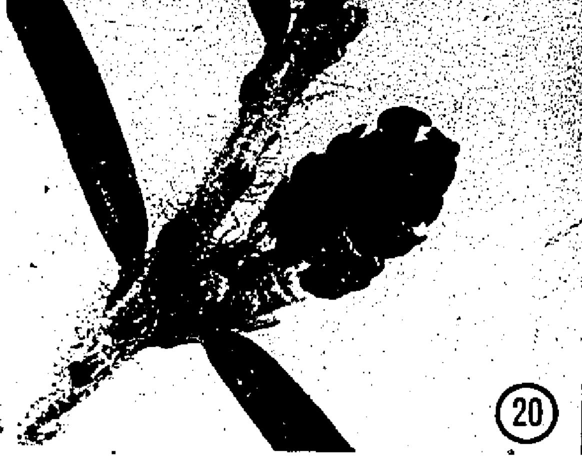

⑮

⑯

⑰


⑱

⑲


⑳


㉑

The irregularly thickened cells of the microsporangial wall dried and the microsporangia split open along a predetermined line, resulting in pollen shed, stage 8 (Fig. 20). Pollen was shed in early April, 2 to 10 days after the formation of mature pollen. The duration of pollen shed for the eight clones ranged from 4 to 10 days. Pollen shed was monitored for an additional 52 trees in the clone bank. The empty cones (stage 9) remained on the trees until the next winter (Fig. 21).

A comparison of pollen-cone phenology and cytology is presented in Table 7. Pollen-cone phenology was synchronized with pollen cytology, independent of the collection dates. Pollen shed commenced about eight weeks after the end of pollen-cone bud dormancy, seven weeks after meiosis, five weeks after tetrad separation and three weeks after the first pollen cell division.

Table 7: A comparison of pollen-cone phenology (STAGE) and pollen cytology for eight western hemlock field-grown clones in 1983.

Dates bound the period during which each stage was observed. Cytology was determined from sectioned material collected at each stage.

STAGE	DATES	POLLEN PHENOLOGY	POLLEN CYTOLOGY
1	Jan 10 - Feb 25	Quiescent bud	PMC to the end of meiosis
2	Feb 20 - Mar 5	Swollen bud	Tetrads of microspores to separation of tetrads
3	Mar 3 - Mar 10	Bud burst	1-cell pollen
4	Mar 10 - Mar 17	Cone one-quarter to one-half emerged	1-cell pollen Starch accumulation
5	Mar 13 - Mar 19	Cone one-half to less than fully emerged	2-cell pollen Starch accumulation
6	Mar 19 - Mar 26	Cone fully emerged	3- and 4-cell pollen
7	Mar 26 - Apr 3	Stalk elongation	4- and 5-cell pollen
8	Apr 3 - Apr 14	Pollen shed	5-cell pollen
9	Apr 5 -	Empty pollen cone	

4.2 Effects of Forcing on Pollen Development

4.2.1 Cut-Branch Method

4.2.1.1 PFT1

The pollen-cone buds were quiescent (stage 1) when the branches were collected on February 9, 1983. A distinct uninucleate layer of tapetal cells surrounded the PMC, which were at pachytene of meiosis (Fig. 1). Meiosis resumed and was completed within two days, resulting in four microspores enclosed within the PMC wall. During this time nuclear division resulted in binucleate tapetal cells. The tapetal walls and cytoplasm began to degenerate releasing their contents into the thecal fluid three to four days after collection. Increased microspore size and numerous cell divisions in the axis of the pollen cone caused the bud to enlarge to stage 2. The exine developed very quickly, and by tetrad separation, baculae were present (Fig. 22). The exine attained full size about two days after tetrad separation. Seven to eight days after collection, the pollen cones elongated beyond the bud scales (stage 3).

Severe degeneration of the cells of the microsporangial wall and the microsporophylls were observed about eight to 10 days after collection (Fig. 23). These cones displayed abnormal pollen development.

The first cell division was observed about 10 days after collection. However, very few pollen grains underwent cell division. Most of the pollen grains started to degenerate about 10 to 12 days after collection (Fig. 24). Those that did not degenerate underwent abnormal division, resulting in atypical cell patterns (Fig. 25). Other pollen-grain abnormalities included irregularly shaped grains at all stages of cell division, shrunken cytoplasm and undeveloped microspores (Fig. 26). Any cell divisions that occurred were asynchronous so that within any microsporangium 2- to 5-cell pollen grains could be found.

About 17 days after collection the first pollen was released. Pollen was shed over a ten-day period. Of the total 1900 cones, 1213 cones (63.8%) either aborted (shriveled), or failed to open. All pollen cones that failed to shed pollen had severely degenerated microsporophyll and microsporangial cells. The remaining 687 cones (36.2%) produced less than one-quarter of a ml of pollen. The pollen cones that aborted, or did not dehisce, elongated very little. The cones that released pollen elongated only 2.5 (±.1) mm.

4.2.1.2 PFT2

When the branches were collected on February 23, the pollen-cone buds were swollen (stage 2), microspores were starting to separate and meiosis had been completed under ambient conditions. Baculae were not visible. The tapetal cells were binucleate, large and intact, with distinct cell walls. Within three days the microspores began to expand, baculae appeared and the tapetum started to degenerate. The first cell division was observed five days after collection. By seven days the exine was very thick (5-6 μm), the 2- and 3-cell pollen grains were almost full-size and the pollen cones had elongated to stages 4 or 5. Abnormalities, consisting of severe degeneration of the microsporangial wall cells (Fig. 23), shrunken cytoplasm within the pollen, complete pollen degeneration and abnormal and asynchronous cell divisions (Fig. 24, 25), were observed about 8 days after collection. Pollen at the 3- to 5-cell stage was released first twelve days after collection. Pollen was shed over a five-day period. Of the total 1871 pollen cones, 419 cones (22.4%) did not dehisce and 1452 cones (77.6%) released approximately .5 ml of pollen. The final length of the pollen cones averaged 2.9 (±.1) mm.

4.2.1.3 PFT3

The pollen-cone buds were at bud burst (stage 3) when the branches were collected on March 6. A thick exine was present on the 1-cell microspores. The binucleate tapetal cells lacked cell walls and were degenerating. Starch accumulation and microspore enlargement was observed within two days. The cones elongated and were completely emerged (stage 6) within four days. On this date the first cell division occurred, followed by the second division one day later.

Figs. 22-26. Longitudinal sections through pollen cones showing the variations in pollen and pollen-cone development after forcing on cut branches.

Fig. 22. Section through part of a pollen cone forced before meiosis. Microspores (m) with thick exines (e) are being released from the tetrads. Ubisch bodies (arrow) are visible on the surface of the tapetal protoplasts (tp). x400.

Fig. 23. Degenerated microsporangial and microsporophyllous cells (arrowheads) containing microspores (m). x35.

Fig. 24. Degenerated pollen (p) within a microsporangium (ms). x115.

Fig. 25. Abnormal cell division in the pollen results in atypical cell patterns. The cells of the microsporangial wall (ms) have degenerated. x115.

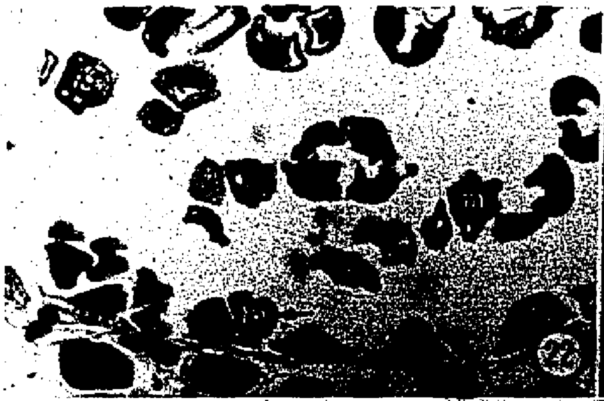
Fig. 26. The cytoplasm (c) has pulled away from the pollen wall. The cells of the microsporangial wall (ms) have begun to degenerate. x110.

Figs. 27-29. Longitudinal sections through microsporangia showing variations in pollen cytology after forcing container-grown ramets of clone 13 at 18°C.

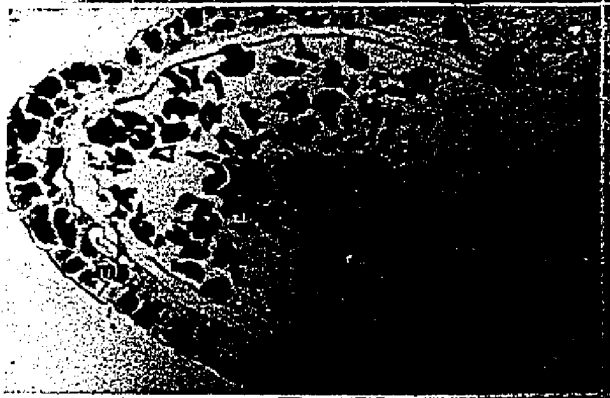
Fig. 27. Cell-wall formation (arrowhead) between two daughter nuclei after meiosis I, results in dyads (d). x180.

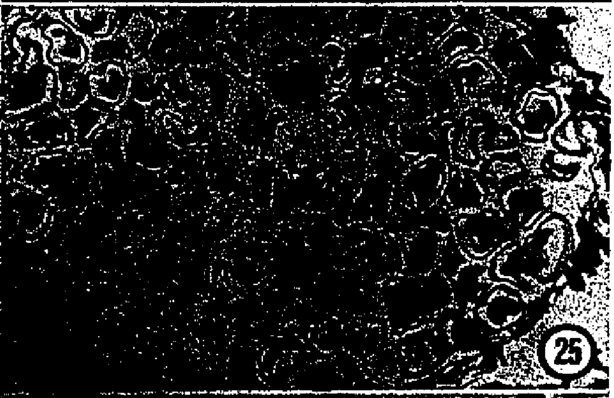
Fig. 28. Various orientations of the tetrads after meiosis II, such as planar (pl), T-shaped (T) and linear (l). x180.

Fig. 29. Proliferation of the microspores during meiosis results in many cells (arrowhead) within the pollen mother cell wall. x165.


㉒

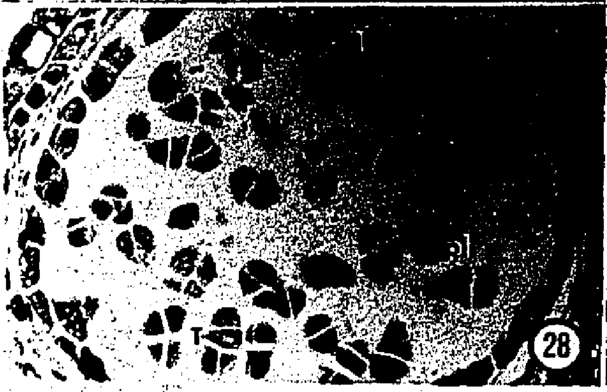

㉓



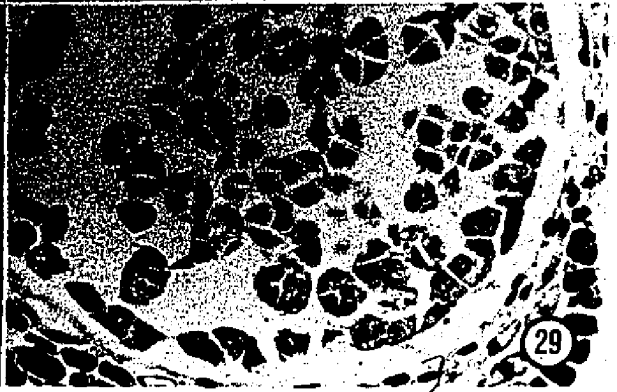

㉕


㉖


㉗



㉘


㉙

The pollen grains had a thick exine, a distinct intine and darkly stained cytoplasm. The first pollen cones started to shed ten days after collection at which time the pollen grains were at the 3- to 5-cell stage. Some abnormalities were observed, such as shrunken cytoplasm, irregular cell division and degenerating pollen and collapsed microsporophyll and microsporangial cells (Fig. 23, 24, 25, 26). The severely degeneration was observed in the cones that failed to shed pollen. Pollen was shed over a five day period. Of the total 1818 pollen cones, 198 (10.9%) did not dehisce and 1620 (89.1%) released pollen from which about 1.0 ml of pollen was collected. The final length of the pollen cones averaged 3.1 (±.1) mm.

4.2.1.4 PFT4

The pollen-cone buds were less than half emerged (stage 4) when the branches were collected on March 13. The 1-cell microspores had a thick exine, a distinct intine and were about one-third to one-half full-size. The tapetal cells had decreased to half size. The first cell division occurred the next day, followed by the second cell division on day three. By the third day the pollen grains were three-quarters to full size and the cones were at stage 6. Pollen was released at the 4- and 5-cell stage seven days after collection and was shed over the next three days. Some cytoplasmic shrinkage and pollen degeneration was observed. Greater than 95% of the approximately 1900 cones released pollen, producing more than 3 ml of pollen. There was very little stalk elongation and the final length of the cones averaged 3.1 (±.1) mm.

4.2.1.5 PFT5

The pollen cones were three-quarters emerged (stage 5) when the branches were collected on March 19. The 2-cell pollen grains were one-half full size and the tapetal cells had degenerated. By day three the pollen grains were at the 3- and 4-cell stage and three-quarters full size. Pollen was shed at the 4- and 5-cell stage, four days after the collections were made. Pollen was released over the next three days. Over 95% of the 1900 cones observed shed pollen, from which more than 3 ml of pollen were collected. The pollen cones averaged 3.3 (±.1) mm.

4.2.1.6 PFT6

On March 30 the pollen cones were at stage 7. The pollen grains were at the mature 5-cell stage. More than 95% of the 1900 cones shed within the next two days from which more than 3 ml were collected. Final cone length averaged 4.1 (±.1) mm. Pollen was mature when branches were collected and began to be shed immediately. PFT6 was considered to be the control for this study.

4.2.1.7 Trends in Pollen Development

Several trends in pollen-cone phenology and pollen cytology were observed between the different pollen-forcing trials. Pollen-cone phenology and pollen cytology at the time of branch collection, the number of days from collection to pollen shed and final cone length are presented for each trial in Table 8.

The number of days until pollen shed decreased with each successive collection ranging from 17 days in PFT1 to one day in PFT6. Regardless of the time of collection, the rate of development with forcing was about three times that observed in the field (Table 8).

There was very little increase in size from the quiescent bud (1.8 (±.1) mm) to the final stage 8 (shedding) pollen cone for cones forced in PFT1 to PFT5. This was due to minimal elongation along the central axis, resulting in little or no separation of the microsporophylls and minimal stalk elongation. The average pollen-cone length of PFT6 (4.1 mm) was significantly higher than that from the first five trials (Table 8). This increased length was due to elongation along the central axis, causing a greater separation of the microsporophylls and a very long stalk.

Table 8: Pollen phenology, cytology and final cone length in the 1983 western hemlock cut-branch pollen-forcing trials (PFT).

Pollen-cone phenology (STAGE) and cytology at the time of collection, and the average number of days until pollen shed from the time of collection under lab and field conditions are presented for the six pollen-forcing trials. Mean pollen cone lengths for 20 cones per trial (LENGTH ± SE) were compared using Student-Newman-Keuls test ($p<.05$). All means followed by the same letter are not significantly different.

TRIAL	STAGE	CYTOLOGY	DAYS UNTIL POLLEN SHED IN LAB AND (FIELD)	LENGTH (±SE)
PFT1	1	pollen mother cells	17 (50)	2.5^{a} (.3)
PFT2	2	separation of microspores	12 (37)	2.9^{b} (.1)
PFT3	3	microspores	10 (30)	3.1^{bc} (.1)
PFT4	4	starch accumulation	7 (22)	3.1^{bc} (.1)
PFT5	5	2-cell pollen	4 (13)	3.3^{c} (.1)
PFT6	7	5-cell pollen	1 (2)	4.1^{d} (.2)

Several trends in pollen production and pollen development were observed (Table 9). The percentage of cones that shed pollen increased from PFT1 (36.2%) to PFT4 (95%). The percentage of cones that shed varied significantly ($p<.05$) between PFT1, PFT2, PFT3 and PFT4. No differences were observed between PFT4, PFT5 and PFT6 where more than 95% of the cones released pollen. A similar trend was observed for the amount of pollen produced in each trial. PFT1 produced only a fraction of a ml of pollen from all cones while greater than 3 ml of pollen were collected in PFT4, PFT5 and PFT6. The pollen amounts collected in PFT1, PFT2 (.5 ml), PFT3 (1.0 ml) and PFT4 (>3 ml) were significantly different ($p<.05$) whereas no differences in pollen production were observed between PFT4, PFT5 and PFT6 (Table 9).

Table 9: Pollen production in the 1983 western hemlock cut-branch pollen-forcing trials (PFT).

The percentage of cones that shed (% SHED) and the quantity of pollen produced (ml) from about 1900 cones were compared using multiple comparisons for proportions ($p<.05$). All values followed by the same letter are not significantly different.

TRIAL	STAGE	% SHED	POLLEN PRODUCED	CYTOLOGY AT POLLEN SHED
PFT1	1	36^{a}	$<.1$ ml^{a}	abnormal
PFT2	2	77^{b}	$.5$ ml^{b}	1-5 cell
PFT3	3	89^{c}	1.0 ml^{c}	3-5 cell
PFT4	4	$>95^{d}$	>3 ml^{d}	4-5 cell
PFT5	5	$>95^{d}$	>3 ml^{d}	4-5 cell
PFT6	7	$>95^{d}$	>3 ml^{d}	5 cell

The cytology of the pollen grains at the time of shedding varied between the trials. PFT1 produced very little pollen, most of which was abnormal. About half of the microspores produced in PFT2 degenerated within the microsporophylls. Pollen that did not degenerate varied between the 1- and 5-cell stage when shed. Less degeneration was observed in PFT3, where pollen was shed at the 3- to 5-cell stage. Pollen was released at the 4- and 5-cell stage in PFT4 and PFT5. With the exception of some cytoplasmic shrinking and pollen-grain degeneration, most of the pollen grains produced in PFT4 and PFT5 appeared normal. The pollen collected from PFT6 was 5-cell.

4.2.1.8 Seed Efficiency

Two clones were pollinated with fresh pollen produced in PFT2 to PFT6. The SEFs produced in each clone were not significantly different so the results were pooled (Table 10). The average SEF increased from 45.4 in PFT2 to 71.3 in PFT6. The results fell into three significant groups ($p<.05$). PFT5 (69.7) was the only trial not significantly different from the control, PFT6 (71.3), suggesting that no detectable loss in fertilization potential occurred when pollen cones were collected at stage 5 and forced to mature under lab conditions. The SEFs produced from pollen collected in PFT2 (45.4), PFT3 (52.2) and PFT4 (56.0) were significantly lower than the control, PFT6 (71.3) implying that a reduction in the pollens' fertilization ability occurred during the forcing procedures. Pollen produced in PFT2 resulted in a significantly lower SEF than pollen produced in PFT3 and PFT4.

Table 10: Seed efficiencies resulting from pollinations of two western hemlock clones with fresh pollen produced in the 1983 cut branch pollen-forcing trial (PFT).

The mean seed efficiencies (SEF ± SE) were determined for 40 cones (20 cones per clone) using pollen produced in PFT2 to PFT6. Means with the same letter are not significantly different as determined by pair-wise comparisons using Wilcoxon 2-sample test ($p<.05$).

TRIAL	N	SEF (±SE)
PFT2	40	45.4^{a} (2.0)
PFT3	40	52.2^{b} (2.0)
PFT4	40	56.0^{b} (2.1)
PFT5	40	69.7^{c} (1.3)
PFT6 (CONTROL)	40	71.3^{c} (1.7)

The pollens' ability to produce filled seed (fertilization potential) was retested after two years storage at 4°C in a 1985 pollination trial (Table 11). The results were classified into two significant groups ($p<.05$). The first group included PFT2 and PFT3 with SEFs of 2.9 and 1.2, respectively. The second group consisted of PFT4, PFT5 and PFT6 with SEFs of 58.3, 59.8 and 64.7, respectively (Table 11).

The 1983 and 1985 SEF results, comparing fresh pollen to pollen stored at 4°C for two years, are presented in Fig. 30. After two years of storage the SEFs resulting from the pollen produced in PFT2 and PFT3 decreased from 45% to near zero while little or no detectable decrease was observed in the SEFs resulting from the pollen collected in PFT4, PFT5 and PFT6. The decrease in SEF indicates a considerable reduction in viability and/or vigor after two years of storage for the pollen produced in PFT2 and PFT3.

Table 11: Seed efficiencies resulting from the pollination of a western hemlock clone with two-year-old pollen produced in the 1983 cut-branch pollen-forcing trial (PFT).

The mean seed efficiencies (SEF ± SE) were determined for N cones in 1985 using pollen stored at 4°C for two years. Means with the same letter are not significantly different as determined by pair-wise comparisons using the Wilcoxon 2-sample test ($p<.05$).

TRIAL	N	SEF (±SE)
PFT2	18	2.9^a (0.7)
PFT3	16	1.2^a (0.4)
PFT4	18	58.3^b (2.3)
PFT5	16	59.8^b (3.7)
PFT6 (CONTROL)	14	64.7^b (4.0)

4.2.2 Whole-Tree Method

4.2.2.1 18°C Growth Chamber Trials

Ramets of three clones (13, 37, 101) were placed into growth chambers maintained at 18°C on three dates (January 12, 22 and February 5). Average pollen-cone phenology (STAGE) for the clones in each trial were plotted versus date (Fig. 31). The pollen cones were at stage 1 (quiescent) when the trees were put into the growth chambers. Within 3 to 4 days, the buds in PFT1-18 and PFT2-18 began to swell (stage 2). The buds on the ramets in PFT3-18 started to swell within 24 hours. On any date the cones within a clone varied in phenology, with the distal cones being one or two stages further advanced than the basal cones. The distal-to-basal-trend was observed in all ramets at 18°C.

Figure 30. Seed efficiency and standard errors for pollinations conducted in 1983 using fresh pollen from the cut-branch pollen forcing trials and in 1985 using the same pollen stored at 4°C for two years.

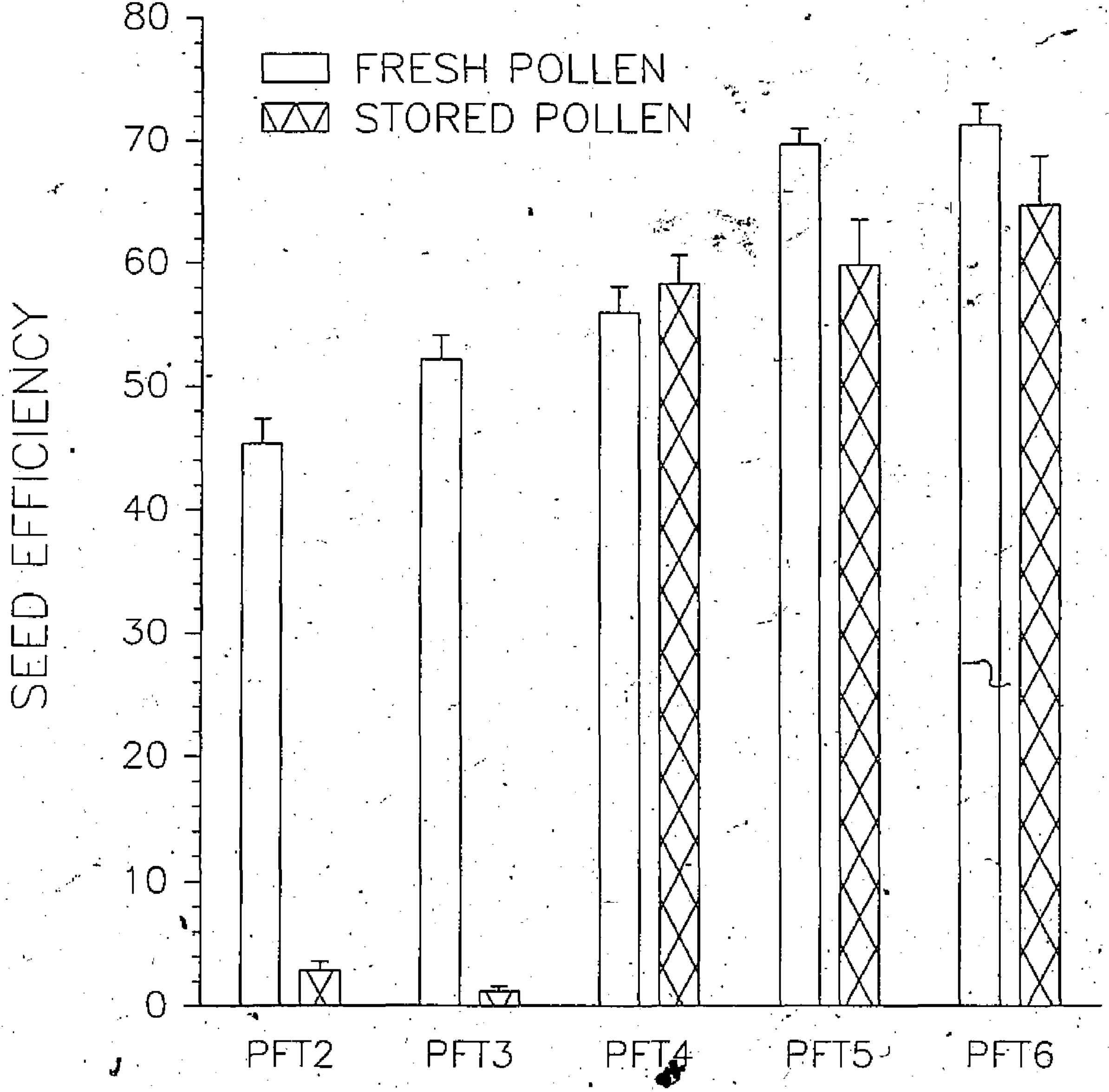
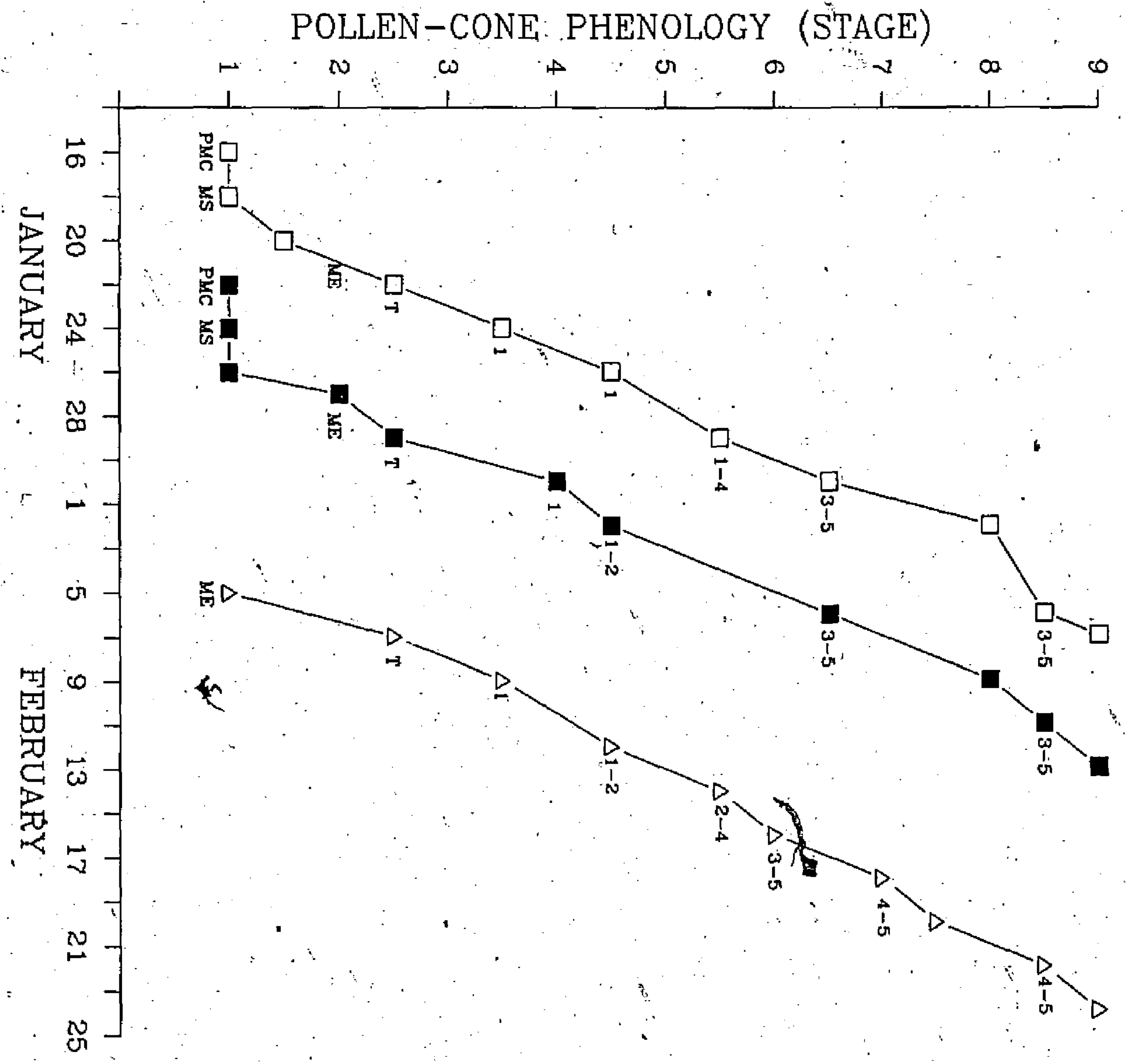

SEED EFFICIENCY
80
70
60
50
40
30
20
10
0
FRESH POLLEN
STORED POLLEN
PFT2
PFT3
PFT4
PFT5
PFT6

Figure 31. Pollen phenology at 18°C in the 1984 whole-tree pollen-forcing trial. Average pollen phenology for the three clones in PFT1-18 (□), PFT2-18 (■) and PFT3-18 (△) were plotted versus date. Pollen cytology at each sampling date is included: pollen mother cells (PMC), start of meiosis (MS), end of meiosis (ME), separation of tetrads (T), 1-cell pollen grains (1), variable number of cells per pollen grain, eg. 2- to 4-cell pollen grains (2-4).


POLLEN-CONE PHENOLOGY (STAGE)
1
2
3
4
5
6
7
8
9
16
20
24
28
1
5
9
13
17
21
25
JANUARY
FEBRUARY
PMC MS
ME
T
1
1
1-4
3-5
3-5
PMC MS
ME
T
1
1-2
3-5
3-5
ME
T
1
1-2
2-4
3-5
4-5
4-5

In the first two trials, PFT1-18 and PFT2-18, the PMCs were arrested at pachytene of meiosis when the ramets were placed into growth chambers. Meiosis resumed within two days in both trials. By day four all stages of meiosis could be found within each pollen cone. The PMCs in the basal microsporangia were further advanced than PMCs in distal microsporangia. When the basal microsporangia contained tetrads of microspores, PMC in the mid-section of the cone ranged between prophase II to telophase II while in the distal microsporangia the PMC ranged between prophase I and metaphase I. The tapetal cells were binucleate and had started to degenerate. Exceptions were found in clone 13, where the reverse was found in two of the six pollen cones sectioned at this stage. Pollen in the distal microsporangia were further advanced than pollen in the basal microsporangia. Another anomaly displayed by clone 13 was dyad formation (cell wall formation between the two daughter nuclei during prophase) II in about one-third of all PMCs (Fig. 27). The second meiotic division resulted in several orientations of the tetrads: in the normal tetrahedron, planar, a linear formation, T-shaped tetrads and many variations of the above (Fig. 28). The unusual orientations did not affect tetrad separation. In one cone a proliferation of cells was observed within the PMC wall (Fig. 29).

The pollen cones in all three clones elongated to stage 2 during meiosis. Meiosis was completed in all regions of the cones by day six. The cones continued to elongate so that by day eight separation of the tetrads coincided with bud burst (stage 3). The cones elongated to stage 4 while the 1-cell pollen grains began to enlarge and accumulate reserves.

In PFT3-18 clones 37 and 101 were placed into the growth chamber at the tetrad stage. The tapetal cells were binucleate and had not started to break down. Within one day the pollen-cone buds began to swell (stage 2) and by the second day the tetrads separated. Clone 13 was still at the overwintering (pachytene) stage when placed into the growth chamber. Meiosis resumed immediately and was completed within three days. Pollen development in clone 13 was similar to that observed for that clone in the first two trials.

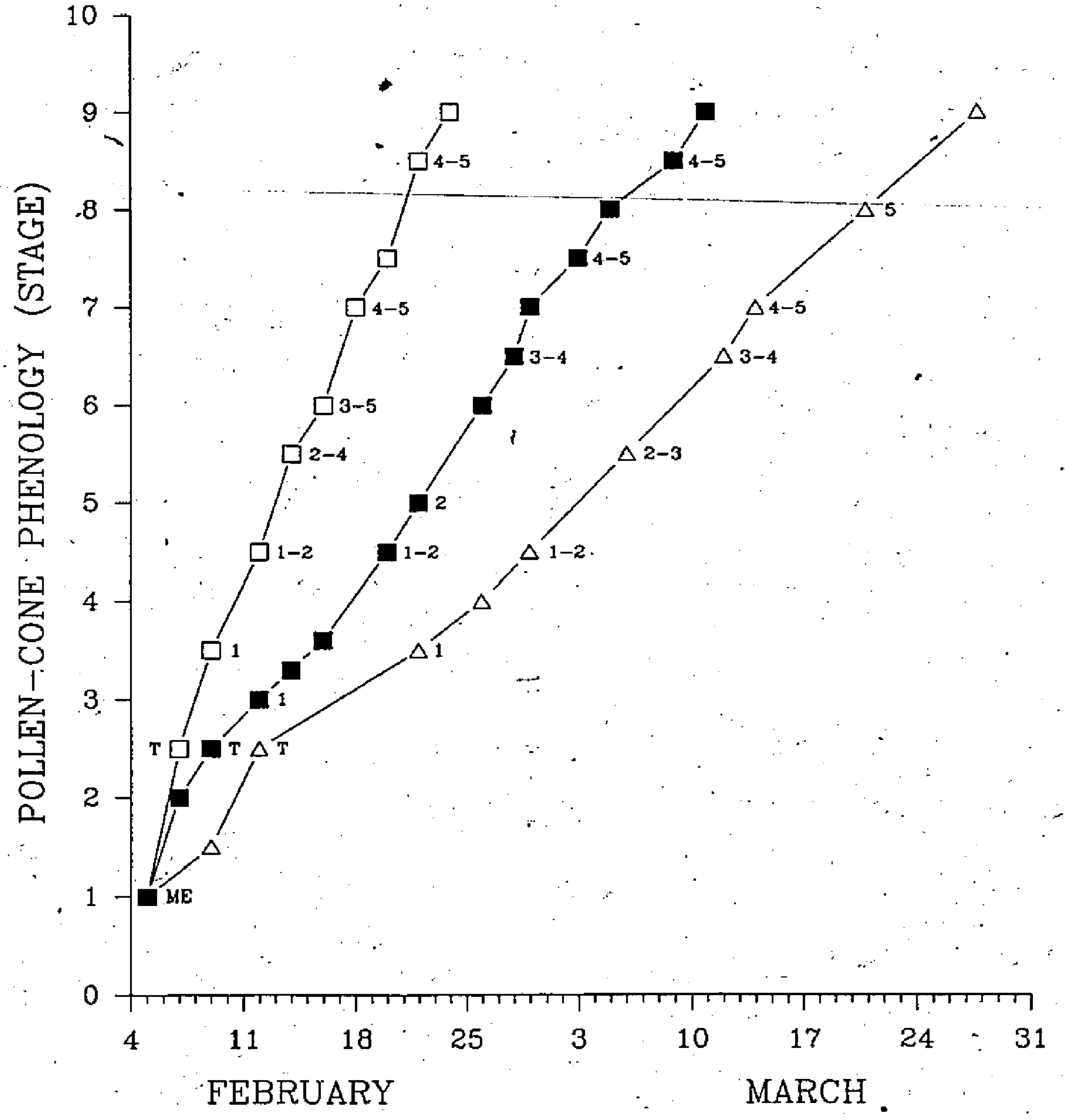
In all clones the 1-cell pollen grains enlarged while the pollen cones elongated to stage 4. The rate of development from the separation of tetrads to pollen shed was similar for all three trials, independent of the dates and bud stages when the ramets were placed into the growth chambers (Fig. 31). It took about 16 days from the time the first pollen-cone buds started to swell until the start of pollen shed.

The average time span between the separation of the tetrads and the first cell division was about five days. The pollen cones were at stage 5 and the pollen grains were one-half to three-quarters full size. Cell divisions were asynchronous, so that within a cone 1- to 3-cell pollen grains could be found. Pollen shed occurred about seven to nine days after the first-cell division. Many pollen grains had a shrunken cytoplasm, a few were irregularly shaped and some pollen-grain degeneration was observed. In PFT1-18, PFT2-18 and clone 13 of PFT3-18, pollen was shed at the 3- to 5-cell stage while the other two clones in PFT3-18 shed pollen at the 4- to 5-cell stage.

4.2.2.2 Effect of Temperature on Pollen Development

Separate ramets of three clones (13, 37 and 101), were placed into growth chambers set at 18°C and 10°C, respectively, on February 5, 1984. Other ramets of the same clones were kept outdoors under ambient conditions. The pollen-cone buds were quiescent (stage 1) on February 5. Tetrads were found in clones 37 and 101, while clone 13 was at pachytene. Average pollen-cone phenology (STAGE) for clones 37 and 101 was plotted versus date (Fig. 32). Clone 13, which consistently lagged eight to 10 days behind the other two clones at all temperatures, will be discussed separately.

Figure 32. Pollen phenology at 18°C, 10°C and ambient temperatures in the 1984 whole-tree pollen-forcing trial. Average pollen phenology for two clones in PFT3-18 (□), PFT3-10 (■) and PFT3-C (Δ) were plotted versus date. Pollen cytology at each sampling date is included: end of meiosis (ME), separation of tetrads (T), 1-cell pollen grains (1), variable number of cells per pollen grain, eg. 2- to 4-cell pollen grains (2-4).


POLLEN-CONE PHENOLOGY (STAGE)
0
1
2
3
4
5
6
7
8
9
10
4
11
18
25
3
10
17
24
31
FEBRUARY
MARCH
ME
T
T
T
1
1
1
1-2
1-2
1-2
2-4
2
2-3
3-5
3-4
3-4
4-5
4-5
4-5
4-5
4-5
5

The rate of pollen development varied with temperature. Tetrad separation in PFT3-18, PFT3-10 and PFT3-C occurred 2, 4 and 7 days later, respectively. At tetrad separation the pollen cones in PFT3-18 were at stage 3 while in PFT3-10 and PFT3-C the pollen cones were still at stage 2. The exine was fully developed 3, 8 and 13 days, respectively after tetrad separation. The time between the separation of tetrads and first cell division was 5, 11 and 15 days, in PFT3-18, PFT3-10 and PFT3-C, respectively. The pollen cones in each trial were at stage 5 when the first cell division occurred.

Cell divisions were asynchronous at 18°C. One- to 3-cell pollen were found within stage 5 pollen cones. At 10°C and ambient temperatures cell division was synchronous. Two-cell pollen grains generally were found within the stage 5 cones. By stage 6, 3- and 4-cell pollen grains were observed in the three trials. During stage 7, mature 5-cell pollen grains were found in PFT3-10 and PFT3-C, while 4- and 5-cell pollen grains were observed in PFT3-18. Pollen shed in the three trials started about 8, 13 and 22 days respectively, after the first cell division in PFT3-18, PFT3-10 and PFT3-C. In PFT3-18, a few degenerated pollen grains and some with shrunken cytoplasm were observed and pollen shed started 16 days after the trees were placed in the growth chamber. In PFT3-10 and PFT3-C, pollen shed started 30 and 44 days, respectively, after the start of the experiment.

As mentioned previously, clone 13 lagged behind the other clones. Meiosis was completed 3, 7 and 11 days respectively, after being placed in the growth chamber in PFT3-18, PFT3-10 and PFT3-C. After tetrad separation, pollen development occurred at the same rate in clone 13 as in clones 37 and 101 except that it lagged 8 to 10 days behind. At 18°C, a cell wall formed between approximately one-third of two daughter nuclei after meiosis I, resulting in the unusual orientation of the tetrads within the PMC walls. The cell wall did not form at 10°C or under ambient conditions.

4.2.2.3 Seed Efficiency

The first pollination trial in 1984 using freshly collected, pooled pollen was incomplete due to root weevil infestation. The cones aborted and later the ramets died so no SEF data could be collected. The air-dried pollen was stored at 4°C and the pollinations repeated one year later. The SEFs produced in the 1985 pollinations are presented in Table 12.

Table 12: Seed efficiency results using pollen stored for one year at 4°C, in the whole-tree pollen-forcing trials.

TRIAL	N	SEF (±SE)
PFT1-18	16	3.3^{a} (0.9)
PFT2-18	13	5.3^{a} (1.7)
PFT3-18	15	11.8^{b} (2.1)
PFT3-10	13	40.1^{c} (3.7)
PFT3-C	12	51.9^{c} (2.5)

After one year of storage at 4°C, pollen forced at 18°C produced low SEFs, with values of 3.3, 5.3 and 11.8, respectively for PFT1-18, PFT2-18 and PFT3-18. Pollen produced in PFT3-10 resulted in an average SEF of 40.1 while the highest average SEF of 51.9 was obtained from pollen produced in PFT3-C. In PFT3-18, where the pollen was forced after the completion of meiosis, the average SEFs were significantly higher ($p<.05$) than the average SEFs resulting from the pollinations by pollen produced in PFT1-18 and PFT2-18, where forcing started before meiosis. It must be remembered that about one-third of the pollen in PFT3-18 was derived from clone 13, which was forced before meiosis. Even with this pollen, the SEFs produced by the PFT3-18 pollen were significantly higher than those produced by the first two trials. Pollen forced at 10°C produced a SEF (40.1) that was significantly higher than the SEFs produced by the 18°C trials. Pollen collected from the trees grown under ambient conditions produced the highest SEF (51.9), which was significantly higher than that from the 18°C growth-chamber trials, but not the 10°C trial. From these results it cannot be determined whether forcing pollen or storing it at 4°C for one year caused the reduction in SEF. Since the SEF results of freshly collected pollen are unavailable, the separate effects of forcing and storage cannot be determined from this trial.

4.3 Pollination Mechanism

Cone development in western hemlock was divided into nine phenological stages as described previously in Table 4. These included the quiescent seed-cone bud through cone development until cone closure (Figs. 33-41).

Western hemlock seed-cone buds were located terminally on distal, more vigorous branches in the middle to upper crown. Seed cones were collected before, during and after pollination in 1983 and 1984. Quiescent (stage 1) seed-cone buds averaged 2.5 (±.4) mm in length (Fig. 33). Each bud was enclosed by bud scales and consisted of spirally arranged bracts (Fig. 42). In the axil of most bracts was a small swelling which would develop into an ovuliferous scale (Fig. 43). Most ovuliferous scales bore two ovule primordia. The first three or four basal bracts were sterile since they lacked ovuliferous scales. The next four to eight ovuliferous scales were also sterile because they lacked ovule primordia. The distal one to four ovuliferous scales also lacked ovule primordia. The average number of fertile ovuliferous (±SE) scales was 16.4 (±2.5) ranging from 14 to 22.

Enlargement of the seed-cone buds to stage 2 was observed in late February and early March (Fig. 34). Cell division and elongation in the central axis of the cone as well as in the bracts, ovuliferous scales and ovules caused the cone to increase in width and length. The cells surrounding the nucellus divided and enlarged, forming the integument, a ring of meristematic tissue (Fig. 44). The integument elongated and overgrew the nucellus to form a short funnel with a wide opening, the micropyle (Figs. 44, 45). Stage 3, bud burst, resulted from elongation along the central axis, bracts and ovuliferous scales. This caused the bud to increase in size and the distal bracts to protrude through the bud scales (Fig. 35).

Figs. 33-41. Seed-cone phenology, stages 1 to 9.

Fig. 33. Stage 1; quiescent seed-cone bud, completely enclosed by bud scales (bs) collected in mid-February. x12.

Fig. 34. Stage 2; swollen seed-cone bud, collected in mid-March. x10.

Fig. 35. Stage 3; bud burst, less than one-quarter of the cone has emerged through the bud scales. x10.

Fig. 36. Stage 4; one-quarter to one-half of the cone has emerged through the bud scales exposing bracts (b) and ovuliferous scales (os) in mid- to late-March. x8.

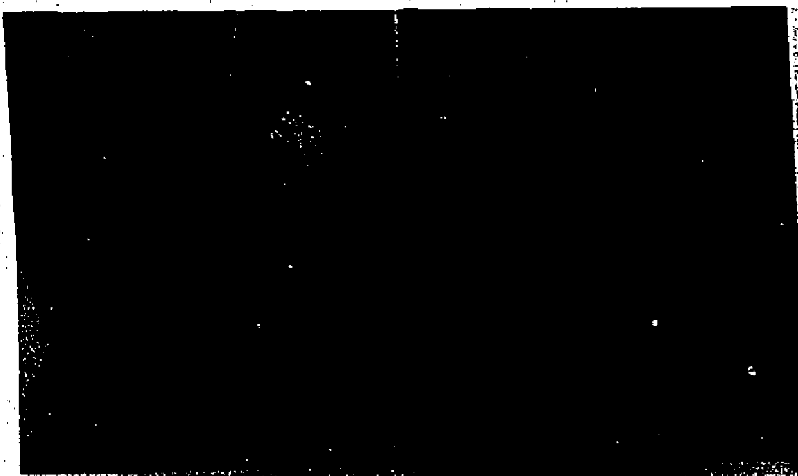
Fig. 37. Stage 5; three-quarters of the cone has emerged through the bud scales. x8.

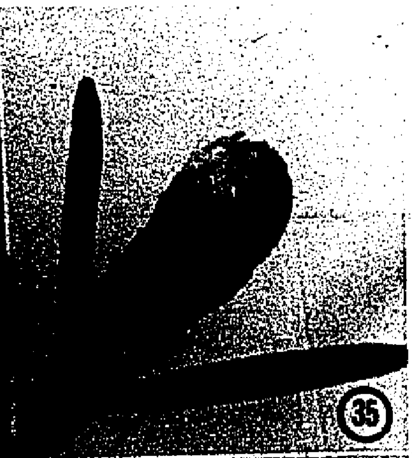
Fig. 38. Stage 6; seed-cone completely emerged through bud scales in late March to early April. x8.

Fig. 39. Stage 7; ovuliferous scales have started to elongate over the bracts. Less than 50% of the bract covered. x7.

Fig. 40. Stage 8; bracts more than half covered by ovuliferous scales. x8.

Fig. 41. Stage 9; closed cone, bracts completely covered by ovuliferous scales. x6.





35

37

38

39

40

41

Figures 42-47. Scanning electron micrographs (SEM) and light micrographs of sections of seed cones, bracts (b), ovuliferous scales (os) and ovules (o) during post-dormancy development.

Fig. 42. SEM of a quiescent seed-cone bud consisting of spirally arranged bracts. x30.

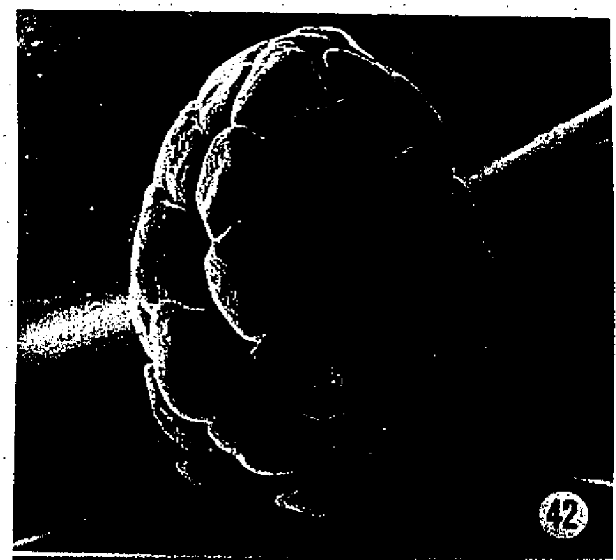
Fig. 43. Longitudinal section through a quiescent seed-cone bud showing bud scales (bs), bracts, ovuliferous scales and ovule primordia (op). x35.

Fig. 44. SEM of developing ovule on an ovuliferous scale showing nucellus (n), elongating integument (i), ovule and part of the ovuliferous scale. x100.

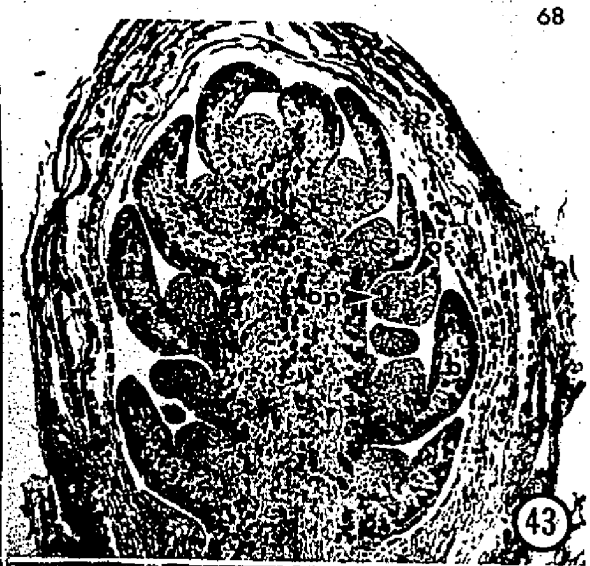
Fig. 45. Longitudinal section through stage 2 seed-cone bud showing a portion of the bract, ovuliferous scale and an ovule with developing nucellus, integument and micropyle, (m). x80.

Fig. 46. SEM of a fully enlarged ovule on an ovuliferous scale where the integument has elongated, forming a short micropyle (m). The nucellus is visible at the base of the short micropyle. x100.

Fig. 47. Longitudinal section through stage 6 seed cone showing a portion of the bract, ovuliferous scale and an ovule at pollination. The integument has elongated forming a short micropylar canal within which is the nucellus . x80.



42



43

45

46

47

At bud burst the ovuliferous scales were about the same length as the bracts. As a result of cell divisions in the integument's tip, the micropyle increased in length (Figs. 46, 47).

The seed cones continued to elongate and emerge from the bud scales over the next several weeks. Cones were considered stage 4 when 25 to 50% of the cone had emerged (Fig. 36). Stage 5 cones were greater than 50% emerged but less than fully emerged (Fig. 37). Stage 6 cones were emerged fully (Fig. 38). All but a few basal bracts were exposed. The ovuliferous scales elongated more than the bracts so that by stage 6, the ovuliferous scales were slightly longer than the bracts. The bracts reached maximal length by stage 6.

During stage 6 the bracts and scales often reflexed, however not sufficiently so that the ovules were exposed. About one week after the cones reached stage 6 the ovuliferous scales started to elongate beyond the bracts. Cones were considered in stage 7 if less than half of the bract was covered by the ovuliferous scale (Fig. 39) and at stage 8 if bract coverage was between 50 and 100%. Cones were at stage 9 after the scales completely covered the bracts (Fig. 41).

No structural changes were observed in the ovules other than an increase in size between stages 3 and 9. All cones had closed by the end of April in 1983 and by the first week of April in 1984. Once the cones closed they elongated to full size over the next 5 to 6 weeks, increasing from an average of 5 mm to about 24 mm.

Since the bracts and scales never reflexed enough to allow pollen to filter down to the ovules, pollen was not observed near the integument or in the micropyle. Pollen was found consistently on the surface of the bracts (Fig. 48), which was covered with a thick, thread-like, waxy layer (Fig. 49). The outer, exposed edge of the ovuliferous scale also had a similarly thick layer, while only a thin waxy layer was found on the rest of the ovuliferous scale (Fig. 50).

The surface of western hemlock pollen is roughly sculptured and contains many spines and tecta in a verrucate pattern which became entangled in the waxy layer of the bracts, firmly attaching the pollen to the bracts (Fig. 51, 53) where it remained. Eventually the ovuliferous scale elongated over the bracts, trapping the pollen between the bracts and scales (Fig. 52).

Shortly after cone closure, several changes were observed on the surface of the bracts. The epidermal cells along the margin of the bracts developed long projections. About one week after cone closure each adaxial epidermal cell produced a single smooth projection which erupted through the waxy layer (Fig. 53, 54). They first appeared along the mid-rib of the bract and then spread toward the margins. The longest projections were observed along the centre of the bract (Fig. 55). Long projections were observed also arising from the epidermal cells on the margin of the ovuliferous scales, where they overlapped, forming a seal between the scales (Fig. 56). On the bracts the projections developed around any pollen grains adhering to the surface (Fig. 57).

Shortly after the projections were observed the pollen grains began to hydrate and swell and within a few days pollen tubes were visible (Fig. 58). The pollen tubes grew along the bract surface (Fig. 59) towards the micropyles. It often appeared that pollen grains located on the margin of the bract furthest from the ovules and cone axis grew pollen tubes in random directions while pollen closer to the micropyle grew tubes towards the micropyle. About six weeks after pollination and four to seven days after pollen germination, pollen tubes grew into the micropyles (Fig. 60). Usually 1 to 6 pollen tubes were observed within each micropyle but on several occasions up to 10 pollen tubes were found.

4.4 Optimal Time of Pollination

4.4.1 Cobble Hill - 1983

A relationship was observed between seed-cone phenology, and SEF for the two clones in 1983 (Fig. 61). The SEF results for the two clones are also presented in Table 13. For both clones 7-10 and 9-3 the average SEF increased with phenological stage. Since phenological class was determined by the amount of cone exposed it was related to the number of bracts accessible to pollen. The pollinations were conducted by date, rather than stage, so the cones within each bag were at different phenological stages.

Figures 48-54. Scanning electron micrographs (SEM) and light micrographs of sections of seed cones at pollination.

Fig. 48. Pollen (p) adhering to the exposed bracts (b) of a stage 6 seed cone. x10.

Fig. 49. SEM of epicuticular waxes on the surface of the bract at pollination. x1650.

Fig. 50. SEM of epicuticular waxes on the surface of the ovuliferous scale at pollination. x1650.

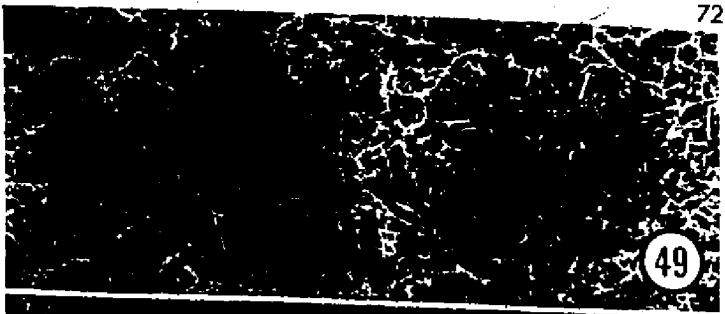
Fig. 51. SEM of a pollen grain entangled in the epicuticular waxes (w) on a bract. x950.

Fig. 52. Longitudinal section of pollen trapped between a bract and an ovuliferous scale (os) after cone closure. x88.

Fig. 53. SEM of epidermal projections (ep) erupting through the waxy layer (w) next to a pollen grain. The pollen grain is entangled in the wax fibres. x3500.

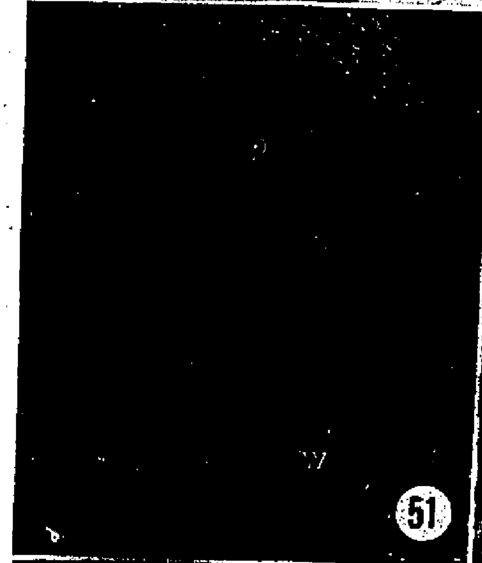
Fig. 54. SEM of epidermal projections (ep) developing from the epidermal cells of the bract. x510.

48



49

50



51

52

53

54

Figures 55-60. Scanning electron micrographs and light micrographs of sections of post-pollination seed cone, and pollen tube development.

Fig. 55. SEM of the epidermal projections on the surface of a bract. x50.

Fig. 56. Longitudinal section showing projections (pr) between ovuliferous scales (os). x88.

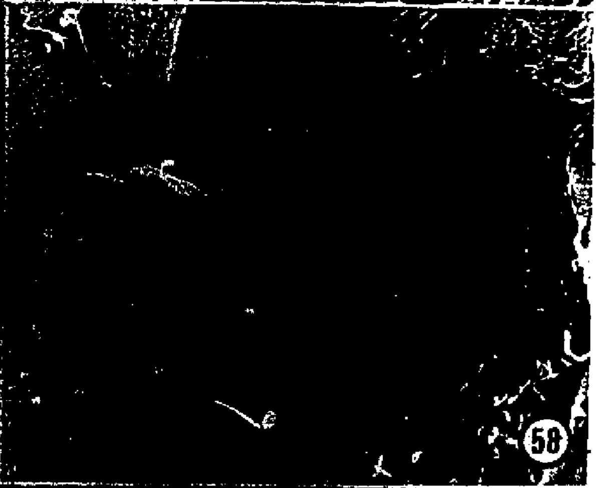
Fig. 57. SEM of epidermal projections (ep) around pollen (p) on the surface of a bract. x150.

Fig. 58. SEM of a partially dissected seed cone showing two ovuliferous scales (os), two ovules (o) and three bracts (b) with developing pollen tubes (pt). x35.

Fig. 59. SEM of pollen tubes growing along the surface of the bracts. x130.

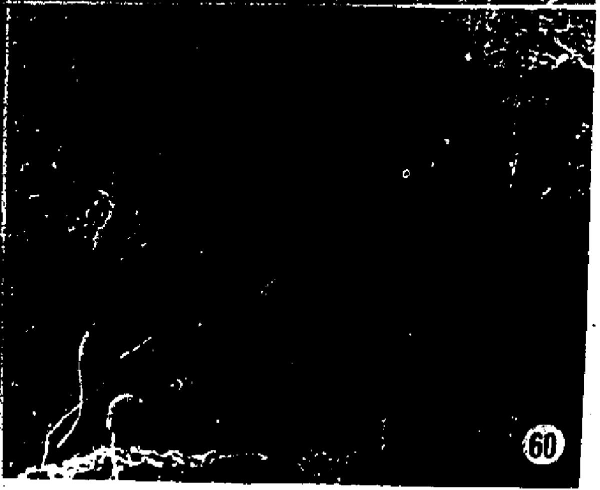
Fig. 60. SEM showing a bract, an ovuliferous scale and pollen tubes growing into the micropyle of an ovule. x40.

56



58

59



60

Table 13: Seed efficiency results for two western hemlock clones at Cobble Hill in the 1983 optimal-time-of-pollination study.

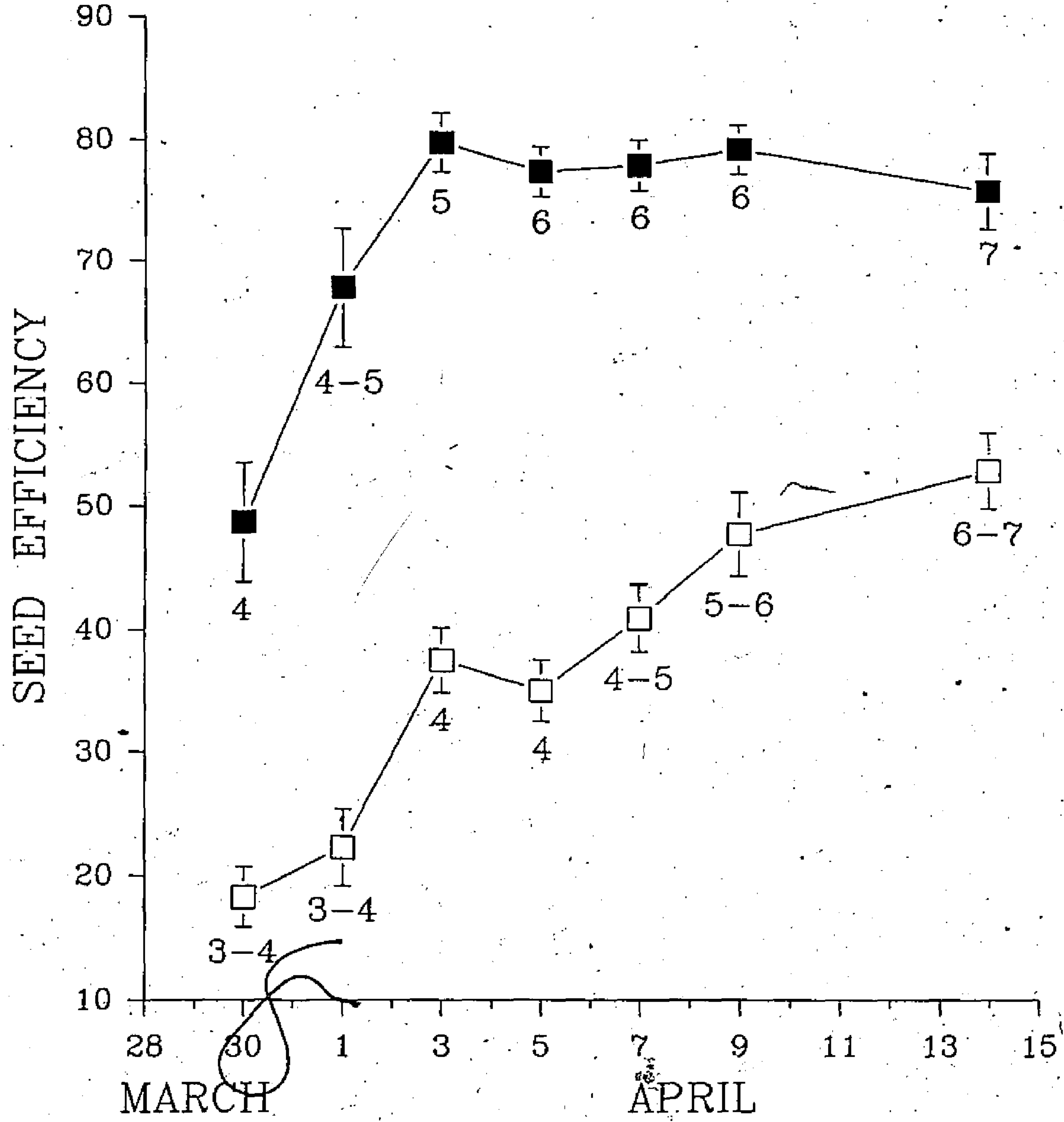
Pollination dates, phenological stages, average seed efficiencies (SEF ± SE) for N cones were determined for two clones.

	CLONE 7-10				CLONE 9-3			
DATE	STAGE	N	SEF	(±SE)	STAGE	N	SEF	(±SE)
March 30	3-4	20	18.3	(2.4)	4	20	48.7	(4.9)
April 1	3-4	20	22.3	(3.1)	4-5	20	67.8	(4.8)
April 3	4	19	37.5	(2.6)	5	20	79.7	(2.4)
April 5	4	19	35.0	(2.5)	6	20	77.3	(2.0)
April 7	4-5	20	40.9	(2.7)	6	20	77.8	(2.1)
April 9	5-6	19	47.7	(3.4)	6	20	79.1	(2.0)
April 14	6-7	19	52.8	(3.1)	7	20	75.6	(3.1)

In 1983, the pollinations were started after the seed cones became receptive, the stage of development differed between the two clones. The cones were at stage 4 (25 to 50% emerged) in clone 9-3 and between bud burst and 50% emerged (stages 3-4) in clone 7-10 (Table 13, Fig. 61). In clone 9-3, a significant increase ($p<.05$) in SEF was observed from March 30 to April 1 (Fig. 61). No significant differences in SEF were found between April 1 and April 14, during which time seed-cone phenology ranged from stage 4-5 to stage 7. In clone 7-10, an increase in SEF was found between March 30 and April 7, corresponding to changes in seed-cone phenology from stages 3-4 to stages 4-5. Maximum SEF occurred from stages 4-5 (April 7) to stages 6-7 (April 14) in clone 7-10. For both clones maximal SEF was observed when the cones reached stage 4-5.

Maximal SEF was lower in clone 7-10 than 9-3 due to a higher level of pre-pollination ovule abortion. Up to 35% of the potential seed per cone in clone 7-10 aborted early in development resulting in small, rudimentary seed attached to a seed wing in mature cones, compared to 7% ovule abortion in clone 9-3.

Figure 61: Seed efficiency versus date of pollination for two western hemlock clones at Cobble Hill, in the 1983 optimal time of pollination study. Average phenological stage and standard errors are presented for each pollination date for clone 7-10 (□) and 9-3 (■).


SEED EFFICIENCY
90
80
70
60
50
40
30
20
10
28
30
1
3
5
7
9
11
13
15
MARCH
APRIL
4
4-5
5
6
6
6
7
3-4
3-4
4
4
4-5
5-6
6-7

4.4.2 Lost Lake - 1984

A relationship between stage of development and SEF was observed in four clones at Lost Lake Seed Orchard in 1984. The mean SEF for each clone and stage, as well as the average SEF for each stage, is presented in Table 14.

Table 14: Seed efficiency results for four western hemlock clones at the Lost Lake Seed Orchard in the 1984 optimal-time-of-pollination study.

The average seed efficiencies (SEF) for N cones were determined for each clone by phenological stage at pollination. For each stage the total N, and mean SEF (± SE) were calculated.

	CLONE 39		CLONE 142		CLONE 143		CLONE 190		TOTAL	
STAGE	N	SEF	N	SEF	N	SEF	N	SEF	N	SEF (±SE)
2	8	0.0	-	—[1]	-	—	11	0.0	19	0.0 (0)
3	3	0.0	3	13.7	6	3.8	10	17.0	22	10.6 (2.3)
4	-	—	10	53.9	8	63.7	-	—	18	58.3 (3.0)
5	5	76.8	8	81.1	-	—	-	—	13	79.5 (1.7)
6	-	—	10	81.1	4	79.6	9	73.7	23	77.9 (1.2)
7	12	72.6	5	78.6	10	78.8	4	72.3	31	75.6 (1.0)
8	7	62.9	3	70.2	4	71.0	-	—	14	66.8 (2.6)
9	-	—	9	0.0	-	—	-	—	9	0.0 (0)

[1] No values for these stages

The relationship between seed-cone phenology and SEF can be related to the number of bracts exposed. A stage 2 cone was completely enclosed within the bud scales. Pollination of stage 2 cones resulted in a SEF of zero. Stage 3 cones are less than one quarter exposed and the resulting SEF when these cones were pollinated averaged 10.6 and varied between 0 and 17. The average number of sterile distal scales varied between the clones. In clones 39 and 143, two to four of the distal scales were sterile, so most of the exposed bracts of the stage 3 cones were associated with sterile scales. This accounts for the SEFs of 0 and 3.8. In clones 142 and 190, only one or two of the distal scales were sterile so some bracts with fertile scales were exposed in the stage 3 cones, which gave SEFs of 13.7 and 17.0, respectively. By stage 4 the cones were about half emerged and the SEF averaged 58.3, ranging from 53.9 to 63.7. Maximum SEF of 79.5 was attained during stage 5 when the cones were almost completely beyond the bud scales. The cones averaged 29.1 total scales, ranging from 26 to 31. The number of basal sterile scales ranged between 7 to 10 so the basal one-quarter of each cone was sterile. The maximum SEF rate was maintained during stage 6 and 7, which averaged 77.9 and 75.6, respectively. The average SEF decreased to 66.8 during stage 8, when half or more of each bract was covered by elongating scales. At stage 9, the ovuliferous scales completely covered the bracts and the SEF was zero.

The relationship between phenological stage and SEF is presented in Figure 62. The SEF increased after the cones elongated beyond the bud scales, reaching a maximum at stage 5, which was maintained until stage 7. A slight decrease was observed between stages 7 and 8, followed by a large decrease to zero as the ovuliferous scale completely covered the bracts (cone closure) at stage 9.

The high rate of cone abortion observed at pollination in 1984 may have been due to below-freezing temperatures. Temperatures in the Seed Orchard dropped to below 0°C on at least three nights during the pollination period.

4.5 Seed-Cone Receptivity in 1983 and 1984

Differences were observed in seed-cone phenology between 1983 and 1984. Average seed-cone phenology for eight clones at the Cobble Hill clone Bank (1983) and four clones at Lost Lake Seed Orchard (1984) were plotted versus date in Figure 63. In 1984 bud burst (stage 3) occurred about eight days before bud burst in 1983. The average time between bud burst and cone closure (stage 9) was 34 days in 1983 but only 23 days in 1984. If optimal seed-cone receptivity is considered to be between stages 4 and 8, then seed-cone receptivity occurred during approximately 23 days in 1983 and 14 days in 1984. The differences may be clonal, and/or environmental because different clones and sites were used in the two years.

Figure 62: Seed efficiency versus cone phenology in western hemlock at the Lost Lake Seed Orchard in 1984. Vertical bars represent standard error. The average seed efficiency (±SE) for each stage is included in parentheses.

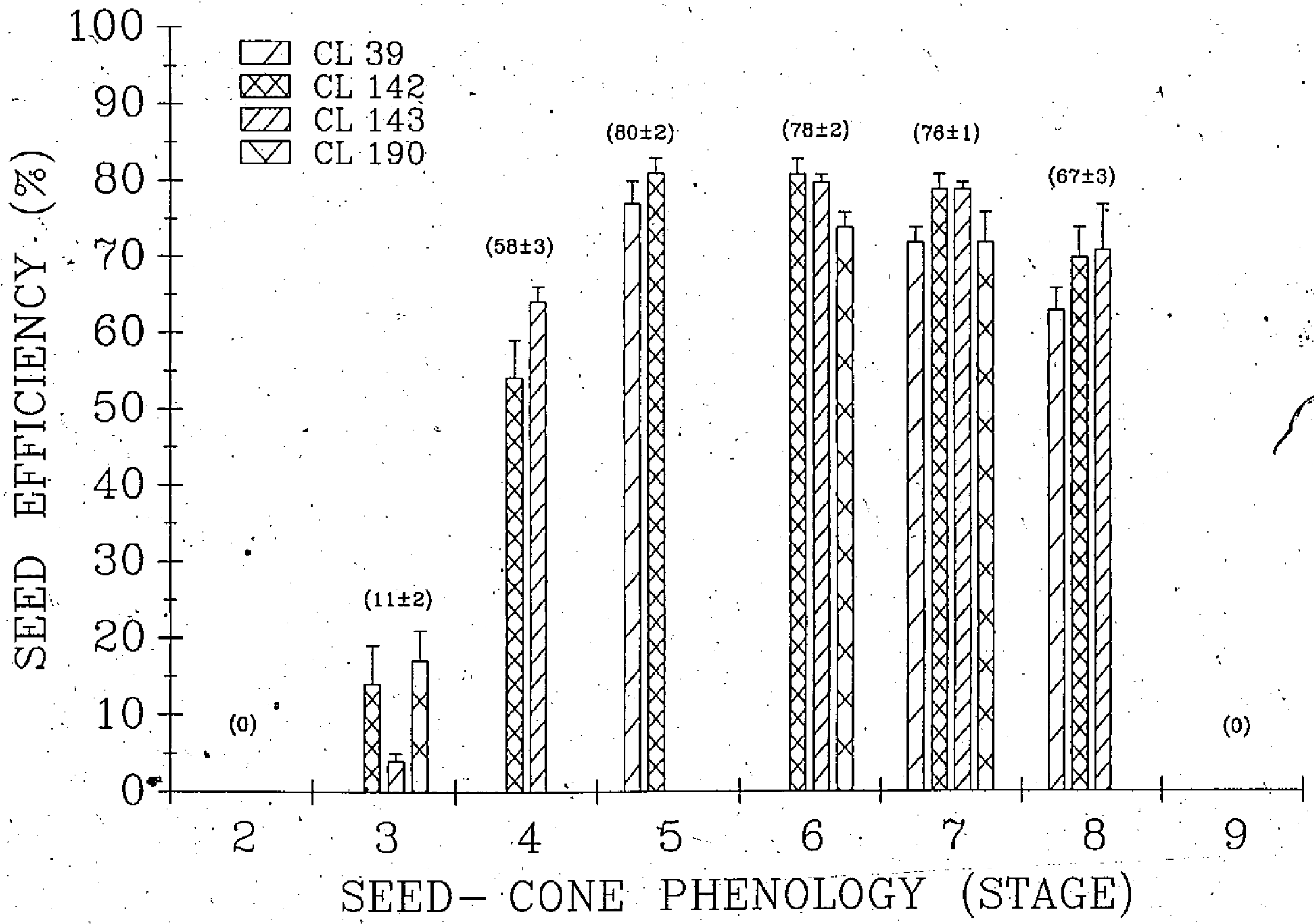
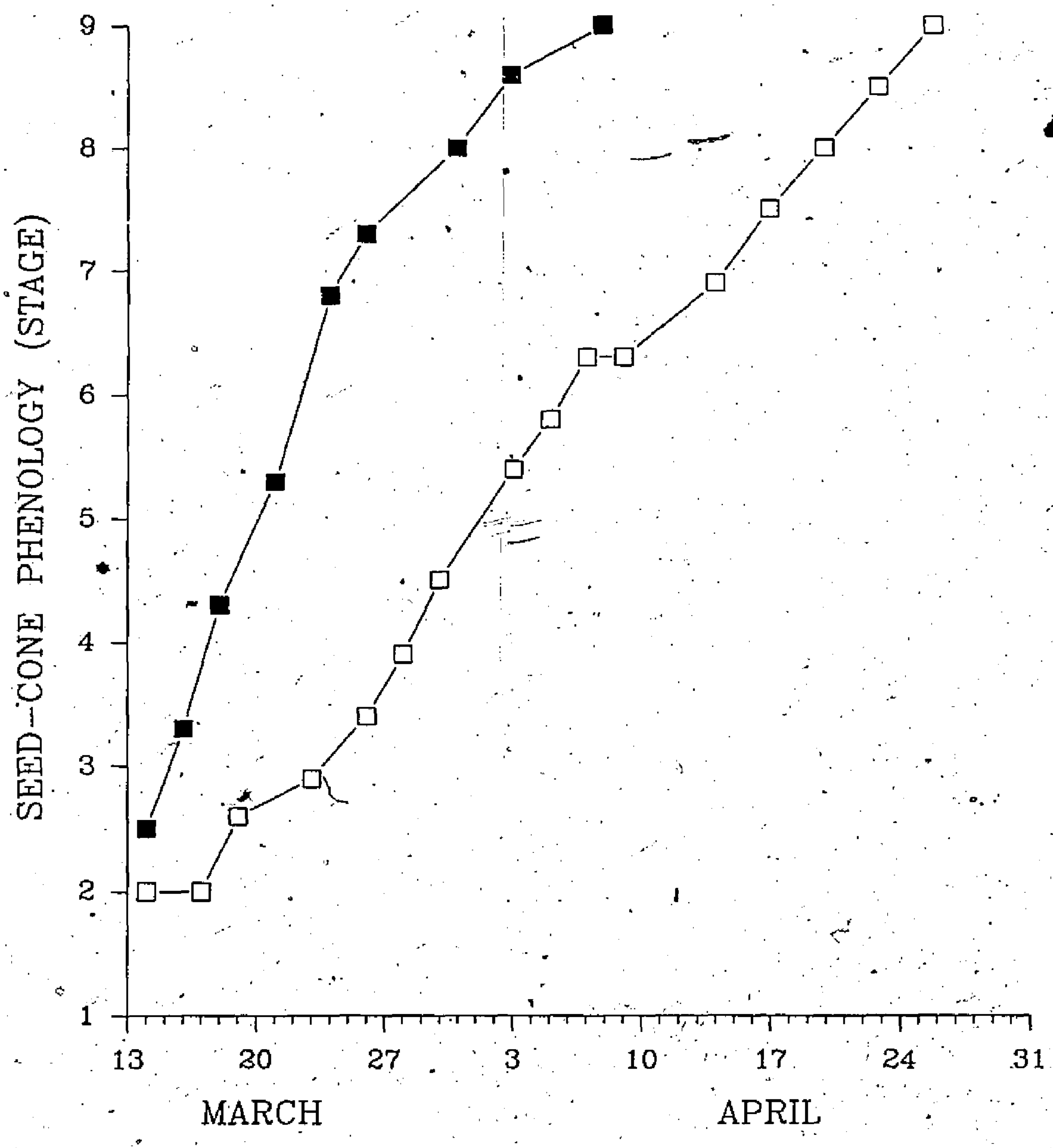

CL 39
CL 142
CL 143
CL 190
SEED EFFICIENCY (%)
100
90
80
70
60
50
40
30
20
10
0
(0)
(11±2)
(58±3)
(80±2)
(78±2)
(76±1)
(67±3)
(0)
2
3
4
5
6
7
8
9
SEED- CONE PHENOLOGY (STAGE)

Figure 63: Seed-cone phenology for western hemlock in 1983 and 1984. Average phenology is plotted versus date for eight clones in 1983 (□) at the Cobble Hill Clone Bank and four clones in 1984 at Lost Lake Seed Orchard (■).


SEED-CONE PHENOLOGY (STAGE)
1
2
3
4
5
6
7
8
9
13
20
27
3
10
17
24
31
MARCH
APRIL

4.6 Seed-Cone Receptivity Versus Pollen Shed in 1983

Both seed- and pollen-cone phenology were monitored for 60 clones at the Cobble Hill clone Bank in 1983 until seed cones were no longer receptive. The number of receptive clones (between stages 4 and 8) and the number of clones shedding pollen (stage 8) were plotted versus date in Figure 64. One clone had stage 4 seed cones on March 19, so was considered receptive. By March 26 ten clones had receptive seed cones. Pollen shedding was observed first in three clones on April 3, by which time 52 clones had receptive seed cones. The maximum number of clones shedding pollen at one time was 48, which occurred from April 7 to April 10. Three clones released pollen before April 7 and nine clones did not start pollen shed until after April 10. All 60 clones had receptive seed cones between April 7 and April 14. The number of clones shedding pollen decreased to 24 by April 14, to four by April 17 and ~~to~~ zero by April 22. Seed-cone receptivity decreased after peak pollen shed. On April 17, 59 clones were still receptive. This decreased to 33 by April 20, to five by April 26 and to zero on April 28.

Seed-cone receptivity occurred before pollen release and continued for about one week after all the pollen was shed. The average clone was receptive for 23 days. Variability in pollen-cone phenology resulted in the average clone shedding pollen over four to seven days.

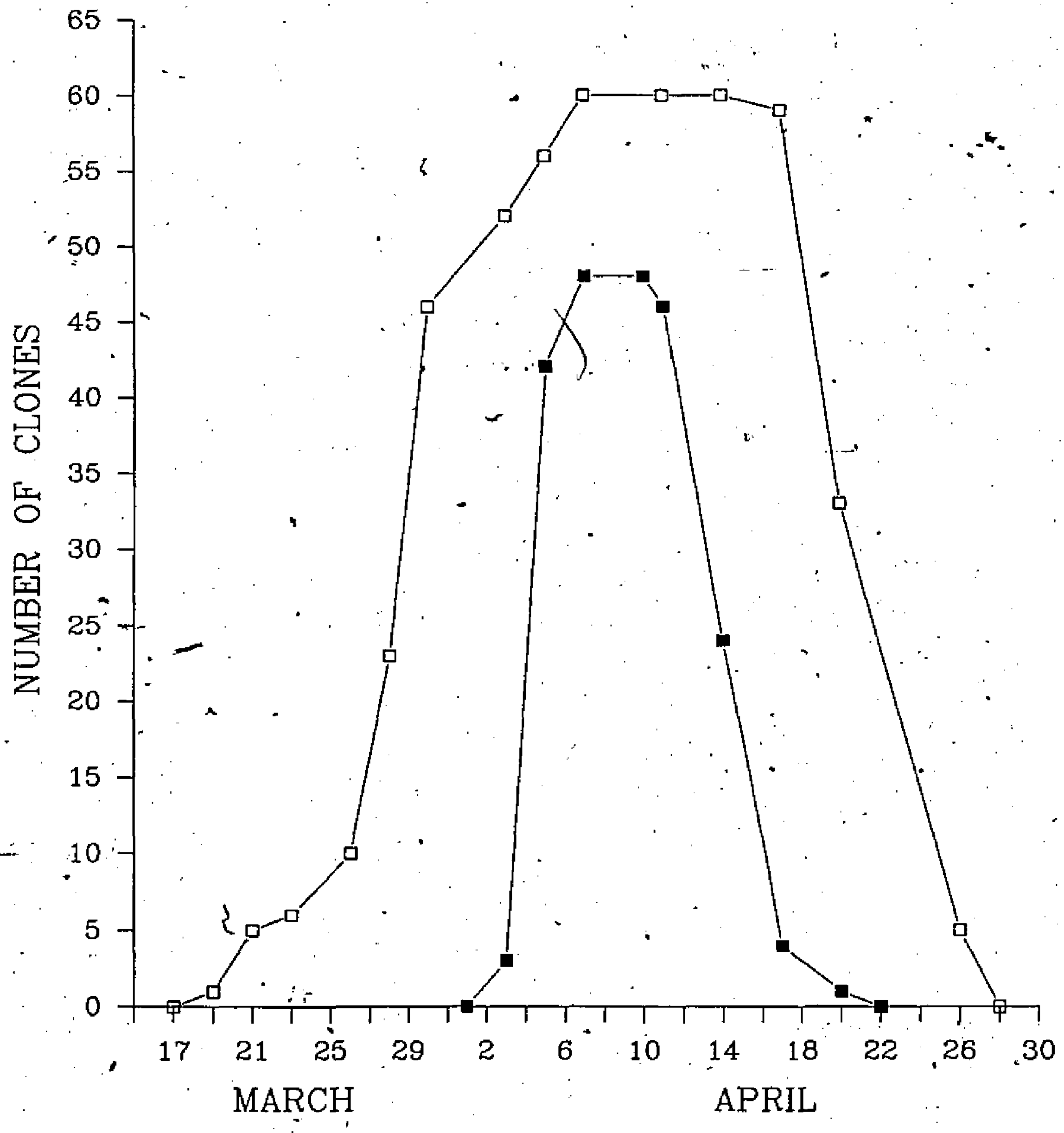
4.7 Seed Development in Field-Grown Clones

4.7.1 Seed Development After Wind-Pollination

4.7.1.1 Post-dormancy Development

Stage 1, or quiescent, seed-cone buds consisted of bracts, ovuliferous scales and ovule primordia which were initiated before dormancy. Within each ovule primordium was a large megaspore mother cell (MMC) surrounded by layers of smaller cells forming the megasporangium or nucellus. Each premeiotic MMC had darkly staining cytoplasm and a large nucleus with a distinct nuclear membrane, several prominent nucleoli and lightly staining chromatin (Fig. 65).

Figure 64: Seed cone receptivity (□) versus pollen shed (■) for 60 western hemlock clones at the Cobble Hill Clone Bank in 1983.


NUMBER OF CLONES
0
5
10
15
20
25
30
35
40
45
50
55
60
65
17
21
25
29
2
6
10
14
18
22
26
30
MARCH
APRIL

Cell divisions were observed first on February 12, 1983. Random divisions were found in the ovules, ovuliferous scales, bracts and cone bud axis. Between February 12 and 20 the MMC enlarged and proceeded quickly through leptotene to zygotene. Very few cells were observed at leptotene, so it was assumed that this stage was relatively short. The MMC remained at zygotene for the next three to four weeks. During this time the bracts, ovuliferous scales, ovules and cone axis enlarged through cell division, causing the buds to swell to stage 2. The integument developed and the micropyle was formed. During the first two weeks in March, meiosis resumed and the MMC went quickly through the rest of prophase I (pachytene, diplotene and diakinesis), metaphase I, anaphase I and telophase I with no subsequent cell wall formation, producing two nuclei within the MMC wall. Within a few days a second meiotic division produced four nuclei (Fig. 66). Cell wall formation resulted in a triad, a linear arrangement of three cells with a binucleate center cell. The binucleate cell and the cell closest to the micropyle degenerated, leaving the uninucleate cell farthest from the micropyle as the functional megaspore (Fig. 67). Functional megaspore formation coincided with bud burst (stage 3). The functional megaspore was vacuolate and surrounded by developing tapetal cells. By late March the functional megaspore started free-nuclear division (Fig. 68). The start of free-nuclear division coincided with pollination and continued for the next four to five weeks.

Several ovules aborted or ceased development before pollination and remained as small, rudimentary structures at the base of the developing seed wing. At cone maturity these ovules were identified as small, flat seeds at the base of the seed wing. The prepollination ovule abortion rate, based on the number of flat seed in mature cones, was determined for 23 clones which were included in several of the studies at the Cobble Hill in 1983 (Table 15).

Table 15: Estimates of the prepollination ovule abortion rate (%) for 23 clones at the Cobble Hill Clone Bank in 1983 based on flat rudimentary seeds in mature cones.

The ovule abortion rate (± SE) was calculated for 20 cones per clone.

CLONE	% ABORTION	CLONE	% ABORTION
2-05	13.9 (1.6)	9-03	7.4 (1.0)
2-09	9.5 (1.1)	9-10	10.6 (1.3)
3-04	7.9 (2.0)	10-04	9.7 (0.6)
4-01	8.4 (1.2)	10-07	7.9 (1.2)
5-08	13.7 (1.7)	10-09	11.5 (2.0)
5-10	11.7 (1.6)	11-02	8.2 (1.2)
6-03	15.0 (1.8)	11-08	5.9 (0.7)
6-06	6.0 (1.2)	12-03	15.9 (1.4)
7-04	40.8 (2.5)	12-10	5.5 (1.0)
7-06	6.9 (1.4)	16-02	5.9 (0.8)
7-10	33.4 (2.3)	16-06	7.6 (1.7)
9-01	7.8 (1.0)		
		TOTAL	11.8 (1.8)

By late April, all cones were closed and elongating. Stage 9 coincided with the final stages of free nuclear division. The first cell walls in the developing megagametophyte were observed by the end of April (Fig. 69). Cell-wall formation resulting in primary prothallial cells began at the periphery and proceeded centripetally. Several of the primary prothallial cells at the micropylar end enlarged, forming archegonial initials (Fig. 70). The cells of the megagametophyte continued to divide and enlarge within the megaspore cell wall. The archegonial initials elongated over the next two weeks. Small cells surrounding the archegonial initials divided periclinally, forming an archegonial jacket around each archegonial initial.

By mid-May the archegonial initials divided unequally, forming a small primary neck cell at the micropylar end and a large vacuolate central cell (Fig. 71). The primary neck cell divided once, anticlinally, producing two neck cells. The nucleus of the central cell remained at the micropylar end while the cytoplasm continued to expand and accumulate densely staining material. The neck cells elongated and accumulated starch. The cells of the megagametophyte divided and enlarged overarching the neck cells, forming a slight depression or archegonial chamber.

The central cell divided once, producing a small lens-shaped ventral canal cell adjacent to the neck cells and a large egg cell. The nucleus of the egg cell migrated to the center of the cell. Both small and large cytoplasmic inclusions became prominent in the egg cell (Fig. 72). At about the time the egg cell formed in mid-May, cell division and enlargement in the megagametophyte stopped (Fig. 73). Two to five mature archegonia were observed within the ovules.

Between cone closure (stage 9) in late April and the formation of the mature megagametophyte in late May the seed cones increased from an average of 5 mm to 24 mm in length. The cones elongated at an average rate of 0.5 mm per day. The increase in size was due to cell elongation along the central axis and the ovuliferous scales. The individual ovules increased from an average length of 0.5 mm at cone closure (end of pollination) to 2.5 mm at the formation of the mature megagametophyte (fertilization).

4.7.1.2 Fertilization

Pollen tubes penetrated the nucellus in mid-May, about the time the archegonial initials divided to form the primary neck cell and the central cell (Fig. 74). Pollen tubes were visible outside the megaspore cell wall in the neck cell region during or shortly after the central cell divided. It was during this time that the body cell divided to form two unequal-size male gametes (Fig. 75, 76). The pollen tubes pierced the megaspore cell wall and penetrated between, and eventually disrupted the neck cells (Fig. 77). The pollen tube bypassed the ventral canal cell and penetrated between the egg cell and archegonial jacket for a short distance before piercing the egg cell wall and discharging its contents into the egg cytoplasm. A receptive vacuole was usually found in the egg cytoplasm where the pollen tube discharged its contents. The pollen tube contained two male gametes, the stalk cell, the tube nucleus and tube cytoplasm containing starch. Sometimes only the larger male gamete was discharged into the egg cytoplasm. The second male gamete was observed in the pollen tube outside the neck cells, in the neck cell region, inside the receptive vacuole or within the egg cytoplasm (Fig. 78, 79, 80). Similar observations were made for the other components of the pollen tube including the stalk cell, tube nucleus and starch. The larger male gamete migrated to the center of the cell, fused with the egg nucleus and the two haploid sets of chromosomes merged (Fig. 81). Fertilization occurred in mid- to late-May.

Twenty-six ovules were sectioned at fertilization or shortly after. Twenty-one of these (81%) were fertilized. An average of 1.5 zygotes was found, ranging from zero to four zygotes per ovule.

4.7.1.3 Proembryo development

The first zygotic division (Fig. 81) resulted in two free nuclei surrounded by densely staining neocytoplasm. A second division produced four free nuclei (Fig. 82). The four free nuclei, plus some neocytoplasm migrated to the chalazal end of the egg cell, forming one tier of four nuclei. The time between pollen tube penetration of the neck cells and the one-tiered proembryo was 4 to 6 days.

Vertical spindles formed and the four nuclei divided to form two tiers of eight nuclei. A transverse cell wall formed between the two tiers, followed by axial wall formation, leaving the proximal tier open to the egg cytoplasm and the distal primary embryo tier completely walled in (Fig. 83). The open tier divided to form a completely walled rosette, or disfunctional, suspensor tier and an open tier. The primary embryo tier divided to form a suspensor tier and a distal embryo tier (Fig. 84). The mature proembryo consisted of four tiers of four cells with the proximal tier open to the cytoplasm of the egg cell. The time between fertilization and the 16-cell proembryo averaged about 10 days. The cytoplasm at the micropylar end of the egg cell began to degenerate by the time the 16-cell proembryo formed.

Figs. 65-73. Median longitudinal sections of ovules showing post-dormancy megagametophyte development.

Fig. 65. Ovule (o) showing a dormant megaspore mother cell (mmc) in early February. x250.

Fig. 66. Ovule showing four nuclei within the megaspore wall (mw). x190.

Fig. 67. Ovule showing enlarging functional megaspore (fm) and two degenerating megaspores (arrowhead). x200.

Fig. 68. Ovule showing early free nuclear division (fn) of megagametophyte. x270.

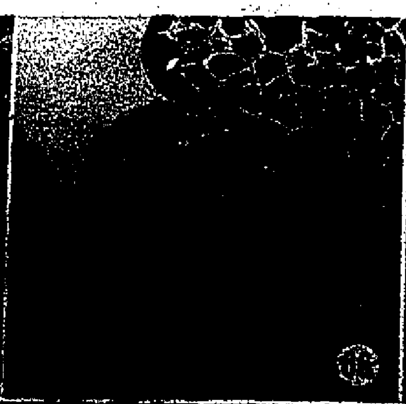
Fig. 69. Megagametophyte at cell-wall formation in late April. x200.

Fig. 70. Megagametophyte in early May showing an archegonial initial (ai). x220.

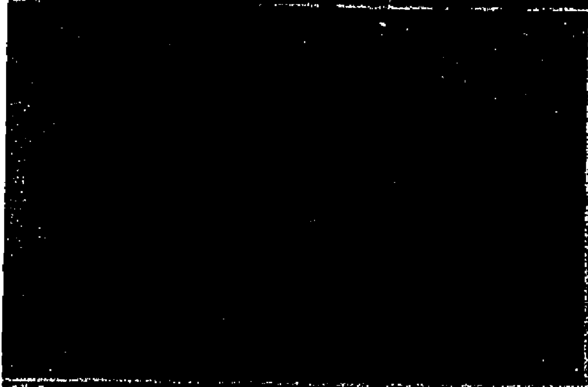
Fig. 71. Megagametophyte in mid-May showing a large central cell (cc), primary neck cell (pnc), jacket cells (Jc) and thick megaspore cell wall. x200.

Fig. 72. Megagametophyte in mid-May enclosed by thick megaspore cell wall (mw) showing the two neck cells (nc), ventral canal cell (vcc), egg cell (ec) with a large egg nucleus (en) and small and large inclusions in the cytoplasm (*). x150.

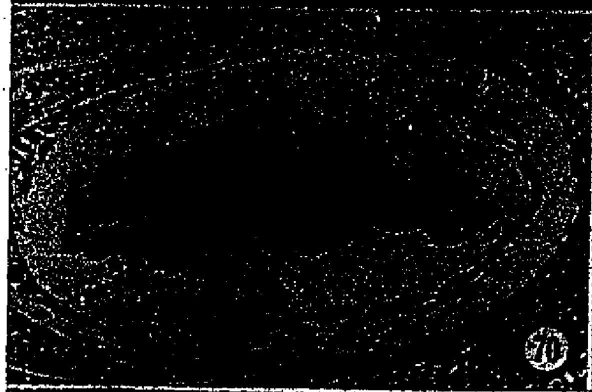
Fig. 73. Longitudinal section through an ovule in mid- to late May just before fertilization showing the mature megagametophyte, megaspore cell wall and nucellus (n) within the developing seed coat (sc). x45.



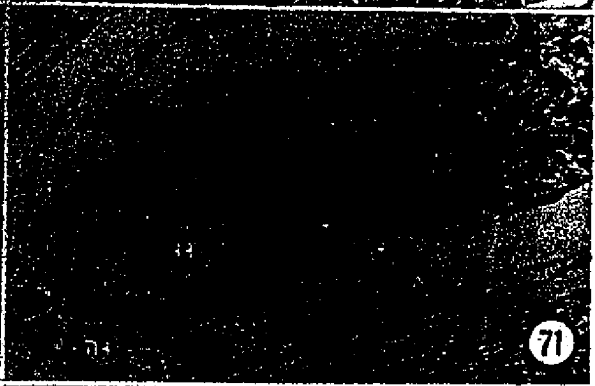
67



69

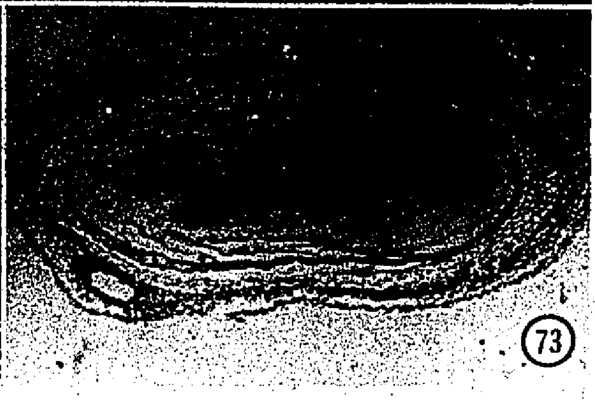


70



71

72



73

Figs. 74-81. Pollen-tube development and fertilization in western hemlock.

Fig. 74. Longitudinal section through the nucellus (n) and integument (i) showing where the pollen tube (pt) has penetrated the nucellus and the megaspore cell wall (mw). x180.

Fig. 75. Section through a pollen tube next to the megaspore cell wall showing the body cell (bc) dividing to form two gametes. x320.

Fig. 76. The body cell has divided within the pollen tube, producing two unequal-sized male gametes (mg) next to the megaspore cell wall. x275.

Fig. 77. Disruption of the megaspore cell wall (mw) and neck cells (nc) by a pollen tube. The egg cell (ec) contains a large nucleus (en) and small and large inclusions in the egg cytoplasm. x340.

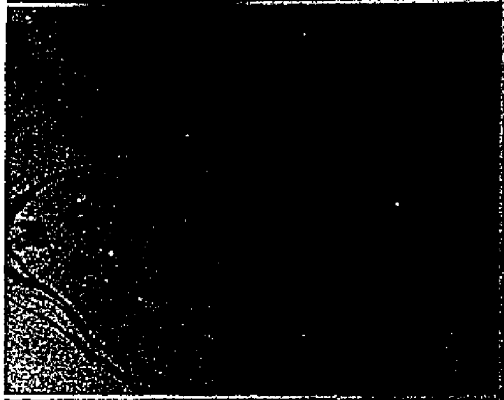
Fig. 78. Section through an egg cell showing the fusion of the large male gamete with the egg nucleus. x330.

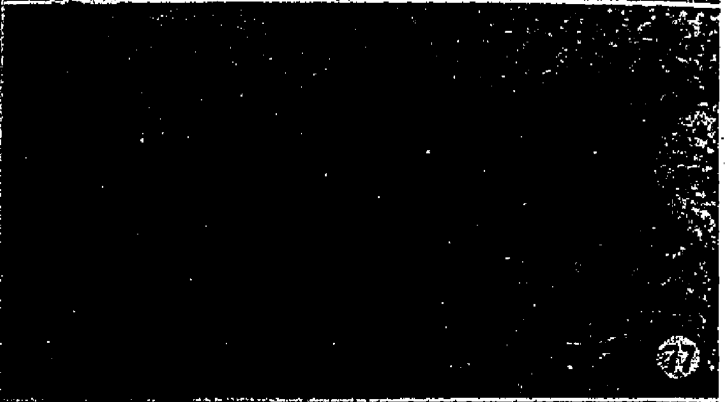
Fig. 79. Section through an egg cell showing the small male gamete (mg) in cytoplasm and the large gamete fusing with egg nucleus (en). x330.

Fig. 80. Supernumerary nuclei (sn) and receptive vacuole (rv) at point of pollen tube entry within the egg cell. x350.

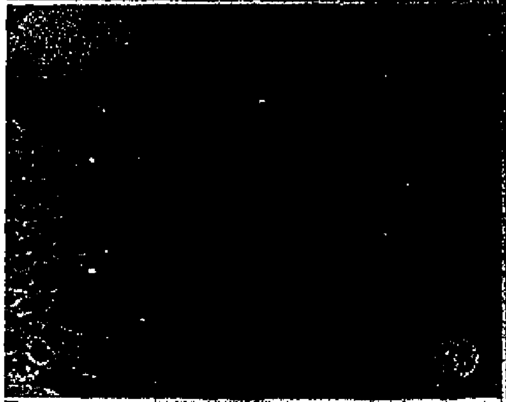
Fig. 81. Section through the egg nucleus showing the first zygotic division with chromosomes at telophase I attached to spindle fibres. x320.

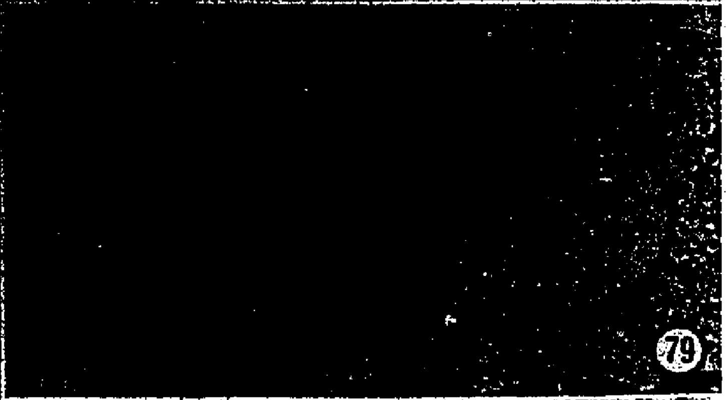
75





77





79

80

81

4.7.1.4 Early Embryo Development

In early June the suspensor tier elongated, pushing the embryo tier deep into the megagametophyte (Fig. 85). The megagametophyte cells around the embryo tier degenerated, producing a small corrosion cavity (Fig. 86). The cells in the path of the corrosion cavity were full of starch which was released when the cells degenerated (Fig. 86). The suspensor cells elongated rapidly, resulting in the suspensors becoming coiled and twisted. The cells of the rosette tier and open tier degenerated soon after suspensor cell elongation (Fig. 85). Division of the rosette cells to form rosette embryos was not observed.

The cells in the megagametophyte, which had been quiescent since the formation of the mature megagametophyte, resumed development shortly after the suspensors elongated. Some of the nuclei underwent mitosis, resulting in binucleate cells. The cells in the megagametophyte enlarged and accumulated cytoplasmic material (Fig. 87). Vascular strands could still be found leading into the developing megagametophyte and the integument at the chalazal end of the ovule (Fig. 87). The three layers of the seed coat arising from the integument were distinguishable (Fig. 87). Maximal seed-cone elongation coincided with elongation of the suspensor, cleavage polyembryony and resumption of megagametophytic development.

When the embryo tier was pushed about three-quarters of the way into the megagametophyte, the embryo(s) underwent cleavage entailing the longitudinal separation of the four cells of the embryo tier (Fig. 88). This produced four separate embryonal units, each with a single apical cell. Between four and 16 developing embryos were observed in fertilized ovules. Some embryos degenerated at this time.

Around the time cleavage occurred, the cells of the integument tip began to expand transversely, closing the micropyle and crushing the distal portion of the nucellus and pollen tubes (Fig. 89). The cells of the nucellus degenerated and were soon pushed against the developing seed coat by the expanding megagametophyte.

The apical cell of each embryo divided in all planes, forming a dome-shaped group of meristematic cells (Fig. 90). Two weeks after suspensor elongation, the embryo furthest into the megagametophyte was usually the largest. One to 6 other embryos could be found in most of the ovules (Fig. 91). The megagametophyte continued nuclear division, reserve accumulation, and cell expansion. Intact vascular strands were still found leading into the ovule.

4.7.1.5 Late Embryo Development

By late June the embryo was a club-shaped mass of cells (Fig. 92). The apical cells enlarged, forming a small dome. The cells around the apical dome divided, giving rise to the cotyledons, or embryonic leaves, leaving a small apical meristem between. A root apical meristem formed in the central portion of the embryo. The embryos were about one-fifth full-size and within the next three weeks increased to almost full size. The embryos elongated by transverse cell divisions in the region below the apical meristem, resulting in a distinct rib meristem between the apical meristem and the proximal region. The cotyledons elongated and provascular tissue appeared (Fig. 93). In the proximal region, the root apex gave rise to a massive root cap. By late July the embryos attained full size and began to accumulate storage products (Fig. 94). Similar cellular storage products were observed in the megagametophyte. This stage, referred to as seed maturation, continued until late August.

Approximately 10% of the sectioned ovules contained aborted or degenerating megagametophytes and/or embryos. A few of these appeared to have degenerated before fertilization and others after partial embryo development. All ovules that degenerated after pollination had completely developed seed coats. These could not be visually distinguished from seed containing an embryo while ovules which degenerated before pollination were considerably smaller at maturity (Fig. 95).

Figs. 82-87. Longitudinal sections of ovules showing post-fertilization proembryo, embryo and megagametophyte development.

Fig. 82. The free nuclear stage of embryo showing three of the four free nuclei (fn) within the egg cell (ec). x130.

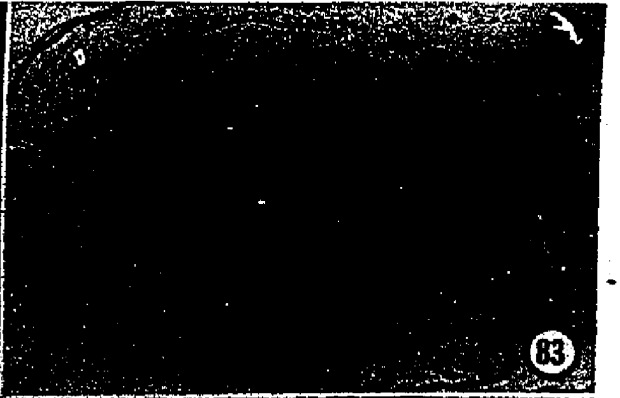
Fig. 83. Section through two archegonia; The upper one is undergoing mitosis in the one tier, while the lower one contains a two-tiered, eight-cell proembryo after transverse cell wall formation. x115.

Fig. 84. Two four-tiered, 16-celled proembryos, from an ovule collected in early June showing the apical or embryo tier (et), suspensor tier (st), rosette tier (rt) and open tier (ot). x170.

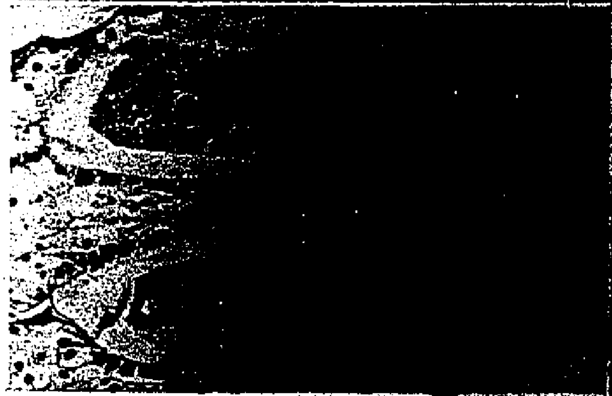
Fig. 85. Early embryo in the megagametophyte (meg) during suspensor tier elongation showing the open tier, rosette tier and embryo tier. x125.

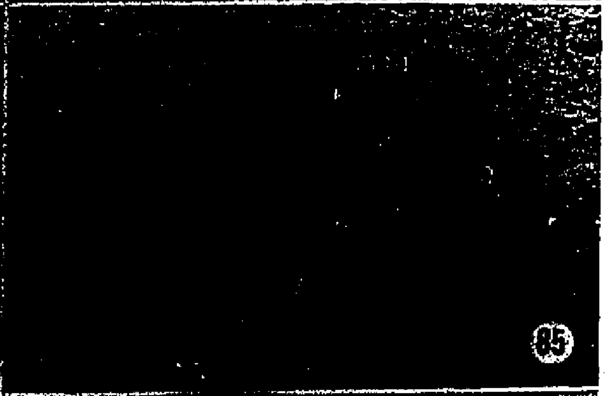
Fig. 86. The embryo tier has divided once after suspensor tier elongation. The cells of the megagametophyte (meg) which break down to form the corrosion cavity contain starch (arrowhead). x115.

Fig. 87. Section through part of the ovule showing the corrosion cavity (cor), vascular strands (vs) penetrating the seed coat and the three layers of seed coat (sc). x45.

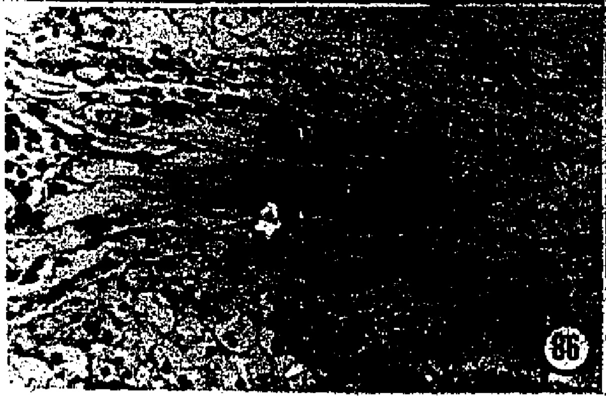


83

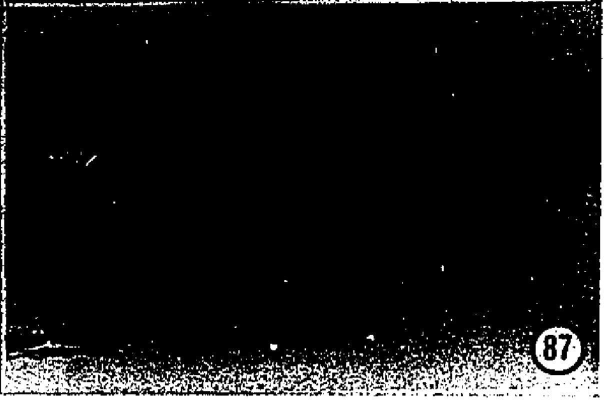




85



86



87

Figs. 88–95. Longitudinal sections through ovules showing embryo and seed development.

Fig. 88. Section through the megagametophyte (meg) showing the corrosion cavity (cor) and the embryo tiers (e) undergoing cleavage. x110.

Fig. 89. Expanded cells of the integument (i) close the micropyle, crushing the cells of the nucellus (n). x60.

Fig. 90. Multicellular embryo (e) within the megagametophyte in mid-June. x50.

Fig. 91. Two larger multicellular embryos and the large corrosion cavity within the megagametophyte in mid- to late-June. x40.

Fig. 92. A club-shaped embryo (e) within the large corrosion cavity in late June and a smaller degenerating multicellular embryo (e) at the micropylar end of the megagametophyte. The cells of the megagametophyte are isodiametric and have accumulated storage products. The three layers of the seed coat (sc) are distinguishable. x50.

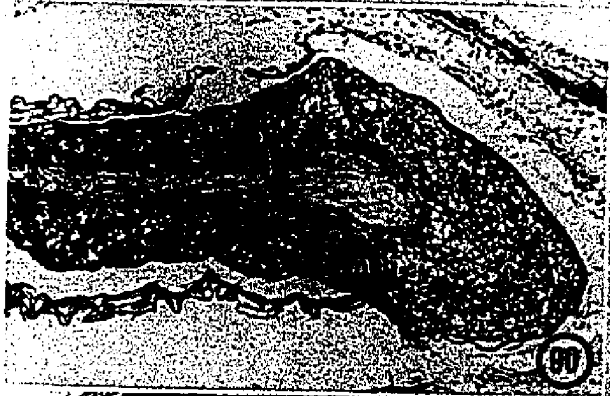
Fig. 93. Median longitudinal section of a developing seed in early July showing megagametophyte and a differentiating embryo showing cotyledons (c) and shoot apex (sa). x50.

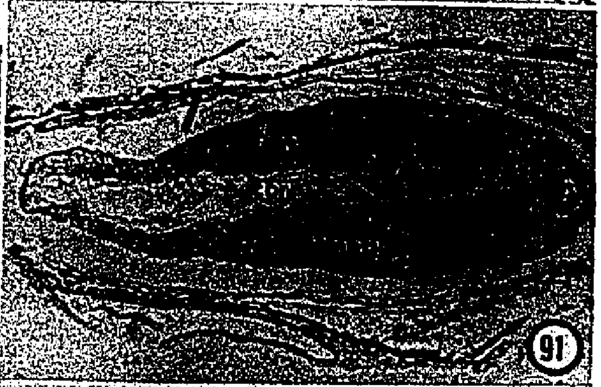
Fig. 94. Median longitudinal section of a mature seed showing megagametophyte with darkly staining storage products and the mature embryo showing cotyledons, shoot apex, procambial strand (pc), cortical initials (ci), root apex (ra) and root cap (rc). x35.

Fig. 95. Mature seeds; a developed full-size seed below (s) with seed wing (sw) and a smaller undeveloped seed due to early ovule abortion (arrowhead). x12.


88

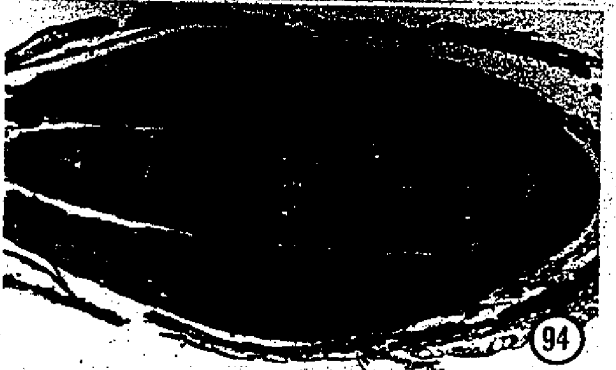

89


90


91


92


93


94


95

4.7.1.6 Seed Efficiencies after Wind Pollination

The average SP and SEF for 20 cones per clone from 58 clones at the Cobble Hill clone Bank were determined for 1983 (Table 16). While 60 clones were monitored, two clones did not have enough mature cones to be included in this study. SP (seed potential) varied among the clones, ranging from 26.6 to 43.5 (SE=.67), with a mean of 32.1. The cones were collected from the upper crown of each of the ramets.

Table 16: Seed potential (SP) and seed efficiencies (SEF) after wind pollination for 58 western hemlock clones at the Cobble Hill Clone Bank, 1983.

SP and SEF were determined for 20 bulked cones per clone. The average standard error (SE) for SP was 0.67.

CLONE	SP	SEF	CLONE	SP	SEF
1-02	38.3	47	7-02	29.3	79
1-05	31.3	39	7-03	29.4	63
2-04	38.3	71	7-04	33.0	38
2-05	37.6	73	7-06	31.8	72
2-06	33.6	66	7-10	38.9	46
2-08	36.1	60	8-01	36.1	53
2-09	29.9	77	8-03	31.9	62
2-10	31.2	77	8-10	32.3	57
3-03	39.7	70	9-01	33.7	67
3-04	40.4	76	9-02	40.1	69
3-05	33.2	55	9-03	29.5	78
3-07	31.2	71	9-05	33.6	75
4-01	36.2	76	9-10	30.6	38
4-02	34.3	74	10-04	34.2	72
4-06	30.0	62	10-06	35.8	75
5-02	41.1	54	10-07	35.3	67
5-03	36.2	65	10-09	37.3	69
5-04	29.4	69	11-02	35.1	61
5-07	39.4	64	11-08	38.1	59
5-08	33.9	68	12-03	32.0	64
5-09	26.9	54	12-04	31.9	73
5-10	34.1	74	12-05	32.0	65
6-02	38.7	60	12-09	32.1	67
6-03	33.6	43	12-10	34.6	71
6-04	26.6	63	16-01	32.0	58
6-05	28.3	75	16-02	35.7	71
6-06	34.2	59	16-03	30.0	41
6-07	32.9	58	16-05	35.8	80
6-09	43.5	59			
6-10	27.6	73	TOTAL	34.0	63
			(SE)	(0.5)	(1.7)

The SEFs resulting from wind pollinations also varied among the clones, ranging from 37.8 to 79.8 with a mean of 66.7. The SEFs were determined from bulked samples, so the variation within a clone is not known.

4.7.2 Seed Development After Cross Pollination

Seed development in the bagged and pollinated cones was similar to that observed in the wind-pollinated clones. Seed-cone phenology was slightly accelerated in the bagged cones relative to unbagged wind-pollinated cones. During the receptive period, the cones in the bags were about one stage further advanced than the cones on the nearest unbagged branch. The bagged cones reached stage 9 (cone closure) about four days before the unbagged cones. Pollen germination was observed in the bagged cones on May 7, four days before pollen germination occurred in the unbagged cones. The subsequent stages of seed development occurred at about the same time after the pollination bags were replaced with insect bags.

Twenty-two ovules were sectioned at fertilization or shortly thereafter. Twenty of these (91%) were fertilized. An average of 2.5 zygotes was found in the sectioned ovules, ranging from zero to four zygotes per ovule.

The SEFs were determined for 20 cones per clone for each of the 16 cross-pollinated clones. No significant differences were observed when SEFs were compared between the cross-pollinated (69%) and wind-pollinated (66%) cones (Table 17). Clones 7-04 and 6-03, which had the lowest SEFs after cross-pollination, with values of 43 and 48, respectively also had the lowest SEFs in the wind-pollinated cones with values of 38 and 43, respectively. Clones 9-03, 3-04 and 16-06 had the highest SEFs of the 16 cross-pollinated clones, with values of 80, 81 and 83, respectively. Clones 9-03 and 16-06 had the highest SEFs after wind-pollination, with values of 78 and 80, respectively. Clone 3-04 ranked fourth after wind-pollination with an average SEF of 76.

Table 17: Seed efficiencies in 16 cross- and wind-pollinated western hemlock clones at the Cobble Hill Clone Bank, 1983.

The seed efficiencies (SEF) were averaged from 20 cones per clone for the cross-pollinated cones (CROSS). SEFs were based on 20 bulked cones per clone for the wind-pollinated cones (WIND). The average standard error was 2.1.

CLONE	CROSS SEF	WIND SEF
2-05	74	73
2-09	73	77
3-04	81	76
5-08	68	68
6-03	47	43
7-04	43	38
7-06	77	72
7-10	54	46
9-01	72	67
9-03	80	78
10-04	77	72
10-07	77	67
10-09	73	69
11-02	61	61
12-03	70	64
16-06	83	80
AVERAGE (SE)	69 (2.9)	66 (3.1)

4.7.3 Seed Development After No Pollination

Ovule development in the unpollinated cones was similar to that observed in the wind-pollinated cones to the mature-megagametophyte stage. The archegonia of unpollinated ovules began to degenerate about two weeks after the expected time of fertilization. The egg cell filled with large, prominent, darkly staining bodies. This coincided with proembryo formation in the pollinated ovules of the same clone. The cells that would have degenerated to form the corrosion cavity contained starch but did not break down. In the pollinated ovules the corrosion cavity formed at the time the suspensor cells elongated. The megagametophyte of unpollinated ovules did not resume development and started to degenerate 1 to 2 weeks after the archegonia, at about the time the apical cells of the embryos would have started cell division.

The seed coat was well developed by the time the unfertilized ovules degenerated. Unpollinated, empty seeds could not be distinguished externally from filled seeds. The average number of round seed per cone, based on 20 cones per sample, was 25.8 (SE=.68) for the pollinated cones and 27.1 (SE=.62) for the unpollinated cones. The average SEFs for the 20 pollinated and unpollinated cones were 73.6 (SE=1.93) and 0, respectively.

4.7.4 Seed Development After Self-Pollination

Ovule development in the self-pollinated clones was similar to that observed in the wind-pollinated cones up to fertilization and proembryo development. Cone closure was completed by April 26 for the ten self-pollinated clones at the Cobble Hill Clone Bank. Pollen germination on the bracts was first observed on May 7. By May 16, pollen tubes were found growing on the bracts, in the micropyles and into the nucellus. With the exception of clone 6-06, where less than 5% of the pollen germinated, the germination percentage in the other nine clones was greater than 75%. In early June, about three weeks after pollen germination, the cells of the integument had elongated, closing the micropyle and crushing the pollen tubes and nucellar tips.

A few ovules aborted shortly after meiosis resumed, but before pollination. When abortion occurred at this early stage, the ovules remained as small rudimentary structures at the base of the ovuliferous scales. As in the wind-pollinated study, these were identified as small flat seed in mature cones. A few other ovules aborted during megagametophyte development after pollination but before fertilization, usually between the archegonial-initial stage and the mature archegonial stage. In several cases, pollen tubes were observed outside the megaspore cell wall of ovules that contained degenerating archegonia (Fig. 96). These resulted in fully developed but empty seeds. Ovule abortion after pollination also resulted in such seed.

Fertilization occurred between May 18 and 23 in 1983. Thirty-seven ovules were sectioned at fertilization or shortly thereafter. Thirty-one of these (84%) were fertilized. An average of 2.2 zygotes was found in the sectioned ovules, ranging from zero to four zygotes per ovule. In four of the unfertilized ovules, no evidence of pollen tube penetration was found in the micropyle or nucellus. This may have resulted from lack of pollen germination or inadequate pollen-tube growth. Three of the four unfertilized ovules were sampled from clone 6-06. The remains of pollen tubes were found in the micropylar region and the nucellus of two of the unfertilized ovules (Fig. 97). In these, pollen-tube arrestment within the nucellus accounted for the lack of fertilization. Pollen-tube arrestment within the nucellus was not observed in the wind-pollinated ovules, but this may have been due to the small number of ovules sectioned at this stage.

Embryo degeneration was observed during several stages of embryo development in the majority of the selfed ovules that were successfully fertilized. No degeneration or irregularities were found during fertilization, proembryo development or early suspensor elongation. The first case where complete degeneration of all developing embryos was found was in early June, after cleavage (Fig. 98). These embryos were typified by densely staining, shrunken cells compared to larger, lighter staining, healthy cells.

Many embryos developed past cleavage but degenerated during early embryo development after a few cell divisions, while others did not degenerate until later in development (Fig. 99). Frequently, the embryo deepest into the female gametophyte, which often was the largest embryo, degenerated, leaving several smaller, healthy, embryos at the micropylar end. These micropylar embryos had become arrested during early development and never attained full size.

In a few ovules, the megagametophyte expanded, but without the corresponding increase in reserves, and eventually degenerated, even though several small healthy embryos were present in the corrosion cavity (Fig. 100). In others the megagametophyte failed to expand (Fig. 101). In a few ovules the embryos became quite large, even though the cells of the megagametophyte were quite vacuolate and lacked the characteristic storage products (Fig. 102). In both cases the embryos eventually degenerated.

Figs. 96-102. Longitudinal sections through ovules showing stages during ovule, embryo and megagametophyte development which result in empty seed.

Fig. 96. Pollen tube (pt) containing a male gamete (mg) outside a degenerated archegonium (a). x300.

Fig. 97. Pollen tubes in the nucellus (n) of unfertilized ovules still at the egg cell (ec) stage after fertilization should have occurred. x170.

Fig. 98. Degeneration of all cleavage embryos (e) within the megagametophyte (meg). x160.

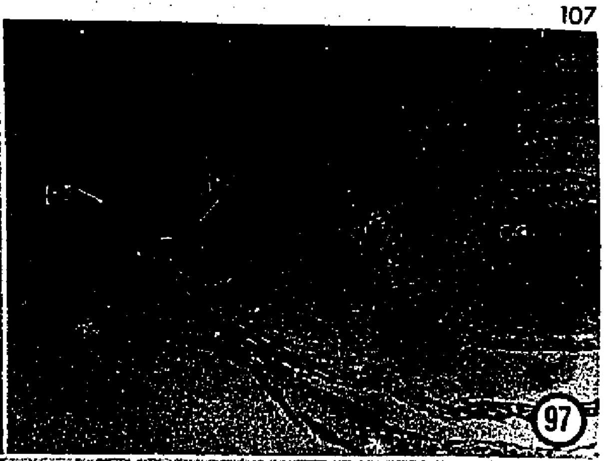
Fig. 99. Degeneration during late embryo development. The cells of the megagagametophyte have accumulated storage products. x120.

Fig. 100. Section through an ovule showing a degenerated megagametophyte with several healthy embryos at the micropylar end. x130.

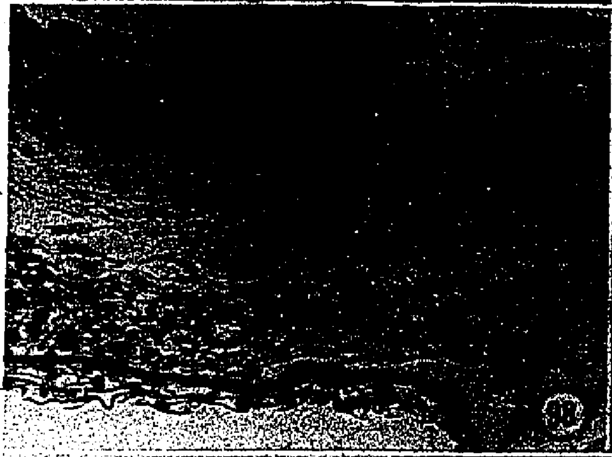
Fig. 101. Section through an ovule where the cells of the megagametophyte failed to accumulate storage products. A healthy embryo is found within the corrosion cavity. x125.

Fig. 102. A section through an ovule where degenerating cells of the megagagametophyte have accumulated some storage products. A multicellular embryo is found within the corrosion cavity (cor). x120.

(96)

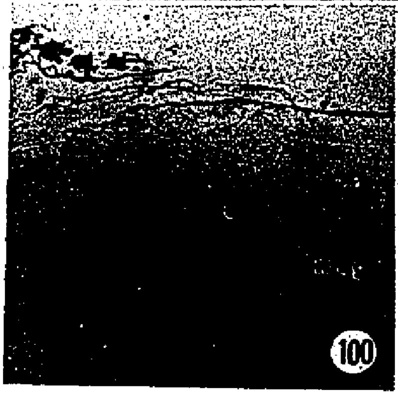


(97)

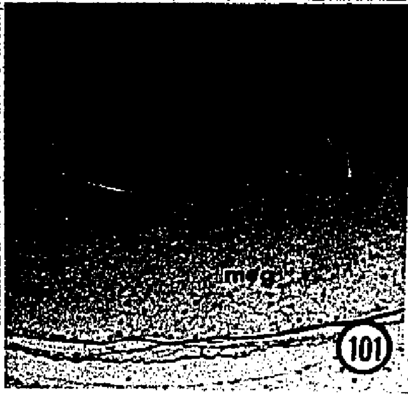


(98)

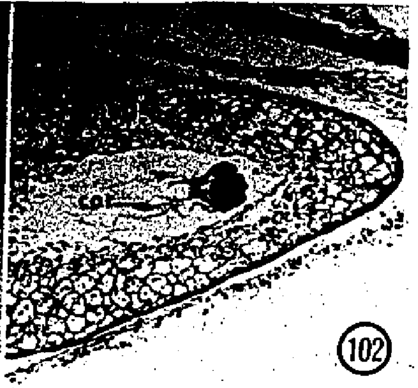
(99)



(100)



(101)



(102)

While more than 80% of the ovules sectioned had been fertilized, very few selfed ovules had embryos that developed past the club-shaped stage. Clone 6-06 had very few (<10%) fertilized ovules, probably due to low pollen germination. No embryo degeneration was observed after the embryos started to differentiate. Approximately 80% of all fertilized ovules degenerated. By late July a few healthy, full-sized embryos were found.

4.7.4.1 Effect of Self- and Wind-Pollination on Seed Efficiency

Selfing decreased SEF in all 10 clones relative to wind pollination (Table 18). The effect of selfing on SEF varied between the clones, ranging from a SEF of 1 in clone 5-10 to 43 in clone 12-10. The average SEF for the 10 clones after self-pollination was 17, compared to an average SEF of 66 when wind-pollinated. Clones that produced a low SEF when selfed did not necessarily produce a low SEF when crossed.

Table 18: Seed efficiencies in ten self- and wind-pollinated western hemlock clones at the Cobble Hill Clone Bank, 1983.

The seed efficiencies (SEF) were averaged for 20 cones per clone for the self-pollinated cones (SELF). The SEFs for the wind-pollinated cones were determined from 20 bulked cones per clone. The average standard error for SEFs after selfing was 1.7.

CLONE	SELF SEF	WIND SEF
2-05	26	73
4-01	23	76
4-02	20	74
5-10	1	74
6-06	3	59
9-10	16	38
11-08	7	59
12-03	13	64
12-10	43	71
16-02	15	71
AVERAGE (SE)	17 (3.8)	66 (3.6)

4.8 Seed Development in Container-Grown Clones

A detailed study of the factors affecting seed development after self- and cross-pollination was undertaken in 1984. Four container-grown clones (11-7, 3-5, 6-8 and 6-9), consisting of two ramets per clone, were pollinated with self and unrelated pollen. Pollen development, fertilization rate, embryo development and factors affecting seed yield after selfing and crossing were compared between clones.

As in the 1983 seed-development study, several stages were found during which a reduction in potential seed yield occurred. These included ovule abortion before pollination, ovule abortion before fertilization, failure of pollen tubes to reach the nucellus, pollen-tube arrestment in the nucellus, and embryo degeneration. Embryo degeneration was further subdivided into three stages: degeneration during or after cleavage, degeneration during early embryo development and ovules in which megagametophyte degeneration preceded embryo degeneration. These stages were found in both the selfed and crossed ovules, but the frequency with which each occurred varied between the two trials.

4.8.1 Ovule Abortion

Ovule abortion before pollination and fertilization was observed in both selfed and crossed cones (Table 19). Very few ovules degenerated before pollination, so pre- and post-pollination ovule abortion were combined to determine total pre-fertilization abortion. Ovules that aborted before pollination resulted in the degeneration of the entire ovule. These ovules had a developed integument but degenerated nucellar or megagametophytic tissue. Post-pollination ovule abortion occurred around the time the archegonial initials began to differentiate, just after the completion of free-nuclear division and cell wall formation. In these ovules the seed coat continued to develop, resulting in full-size empty seeds. The cells of the seed coat expanded to fill in the cavity formed by the degenerated megagametophyte. No differences were observed between the selfed and crossed ovules within each clone, implying that ovule abortion before fertilization may be a maternally controlled characteristic. Ovule abortion was more frequent in clones 11-7 and 6-9, averaging 7% and 9%, respectively. A low level of ovule abortion (2% and 1%) was found in clones 3-5, and 6-8, respectively. The pooled ovule abortion frequency for the four selfed and crossed clones (TOTAL) was 15/341 and 15/299, respectively, reducing the potential seed yield by 4% in the selfed trial and 5% in the crossed trial. Overall ovule abortion approached 5% (30/640).

Table 19: Ovule abortion in four self- and cross-pollinated western hemlock clones, 1984.

From the total number of sampled ovules (OV), pre- and post-pollination ovule abortion (PRE and POST) and total percent abortion (%ABRT) were determined for each clone. The average abortion rate per clone (AVE. %ABRT) combines the selfed and crossed results. The standard error for the average rate was 2.0.

	SELF				CROSS				
CLONE	OV	PRE	POST	%ABRT	OV	PRE	POST	%ABRT	AVE. %ABRT
11-7	73	1	5	8	78	1	4	6	7
3-5	83	0	1	1	73	1	1	3	2
6-9	99	2	6	8	72	2	5	10	9
6-8	86	0	0	0	76	0	1	1	1
TOTAL	341				299				
AVERAGE		3	12	4		4	11	5	5

4.8.2 Fertilization

Fertilization occurred between May 8 and 12, 1984, approximately six weeks after pollination. The ovules collected and sectioned after fertilization were divided into three groups: unfertilized ovules with no pollen tubes in the nucellus (NT), unfertilized ovules with pollen tubes visible in the nucellus (TNF) and fertilized ovules (FERT). Aborted ovules were included in the total count but not in any of the three groups (Table 20). The average number of zygotes per ovule was 1.9 in the selfed clones and 2.0 in the crossed clones, ranging from zero to four zygotes per ovules in both cases.

Table 20: Fertilization status in ovules sampled from four self- and cross-pollinated western hemlock clones, 1984.

The sectioned ovules (OV), were divided into three groups for each selfed and crossed clone: unfertilized ovules with no pollen tubes in the nucellus (NT), unfertilized ovules with pollen tubes in the nucellus (TNF) and fertilized ovules (FERT). Each variable is followed parenthetically by its percent value.

	SELF				CROSS			
CLONE	OV[1]	NT	TNF	FERT	OV	NT	TNF	FERT
11-7	40	7(18)	7(18)	24(60)	54	2(6)	2(6)	46(85)
3-5	50	3 (6)	11(22)	35(70)	49	4(8)	2(4)	42(86)
6-9	59	2 (3)	5 (8)	46(78)	48	1(2)	0(0)	44(92)
6-8	50	3 (6)	6(12)	41(82)	50	1(2)	1(2)	48(96)
TOTAL	199	15	29	146	201	9	5	182
AVERAGE		8	15	73		4	2	91

[1] OV includes post-pollination aborted ovules

Among the selfed ramets, NT ovules ranged from 3% to 18% in clones 6-9 (2/59) and 11-7 (7/40), respectively. The number of NT ovules in the crossed ramets varied from 2% to 8% in clones 6-8 (1/50) and 3-5 (4/49). When the proportion of selfed NT ovules was compared to crossed NT ovules by clone using chi-squared analysis, no significant differences ($p<.05$), were found between the clones. In the selfing trial each clone was pollinated with a different pollen while the same pollen lot collected from four clones was used to pollinate all cones in the crossing trial The NT ovules reduced the potential seed yield in the self-and cross-pollinations by an average of 8% and 4%, respectively.

Among the selfed ramets, TNF ovules ranged from 8% (clone 6-9) to 22% (clone 3-5) averaging 15% (Table 20) and from 0% (clone 6-9) to 6% (clone 11-7), averaging 2% in the crossed ramets. Chi-squared analysis of the frequency of TNF ovules found that a significantly higher ($p<.05$) number of pollen tubes ceased development in the nucellus of selfed ovules than in the nucellus of crossed ovules.

Chi-squared analysis was used to determine the relationship between fertilized and unfertilized ovules in the selfed and crossed clones. Significant differences ($p < .05$), were found in all four clones. The fertilization rate varied from 60 to 82% in the selfed ramets and 85 and 96% in the crossed ramets.

A total of 199 selfed ovules were analyzed from the pooled results of the four clones, of which 8% (15) were unfertilized because of no pollen tubes, 15% (29) were unfertilized even though pollen tubes were visible in the nucellus and 73% (146) had one or more fertilized archegonia. Of the 201 crossed ovules pooled from the same four clones, 4% (9) were unfertilized due to lack of pollen tubes, 2% (5) were unfertilized even though pollen tubes penetrated the nucellus and 91% (182) had one or more fertilized archegonia. Chi-squared analysis of the pooled results found significant differences between TNF and FERT rates of selfed and crossed ramets, while no differences were observed between the NT results. The potential seed yield for the four clones was decreased by approximately 27% from selfing compared to a reduction of 9% following outcrossing (Table 20).

4.8.3 Embryo Development

No irregularities or degeneration were observed during fertilization and proembryo development in either the self- or cross-pollinated ovules. Fertilization and proembryo development appear to be stages infrequently affected by abortion or degeneration. However, embryo degeneration was observed at several stages during embryo development in both the selfed and crossed clones and the degree of degeneration was much higher in the selfing trial.

Many embryos degenerated during or just after cleavage but before the apical cell division. The megagametophyte continued to develop for about one week after the embryos degenerated. During this time many mitoses were observed in the megagametophyte and the cells of the megagametophyte expanded and started to accumulate reserves. After about one week the megagametophyte ceased nuclear division, cell expansion and reserve accumulation and started to degenerate.

Other embryos developed past cleavage only to degenerate during early embryo development. Some embryos degenerated after a few cell divisions while others did not degenerate until later in development. The megagametophyte continued to develop for only a few days after the embryos degenerated. Very few embryos that attained the club-shaped stage degenerated and no embryos degenerated once cotyledon initiation began.

In some ovules with apparently healthy embryos, the megagametophyte ceased development during the early in the reserve-accumulation stage. Nuclear divisions and cell expansion also stopped. The embryos continued cell division at a reduced rate over the next several weeks. In mid-June, ovules with healthy megagametophytes contained embryos that were starting to initiate cotyledons, while ovules in which megagametophyte development ceased had very small embryos. By late June to early July the embryos in these ovules degenerated.

For each self- and cross-pollinated clone the total number of ovules sectioned during June and early July were divided into fertilized (FERT) and unfertilized ovules. The number of fertilized ovules in which total degeneration of the embryos or megagametophyte occurred (DEG) was determined. The proportion of fertilized ovules that degenerated (DEG/FERT) also was calculated (Table 21).

The cross-pollinated ovules had a lower degeneration percentage than the self-pollinated ovules. Percent degeneration in the self-pollinated clones ranged from 51% (clone 6-9) to 71% (clone 6-8), averaging 59%. Percent degeneration in the cross-pollinated clones ranged between 13% (clone 6-9) and 22% (clone 11-7), averaging 19%. The proportion of fertilized ovules that degenerated (DEG/FERT) was much higher after self-pollination. This ranged from 70% (clone 6-9) to 91% (clone 6-8) after selfing and 15% (clone 6-9) to 26% (clone 11-7) after crossing, with an average of 84% and 21% after selfing and crossing, respectively.

Table 21: Embryo degeneration in ovules sampled from four self- and cross-pollinated western hemlock clones, 1984.

From the total number of ovules sampled during late-embryo development fertilization and embryo degeneration rates and the percentage of fertilized ovules which degenerated (% DEG/FERT) were determined for each clone. Each variable is followed parenthetically by its percent value.

	SELF				CROSS			
CLONE	OV	FERT	DEG	% DEG/FERT	OV	FERT	DEG	% DEG/FERT
11-7	51	30(59)	26(51)	87	45	38(84)	10(22)	26
3-5	60	42(70)	36(60)	86	39	32(82)	8(21)	25
6-9	59	44(75)	31(53)	70	46	40(87)	6(13)	15
6-8	55	43(78)	39(71)	91	52	49(94)	10(19)	20
TOTAL	225	132	132		182	159	34	
AVERAGE		71	59	84		87	19	21

The DEG ovules (Table 21) were divided into the three classes, degeneration at cleavage (CLV), degeneration during early embryo development (EMB) and megagametophyte degeneration (MEG) before embryo degeneration (Table 22). The stages where maximal degeneration occurred varied among the selfed clones. The number of CLV ovules was the lowest in clone 11-7, averaging 12% (3/26) of all degenerated ovules. This was followed by clone 6-9 where 29% (9/31), of all degenerated ovules were in the CLV class. In the other clones, CLV ovules averaged about 39% of the total degenerated ovules. The number of EMB ovules averaged about half of the total degenerated ovules in all selfed clones except clone 11-7, where 85% (22/26) of the degenerated ovules were in this class. A low level of MEG ovules (2%), was observed in clones 11-7, while in the remaining clones there were about 16% MEG ovules. The majority of the degenerated crossed ovules (>80%) was found in the EMB class. Three clones contained one or more CLV ovules while only one clone (6-8) had MEG ovule.

Table 22: Embryo degeneration classifications in ovules sampled from four self- and cross-pollinated western hemlock clones, 1984.

The post-fertilization degenerated ovules (DEG) were divided into three groups: degenerated at cleavage (CLV), degenerated during embryo development (EMB), and ovules where megagametophyte degeneration preceded embryo degeneration (MEG). Each variable is followed parenthetically by its percent value.

	SELF				CROSS			
CLONE	DEG	CLV	EMB	MEG	DEG	CLV	EMB	MEG
11-7	26	3 (12)	22 (85)	1 (4)	10	1 (10)	9 (90)	0 (0)
3-5	36	14 (39)	16 (44)	6 (17)	8	1 (13)	7 (87)	0 (0)
6-9	31	9 (29)	17 (55)	5 (16)	6	0 (0)	5 (83)	1 (17)
6-8	39	15 (38)	18 (46)	6 (15)	10	2 (20)	8 (80)	0 (0)
TOTAL	132	41	73	18	34	4	29	1
AVERAGE		31	55	14		12	85	3

4.8.4 Seed Yield

The number of ovules with mature embryos was determined from ovules sampled in mid-July. Percent filled seed (%FILL), was calculated for both the self- and cross-pollinated clones (Table 23). The %FILL ranged from 6 (clone 6-8) to 24% (clone 6-9), averaging 13% for the four selfed clones. A higher %FILL was found in the outcrossed ramets, ranging between 65 (clone 11-7) and 73% (clone 3-5), averaging 70%, for the four ramets.

4.9 Summary of Seed Development in 1983 and 1984

The different stages influencing seed development and filled seed yield for the self- cross- and wind-pollinated clones in 1983 and for the four self- and cross-pollinated clones in 1984 are summarized in Fig. 103. In 1983, pre-pollination ovule abortion accounted for an average of 12% reduction in seed yield for all pollinations. Unfertilized ovules reduced filled yield by about 14, 16 and 9 percent for self, wind and cross pollinations, respectively. The average SEF was 17, 66 and

69 percent, for self, wind and cross pollinations, respectively. The proportion of ovules lost due to embryo and megagametophyte degeneration was extrapolated from the above results and accounts for about 57, 6 and 10 percent of the reduction in filled seed yield for the three pollinations, respectively.

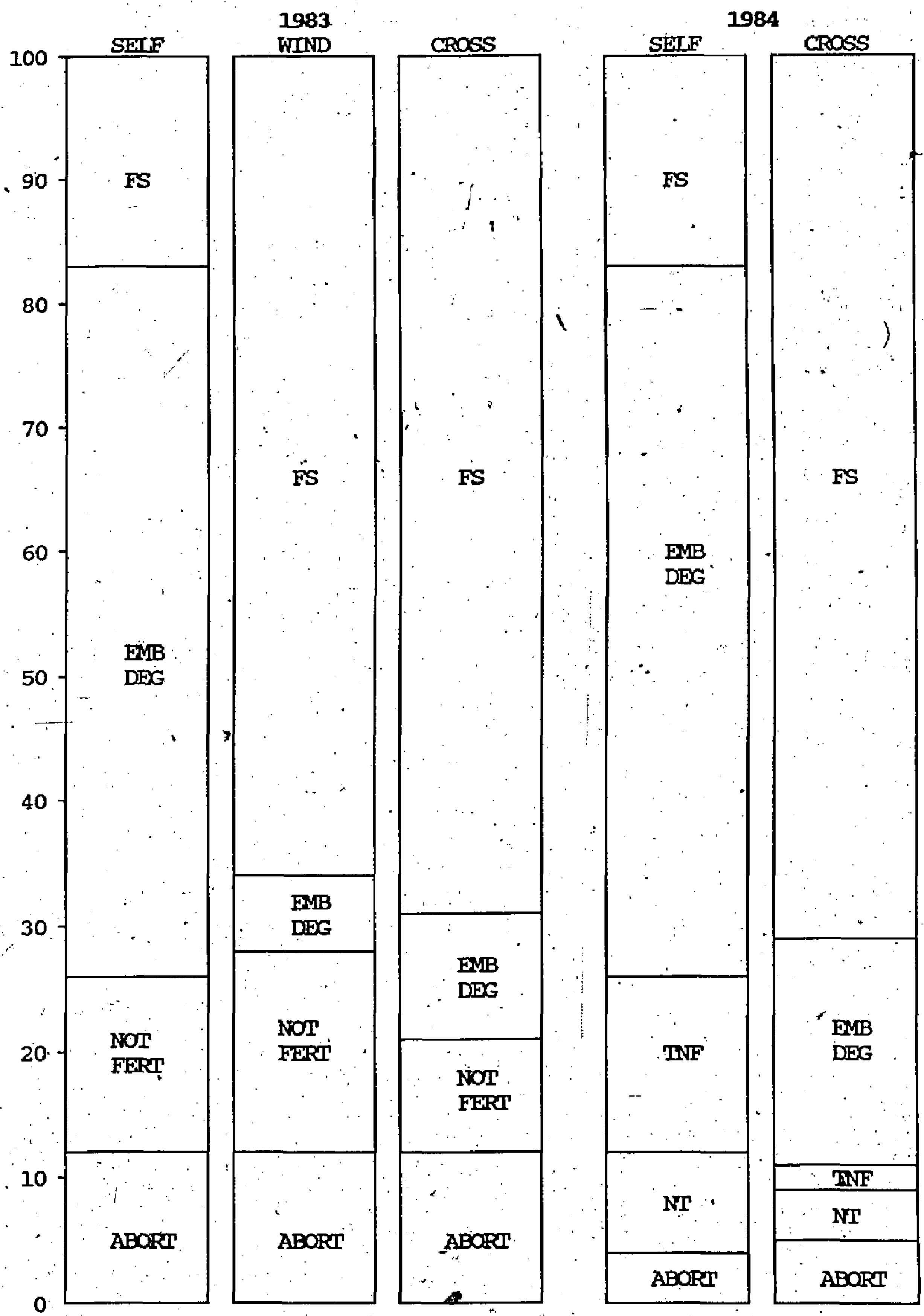
Table 23: Mature embryos in ovules sampled from four self- and cross-pollinated western hemlock clones, 1984.

Percent filled seed (%FILL) was determined from the total number of ovules sectioned at this stage.

	SELF			CROSS		
CLONE	OVULES	EMBRYOS	%FILL	OVULES	EMBRYOS	%FILL
11-7	47	4	9	49	32	65
3-5	48	7	15	45	33	73
6-9	54	13	24	50	36	72
6-8	53	3	6	49	34	69
TOTAL	202	27		193	130	
AVERAGE			13			70

In 1984, ovule abortion, which included both pre- and post-pollination, abortion accounted for a 4 and 5% reduction in potential seed yield for the selfed and crossed ramets, respectively. Unfertilized ovules with no pollen tube (NT) accounted for 8 and 4% of all reduction for the selfed and crossed ramets, respectively. The number of unfertilized ovules with tubes present in the micropyle and nucellus was much higher in the selfed ovules than in the crossed ovules. These accounted for 14 and 2% of all losses, respectively. Embryo degeneration was observed in 57% of all selfed ovules compared to 18% of all the crossed ovules. This was subdivided into 8% MEG, 32% EMB and 17% CLV ovules in the selfed trial and 1% MEG, 15% EMB and 2% CLV ovules in the crossing trial. The predicted filled seed rate (FILL) was 17 and 71% for the selfed and crossed clones, respectively. This is very close to the actual filled rate of 13 and 70%, respectively (Table 23).

Figure 103: Summary of seed-loss causes in 1983 after self, cross and wind pollination and in 1984 after self and cross pollination in western hemlock. In 1983, seed losses were divided into aborted ovules (ABORT), unfertilized ovules (NOT FERT), embryo degeneration (EMB DEG) and filled seed (FS). In 1984, unfertilized ovules were further divided into ovules containing no pollen tubes (NT) and unfertilized ovules with pollen tubes in the nucellus (TNF).


1983
1984
SELF
WIND
CROSS
SELF
CROSS
100
90
80
70
60
50
40
30
20
10
0
FS
EMB DEG
NOT FERT
ABORT
FS
EMB DEG
NOT FERT
ABORT
FS
EMB DEG
NOT FERT
ABORT
FS
EMB DEG
TNF
NT
ABORT
FS
EMB DEG
TNF
NT
ABORT

Chapter V

DISCUSSION

5.1 Pollen Development

Pollen-cone phenology was synchronized closely with pollen development, independent of collection date in eight field-grown clones in 1983 and three containerized clones in 1984, so phenology seems an accurate indicator of cytology. Variability was observed in both studies as to the time dormancy ended and development resumed. This initial variability was maintained throughout the study in any one year because the rate of development remained constant. On each collection date pollen-cone phenology varied both between and within the clones, with some clones up to 10 days further advanced than others. Within the trees, axillary pollen cones in the proximal region of a shoot were further advanced than the more distal cones.

These results amend an earlier paper by Ho and Owens (1974b), where pollen was reported to be mature and shed at the 4-cell stage. Either their pollen was not sampled during stalk elongation, or, due to the squash technique used, the fifth cell was not observed. During both years of the present study, a fourth cell division occurred during stalk elongation, resulting in 5-cell pollen. Mature pollen consisted of two prothallial cells, a stalk cell, a body cell and a tube cell.

Other phenological and cytological relationships were observed. Between the end of dormancy and enlargement of the tetrads, many structural changes were observed in the tapetal cells. These were associated closely with pollen-wall development. The tapetal cells began to enlarge prior to meiosis. During stage 2, the tetrads separated and the tapetal cell walls degenerated. Baculae appeared on the surface of the microspores several days after tetrad separation. Development of the exine was associated with the release of the Ubisch bodies from the tapetal cytoplasm. The baculae increased in size and fused, forming the tectum. The intine became visible within the microspores about the time the pollen grains began to expand. Ultrastructural studies in *Pinus* (Dickinson and Bell 1972, 1976) and other gymnosperms (Singh 1978; Moitra and Bhatnagar 1982) have shown that the tapetal cells are responsible for the synthesis of material composing the pollen wall, including sporopollenin and various coatings (orbicules) found on mature pollen. The relationship between the degeneration of the tapetum and pollen-wall development in western hemlock is in agreement with these observations.

Changes in the tapetum were associated also with increase in pollen size in western hemlock. During early microspore development and after the degeneration of the tapetal cell walls, the amount of tapetal cytoplasm decreased. This corresponded to an increase in pollen size, perhaps due to the accumulation of reserves, the most prominent being starch. Accumulation of reserves occurred over a two-week period during stages 4, 5 and 6. The tapetum was no longer visible after the third cell division during stage 6, at which time the pollen grains were full sized. The decrease in tapetal cytoplasm, which coincided with the increase in size of the pollen grains, suggests that the tapetal cells are a nutritive source for the developing pollen in western hemlock. This is in agreement with the role of the tapetum in microsporogenesis and pollen development as reviewed by Singh (1978) and Moitra and Bhatnagar (1982). The tapetum, which is very active in RNA, protein and lipid synthesis, is believed to be the source of nutrients for sporogenous cells and young microspores (Dickinson and Bell, 1976). Tapetal cells are rich in starch and polysaccharides before meiosis, but after meiosis the cells become depleted, with a corresponding increase of these nutrients in the thecal fluid, in which the tetrads, microspores or pollen grains are suspended (Dickinson and Bell 1976).

Pollen development in western hemlock was similar to that observed in mountain hemlock (Owens and Molder 1975b). Both species began meiosis in the fall and overwintered during pachytene. In the spring meiosis resumed about one week after the breaking of dormancy. In mountain hemlock meiosis was completed in 2 to 3 weeks, compared to the 6 to 10 days documented here in western hemlock. Indirect evidence in mountain hemlock suggests that meiosis is completed before or during early bud swelling (Owens and Molder 1975b), as in western hemlock. While the authors did not directly compare pollen-grain cytology to pollen-cone phenology in mountain hemlock, they did observe that meiosis resumed in late March, tetrads were present by mid-April and visible swelling of the pollen-cone buds was observed by mid-April, coinciding with the end of meiosis. Tetrads separated less than one week after the completion of meiosis in western hemlock, whereas mountain hemlock remained at the tetrad stage for about three weeks. In mountain hemlock, the exine and the sacci began to develop on the microspores while the tetrads were still confined within the PMC wall. In western hemlock baculae did not appear until several days after tetrad separation.

Shortly after the exine developed and before microspore expansion the exine separated, forming vestigial wings in western hemlock. Fossil evidence indicates that the nonsaccate *Tsuga* pollen of the *Micropeuce* subgenus, which contains western hemlock, was derived from saccate ancestors (Wodehouse 1959). Pleistocene bogs in western Washington contain *Tsuga* pollen with and without sacci and all gradations in between (Hansen 1941). The vestigial wings that appear on western hemlock pollen during development may indicate that it evolved from winged ancestors. The vestigial wings formed where sacci would develop in winged species. From this study it was not possible to determine if the vestigial wings formed by a separation of the sexine from the nexine, as occurs in winged species. An ultrastructural study would be required to determine this.

Western hemlock pollen grains began reserve accumulation soon after tetrad separation, while in mountain hemlock this began while the microspores were still confined within the PMC wall. In both species the first cell division occurred 2 to 3 weeks after tetrad separation. By this stage the exine was fully developed but the intine was still forming. Cell division lasted about two weeks and pollen was shed at the 5-cell stage in both species. Pollen was shed less than two months after the resumption of meiosis in western hemlock. However, the same process took almost three months in mountain hemlock. Much of the variation between the two species may be attributed to environmental effects, such as temperature. Mountain hemlock usually grows at much higher elevations (Fowells 1965) and may be exposed to lower temperatures during pollen development.

The stage at which sporogenous tissues overwinters may influence the relationship between PMC meiosis and pollen-cone phenology. Western and, possibly, mountain hemlock (Owens and Molder 1975b), which overwinter during pachytene, complete meiosis before pollen-cone buds begin to swell. Recent work has demonstrated a similar relationship in *Thuja plicata* (Owens *et al.* unpublished). *Pseudotsuga* (Allen and Owens 1972) and *Larix* (Eriksson 1968; Owens and Molder 1979b; Hall 1982) pollen cones overwinter at the diffuse diplotene stage; their pollen cones did not begin to swell until well after meiosis in the former and during meiosis in the latter. In *Picea* and *Abies* (Mergen and Lester 1961; Moir and Fox 1975; Owens and Molder 1980a), pollen cones overwinter at the premeiotic PMC stage and meiosis resumes about two weeks after the resumption of development, resulting in early premeiotic swelling of pollen-cone buds. Meiosis occurs more than one month after the breaking of dormancy in *Pinus*. In *P. contorta* (Owens *et al.* 1981a) and other hard pines (Konar 1960), which overwinter at the sporogenous cell stage, the PMCs increase in size before meiosis, which coincides with the emergence of the small green cones through the cataphylls. In *P. monticola* (Owens and Molder 1977c) and other soft pines (Konar 1960; Owston 1969), which overwinter at the microsporophyll stage, before the formation of sporogenous cells, meiosis occurs more than one month after the breaking of dormancy by which time the pollen cones have emerged beyond the bud scales and between the basal cataphylls and turned green to yellow (Owens and Molder 1977c). Pollen-cone phenology can be used to predict the approximate time of meiosis for each species if the overwintering stage is known.

Post-meiotic stages of pollen development are similar in the Pinaceae studied to date, but minor variations occur in the duration of these stages. These variations occur often between species within a genus, as was observed in western and mountain hemlock. Generally, tetrad separation occurs 1 to 3 weeks after meiosis, the exine differentiates and the microspores start to accumulate reserves before cell division. Cell divisions in pollen usually are completed within two weeks and pollen is shed at the 4- or 5-cell stage, depending on the species.

This study has demonstrated that relating stages of microsporogenesis and pollen development to pollen-cone phenology are valid and more useful than using calendar dates, since weather conditions plus clonal variation can change the time and rate of development by several weeks from one year to another. This information would be useful for pollen collections in seed orchards and wild stands. Pollen-cone phenology can be used to estimate all stages of development and to determine when pollen is mature enough to be safely collected without having to make microscopic observations. Similar studies would be useful for other conifers.

5.2 Effect of Forcing on Pollen Development

Forcing pollen before or during bud burst on cut branches (PFT1, PFT2, PFT3) decreased the number of cones that matured, reduced pollen yield and increased the proportion of abnormal pollen relative to results from forcing buds collected at later stages of development (PFT4, PFT5) or non-forced pollen (PFT6). Forcing buds collected during early stages, but using whole trees in growth chambers at 18°C did not reduce the number of cones that matured or their pollen yield, or increase abnormal pollen frequency relative to development at ambient temperatures. Sarvas (1972) found abnormal pollen development in cut branches of *Populus tremula* above 11°C. Luomajoki (1977) observed normal development in field grown trees at average temperatures of 13°C. The disagreement between the results was explained by the fact that Sarvas forced pollen on cut branches while Luomajoki observed pollen development on whole trees (Luomajoki 1977). Longterm stresses, such as water deficiency, can cause abnormal development in pollen from cut branches (Luomajoki 1977, 1986). Snyder and Clausen (1974) found that the collection of unripe pollen cones resulted in low pollen yields and pollen of low viability. In these western hemlock cut-branch trials, abnormalities and pollen degeneration were not observed until eight to ten days after collection. Collapse and degeneration of the microsporophyll and microsporangial wall cells, usually preceded pollen degeneration. This may have been the time required for physiological stresses such as water and nutrient deficiencies to build up to levels detrimental for pollen development. Abnormal pollen development in the early trials may have been caused primarily by desiccation, since most pollen cones that degenerated had collapsed microsporangial cells. Similar degeneration was observed in all cones that developed past bud burst but failed to shed pollen. Degeneration of the cells in the dehiscence layer would have prevented the microsporangia from opening properly and releasing the pollen. Pollen in PFT4, which matured within seven days, did not have degenerated microsporangial cells. It appears that in the cut-branch method, forcing *per se* did not cause abnormal development but rather that pollen development may have been affected by physiological stresses caused by the cut-branch technique. Successful cone maturation was unaffected by temperature in the whole-tree pollen-forcing trial, suggesting that many of the problems associated with the cut branch technique were due to physical stresses. This is contrary to the results of Ross (1988) for containerized *Picea engelmannii* ramets, where high temperatures in polyhouses increased proportions of under-developed and rotted pollen cones and decreased pollen yields. The temperatures in the spruce study, 24:18°C, day:night, respectively, were higher than the temperatures used in the western hemlock study. Also humidity, which was low in the growth chambers, was very high in the polyhouse. A combination of high heat and humidity was believed to have contributed to the high incidence of pathogen-infected pollen cones. A more recent study in western hemlock (Colangeli, unpublished data), using 18°C growth chambers and high humidity caused many of the pollen cones to case harden and not shed. The effect differed by clone, with some clones being unaffected by the high humidity and others not dehiscing properly.

Several studies have observed the effect of temperature on cone phenology (Sarvas 1967; Boyer 1981; Ross 1988). Others have attempted to correlate temperature and the rate of meiosis (Luomajoki 1977, 1986). In the present study not only was pollen-cone phenology and pollen cytology dependent on temperature, but also the relationship between phenology and cytology was affected by temperature. At 18°C, pollen cytology lagged behind cone phenology relative to development at 10°C and ambient temperatures. Also, the duration of each stage was significantly shorter at the higher temperatures.

In the cut-branch trial, the fertilizing potential of pollen was affected by forcing. Considering that the pollen produced in PFT2 yielded about one-sixth the quantity produced by the control (PFT6), and it was shed at the 1- to 5-cell stage, it is noteworthy that the resulting SEF (45.4) was still two-thirds the control SEF (71.3). This implies that the small amount of mature viable pollen that was produced was capable of fertilization.

Unfortunately, no data are available for the SEFs resulting from freshly collected, whole-tree-forced pollen. Since high SEFs were produced using the cut-branch technique, even though many abnormalities developed and there was a high incidence of pollen degeneration possibly due to stresses imposed by this method, it may be reasonable to assume that the SEFs produced by freshly collected pollen forced on containerized ramets would not differ significantly from the control.

A relationship was found between pollen forcing and longterm (2 years) storage effects. High temperatures during certain stages of pollen development affected storability, viability and/or vigor of the pollen. In the cut-branch trial, pollen forced prior to stage 4, before significant pollen enlargement and reserve accumulation occurred, resulted in very low SEFs after two years' storage. The SEF of pollen forced after this stage was unaffected by storage. The high temperatures which caused a three-fold acceleration in development may have hindered the accumulation of storage products. The active period of reserve accumulation was reduced to 4 to 5 days, compared to 2 weeks or more at ambient temperatures. There may have been sufficient reserves in the pollen to achieve fertilization when fresh pollen was used, as indicated by the resulting SEFs in the 1983 pollination trial, but not enough reserves after storage. A low level of metabolic activity is maintained during storage at 4°C (Stanley and Poostche 1962; Stanley and Linskens 1974). A constant draw on these reserves may have left the pollen with insufficient reserves to achieve fertilization after storage. The SEFs after one and two years' storage suggests that pollen forced before pollen enlargement and starch accumulation had a reduced fertilizing potential. Similar results were observed in the whole-tree trial. Pollen forced at 18°C, before reserve accumulation, resulted in significantly lower SEFs after one year's storage than pollen forced at 10°C or at ambient temperatures.

Another stage that was affected by the higher temperatures was meiosis. In the whole-tree pollen-forcing study, pollen from PFT1-18 and PFT2-18, which were forced before meiosis, resulted in a significantly lower SEF after one year of storage than did pollen from PFT3-18, in which two of the three clones were forced after meiosis. A high correlation between meiotic irregularities, sterile pollen and low temperatures have been observed in *Larix* (Christiansen 1960; Ekberg and Eriksson 1967; Ekberg *et al.* 1968; Eriksson 1968, 1970; Andersson *et al.* 1969), *Abies* (Mergen and Lester 1961) and *Picea* (Andersson 1965, 1980; Jonsson 1974). High temperatures also have been implicated as the cause of meiotic irregularities. Temperatures above 15°C (Chira 1965) and 20°C (Eriksson *et al.* 1970a) were believed to be responsible for the induction of meiotic irregularities in *Picea* and a temperature of 30°C for eight hours caused total pollen sterility in *Taxus baccata* (Chira 1964).

Clone 13, in the 1984 whole-tree-pollen-forcing study displayed a cytological temperature-sensitive phenomenon. At 10°C or at ambient temperatures, meiosis was identical to the other clones. At 18°C, meiosis occurred basipetally rather than acropetally in about one-third of the pollen cones. Dyad formation in about one-third of the PMCs after meiosis I resulted in some irregular orientations of the four microspores within the tetrad after meiosis II. Cell proliferation was observed within a few of the PMCs but, due to the low frequency of this occurrence the subsequent fate of these cells is unknown. None of these phenomena were observed in the other clones at any temperature. Christiansen (1960) found that cell-wall formation at the dyad stage was induced by low temperatures in *Larix decidua*. If temperature extremes can cause structural changes such as dyad formation, then other, more-subtle stages of meiosis may also be affected.

Many factors could have led to the reduction in the SEF after pollen storage. Mature pollen grains have been found to contain enzymes and isozymes encompassing six classes of enzymes (Stanley and Linskens 1974), plant growth regulators (Sweet and Lewis 1969, 1971; Kamienska and Pharis 1975; Kamienska *et al.* 1976a, 1976b), ATP (Ching *et al.* 1975), soluble proteins (Pettitt 1985) and lipids (Andrikopoulous *et al.* 1985), all of which may play an important role in pollen germination and pollen-tube development. Any of these cellular products could be affected by the higher temperatures and accelerated rate of development. The moisture content of the pollen was not determined before storage. Even though all pollen lots were stored under the same conditions, the moisture content of the pollen may have varied.

The cut-branch technique can be used efficiently when the cones are three-quarters emerged (stage 5) and at the 2-cell stage. Forcing earlier than this is possible, but it causes a reduction in SEF. Forcing earlier than stage 4 not only reduces SEF, but also drastically decreases pollen yield. Common horticultural techniques such as recutting the branches underwater every few days and using a nutrient-mineral solution may prolong the time within which pollen could be forced successfully. Snyder and Clausen (1974) suggested cutting branches at a slant, changing the water daily and using cut-flower preservatives with antiseptic substances to increase the number of cones that mature. The cut-branch technique is useful on a small scale, but for large pollen collections would be too inefficient of time and space.

In the whole-tree pollen-forcing trial, trees maintained at 18°C displayed a three-fold acceleration in phenology relative to trees maintained at ambient temperatures. Cone development is especially sensitive to temperature (Sarvas 1967; Boyer 1981; Ross 1988). This suggests that temperature may be used to synchronize pollen maturation in containerized clones, where late-shedding clones can be accelerated by heat treatment and early-shedding clones could be delayed by cooling (Bower *et al.* 1986; Ross *et al.* 1986).

Before temperature is used to accelerate or retard pollen development, more research is required to determine optimal temperatures and possibly humidity for forcing that are not detrimental to the pollens' fertilizing potential. Through the use of controlled-environment chambers and containerized ramets, the different factors affecting pollen development can be studied. Future studies that would help to elucidate why elevated temperatures reduce pollens' storability could involve forcing pollen at different stages and temperatures combined with determination of cytological development, starch, total carbohydrates and lipid content in the freshly collected pollen, and again at regular time intervals after storage. Respiration and germination tests could be conducted to determine pollen viability. An ultrastructural study of microsporogenesis might uncover temperature-sensitive irregularities in organelle development. It is quite likely that many factors are affected by increased temperature and future studies could concentrate on determining what these may be.

5.3 Pollination Mechanism

The pollination mechanism observed in western hemlock is different than that observed in most other conifers and is characterized by germination outside the micropyle where the roughly-sculptured pollen interacted with the epicuticular layer of the exposed bracts and, to a lesser extent, the outer edge of the ovuliferous scales. The pollen remained on the bracts while the ovuliferous scales elongated, trapping the pollen between the bracts and scales. The bracts and scales did not reflex sufficiently to allow pollen to filter down to the ovules, and at no time was the pollen observed on or near the integument tip or micropyle, contrary to the reports of Stanlake and Owens (1974) and Owens and Blake (1983). The pollination mechanism in western hemlock observed here is similar to that described for *Tsuga canadensis* (Doyle and O'Leary 1935b).

Shortly after cone closure the pollen hydrated and germinated. The mechanism of pollen hydration is uncertain. The pollen was tightly sealed between the bracts and scales. The epidermal projections which developed on the bracts and ovuliferous scales after cone closure may offer a suitable environment for pollen hydration and germination.

Pollen located on the margin of the bract furthest from the ovules produced tubes that appeared to grow in all directions. Pollen closer to the micropyle grew tubes oriented less randomly with most of the tubes growing towards the micropyle. Whether the ovules secreted chemotropic substance or whether it was the physical orientation of the bracts, scales and ovules forming a small chamber or to guide the developing pollen tubes (thigmotropism) that altered tube direction could not be determined in this study. The pollen tubes were packed tightly between the bracts and scales, so dissection tended to disturb the orientation and position of the tubes. This is very similar to the observations of Doyle and O'Leary (1935b) for eastern hemlock, where tubes were found growing in masses, sometimes in straight parallel bands and other times twisted and intertwined.

Eastern and western hemlock are the only members of the Pinaceae found to date in which the pollination mechanism involves non-micropylar germination. This type of mechanism has been observed in *Araucaria* and *Agathis* of the Araucariaceae (Doyle 1945; Haines *et al.* 1984) and *Saxegotheae* of the Podocarpaceae (Doyle 1945). *Araucaria* is the only other genus where a similar pollination mechanism has been studied in detail (Haines *et al.* 1984). Although the pollen germinates outside the micropyle, the mechanism of pollen capture and tube growth is different from that observed in eastern and western hemlock. Several species of *Araucaria* have a stoma free furrow on the bract/scales which directs pollen onto a thin flange on the tip of the ovuliferous scales. Other species of *Araucaria* lack the furrow but have a broader, scalloped bract/scale that traps the pollen in a band across both the fused bract and ovuliferous scales on the upright, reflexed cones. After the receptive period is over the bract/scales swell and elongate, trapping the pollen between the fused bract/scales. Unlike western hemlock, in which the roughly sculptured pollen grains come in contact and adhere to the epicuticular layer of the bract, *Araucaria* has a gravity-dependent pollen-transfer mechanism (Haines *et al.* 1984). The mechanism found in *Saxegotheae* appears to be a modified version of the *Araucaria* type, where the pollen is caught between the bracts and later germinates (Doyle 1945). As in western hemlock, it was difficult to speculate whether the ovules secrete a chemotropic substance aiding the directional growth of the pollen tubes (Haines *et al.* 1984). After germination, the pollen tubes of *Araucaria* penetrate the epidermis of the fused bract and ovuliferous scales and travel for a short distance just below the epidermal layer in the general direction of the micropyle. After emerging from the bract/scale, the tubes grow on the scale in the general direction of the cone axis and micropyle. Western hemlock pollen tubes grow approximately 1-2 mm in about one to two weeks, while *Araucaria* pollen tubes must grow 10-12 mm over an 18-month period between germination and fertilization, during which time the pollen tubes are dormant for several months (Haines *et al.* 1984).

5.4 The Optimal Time of Pollination

The optimal time of pollination was closely related to the stage of seed-cone development and the number of bracts exposed. As the number of exposed bracts increased, the SEF increased. This trend was observed in both the field-and container-grown ramets in 1983 and 1984 and relates well to a pollination mechanism where pollen adheres to the bracts. Pollinations conducted before stage 3 (bud burst) or after stage 8 (cone closure), resulted in no filled seed because no bracts were exposed. Maximum SEF was attained by stages 4-5 and maintained until stage 8, when a slight reduction in SEF was observed. At this stage the elongating ovuliferous scales covered more than 50% of the bracts.

Seed-cone receptivity varied among clones and between the two years of observations. As found in other studies (Doyle 1945; Dogra 1964) the optimal time of receptivity in western hemlock was affected by site and weather. In a cool, wet spring, as occurred in 1983, the average time between stages four and eight was approximately 23 days, while in a warmer, drier year, as in 1984, the period was reduced to 14 days. Since two different sites and clones were used, this may have contributed to the differences. The duration of seed-cone receptivity in western hemlock may be predicted from environmental conditions at the time of pollination.

This relationship may be used to accelerate late-flowering trees or retard early-flowering container-grown trees by moving trees into or out of greenhouses.

In western hemlock the period of receptivity occurs over a much longer period than in other conifers, due mainly to the much different pollination mechanism in this species. In *Chamaecyparis* (Owens *et al.* 1980), *Picea* (Owens and Blake 1984), and *Pinus* (Lill and Sweet 1977; Owens *et al.* 1981a; Greenwood 1986; Brown and Bridgwater 1987) individual cones are receptive for about one week or more, while individual ovules are receptive for only about 2-5 days because not all pollination drops are exuded at the same time. In *Pseudotsuga menziesii* (Owens *et al.* 1981b), which has a stigmatic integument tip, individual cones are receptive for 4-6 days. The long period of receptivity per cone in western hemlock should ensure a higher seed crop, especially in years with abundant pollen production. A comparison of SEFs produced by controlled crosses and wind-pollinated crosses in 1983 (Table 16) found no significant differences between the SEFs of the cones that were bagged and pollinated and those that were wind-pollinated. This is probably a reflection of both the extremely heavy pollen crop and the long period of receptivity that year. In poor pollen years even the long period of receptivity would not likely overcome low seed set. In such years supplemental pollinations should be considered.

Other factors being equal, the pollination mechanism in western hemlock may favour higher seed efficiencies than those determined for other conifers because this mechanism allows a large number of pollen grains to adhere to the bracts and increases the probability of fertilization. Micropylar capacity has been considered to be an important factor in limiting the amount of pollen taken into the ovule (Sarvas 1962, 1968; Lill and Sweet 1977; Bridgwater and Trews 1981). A maximum of 6 or 7 pollen grains were taken into the micropyle of *Picea*, even though as many as 20 pollen grains were found on the micropylar arms (Owens and Blake 1984). In controlled crosses of *Pseudotsuga menziesii* an average of 3 to 4 pollen grains were taken into the micropylar canal, even though a minimum of 11 pollen grains adhered to each stigmatic tip (Owens *et al.* 1981b). An average of 3 to

4 pollen grains were found in the micropyles of *Larix decidua* (Hall and Brown 1976). In several species of *Pinus* the number of pollen grains found within the micropyle ranged between 1 and 5 with a maximum of 7 (Sarvas 1962; Lill and Sweet 1977; Owens *et al.* 1981a; Greenwood 1986). The bracts of western hemlock are capable of trapping over 100 pollen grains. The average number of pollen grains per bract after controlled crosses was 34, ranging from 2 to 116. A possible disadvantage to this pollination mechanism is that the pollen tubes must grow a much longer distance to achieve fertilization than in other conifers, where the pollen is deposited in the micropyles. With its long period of receptivity and the potentially large number of pollen grains per bract, the pollination mechanism found in western hemlock may increase genetic efficiency in a seed orchard.

The relationship between pollen shed and seed-cone receptivity in western hemlock is quite different than that observed in other species. In 1983 the seed cones became receptive 4 to 7 days before and remained receptive 4 to 8 days after the peak period of pollen shed for 60 clones at the Cobble Hill clone bank (Fig. 63). In other species, the pollen starts to shed several days before seed cone receptivity and usually is finished at the same time or a couple of days after the seed cones are no longer receptive (Orr-Ewing 1954; O'Reilly *et al.* 1982; El-Kassaby *et al.* 1984). Whether the pollination mechanism found in western hemlock ensures greater panmixis in a seed orchard has not been demonstrated but since clones are receptive for such a long time relative to the pollen cloud and because of the large numbers of pollen grains capable of adhering per bract, out-crossing may be facilitated.

5.5 Factors Affecting Seed Development in Western Hemlock

Megagametophyte, ovule and embryo development have been studied in western hemlock (Stanlake and Owens 1974), mountain hemlock, *T. mertensiana* (Owens and Molder 1975b) and, to a lesser extent, in *T. canadensis* (Murrill 1900; Sterling 1948) and *T. caroliniana* (Buchholz 1931). The pattern of megagametophyte and embryo development found in this study agrees generally with previous reports, however some variations occur. Unlike earlier studies, which were concerned primarily with normal seed development, the present studies identified the different stages in the reproductive life cycle of western hemlock where a reduction in potential and actual seed yield occurred.

Many factors affected seed development and seed yield. These included pre- and postpollination ovule abortion, cone abortion, insufficient pollen, pollen inviability, pollen-tube degeneration and embryo and megagametophyte abortion. These factors can be divided into two classes: prefertilization and postfertilization factors. Self pollination resulted in a greater reduction in seed yield relative to wind or cross pollination by increasing the proportion of degeneration or abortion at the some of the stages described above.

5.5.1 Prefertilization Factors

The quiescent seed-cone bud consisted of bracts, ovuliferous scales, ovule primordia and premeiotic MMCs which were initiated the year prior to pollination and seed maturation (Owens and Molder 1974a). This is the most-common pattern observed among the native genera (Owens and Blake 1985). Meiosis was completed in early March with the formation of a triad of megaspores. Triads are produced by the formation of only two transverse cell walls at telophase II, resulting in a binucleate center cell. Tetrads are the most common pattern among conifers (Singh 1978). Triads have been observed in mountain hemlock (Owens and Molder 1975b) and *Abies* (Dogra 1967; Owens and Molder 1977d; Singh and Owens 1981b, 1982).

Prepollination ovule abortion, which occurred between the MMC stage and early free nuclear division, resulting in small flat seed, accounted for an average of 12% (5 to 41%) of the reduction in seed yield in the field-grown clones in 1983, an average of 4% (2 to 6%) in field-grown clones in 1984 and an average of 5% of seed yield in the containerized clones in 1984, (1 to 10%). Prepollination ovule abortion has been reported in the Pinaceae and Cupressaceae. Several theories have been postulated for the causes, which include both environmental and genetic components. Environmental components include: competition for nutrients (Lyons

1956; Burdon and Low 1973), drought (Simak and Gustafsson 1954; Sarvas 1962; Dogra 1967) and low temperatures (Dogra 1967; Sweet and Bollmann 1972; Owens and Molder 1980b) at the time of megagametophyte development. Orr-Ewing (1977) found a *Pseudotsuga menziesii* clone which was completely female sterile; all seeds were flat due to prepollination ovule abortion. Several ramets of the clone were planted in different sites and all were female-sterile, implying that, for this clone, female sterility due to ovule abortion was inherited. The extreme variability in the proportion of flat seed between the field-grown clones and low variability within clones in 1983 also suggests a genetic component. Studies are underway to determine if the proportion of flat seed per clone varies or remains relatively constant between years. Observing the clones over several growing seasons and under different environmental conditions would help determine if ovule abortion is genetically and/or environmentally controlled.

Cone abortion at pollination in 1983 at the Cobble Hill clone bank was not observed, whereas cone abortion at this stage severely reduced the number of developing cones at the Lost Lake Seed Orchard in 1984. Unaborted cones had high seed efficiencies (average 78%) and a low proportion of flat seed (< 4%). As previously discussed, flat seed are caused by prepollination ovule abortion, so whatever factors resulted in whole cone abortion occurred during or after pollination. Frosts during or shortly after pollination may have been responsible for cone abortion, since temperatures decreased to below freezing on three separate occasions during this period in 1984. It was not determined if the cold temperatures had any effect on seed development. Studies are underway to determine the effect of freezing and near-freezing temperatures on seed and seed-cones before and during pollination.

Postpollination ovule abortion occurred before fertilization in a few of the ovules. The proportion of ovules that aborted at this stage was very low, (< 4% or less than one seed per cone) at Cobble Hill in 1983 and in the containerized ramets in 1984. Most of these aborted between free-nuclear division and central-cell formation. Postpollination ovule abortion does occur in some genera if pollen is not present. These include all species of *Pinus* (Sweet 1973; Plym-Forshell 1974; Owens *et al.* 1981a; Owens *et al.* 1982) *Picea* (Sarvas 1968; Mikkola 1969; Kossuth and Fechner 1973; Owens and Blake 1984) and *Thuja* (Owens *et al.* 1989). In these genera pollen is essential for the continued development of the prefertilization megagametophyte. Pollen is not required for cone or ovule development in other conifers (Owens and Blake 1985), including western hemlock.

Insufficient pollen at pollination has been cited as a principal reason for low seed yields (Sarvas 1962; Bramlett 1974, 1981; Hall and Brown 1976, 1977; Daniels 1978; Kozinski 1987; Colangeli and Owens 1989). Hall and Brown (1976) found that in *Larix* 24% of the ovules contained no pollen in the pollen chambers, even after supplemental pollination. Insufficient pollen did not make a significant contribution to seed losses from controlled crosses in the studies reported here. This can be attributed largely to the pollination mechanism in this species. Inspection of the bagged cones after pollination established that pollen was found on most bracts. In 1983, approximately 10% of the cross-pollinated ovules (2 to 3 per cone) and 16% of the wind-pollinated ovules were unfertilized with no evidence of pollen in the micropyles. An ovule would remain unfertilized if (1) pollen did not land on the corresponding bract, (2) pollen did not germinate on the corresponding bract, or (3) pollen germinated but the pollen tubes failed to reach the micropyle and nucellus. No significant differences were found between the SEFs in 16 cross- and wind-pollinated western hemlock clones in 1983. This may be due to the pollination mechanism and the heavy pollen-cone crop that year. In poor pollen years, insufficient pollen probably would be a major factor contributing to low seed yields. In such years, western hemlock seed orchards would be ideal for supplemental pollination because of the ease with which the receptive period can be identified.

Low pollen germination (low pollen viability), or pollen germination but inadequate tube growth (low pollen vigor), was found to be a contributing factor for reduced seed yield. In the 1983 selfing study, clone 6-06 had a fertilization rate of less than 10%, because less than 5% of the pollen on the bracts germinated. The germination rate was greater than 75% in the other nine clones in the selfing trial, as well as in the cones pollinated with a polymix. Empty seed resulting from no pollen-tube penetration of the nucellus was found in 6% of the ovules sectioned in the 1984, corresponding to about 2 seeds per cone. While greater than 75% of the pollen grains germinated, many produced very short pollen tubes before development ceased, while others grew long tubes but failed to reach the nucellus. In 1984 an average of 34 pollen grains were found per bract, from which only about 3 or 4 pollen tubes per ovule penetrated the nucellus. Clone 11-7 had the highest proportion of unfertilized ovules due to the absence of pollen, (7/40) constituting 18% of all the ovules observed at this stage.

The pollen used in the different crossing trials was a polymix of many clones so it could not be determined if any clones produced pollen of low-viability or vigor. In both the 1983 and 1984 cross-pollination studies about 4% of the total seed was empty because no pollen tubes reached the micropyles. Low pollen germination has been implicated as a contributing factor to reduced seed yield in several studies (Hall and Brown 1976, 1977; Kozinski 1987; Owens *et al.* 1989). Pollen viability and vigor may play a role in seed yield but, unfortunately, pollen germination tests or other pollen viability tests were not conducted to determine the state of the various pollen lots.

In approximately 2% of the cross-pollinated ovules and 14% of the selfed ovules in 1984, pollen tubes ceased development within the nucellus (TNF) resulting in unfertilized seed. The higher proportion of TNF ovules after self-pollination suggests a self-incompatibility reaction. Pre-zygotic self-incompatibility has not been demonstrated in conifers and it is generally believed that gymnosperms lack the ability for prezygotic detection of the pollen by the maternal sporophyte, (nucellus), (Sarvas 1962, 1968; reviewed by Hagman 1975). In *Picea glauca* (Mergen *et al.* 1965) and *Pseudotsuga menziesii* (Orr-Ewing 1957) no differences between self- and cross-pollinated ovules were observed from pollen germination to the two-tiered embryo. Pollen-tube arrestment within the nucellus of both self- and cross-pollinated ovules was observed in *Pinus peuce* (Hagman and Mikkola 1963). Pollen tube arrestment within the nucellus has been observed in interspecific crosses in *Picea* (Mikkola 1969; Kossuth and Fechner 1973) and *Pinus* (Hagman and Mikkola 1963; Kriebel 1972; Karmutak 1984).

In angiosperms two types of genetic incompatibility occur: (1) sporophytic incompatibility, in which the maternal sporophytic tissue rejects the pollen based on the genotype of the pollen-producing parent and (2) gametophytic incompatibility, in which the maternal sporophyte rejects pollen based on the genotype of the pollen. In plants with sporophytic incompatibility, rejection occurs usually between the pollen grains and the stigma, while in plants with gametophytic incompatibility rejection occurs between the pollen tube and the style or ovary (Heslop-Harrison 1975, 1978; Lewis 1976; Nettancourt 1977; Seavey and Bawa 1986).

Interspecific incompatibility observed in conifers may be similar to the gametophytic incompatibility of angiosperms. Several mechanisms have been presented to explain why pollen-tube growth ceased in *Picea* and *Pinus* after interspecific crossing. These include: (1) inhibition of pollen tube growth by a reaction between specific protein(s) produced by the pollen tube and protein(s) produced by the nucellus, (2) inability of the pollen tube to penetrate between the cells of the nucellus due to the lack of some essential or specific enzymes and (3) inability of the pollen tubes to assimilate nutrients efficiently from the nucellar tissue (Mikkola 1969; Karmutak 1984).

A self-incompatibility reaction may occur between the male gametophyte (pollen tube) and the female sporophyte (nucellus) in western hemlock. If it does, it is either a very weak reaction, since only about 14% of the ovules were affected, or the reaction can be overcome by several pollen tubes penetrating the nucellus.

Pollen-tube degeneration within the nucellus may also be due to low pollen vigor. The pollen may have had enough reserves to germinate but not enough to complete tube elongation. Western hemlock pollen tubes must grow a considerable distance to reach the egg relative to other conifers where the pollen is deposited in the micropyle or on the nucellus. All of the factors contributing to pollen-tube growth are not known. In 1984 the pollen was collected from four clones located at the Lost Lake Seed Orchard. The proportion of TNF ovules in the four crossed clones did not differ significantly, ranging from 0 to 4%. The proportion of TNF ovules in the four selfed, containerized clones in 1984 ranged from 9 to 22%. It is possible that the pollen collected from the four containerized clones may have had a slightly lower viability than the pollen collected from the four orchard clones. Alternatively, the vigor of the pollen used in the selfing trial may have been lower than that of the polymix. Differences in individual pollen vigor would be masked in the polymix. Moran and Griffin (1985) found that certain pollen parents in a *Pinus radiata* polymix were more successful than others in contributing to the viable-embryo population. The competitive success of each clone depended on the other males present.

In the prezygotic phase, gametic selection between clones may occur during pollen germination and pollen tube growth (Willson and Burley 1983). Since germination and other pollen-viability tests were not conducted in the western hemlock study, the viability and vigor of the respective pollen lots is unknown. To determine if pollen-tube degeneration within the nucellus of western hemlock is due to a possible self-incompatibility reaction or low pollen vigor, pollen from individual clones could be applied to self cones and unrelated cones. If a greater proportion of pollen tubes degenerate within the selfed ovules some sort of self-incompatibility reaction may be involved. If no differences are found between the selfed and outcrossed ovules, pollen-tube degeneration may be a measure of pollen vigor.

An interesting observation made at this stage was that, even though fewer selfed ovules were fertilized, three-quarters of all the fertilized ovules in both the selfed and crossed cones had multiple fertilizations. The average number of fertilized archegonia per ovule was 1.9 in the selfed cones and 2.0 in the crossed cones. Even though some of the self pollen may have been inhibited by a self-incompatibility reaction or because of lower pollen vigor, most of the ovules in which fertilization occurred had multiple fertilizations. It might be expected that, if pollen tubes were being arrested within the nucellus, due either to incompatibility or low vigor, the average number of fertilizations per ovule would be lower in the selfed ovules than in the crossed ovules. This was not found. The pollination mechanism in western hemlock may play an important role in the high frequency of multiple fertilizations observed in this species. The large number of pollen grains per bract capable of penetrating the nucellus and effecting fertilization may minimize the effect of self-incompatibility and pollen vigor.

Similar observations were made in *Pinus peuce* (Hagman and Mikkola 1963). Due to the small sample size they were unable to determine if pollen tube arrestment within the nucellus was an incompatibility reaction or a measure of reduced pollen vigor. It was stated that, if a self-incompatibility reaction was involved, then cases should exist in *Pinus* where all pollen cease growth within the nucellus when selfed and function normally when crossed. To date, no such experiments have been made. As in the present western hemlock study, Hagman and Mikkola (1963) did not do any pollen testing on the different pollen lots. They suggested that reduced vigor due to the pollen itself, or due to improper handling, could have accounted for the pollen tube arrestment within the nucellus.

5.5.2 Postfertilization Factors

5.5.2.1 Ovule development after no pollination

The megagametophyte ceased development at about the time the egg cells formed. Following fertilization, suspensor elongation was necessary for the megagametophyte to resume development. If this failed to occur, the archegonia, followed by the megagametophyte, degenerated within two weeks of potential fertilization. Suspensor elongation, pushing the embryo tier(s) into the megagametophyte apparently was necessary to stimulate the megagametophyte to resume development. The embryo/megagametophyte interaction observed here has been observed in all conifers studied to date; if fertilization does not occur or all embryos degenerate the megagametophyte degenerates (reviewed by Singh 1978; Owens and Blake 1985).

In western hemlock, intact vascular strands were observed leading from the ovuliferous scale into the chalazal end of the nucellus. The seed coat differentiated around the vascular strands, which were not cut off until well into embryo development, after the megagametophyte had accumulated abundant starch and lipoproteins. In other conifers it is believed that the vascular connections are severed by the time fertilization occurs (Owens *et al.* 1982; Singh and Owens 1981a, b, 1982). In western hemlock, the ovules, and perhaps the megagametophytes, appear to be supplied by nutrients from the ovuliferous scale after fertilization. Studies using labelled compounds would be necessary to determine if the megagametophyte actually receives nutrients from the ovuliferous scale after fertilization.

In some conifers a corrosion cavity forms, even if the ovule remains unfertilized (Owens and Blake 1985) and it has been suggested that cavity formation may be caused by the release of substances from the degenerating archegonia (Singh and Owens 1981b, 1982). In western hemlock, a corrosion cavity did not form in the unfertilized ovules, even though the cells that would have degenerated to form the corrosion cavity were quite distinct. Embryos were necessary for cavity formation, and a corrosion cavity did not form unless the embryos came in contact with the cells of the megagametophyte. The first embryos to penetrate the megagametophyte appeared to be embedded within the cells of the megagametophyte, which degenerated around the young embryos. An embryo/megagametophyte interaction apparently was necessary to stimulate first the resumption of megagametophytic development and, second, the corrosion cavity.

5.5.2.2 Ovule development after fertilization

Fertilization and proembryo development were stages that did not contribute to a reduction in filled-seed yield. In all the ovules sectioned, whether self-, cross- or wind-pollinated, if fertilization occurred, proembryo development followed.

Immediately after fusion, the zygote underwent two quick divisions, producing four free nuclei. Each nucleus was surrounded by darkly staining neocytoplasm. Ultrastructural studies have shown that the neocytoplasm around each nucleus contains, mitochondria contributed from both the male and female parents (Camefort 1969; Thomas and Chesnoy 1969) and plastids from the male parent (Camefort 1968; Chesnoy and Thomas 1971; Ohba *et al.* 1971; Neale *et al.* 1986). It has been demonstrated in *Larix* (Camefort 1968; Chesnoy and Thomas 1971), and *Pseudotsuga* (Thomas and Chesnoy 1969) that the pollen tube releases a small amount of cytoplasm containing plastids and mitochondria into the egg cell. Paternal inheritance of chloroplast DNA in *Pseudotsuga* was demonstrated using a restriction fragment length polymorphism as a marker (Neale *et al.* 1986). In the present study not only were the male gametes discharged into the egg cytoplasm, but also the stalk cell, tube nucleus and some contents of the tube cytoplasm, which included large amounts of starch. Ultrastructural studies are required to determine the amount, integrity and fate of mitochondria, plastids and other cytoplasmic organelles that may be discharged into the egg cytoplasm. These may have a potential role in self-incompatibility or self-inviability.

Proembryo development has been described for western hemlock (Stanlake and Owens 1974), mountain hemlock (Buchholz 1931; Owens and Molder 1975b), *T. canadensis* and *T. caroliniana* (Buchholz 1931). Proembryo development in western hemlock observed here was similar to that observed previously and in other members of the genus *Tsuga*.

The next stage that accounted for a reduction in filled seed was embryo degeneration. Embryo degeneration occurred just after cleavage or during early embryo development, but well before the club-shaped stage. These resulted in full-size but empty seed.

Western and mountain hemlock have both simple and cleavage polyembryony (Stanlake and Owens 1974; Owens and Molder 1975b). Cleavage is indeterminate, in that there are no indications that any one of the four embryos derived from the single zygote has a distinct advantage during development. Cleavage occurs during suspensor elongation but before embryonal-tube formation. Cleavage polyembryony has been observed also in *Pinus* (Lill 1976; Owens and Molder 1977c; Owens *et al.* 1982) and *Chamaecyparis* (Owens and Molder 1975b).

In *Pinus* the embryo tier divides before cleavage. The four files of cells do not develop equally, with one or more of the files overgrowing the others, resulting in unequal-sized embryos at cleavage (Owens *et al.* 1982). There is some controversy over the advantages and disadvantages of simple and cleavage polyembryony. Buchholz (1929) argued that a single large proembryo, as is found in simple polyembryony, should be able to prevail over a small cleavage embryo and considered cleavage the most primitive form of embryo development in conifers. On the other hand, Doyle and Brennan (1971) found that cleavage embryos grew faster. Willson and Burley (1983) proposed that cleavage embryos may assimilate nutrients faster than a single large embryo.

A suggested role for simple polyembryony is to maintain heterozygosity even when high frequencies of self-pollination are present (Lindgren 1975). Simple polyembryony can reduce losses due to selfing and inbreeding. If an embryo that has originated through selfing dies, a normal seed may develop if the ovule contains, in addition to the inbred embryo, one or several embryos originating through cross fertilization (Hagman 1975; Lindgren 1975; Sorensen 1982). Polyembryony provides an opportunity for selection between embryos during the early stages of embryo development (Sorensen 1982).

Embryo abortion was a major contributing cause in reduction of filled seed yield in all studies whether selfed, crossed or wind-pollinated. Ovules that degenerated at this stage were divided into three groups: (1) ovules where all embryos degenerated at cleavage, (2) ovules where embryos degenerated during early development and (3) ovules where megagametophytic degeneration preceded embryo degeneration. All three stages were observed in selfed and crossed ovules, but these constituted a much greater proportion of the selfed ovules.

Degeneration at cleavage or during early embryo development could be due to the impact of lethal alleles. Degeneration at fertilization or during proembryo development has not been observed, so it can be suggested that the lethal alleles may be involved with the development of the embryo tier. Failure at this early stage may be due to physiological incompatibility between embryonic and megagametophytic tissue (Orr-Ewing 1957; Mergen *et al.* 1965) or due to increased homozygosity of deleterious recessive genes which result in the inability to effect cell division, or lack of some key enzymes required for nutrient assimilation. The variation in the proportion of degeneration at cleavage and during early embryo development among the four selfed clones in 1984 suggests that several lethal alleles which manifest themselves at different stages of development may be involved. In clones 11-7 and 6-9, most of the degeneration occurred during early embryo development, while in clones 3-5 and 6-8, degeneration occurred with the same frequency at both stages. In the crossed clones, 85% of the degeneration occurred during early embryo development.

A proportion of empty seed due to embryo abortion (18%) occurred after outcrossing which cannot be attributed to selfing effects. If homozygous recessives do occur in a population, then it is quite likely that different clones may contain the same homozygous recessives, which could combine through outcrossing as well as selfing. Koski (1971) suggested that the yield of empty seed after outcrossing can be ascribed to the action of common lethal alleles. He calculated that if, after selfing, there is an individual range of 2-20 recessive embryonic lethals with 9.4 as the average, then there are approximately 70-80 lethals in the population. Bramlett and Pepper (1974) estimated that 150-180 such genes would provide the most satisfactory explanation for the frequency of empty seed after individual crosses in *Pinus virginiana.* Griffin and Lindgren (1985) believe that empty seed after outcrossing is predominantly environmental. Johnsson (1976) found in a 6x6 diallel that some individuals produced greater amounts of empty seed as females and other individuals resulted in more empty seed as males. In the first case it was speculated that certain recessive genes may have a lethal effect on the haploid megagametophyte. Variation in seed set (SEF) after cross-pollination of different clones with the same pollen is abundant in the literature. Sorensen *et al.* (1976) found that seed set from the outcrossing of 10 *Abies procera* clones ranged from 20 to 70% (average 51%); seed set by 10 *Picea glauca* clones (Fowler and Park 1983) ranged from 29 to 82% (average 57%) and in 16 clones of *Picea mariana* (Park and

Fowler 1984) seed set ranged from 43 to 96% (average 74%). These results are comparable to SEFs of 43 to 83% in 16 cross-pollinated clones (average 69%) found in western hemlock in 1983.

The megagametophyte failed to develop in ovules where fertilization did not occur. An interaction between suspensor elongation and megagametophyte development was observed in fertilized ovules. In 1984, the megagametophyte either failed to resume development or the cells expanded but failed to accumulate reserves even though apparently healthy embryos were found in the corrosion cavity in 14% of the degenerated selfed ovules. The failure to resume development may be due to many reasons, such as inactivation or lack of enzymes or growth regulators produced by the developing embryo that stimulate the megagametophyte to resume development.

Chapter VI

SUMMARY AND CONCLUSIONS

Several conclusions were drawn from the present study with respect to pollen development, pollen forcing, the pollination mechanism, the optimal time of pollination, and factors affecting ovule and seed development after self-, cross-, wind- or no pollination in western hemlock. These are summarized as follows:

(1) Pollen phenology proved to be an accurate indicator of cytology, independent of collection dates and rate of development under ambient conditions. The breaking of dormancy, the resumption of development and meiosis occurred during stage 1 (quiescent). During stage 2 (swollen bud) the tetrad of microspores separated. The exine was completely developed during stage 3 (bud burst). The 1-cell microspores expanded during stage 4, when the pollen cones were one-quarter of one-half emerged through the bud scales. The first cell division occurred during stage 5 when the cones were greater than half emerged but less than fully emerged. The second and third cell divisions occurred during stage 6, after the cones had emerged completely beyond the buds scales. The fourth and final cell division, resulting in mature 5-cell pollen grains occurred during stage 7 (stalk elongation). Mature pollen consisted of two prothallial cells, a stalk, body and tube cell. Pollen shed occurred at stage 8. The empty cones (stage 9) remained on the trees until the following winter.

(2) Pollen can be forced successfully on cut branches after stage 4 without decreasing yield or fertilizing potential relative to unforced pollen. Forcing pollen on cut branches at early phenological stages (before stage 4) reduced the number of cones that matured, decreased the quantity and quality of pollen produced, increased pollen abnormalities and reduced the pollens' fertilizing potential.

(3) Forcing pollen on containerized ramets in growth chambers at 18°C accelerated development three-fold relative to ambient temperatures and altered the relationship between pollen phenology and cytology. In one clone, high temperatures resulted in abnormal development.

(4) Longterm storage of pollen forced at above ambient temperatures reduced the fertilizing potential relative to stored, unforced pollen.

(5) Western hemlock has a pollination mechanism that does not require pollen to be deposited near or in the micropyle. The pollination mechanism involves an interaction between the roughly sculptured pollen grains and the long epicuticular waxes on the surface of the bracts.

(6) Seed cones are receptive to pollen soon after bud burst when the bracts become exposed and remain receptive as long as bracts are exposed. The optimal time for pollination occurs when the cones are one-half or more emerged beyond the bud scales, when all the bracts associated with fertile ovuliferous scales are exposed, and continues until the ovuliferous scales elongate and grow over the bracts, trapping the pollen between the bracts and scales.

(7) The optimal date for pollination varies among clones from one year to another depending on site and weather conditions, ranging from two to over three weeks.

(8) Several weeks after pollination and cone closure, the pollen germinates and forms long pollen tubes which grow toward and into the micropyles. Fertilization occurs approximately six weeks after pollination.

(9) Pollen is not required for normal cone development in western hemlock (parthenocarpy). Pollen and fertilization resulting in embryo development are

required for the resumption of development of the megagametophyte.

(10) Several stages which affect seed development and resulted in a reduction in seed yield were revealed. These stages were divided into two major classes: pre-fertilization and post-fertilization stages. Pre-fertilization losses included pre- and post-pollination ovule abortion, inadequate pollination, pollen inviability and low pollen vigor. Post-fertilization losses included embryo degeneration and megagametophyte degeneration. Embryo degeneration was subdivided into degeneration at cleavage and during early embryo development.

(11) Ovule abortion accounted for a measurable reduction in seed yield. Several clones had a very high ovule abortion rate. It is not known whether this is a genetically or environmentally controlled factor or a combination of both.

(12) Inadequate pollination was not a major contributing factor in reducing seed yield after self- or cross-pollination. Low seed yield due to lack of pollen would be a major factor in reducing yield in a wind-pollinated orchard in poor pollen years.

(13) Pollen vigor and viability influence final seed yield. The importance of these factors depends on the specific pollen lot. Several studies identified low pollen viability, as exemplified by pollen tube growth but no fertilization, as a contributing factor to yield reduction.

(14) Self-incompatibility, which is identified by prezygotic, selection has not been demonstrated previously in conifers. A higher proportion of pollen-tube arrestment within the nucellus was found after self-pollination than after cross-pollination. This may indicate a self-incompatibility reaction between the paternal gametophyte and the maternal sporophyte.

(15) Self-inviability, which is identified by postzygotic selection, manifested in the form of embryo degeneration, was the most-prevalent factor reducing seed yield after self-pollination.

(16) Many factors contribute to a reduction in filled seed yield. These factors are affected by genetics and environment, and will vary from one year to another and one site to another.

LITERATURE CITED

Allen, G.S. 1942. Parthenocarpy, parthenogenesis and self-sterility of Douglas fir. J. Forestry 40: 642-644.

___ 1963. Origin and development of the ovule in Douglas fir. For. Sci. 9: 386-393.

Allen, G.S. and J.N. Owens. 1972. The life history of Douglas fir. For. Serv. Environment Canada 139 pp.

Allen, P.H. and K.B. Trousdell. 1961. Loblolly pine seed production in the Virginia-North Carolina coastal plain. J. For. 59: 187-190.

Andersson, E. 1965. Cone and seed studies in Norway spruce (*Picea abies* (L.) Karst). Stud. For. Suec. 23: 1-214.

___ 1980. Temperature-conditioned irregularities in pollen mother cells of *Picea abies* (L.) Karst. Hereditas 92: 27-35.

Andersson E., I. Ekberg and G. Eriksson. 1969. A summary of meiotic investigations in conifers. Stud. For. Suec. 70: 1-19.

Andrikopoulos, N.K., A. Siafaka-Kapadai, C.A. Demopoulos and V.M. Kapoulas. 1985. Lipids of *Pinus halepensis* pollen. Phytochemistry 24: 2953-2957.

Barner, H. and H. Christiansen. 1960. The formation of pollen, the pollination mechanism and the determination of the most favourable time for controlled pollination in *Larix*. Silvae Genet. 9: 1-11.

___ 1962. The formation of pollen, the pollination mechanism and the determination of the most favourable time for controlled pollination in *Pseudotsuga menziesii*. Silvae Genet. 11: 89-102.

Beers, W.L., Jr., J. Bivens and J.E. Mocha. 1981. Pollen collection. Chapter 6 in "Pollen Management Handbook", F.E. Carlyle (Ed.) Agric Handbook, 587, Wash. D.C. U.S. Dept. Agric.

Berlyn, G.P. and J.P. Miksche. 1977. Botanical Microtechnique and Cytochemistry. Iowa State University Press.

Bingham, R.T. and A.E. Squillace. 1955. Self-incompatibility and effects of self-fertility in western white pine. For. Sci. 1: 121-129.

Bower, R.C., S.D. Ross and A.M. Eastham. 1986. Management of a western hemlock containerized seed orchard. In: Proc. IUFRO conf. on Breeding Theory, Progeny Testing and Seed Orchards. Oct. 12-17, 1986, Williamsburg, Virginia. *Edited by*: R.J. Weir, N.C. State Univ., Raleigh, N.C. pp. 604-612.

Boyer, W.D. 1981. Pollen production and dispersal as affected by seasonal temperature and rainfall patterns. Pages 2-9 *in* Franklin E.C., ed. Pollen management handbook. USDA Forest Serv. Agric. Handbook No. 587.

Boyer, W.D. and F.W. Woods. 1973. Date of pollen shedding by Longleaf pine advanced by increased temperatures at strobili. For. Sci. 19: 315-318.

Bramlett, D.L. 1974. Seasonal development and loss of Virginia pine ovules and seed. Seed Sci. & Technology 2: 285-292.

___ 1981. Effectiveness of wind pollination in seed orchards. Pages 10-14 *in* Franklin E.C., ed. Pollen Management Handbook USDA For. Serv. Agric. Handbook No. 587.

Bramlett, D.L., E.W. Belcher, G.L. DeBarr, G.D. Hartel, R.P. Karrfalt, C.W. Lantz, T. Miller, K.D. Ware and H.O. Yates. 1977. Cone analysis of southern pines. A guidebook. USDA Forest Serv. Gen. Tech. Rep. No. SE-13, 28p.

Bramlett, D.L. and W.D. Pepper. 1974. Seed yield from a diallel cross in Virginia pine., Colloquium on Seed Yield for Southern Pine Seed Orchards. Proceedings, April 2-3, 1974, Macon, GA pp. 49-55.

Bramlett, D.L. and T.W. Popham. 1971. Model relating unsound seed and embryonic lethal alleles in self-pollinated pines. Silvae Genet. 20: 191-192.

Bridgwater, F.E. and D.L. Bramlett. 1982. Supplemental mass pollination to increase seed yields in loblolly pine seed orchards. South J. Appl. For. 6: 100-104.

Bridgwater, F.E. and I.F. Trew. 1981. Supplemental mass pollination. Pages 52-57 *in* Franklin, E.C., ed. Pollen management handbook. USDA Forest Serv. Agric. Handbook No. 587.

Brix, H. and H. Barker. 1975. Rooting studies of western hemlock cuttings. CFS/PFRC Report BC-X-131.

Brix, H. and F.T. Portlock. 1982. Flowering response of western hemlock seedlings to gibberellin and water-stress treatments. Can. J. of For. Res. 12(1): 76-82.

Brown, I.R. 1971. Flowering and seed production in grafted clones of Scots pine. Silvae Genet. 20: 121-132.

___ 1973. Factors affecting the yield from Scots pine seed orchards. Scot. For. 27: 296-307.

Brown, S.D. and F.E. Bridgwater. 1987. Observations on pollination in loblolly pine. Can. J. For. Res. 17: 299-303.

Buchholz, J.T. 1929. The embryogeny of the conifers. Pages 359-392 *in* Proc. Fourth Internat. Bot. Congr.

___ 1931. The pine embryo and embryos of related genera. Trans. Ill. State Acad. Sci. 23: 117-125.

Burdon, R.D. and C.B. Low. 1973. Seed production of *Pinus radiata* clones on four different sites. N.Z.J. For. Sci. 3: 211-219.

Butler, V., Ch. Bornman and W.A. Jensen. 1979. *Welwitschia mirabilis*: Fine structure of the germinating seed. III. The quiescent gametophyte. Z. Pflanzenphysiol. Bd. 91: 399-413.

Camefort H. 1968. Sur l'organisation du nocytoplasme dans les proembryons tetranucles du *Larix decidua* Mill. (*Larix europea* D.C.) et l'origine des mitochondries et des plastes de l'embryon chez cette espce. C.R. Acad. Sc. Paris Srie D. 266: 88-91.

___ 1969. Fecondation et proembryognse chez les Abitaces (notion de neocytoplasme). Rev. Cytol. Biol. Vegt. 32: 253-271.

Cecich, R.A. 1979. Ovule development and abortion in *Pinus banksiana*. In: Flowering and seed development in trees: a symposium. Mississippi State University May 15-18, 1978, p. 33-40 USDA For. Serv. New Orleans LA, 1978.

___ 1984. The histochemistry and ultrastructure of jack pine microsporangia during the winter. Am. J. Bot. 71: 851-864.

Chamberlain, C.J. 1935. Gymnosperms: structure and evolution. University of Chicago Press, Chicago (Reprint, 1966. Dover Pub. Inc. N.J.). 484 p.

Chandler, C. and S. Mavrodineanu. 1965. Meiosis in *Larix laricina* Koch. Boyce Thompson Inst. for Plant Res. 23: 67-75.

Chesnoy, L. and M.J. Thomas. 1971. Electron microscopy studies on gametogenesis and fertilization in gymnosperms. Phytomorphology 21(1): 50-63.

Ching, T.M., M.W. Ranzoni and K.K. Ching. 1975. ATP content and pollen germinability of some conifers. Plant Sci. Lett. 4: 331-333.

Chira, E. 1964. Einfluss der temperatur auf den Verlauf der meiosis der pollenmutterzellen von *Taxus baccata* L. Biologia (Bratislava), 19: 235-244.

___ 1965. On some biological questions concerning *Picea excelsa* (Lom.) Link. pollen. Biologia 20: 641-653.

Chowdhury, C.R. 1961. The morphology and embryogeny of *Cedrus deodora* (Roxb) Loud. Phytomorph 11: 283-304.

Christiansen, H. 1960. On the effect of low temperature on meiosis and pollen fertility in *Larix decidua* Mill. Silvae Genet. 9: 72-78.

Colangeli, A.M. and J.N. Owens. 1989. Seed production in western red cedar (*Thuja plicata* Donn): I. The role of the pollination mechanism and the optimal time of pollination on pollination efficiency. Can. J. For. Res. (in press).

Coles, J.F. and D.P. Fowler. 1976. Inbreeding in neighboring trees in two white spruce populations. Silvae Genet. 25: 29-34.

Conn, H., J.M.A. Darrow and V.M. Emmel. 1960. Staining procedures used in Biological Stain Commission. William and Wilkins, Baltimore, MD.

Cram, W.H. 1984. Some effect of self-, cross-, and open-pollinations in *Picea pungens*. Can. J. Bot. 62: 392-395.

Daniels, J.D. 1978. Efficacy of supplemental mass pollination in a Douglas fir seed orchard. Silvae Genetica 27(2): 52-58.

Dickinson, H.G. and P.R. Bell. 1972. The role of the tapetum in the formation of sporopollenin-containing structure during microsporogenesis in *Pinus banksiana*. Planta 107: 205-215.

___ 1976. The changes in the tapetum of *Pinus banksiana* accompanying formation and maturation of the pollen. Ann. Bot. 40: 1101-1109.

Dogra, P.D. 1964. Pollination mechanisms in gymnosperms. Chap. 7 in "Advances in Palynology", Nat. Bot. Gard. Lucknow Ann. Rep. pp 142-175.

___ 1967. Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae. Stud. For. Suec. 45: 97p.

Dorman, K.W. 1976. The genetics and breeding of southern pines. U.S. Dep. Agric., Agric. Handb. 471.

Doyle, J. 1926. The ovule of *Larix* and *Pseudotsuga*. Roy. Irish Acad. Proc. Sect. B 37: 170-180.

___ 1945. Developmental lines in pollination mechanisms in the Coniferales. Scient. Proc. R.D.S. 24(5): 43-62.

___ 1963. Proembryogeny in *Pinus* in relation to that in other conifers - a survey. Proc. Roy. Irish Acad. 62B: 181-216.

Doyle, J. and M. Brennan. 1971. Cleavage polyembryony in conifers and taxads - a survey. I. Podocarps, taxads and taxodoids. Sci. Proc. Roy. Dublin Soc. 4A: 57-88.

Doyle, J. and A. Kane. 1943. Pollination in *Tsuga pattoniana* and in species of *Abies* and *Picea*. Scient. Proc. R.D.S. 23(7): 57-71.

Doyle, J. and M. O'Leary. 1935a. Pollination in *Pinus*. Scient. Proc. R.D.S. 21(20): 180-190.

___ 1935b. Pollination in *Tsuga*, *Cedrus*, *Pseudotsuga* and *Larix*. Scient. Proc. R.D.S. 21(21): 191-204.

Ehrenberg, C.E., A. Gustafsson, C. Plym-Forshell and M. Simak. 1955. Seed quality and the principles of forest genetics. Hereditas 41: 292-365.

Ekberg, I. and G. Eriksson. 1967. Development and fertility of pollen in three species of *Larix*. Hereditas 57: 303-311.

Ekberg, I., G. Eriksson and A. Jonsson. 1972. Meiosis in pollen mother cells of *Pinus contorta*. Hereditas 71: 313-324.

Ekberg, I., G. Eriksson and Z. Sulikova. 1968. Meiosis and pollen formation in *Larix*. Hereditas 59: 427-438.

El-Kassaby, Y.A., A.M.K. Fashler and O. Sziklai. 1984. Reproductive phenology and its impact on genetically improved seed production in a Douglas fir Seed Orchard. Silvae Genet. 33: 120-125.

El-Kassaby, V.A., F.C. Yeh and O. Sziklai. 1981. Estimation of the outcrossing rate of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) using allozyme polymorphisms. Silvae Genet. 30: 182-184.

Eriksson, G. 1968. Temperature response of pollen mother cells in *Larix* and its importance for pollen formation. Stud. For. Suec. 63: 1-131.

___ 1970. Low temperature induced irregularities in pollen mother cells of *Larix leptolepis*. Stud. For. Suec. 85, 14p.

Eriksson, G., I. Ekberg and A. Jonsson. 1970a. Meiotic investigations in pollen mother cells of Norway spruce cultivated in a plastic greenhouse. Hereditas 66: 1-20.

___ 1970b. Further studies on meiosis and pollen formation in *Larix*. Stud. For. Suec. 87: 1-65.

Favre-Ducharte, M. 1956. Contribution l'tude de la reproduction chez *Ginko bilboa*. Rev. Cytol. Biol. Vgt. 17: 1-218.

Fechner, G.H. 1979. The biology of flowering and fertilization. Pages 1-24 in Bonner, F., ed. Proc. Symp. Flowering and seed development in trees. IUFRO, 1978. Miss. State Univ. USDA Forest Serv. Southern Forest Exper. Stn.

Foster, G.S., R.K. Cambell and W.T. Adams. 1984. Heritability, gain and C effects in rooting of western hemlock cuttings. Can. J. For. Res. 14: 628-638.

Fowells, H.A. 1965. (Comp.) Silvics of forest trees of the United States. U.S. Dep. Agric., For. Serv. Agric. Handb. No. 271.

Fowler, D.P. 1964. Pre-germination selection against a deleterious mutant in red pine. For. Sci. 10: 335-336.

___ 1965. Effects of inbreeding in red pine, *Pinus resinosa* Ait. III. Factors affecting natural selfing. Silvae Genet. 14: 37-46.

Fowler, D.P. and Y.S. Park. 1983. Population studies of white spruce. I. Effects of self-pollination. Can. J. For. Res. 13(6): 1133-1138.

Frampton, Jr., L.J., K.R. Roeder, D.L. Rockwood and C.A. Hollis, III. 1982. Genetic variation in Chocta-Whatchee Sand Pine Pollen Shed and viability. Southern J. of Appld. For. 6(4): 225-228.

Franklin, E.C. 1970. Survey of mutant forms and inbreeding depression in species of the family Pinaceae. U.S.D.A. For. Sev. SE For. Exp. Sta. SE-61.

___ 1971. Estimates of frequency of natural selfing and of inbreeding coefficients in loblolly pine. Silvae Genet. 20: 194-195.

Freeman, M.F. and J.W. Tukey. 1950. Transformations related to the angular and the square root. Ann. Math. Statist. 21: 607-611.

Greenwood, M.S. 1986. Gene exchange in loblolly pine: the relation between pollination mechanism, female receptivity and pollen availability. Amer. J. Bot. 73: 1443-1451.

Griffin, A.R. and D. Lindgren. 1985. Effect of inbreeding on production of filled seed in *Pinus radiata* - experimental results and a model of gene action. Theor. Appl. Genet. 71: 334-343.

Hadders, G. and V. Koski. 1975. Probability of inbreeding in seed orchards. In: Seed Orchards. Edited by R. Faulkner. Br. For. Comm. Bull. No. 54 pp. 108-117.

Hagman, M. 1964. Incompatibility in *Pinus*. Hereditas 52: 245-262.

___.1975. Incompatibility in Forest Trees. Proc. R. Soc. Lond. B. 188: 313-326.

Hagman, M. and L. Mikkola. 1963. Observations on cross-, self-, and interspecific pollinations in *Pinus peuce*. Grisbe. Silvae Genet. 12: 72-79.

Haines, R.J., N. Prakash and D.G. Nikles. 1984. Pollination in *Araucaria* Juss. Aust. J. Bot. 32: 583-594.

Hkansson, A. 1956. [Seed development of *Picea abies* and *Pinus sylvestris*.] Medd. Statens Skogforskningsinst 46: 1-23.

Hall, J.P. 1982. Microsporogenesis in *Larix laricina*. Can. J. Bot. 60: 797-805.

Hall, J.P. and I.R. Brown. 1976. Microsporogenesis, pollination and potential yield of seed of *Larix* in N.E. Scotland. Silvae Genet. 25: 132-137.

___ 1977. Embryo development and yield of seed in *Larix*. Silvae Genetica 26: 77-84.

Hansen, H.P. 1941. Further pollen studies of post-Pleistocene bogs in the Puget Lowland of Washington. Bull. Torrey Bot. Club, 68: 133-148.

Hanson, P. 1985. Seed Orchards of British Columbia. Ministry of Forests Publication, Victoria, B.C. 117pp.

Harrison, D.L.S. and J.N. Owens. 1983. Bud development in *Picea engelmannii* I. Vegetative bud development, differentiation, and early development of reproductive buds. Can. J. Bot. 61: 2291-2301.

Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Phys. 26: 403-425.

___ 1978. Genetics and physiology of angiosperm incompatibility systems. Proc. R. Soc. Lond. 202: 73-92.

Ho, R.H. 1980. Pollination mechanism and seed production potential in Douglas-fir. For. Sci. 26(4): 522-528.

Ho, R.H. and J.N. Owens. 1974a. Microsporogenesis and pollen formation in lodgepole pine. Can. J. Bot. 52: 1669-1674.

___ 1974b. Microstrobilate morphology, microsporogenesis and pollen formation in western hemlock. Can. J. For. Res. 4: 509-517.

Hohtola, A., S. Kupila-ahvenniemi and R. Ohtonen. 1984. Seasonal changes in the cytoplasmic structures of sporogenous cells of the Scotch pine. Ann. Bot. Fenn. 21: 143-149.

Johansen, D.A. 1940. Plant microtechnique. McGraw-Hill Book Co. Inc., New York.

Johnsson, H. 1976. Contributions to the genetics of empty grains in the seed of pine (*Pinus silvestris*). Silvae Genetica 25(1): 10-15.

Jonsson, A. 1974. A study on the temperature response of pollen mother cells in Norway spruce. Stud. Forest. Suec. 116: 1-32.

Kamienska, A., R.C. Durley and R.P. Pharis. 1976a. Isolation of gibberellins A_3, A_4 and A_7 from *Pinus attenuata* pollen. Phytochemistry 15: 421-424.

___ 1976b. Endogenous Gibberellins of Pine Pollen. III. Conversion of 1,2-$[^3]GA_4$ to Gibberellins A_1 and A_{34} in germinating pollen of *Pinus attenuata* Lemm. Plant Physiol. 58: 68-70.

Kamienska, A., and R.P. Pharis. 1975. Endogenous Gibberellins of Pine Pollen. II. Changes during germination of *Pinus attenuata*, *P. coulteri* and *P. ponderosa* pollen. Plant Physiol. 56: 655-659.

Karmutak, A. 1984. Some cytological and biochemical aspects of interspecific incompatibility in pines (*Pinus* sp.). Acta Dendrobiologica 7: 1-92.

Konar, R.N. 1960. The morphology and embryology of *Pinus roxburghii* Sar with a comparison with *Pinus wallichiana* Jack. Phytomorph. 10: 305-319.

Konar, R.N. and Y.B. Oberoi. 1969. Recent work on reproductive structures of living conifers and taxads - A review. Bot. Rev. 35: 89-116.

Koski, V. 1971. Embryonic lethals of *Picea abies* and *Pinus sylvestris*. Commun. Inst. For. Fenn 75: 1-30.

Kossuth, S.V. and G.H. Fechner. 1973. Incompatibility between *Picea pungens* Engelm and *Picea engelmannii* Parry. For. Sci. 19: 50-60.

Kozinski, G. 1987. Megagametogenesis, fertilization, and embryo development in *Larix decidua*. Can. J. For. Res. 16: 1301-1309.

Kraus, J.F. and A.E. Squillace. 1964. Inheritance of yellow oleoresin and virescent foliage in slash pine. Silvae. Genet. 13: 114-116.

Kriebel, H.B. 1972. Embryo development and hybridity barriers in white pines (section strobus). Silvae Genet. 21: 39-44.

Kupila-Ahvenniemi, S. Pihakaski and K. Pihakaski. 1978. Wintertime changes in the ultrastructure and metabolism of the microsporangiate strobili of the Scotch Pine. Planta 144: 19-29.

Kupila-Ahvenniemi, S., A. Taanila and A. Hohtola. 1980. Structure of the strobili of Scotch Pine from initiation to opening. Aquilo Ser. Bot. 17: 1-10.

Lawson, A.A. 1909. The gametophytes and embryo of *Pseudotsuga douglasii*. Ann. Bot. 23: 163-180.

Lewis, D. 1976. Incompatibility in flowering plants. In: "Receptors and Recognition" Series A. Vol. 2, (P. Cuatrecasas and M.F. Greaves eds.), p. 141-178, Chapman and Hall, London.

Lill, B.S. 1976. Ovule and seed development in *Pinus radiata*: post meiotic development, fertilization and embryogeny. Can. J. Bot. 54: 2141-2154.

Lill, B.S. and G.B. Sweet. 1977. Pollination in *Pinus radiata*. N.Z.J. For. Sci. 7: 21-34.

Lindgren, D. 1975. The relationship between self-fertilization, empty seeds and seeds originating from selfing as a consequence of polyembryony. Stud. For. Suec. 126: 1-24.

Luomajoki, A. 1977. Effects of temperature on spermatophyte male meiosis. Hereditas 85: 33-48.

___ 1982. Temperatures and dates of male meiosis in trees. Hereditas 97: 167-178.

___ 1986. Timing of microsporogenesis in trees with reference to climatic adaptation. A review. Acta For. Fenn. 196: 1-32.

Lyons, L.A. 1956. The seed production capacity and efficiency of red pine cones (*Pinus resinosa* Ait). Can. J. Bot. 34: 37-46.

Maheshwari, P. and M. Sanwal. 1963. The archegonium in gymnosperms: A review. Memoirs Indian Bot. Soc. 4: 104-119.

Makeshwari, P. and H. Singh. 1967. The female gametophytes of gymnosperms. Biol. Rev. 42: 88-130.

McKinley, C.R. and M.W. Cunningham. 1983. Theoretical effects of seed orchard roguing on selfing and percent filled seeds. Can. J. For. Res. 13(1): 187-190.

McWilliam, J.R. 1958. The role of the micropyle in the pollination of *Pinus*. Bot. Gaz 120: 109-117.

Mehra, P.N., F.N.A., and P.D. Dogra. 1975. Embryogeny of Pinaceae I. Proembryogeny. Proc. Indian Natn. Sci. Acad. 41: 486-497.

___ 1977. Embryogeny of Pinaceae II. Late embryogeny in non-cleavage forms: *Abies pindrow* and *Picea smithiana*. Proc. Indian Natn. Sci. Acad. 43: 57-65.

Mergen, F. and D.T. Lester. 1961. Microsporogenesis in *Abies*. Silvae Genet. 10: 146-156.

Mergen, F., J. Burley and G.M. Furnival. 1965. Embryo and seedling development in *Picea gluca* (Moench) Voss, after self-, cross-, and wind pollination. Silvae Genetica 14: 188-194.

Mergen, F, G.R. Stairs and E.B. Snyder. 1963. Microsporogenesis in *Pinus echinata* and *Pinus taeda*. Silvae Genet. 12 127-129.

Mikkola, L. 1969. Observations on interspecific sterility in *Picea*. Ann. Bot. Fenn. 6: 285-339.

Moir, R.B. and D.P. Fox. 1975. Male meiosis in Sitka spruce (*Picea sitchensis* (Bong.) Carr. Silvae Genet. 24: 187-192.

Moitra, A. and S.P. Bhatnagar. 1982. Review Article: Ultrastructure, cytochemical and histochemical studies on pollen and male gamete development in gymnosperms. Gamete Research 5: 71-112.

Moran, G.F. and A.R. Griffin. 1985. Non-random contribution of pollen in polycrosses of *Pinus radiata* D. Don. Silvae Genet. 34: 117-121.

Murrill, W.A. 1900. The development of the archegonia and fertilization in spruce (*Tsuga canadensis* Carr.). Ann. Bot. 14: 583-607.

Neale, D.B., N.C. Wheeler and R.W. Allard. 1986. Paternal inheritance of chloroplast DNA in Douglas fir. Can. J. For. Res. 16: 1152-1154.

Nettancourt, D. de. 1977. *Incompatibility in Angiosperms*. Springer-Verlag, New York.

Ohba, K., M. Iwakawa, Y. Okada and M. Murai. 1971. Paternal transmission of a plastid anomaly in some reciprocal crosses of Sugi, *Cryptomeria japonica* D. Don. Silvae Genetica 20: 101-107.

O'Reilly, C., W.H. Parker and J.E. Barker. 1982. Effect of pollination period and strobili number on random mating in a clonal seed orchard of *Picea mariana*. Silvae Genetica 31: 90-94.

Orr-Ewing, A.L. 1954. Inbreeding experiments with the Douglas fir. For. Chron. 30: 7-16.

___ 1957. A cytological study on the effects of self-pollination on *Pseudotsuga menziesii* (Mirb.) Franco. Silvae Genetica 6: 179-185.

___ 1965. Inbreeding and single crossing in Douglas fir. Forest Science 11: 279-290.

___ 1977. Female sterility in Douglas fir. Silvae Genetica 26: 75-77.

Owens, J.N. 1969. The relative importance of initiation and early development on cone production in Douglas fir. Can. J. Bot. 47: 1039-1049.

___ 1980. Reproductive Biology of Conifers. Proceedings 6th North Am. Forest Biology Workshop. U. of A. B.P. Dancik, K.O. Higginbotham (Eds.), Aug. 1980, p 77-92.

___ 1982. Pollen management through phenological control of development. Proceedings of the 18th meeting of the Canadian Tree Improvement Assoc. Part 2, pp 39-48, Duncan, B.C. Aug. 1981.

___ 1984a. Bud development in mountain hemlock (*Tsuga mertensiana* II. Cone-bud differentiation and predormancy development. Can. J. Bot. 62: 484-494.

___ 1984b. Bud development in grand fir (*Abies grandis*). Can. J. For. Res. 14: 575-588.

Owens, J.N. and M.D. Blake. 1983. Pollen morphology and development of the pollination mechanism in *Tsuga heterophylla* and *T. mertensiana*. Can. J. Bot. 61: 3041-3048.

___ 1984. The pollination mechanism of Sitka spruce (*Picea sitchensis*). Can. J. Bot. 62: 1136-1148.

___ 1985. Seed Production in Forest Trees: A review of the literature and recommendations for research. Can. For. Service, Petawawa 240 pp.

Owens, J.N., A.M. Colangeli and S.J. Simpson. 1989. Seed production in western red cedar (*Thuja plicata* Donn): II. The effect of self-, cross-, and no-pollination on ovule, embryo, seed and cone development. Can. J. For. Res. (submitted).

Owens, J.N. and M. Molder. 1971. Pollen development in Douglas fir. Can. J. Bot. 49: 1263-1266.

___ 1974a. Bud development in western hemlock: II. Initiation and early development of pollen cones and seed cones. Can. J. Bot. 52: 283-294.

___ 1974b. Cone initiation and development before dormancy in yellow cedar (*Chamaecyparis nootkatensis*). Can. J. Bot. 52(9): 2075-2084.

___ 1975a. Pollination, female gametophyte and embryo and seed development in yellow cedar (*Chamaecyparis nootkatensis*). Can. J. Bot. 53(12): 186-199.

___ 1975b. Sexual reproduction of mountain hemlock (*Tsuga mertensiana*). Can. J. Bot. 53: 1811-1826.

___ 1976. Bud development in Sitka spruce. II. Cone differentiation and early development. Can. J. Bot. 54: 766-779.

___ 1977a. Vegetative bud development and cone differentiation in *Abies amabilis*. Can. J. Bot. 55: 992-1008.

___ 1977b. Development of long-shoot terminal buds of western white pine (*Pinus monticola*). Can. J. Bot. 55(10): 1308-1321.

___ 1977c. Seed-cone differentiation and sexual reproduction in western white pine (*Pinus monticola*). Can. J. Bot. 55: 2574-2590.

___ 1977d. Sexual reproduction of *Abies amabilis*. Can. J. Bot. 55: 2653-2667.

___ 1977e. Bud development in *Picea glauca*. II. Cone differentiation and early development. Can. J. Bot. 55: 2746-2760.

___ 1977f. Cone induction in yellow cypress (*Chamaecyparis nootkatensis*) by GA_3 and the subsequent development of seeds within the induced cones. Can. J. For. Res. 7: 299-325.

___ 1979a. Sexual reproduction of white spruce (*Picea glauca*). Can. J. Bot. 57: 152-169.

___ 1979b. Sexual reproduction in *Larix occidentalis*. Can. J. Bot. 57(23): 2673-2690.

___ 1979c. Bud development in *Larix occidentalis*. Cone differentiation and early development. Can. J. Bot. 57: 1557-1572.

___ 1980a. Sexual reproduction in Sitka spruce (*Picea sitchensis*). Can. J. Bot. 58(8): 886-901.

___ 1980b. Sexual reproduction of western red cedar (*Thuja plicata*). Can. J. Bot. 58(12): 1376-1393.

Owens, J.N. and R.P. Pharis. 1967. Initiation and ontogeny of the microsporangiate cone in *Cupressus arizonica* in response to gibberellin. Amer. J. Bot. 54: 1260-1272.

___ 1971. Initiation and development of western red cedar cones in response to gibberellin induction and under natural conditions. Can. J. Bot. 49: 1263-1266.

Owens, J.N. and S. Simpson. 1982. Further observations on the pollination mechanism and seed production of Douglas fir. Can. J. For. Res. 12: 431-434.

Owens, J.N., S.J. Simpson and G.E. Caron. 1987. The pollination mechanism of Engelmann spruce (*Picea engelmannii*). Can. J. Bot. 65: 1439-1450.

Owens, J.N., S.J. Simpson and M. Molder. 1980. The pollination mechanism in yellow cypress (*Chamaecyparis nootkatensis*). Can. J. For. Res. 10(4): 564-572.

___ 1981a. Sexual Reproduction in *Pinus contorta*. I. Pollen development, the pollination mechanism and early ovule development. Can. J. Bot. 59(10): 1828-1843.

___ 1981b. The pollination mechanism and optimal time of pollination in Douglas fir (*Pseudotsuga menziesii*). Can. J. For. Res. 11(1): 36-50.

___ 1982. Sexual Reproduction of *Pinus contorta*. II. Post dormancy ovule, embryo and seed development. Can. J. Bot. 60: 2071-2083.

Owens, J.N. and H. Singh. 1982. Vegetative bud development and the time and method of cone initiation in subalpine fir (*Abies lasiocarpa*). Can. J. Bot. 60: 2249-2262.

Owens, J.N. and F.H. Smith. 1964. The initiation and early development of the seed cone of Douglas fir. Can. J. Bot. 42: 1031-1047.

___ 1965. Development of the seed cone of Douglas fir following dormancy. Can. J. Bot. 43: 317-332.

Owston, P.W. 1969. The shoot apex in eastern white pine: its structure, seasonal development, and variation within the crown. Can. J. Bot. 47: 1181-1188.

Park, Y.S. and D.P. Fowler. 1982. Effects of inbreeding and genetic variances in a natural population of Tamarack (*Larix laricina* (Du Roi), K. Koch) in eastern Canada. Silvae Genet. 31: 21-26.

___ 1984. Inbreeding in black spruce (*Picea mariana* (Mill.) D.S.P.): self-fertility, genetic load and performance. Can. J. For. Res. 14: 17-21.

Pettitt, J.M. 1985. Pollen tube development and characteristics of the protein emission in conifers. Annals of Botany 56: 379-397.

Pharis, R.P. and S.D. Ross. 1984. PINACEAE - Hormonal Promotion of Flowering. In: Handbook of Flowering (A.H. Halevy Ed.) CRC Press. Vol. V.

Peisch, R.F. 1976. Tree improvement in western hemlock. In: Western hemlock management conference. *Edited by* S.W. Atkinson and R.J. Zasoski. College of Forest Resources, Univ. of Wash., Seattle. pp 155-165.

Plym-Forshell, C. 1974. Seed development after self-pollination and cross-pollination of Scots pine, *Pinus sylvestris* L. Stud. For. Suec. Nr. 118: 1-37.

Pollard, D.F.W. and F.T. Portlock. 1984. The effects of photoperiod and temperature on gibberellin $A_{4/7}$ induced strobilus production of western hemlock. Can. J. For. Res. 14: 291-294.

Powell, G.R. 1974. Initiation and development of lateral buds in *Abies balsamea*. Can. J. For. Res. 4: 458-469.

Ross, S.D. 1988. Pre- and post-pollination polyhouse environment effects on pollen and seed development in potted *Picea engelmannii* grafts. Can. J. For. Res. (accepted).

Ross, S.D., A.M. Eastham and R.C. Bower. 1986. Potential for container seed orchards. Presented at Symposium on Conifer Tree Seed in the Inland Mountain West, Missoula, Montana, Aug. 5-7, 1985.

Ross, S.D., R.F. Piesch and F.T. Portlock. 1981. Promotion of cone and seed production in rooted ramets and seedlings of western hemlock by gibberellins and adjunct cultural treatments. Can. J. For. Res. 11(1): 90-98.

Rottink, B.A. 1986. Comparisons of gibberellins A_3 and $A_{4/7}$ for promoting strobili in western hemlock. Can. J. For. Res. 16: 389-390.

Runquist, E. 1968. Meiotic investigations in *Pinus sylvestris* L. Hereditas 60: 77-128.

Sarvas, R. 1962. Investigations on the flowering and seed crop of *Pinus sylvestris*. Inst. For. Fenn. 53: 1-198.

___ 1967. Climatological control of flowering in trees. Internat. Union of Forest Research Organizations Congress, Munich 1967, pp. 15-30.

___ 1968. Investigations on the flowering and seed crop of *Picea abies*. Commun. Inst. For. Fenn. 67, 84p.

___ 1972. Investigations on the annual cycle of development of forest trees. Active period. Commun. Inst. For. Fenn. 76: 1-110.

Seavey, S.R. and K.S. Bawa. 1986. Late-acting self-incompatibility in angiosperms. Bot. Rev. 52: 195-219.

Shaw, D.V. and R.W. Allard. 1982. Estimation of outcrossing rates in Douglas fir using isozyme markers. Theor. Appl. Genet. 62: 113-120.

Simak, M. and A. Gustafsson. 1954. Seed properties in mother trees and grafts of Scots pine. Medd. Stat. Skogforskn-inst. 42: 1-73.

Simola, L.K. 1974. The ultrastructure of dry and germinating seeds of *Pinus sylvestris*. L. Acta. Bot. Finn. 103: 1-31.

Singh, H. 1978. Embryology of gymnosperms. Gebrder Borntraeger, Berlin.

Singh, H. and B.M. Johri. 1972. Development of gymnosperm seeds. Pages 21-75 *in* Kozlowski, T.T. ed. Seed Biology, Vol. 1. Academic Press, New York.

Singh, H. and J.N. Owens. 1981a. Sexual reproduction of Engelmann spruce (*Picea engelmannii*). Can. J. Bot. 59(5): 793-810.

___ 1981b. Sexual reproduction in subalpine fir (*Abies lasiocarpa*). Can. J. Bot. 59(12): 2650-2666.

___ 1982. Sexual reproduction in grand fir (*Abies grandis*). Can. J. Bot. 60: 2197-2214.

Singh, H., J.N. Owens and H.F. Dietrich. 1983. Ultrastructure and development of the microsporangium of *Pseudotsuga menziesii* (Mirb.) Franco. I. Dormant stages. Am. J. Bot. 70(9): 1272-1280.

Snyder, E.B. and K.E. Clausen. 1974. Pollen handling. Pages 75-97 *in* Schopmeyer, C.S., ed. Seeds of Woody Plants in the United States. USDA For. Service Agric. Handbook, No. 450.

Sorensen, Frank. 1969. Embryonic genetic load in coastal Douglas fir *Pseudotsuga menziesii* var. menziesii. The American Naturalist 103: 389-398.

___ 1970. Self-fertility in a central Oregon source of ponderosa pine. USDA For. Serv. Res. Pap. PNW-109, 9 p. Pac. Northwest Forest and Range Exp. Stn., Portland, Ore.

___ 1971. Estimate of self-fertility in coastal Douglas fir from inbreeding studies. Silvae Genetica 20: 115-120.

___ 1973. Frequency of seedlings from natural self-fertilization in coastal Douglas fir. Silvae Genetica 22: 20-24.

___ 1982. The roles of polyembryony and embryo viability in the genetic system of conifers. Evolution 36: 725-733.

Sorensen, F.C., J.F. Franklin and R. Woollard. 1976. Self-pollination effects on seed and seedling traits in Noble fir. For. Sci. 22(2): 155-159.

Sorensen, F.C. and R.S. Miles. 1974. Self-pollination effects of Douglas fir and Ponderosa pine seeds and seedlings. Silvae Genetica 23: 135-138.

Squillace, A.E. and R.E. Goddard. 1982. Selfing in clonal seed orchards of slash pine. For. Sci. 28: 71-78.

Stanlake, E.A. and J.N. Owens. 1974. Female gametophyte and embryo development in western hemlock (*Tsuga heterophylla*). Can. J. Bot. 52: 885-893.

Stanley, R.C. and H.F. Linskens. 1974. Pollen: Biology Biochemistry Management. Springer-Verlag New York, 307pp.

Stanley, R.G. and I. Poostche. 1962. Endogenous carbohydrates, organic acids and pine pollen viability. Silvae Genet. 11: 1-3.

Sterling, C. 1948. Abnormal prothallia in *Tsuga canadensis*. Bot. Gaz. 109: 531-534.

Sweet, G.B. 1973. Shedding of reproductive structures in forest trees. Pages 341-382 *in* Kozlowski, T.T., ed. Shedding of plant parts. Academic Press, New York.

Sweet, G.B. and M.D. Bollman. 1972. Regional variation in Douglas fir seed yields. N.Z.J. For. 17: 74-80.

Sweet, G.B. and P.N. Lewis. 1969. A diffusible auxin from *Pinus radiata* pollen and its possible role in stimulating ovule development. Planta 89: 380-384.

___ 1971. Plant growth substances in pollen of *Pinus radiata* at different levels of germination. New Zealand J. of Botany 9: 146-56.

Takao, A. 1960. Histochemical studies on embryogenesis of *Pinus thunbergii*. Parl. Bot. Mag., Tokyo 73: 379-388.

Thomas, M.J. and L. Chesnoy. 1969. Observations relatives aux mitochondries Feulgen positives de la zone prinucleaire de l'oosphere du *Psuedotsuga menziesii* (Mirb.) Franco. Rev. Cytol. Biol. Vgt. 32: 165-182.

Villar, M. R.B. Knox and C. Dumas. 1984. Effective pollination period and nature of pollen-collecting apparatus in the gymnosperm, *Larix leptolepis*. Ann. Bot. 53: 279-284.

Willemse, M.Th.M. 1971a. Morphological and quantitative changes in the populations of cell organelles during microsporogenesis of *Pinus sylvestris* L. I. Morphological changes from zygotene until premetaphase I. Acta. Bot. Neerl. 20: 261-274.

___ 1971b. Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. II. Morphological changes from premetaphase I until the tetrad stage. Acta. Bot. Neerl. 20: 411-427.

___ 1971c. Morphological and quantitative changes in a population of cell organelles during microsporogenesis of *Pinius sylvestris* L. III. Morphological changes during the tetrad stage and in the young microspore. A quantitative approach to the changes in the population of cell organelles. Acta Bot. Neerl. 20: 298-523.

Willson, M.F. and N. Burley. 1983. Mate choice in plants. Princeton Univ. Press, Princeton, N.J. 251 p.

Wodehouse, R.P. 1959. Pollen grains: their structure, identification and significance in science and medicine. Hafner Publishing Co., New York, pp266-267.

Zar, J.H. 1984. Biostatistical Analysis. Prentice-Hall, Inc, Toronto 718pp.

PARTIAL COPYRIGHT LICENSE

Title of Dissertation

POLLINATION AND POLLEN AND SEED DEVELOPMENT IN WESTERN HEMLOCK.

Author

ANNA M. COLANGELI

April 7, 1989