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An Investigation of Sexual Reproduction in *Picea* (Pinaceae) Including
Prezygotic Stages, Fertilization, and Proembryo Formation.

by

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B.Sc., Carleton University, 1991

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

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ABSTRACT

Sexual reproduction of interior spruce was investigated. In a preliminary study, seeds were produced from $50.5 \pm 20.0\%$ of ovules in the most productive seed orchard trees. Seed production in most trees was far lower. Development between the time of pollination and early embryo formation was studied and reasons for low seed set are described.

Low levels of pollination, as judged by dissection of ovules, was a problem in the seed orchard so the pollination mechanism was studied. The seed orchard is in a hot, dry location and there was a suspicion that pollination drops might not be produced under these conditions. Pollination drops were secreted very late by ovules as seed cones began to close at the end of the receptivity period. Micropylar arms withered and no longer functioned in pollen capture by the time pollination drops were secreted. Pollen move into inverted ovules by floating upwards within the pollination drop. Sacci, the 'wings' of conifer pollen, function as floatation devices in the pollination mechanism. Pollination drops are large and nearly fill the space that the micropyle occupies within the closed seed cone. Pollen adhering to the micropylar arms float within the pollination drop into the micropyle but, as well, pollen adhering to the cone axis near the micropylar arms are scavenged by the large drop. Rain may function in the pollination mechanism of interior spruce and an evolutionary scenario for the pollination drop mechanism is proposed. *Picea orientalis* has saccate pollen that sink into upright ovules. This is different from all other spruce so saccate morphology was investigated. TEM revealed that the exine layer of the sacci is porous when compared to interior spruce. Hydration of the pollen body forces air out of the sacci as observed by confocal microscopy. Ovule orientation and pollen floatation are described as correlated characters.

An anatomical study was made of ovule development from pollination through fertilization and early embryo formation. Many of the observations confirm reports in the literature but examination of resin embedded specimens has allowed for a more detailed description of development. Many abnormalities which prevent fertilization were observed. Because prezygotic failure other than lack of pollen is not generally considered to be a constraint in conifer seed production, abnormal developments are described. Abnormal ventral canal cells and nucelli account for most of the observed prezygotic seed losses. Losses due to lack of pollen, and abnormal development were estimated at 15% each. Self-pollination results in low seed set in conifers so prezygotic development in self-pollinated ovules is described. Two types of prezygotic abnormality which prevent fertilization and that occur only after self-pollination were described and the possibility that some type of self-incompatibility mechanism might occur in conifers is discussed.

Failure at prezygotic stages is common in interior spruce but it is generally only possible to observe these stages by doing careful study of the fertilization period. When seeds without developing embryos are examined after the time of fertilization it is usually impossible to tell if failure was pre-, or postzygotic. Seed losses in conifers are usually described to result from genetic load. The degree of prezygotic failure observed in this study suggests that conifers may not have as many embryonic lethal recessive alleles as estimated.

Recommendations for increasing seed production in the seed orchard through effective use of supplemental pollen and for further studies of a botanical nature are made.

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The whole project is dedicated to my wife Catherine. She made the whole thing fly (get it?).

Chapter 1

General introduction

In 1993, the Canadian forestry industry and government planted over 655 million seedling trees and directly seeded 35,000 hectares of land for reforestation (Anonymous, 1995). Reforestation in British Columbia accounted for approximately 30 percent of this total but 40 percent of harvested land was left to regenerate naturally. Increasing demand for timber and forecasted shortages mean that more harvested areas must be restocked. By the year 2000, approximately 200 million conifer seedlings will be required in British Columbia annually to meet this demand (Anonymous, 1989). The genus in greatest demand in Canada is *Picea* (Spruce). In British Columbia, white spruce (*Picea glauca* (Moench) Voss) and Engelmann spruce (*Picea engelmannii* Parry) hybridize freely at intermediate elevations (600-1500m) in the Interior and along the eastern slopes of the Coast Range mountains (Coates et al., 1991). Collectively, these species and their hybrids are known as interior spruce (fig. 1). Projected annual seedling requirements for interior spruce are in excess of 90 million by the year 2000. More than half of these seedlings should be from genetically improved seed.

To meet the high demand for genetically improved seedlings, the province of British Columbia and industry operate managed seed orchards (fig. 2). Scions of parent trees that have been selected for desirable growth characteristics are clonally propagated by grafting onto rootstocks. Grafted trees begin to produce seed within only a few years of establishment. Continued selection for growth, disease resistance, or other desirable characteristics results in production of genetically improved seed. Demand, however, continues to exceed production so research into effective means of increasing seed orchard yield continues.

Figures 1-2

Fig. 1 White spruce can reach 40 metres tall and 1 metre in diameter when mature. Where it hybridizes with Engelmann spruce in British Columbia, they are collectively called interior spruce.

Fig. 2 Seed orchards for various conifer species are established in British Columbia. In the background, interior spruce trees are being used in control crosses to produce seeds with superior qualities. Pollination control bags prevent fertilization of seed cones by pollen from outside sources.



This study was of interior spruce seed production at the Kalamalka Seed Centre near Vernon, British Columbia during 1991-1993. Sexual reproduction was investigated between pollination and early embryo formation in an attempt to identify reasons for low seed production. Dawkins and Owens (1993) studied pollen development and germination and Krasowski and Owens (1993) studied seed development after embryo formation in white spruce. This study of sexual reproduction in interior spruce includes pollination and ovule development until early embryo formation and so is bracketed by the former two studies with respect to the temporal sequence of development. Many factors which affect seed production have been identified. In particular, prezygotic development within the pollinated ovule was studied in detail.

Chapter 2 is a general review of sexual reproduction within Pinaceae. The pollination mechanism of interior spruce is described in Chapters 3 and 4. Chapter 5 describes pollination in *Picea orientalis* L., a species with an exceptional pollination mechanism for this genus. Taken together, results of Chapters 3 through 5 clarify the interaction between pollination drop and saccate pollen in the pollination mechanism of *Picea* and make possible a description of potential scenarios for evolution of such mechanisms. Chapters 6 and 7 detail development within the ovule after pollination. Observations were similar to those of Owens and Molder (1979a, 1984a) but ultrastructural investigation has allowed description of development in more detail. Observation of normal development within the ovule, as described in Chapters 6 and 7, was required before abnormal developments could be recognized. Chapter 8 describes abnormalities that prevented production of mature seeds. Many of the abnormalities observed precluded fertilization and are therefore described as prezygotic. Reasons for prezygotic failure, other than lack of pollen and very early ovule abortion (Owens et al., 1991), had not been previously described. Self-pollination of *Picea* leads to a large reduction in seed production (Fowler and Park, 1983) and this has been ascribed to the postzygotic effect of lethal allelic

combinations in the embryo. Chapter 9 describes instances of prezygotic failure unique after self-pollination which provide evidence that a late-acting self-incompatibility mechanism might be acting in a gymnosperm.

Significant causes of failure in seed production are presented, and many of them occur prezygotically. Chapter 10 summarizes the results of Chapters 3 through 9 and describes implications for seed production.

Note on format

Chapter 3 has been published as Runions et al. (1995), and Chapter 4 has been published as Runions and Owens (1996). These papers are occasionally cited in subsequent chapters as journal publications but, since no significant alterations have been made, the reader can refer to the chapters within this dissertation.

Chapter 2

Pinaceae: origin, sexual reproduction and tree improvement

Phylogenetic relationships of modern conifers

Fossil evidence supports the origin of modern conifers from the Mesozoic Voltziaceae, the so-called transition conifers, over 200 million years ago (Florin, 1951; Miller, 1982). Modern conifers are distinct from voltziacean species in morphological characters, however, so specific ancestors of modern families remain unknown (Miller, 1976). A generic list of extant conifer families is presented in table 1. The focus of this chapter is on Pinaceae with particular reference to *Picea*.

Pinaceae has 10 genera which are distinguishable by morphological characters of vegetative and reproductive structures (Hart, 1987), nucleotide sequence divergence (Chase et al., 1993), and immunological distance comparison of seed proteins (Price et al., 1987). Figure 3 is a cladogram showing generic relationships within Pinaceae. It is a reconciliation of the morphological analysis (Hart, 1987) and the genetical analysis (Chase et al., 1993). In the morphological study by Hart (1987), *Pseudotsuga* and *Larix* group with the abietoid Pinaceae. In Chapter 4, the pollination drop is discussed as a derived character rather than as an ancestral condition in Pinaceae. If this is the case, *Pseudotsuga* and *Larix* group with the pinoid Pinaceae in support of the results of the nucleotide sequence divergence study (Chase et al., 1993). *Cathaya* was not placed in this cladistic analysis of Pinaceae because DNA was not available for sequencing. Morphologically, it is similar to *Pinus* from which it probably diverged relatively recently. Divergence of *Pinus* and *Picea* occurred approximately 80 million years ago (Nienstaedt and Teich, 1972).

Table 1. Classification of the conifers. Only selected genera have been shown in many families.

Order	Family	Sub-family	Genus
Coniferales	Araucariaceae		<i>Agathis</i> <i>Araucaria</i>
	Cephalotaxaceae		<i>Cephalotaxus</i>
	Cupressaceae †	(Taxodiaceae)	<i>Cryptomeria</i> <i>Metasequoia</i> <i>Sequoia</i> <i>Sequoiadendron</i> <i>Taxodium</i>
		(Cupressaceae)	<i>Chamaecyparis</i> <i>Juniperis</i> <i>Thuja</i>
	Phyllocladaceae		<i>Phyllocladus</i>
	Pinaceae	Abietoideae	<i>Abies</i> <i>Cedrus</i> <i>Keteleeria</i> <i>Tsuga</i> <i>Pseudolarix</i> <i>Cathaya</i>
		Pinoideae	<i>Larix</i> <i>Picea</i> <i>Pinus</i> <i>Pseudotsuga</i>
	Podocarpaceae		<i>Dacrydium</i> <i>Podocarpus</i>
	Sciadopityaceae		<i>Sciadopitys</i>
	Taxaceae		<i>Taxus</i> <i>Torreya</i>

† Recent reviews favour a merger of Taxodiaceae and Cupressaceae (Eckenwalder, 1976; Brunfeld et al., 1994). Naming priority requires that the merged family be called Cupressaceae. Adapted from Gifford and Foster (1987) and Price et al. (1987).

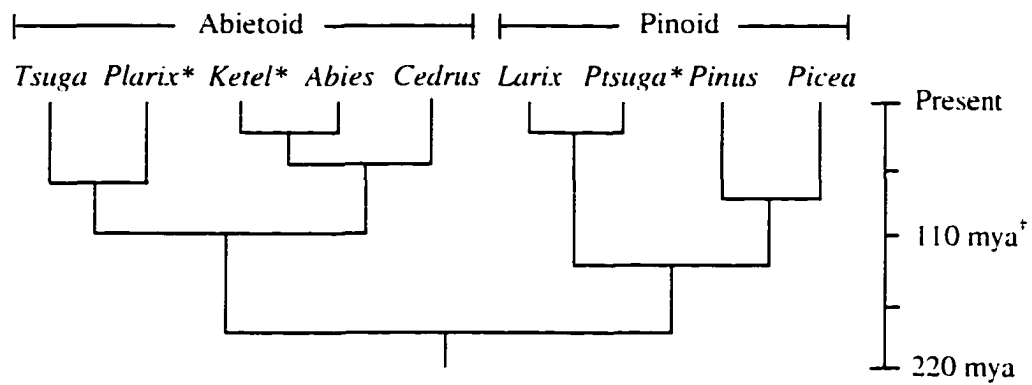


Figure 3. Phylogenetic relationships of Pinaceae. Adapted from Hart (1987) and Chase et al. (1993). † mya - million years ago

* *Plarix* - *Pseudolarix*
Ketel - *Keteleeria*
Ptsuga - *Psudotsuga*

Nienstaedt and Teich (1972) describe the evolution of *Picea* which probably originated in northeast Asia. White spruce (*Picea glauca* (Moench) Voss) is probably the oldest extant member of the genus in North America. Divergence resulted in formation of the western North American complex that includes white spruce, Engelmann spruce (*P. engelmannii* Parry), Sitka spruce (*P. sitchensis* (Bong.) Carr.), and blue spruce (*P. pungens*). White spruce is the most widely distributed conifer in North America, ranging from western Alaska to Newfoundland and extending south of the Great Lakes. Engelmann spruce is restricted to higher elevations in British Columbia, Alberta, and the central Rocky Mountains of the United States. White and Engelmann spruce can be distinguished by morphological features of the ovulate cone such as the appearance of the margin of the ovuliferous scales. In practice, this distinction is difficult to make with certainty so where their ranges overlap and the species hybridize they are collectively called interior spruce. Hybridization between the two species has resulted in their introgression. Khasa and Dancik (1996) have been able to distinguish the parent species and the level of introgression in hybrids using polymorphic DNA markers.

Sexual reproduction in conifers - historical perspective

Reports describing sexual reproduction in conifers are numerous from the mid-nineteenth century onwards. Reviews of early literature are available in more modern sources. Each of the stages mentioned here will be described in greater detail in following sections. Sterling (1963) described the structure of the male gametophyte and proposed a terminology for the cell types contained within. Pollination mechanisms were described by Doyle and O'Leary (1935) and Doyle (1945). Ovule development and embryogeny are extensively described in older literature. Camefort (1968) acknowledges the pioneering work of Hofmeister, Strasburger, and others during the mid-, to late nineteenth century as the basis for our present understanding of gymnosperm reproduction. Maheshwari and

Singh (1967), and Konar and Oberoi (1969) include comprehensive bibliographies of work published in the twentieth century. Megagametophyte development was reviewed, in a comparative manner, for most gymnosperm taxa by Maheshwari and Singh (1967). Konar and Oberoi (1969) briefly describe fertilization in conifers although, prior to the early 1960's, not a great deal of literature existed on this subject. Fertilization of *Pinus* was studied by Haupt (1941) and McWilliam and Mergen (1958) presented the first photomicrographic account of this process. The largest body of literature from the twentieth century describes embryogeny in conifers. Bucholz (1929) dissected embryos and suspensors from seeds and published a detailed account of conifer proembryogeny. Proembryo formation has been reviewed by Chowdhury (1962), and with a concentration in *Pinus*, by Doyle (1963). Doyle provided a detailed terminology of proembryo cell tiers which is generally still in use. Dogra (1967) was concerned with seed sterility resulting from abortion of embryos. Embryological disturbance was described as a significant post-fertilization factor resulting in empty seeds. Environmental, and genetic effects were found to each play a part in embryo mortality which occurred, for the most part, during early embryo development. Proembryo and early embryo development in Pinaceae were later described in a more concise series (Mehra and Dogra, 1975, 1977).

Chamberlain (1935) wrote a comprehensive text on gymnosperm structure and evolution which included sexual reproduction in conifers. The most recent comprehensive text on gymnosperm embryology is by Singh (1978) and includes sporogenesis to seed maturation. Since Singh's book in 1978, much work has been done on sexual reproduction in conifers. A detailed account of pollination and fertilization which includes a review of the literature on cytoplasmic inheritance was provided by Chesnoy (1987). Pennell (1988) has reviewed sporogenesis. Cone production periodicity, reproductive cycles, floral induction, and all stages from pollination through seed development were reviewed by Owens (1991). Misra (1994) has reviewed zygotic embryogenesis and

described biochemical and molecular changes within developing and germinating conifer seed.

Pollen development, pollination, and pollen tube growth

Microsporogenesis has been reviewed by Pennell (1988) and Dawkins (1990). The pollen wall of Pinaceae consists of a cellulosic intine and a sporopollenin-containing exine (Kurmann, 1989a). Exine consists of the outer ektexine and an endexine. Ektexine in *Abies concolor* (Gord.) Hild. forms a tectal layer with inwardly projecting bacula that contact the endexine (Kurmann, 1989b). Pinaceae has saccate and non-saccate pollen. The sacci of *Pinus*, *Picea*, *Abies*, *Cedrus*, and *Hesperopeuce* (*Tsuga mertensiana* (Bong.) Carr.) form because the ektexine separates from the endexine at two distinct points on the distal surface of the pollen (Kurmann, 1992). Saccus expansion occurs once microspores are released from the tetrad within the microsporangium.

Pollen of Pinaceae has five cells at maturity. Owens (1993) summarizes the series of mitosis from microspore to mature pollen. The microspore divides unevenly to form an embryonal cell (or central cell, see Singh, 1978) and a small prothallial cell. Embryonal cell division forms a second, small prothallial cell and an antheridial initial which in turn divides to form the tube cell and the generative cell. Pollen is mature when the generative cell divides to form the stalk and body cells. All conifers are wind pollinated. In Pinaceae, microsporangia dehisce and release the 4 or 5-cell pollen.

Pollination mechanisms vary within Pinaceae (table 2; Owens, 1993). Pollination is similar between *Picea* (Ho, 1984; Owens and Blake, 1984; Owens and Molder, 1984a; Owens et al., 1987) and *Pinus* (Lill and Sweet, 1977; Owens et al., 1981a; Brown and Bridgwater, 1987). Ovuliferous scales of the megasporangiate strobilus reflex in a manner that opens the cone and exposes the micropylar arms, which are extensions of the ovule

Table 2. Pollination mechanisms in Pinaceae.

	Ovule orientation (at pollination)	Exuded pollination drop	Saccate pollen	Genera
i)	Inverted	Yes	Yes	<i>Cedrus</i> <i>Picea</i> <i>Pinus</i>
ii)	Inverted	No	Yes	<i>Abies</i> <i>Micropeuce</i> [†]
iii)	Inverted	Unknown	Yes	<i>Cathaya</i> <i>Hesperopeuce</i> <i>Keteleeria</i> <i>Pseudolarix</i>
iv)	Inverted	No	No	<i>Larix</i> <i>Pseudotsuga</i>
v)	Upright	Yes	Yes	<i>Picea orientalis</i>

[†] *Micropeuce* pollen has vestigial sacchi.

Adapted from, Doyle (1945), Owens and Molder (1984a), Owens (1993), Takaso and Owens (1995), Tomlinson (1994).

integument at the micropyle opening. Ovuliferous scales of white spruce remain reflexed for about 10 days (Ho, 1984) during which time windborne pollen adhere to sticky microdrop secretions on the micropylar arms (Owens et al., 1987). At some point, towards the end of the receptive period, a pollination drop is secreted from the ovule. Timing of pollination drop secretion in seed orchard interior spruce is the subject of Chapter 3 of this dissertation. Through interaction with the pollination drop, pollen move into the pollen chamber in the apical part of the nucellus within the ovule. Pollen either float into the inverted ovule as proposed by Doyle (1945) or are drawn into the ovule as the pollination drop recedes (Owens, 1993). Pollen / pollination drop interaction in the pollination mechanism is investigated in Chapter 4. *Cedrus* has a similar pollination mechanism although the pollination drop is small and more ephemeral (Takaso and Owens, 1995). One anomalous species, *Picea orientalis* L., differs from other *Picea* in that its pollen is saccate but sinks into ovules that open upwards at pollination (Doyle, 1945). Pollination of this exceptional species is investigated in Chapter 5. Several authors have described movement of saccate pollen into ovules by rainwater (eg. in *Pinus*, Greenwood, 1986; Brown and Bridgwater, 1987; in *Cedrus*, Takaso and Owens, 1995). Floatation of pollen into ovules by rainwater may occur in other genera with saccate pollen, eg. *Abies* and *Picea*, but this matter has not been investigated.

Origin of the pollination drop has been debated but it is believed to originate in the nucellus (Ziegler, 1959; Owens et al., 1987). In Engelmann spruce, the pollination drop contains a low concentration solution of various sugars (4.3% glucose, 3.8% fructose; Owens et al., 1987) and provides the aqueous environment required by the pollen for rehydration and germination. Tomlinson (1991, 1994) described pollen scavenging by a voluminous and extended pollination drop in Podocarpaceae. A similar type of observation is described for interior spruce in Chapter 4.

Genera which lack a large, exuded pollination drop may produce an analogous secretion within the ovule so that pollen germination will occur (eg. *Pseudotsuga*, Takaso and Owens, 1994, 1996; *Larix*, Owens et al., 1994).

Abies pollen is saccate but no pollination drop exudes from the ovule (Owens and Molder, 1986). Pollen adhere to microdrops on the integumentary flap that eventually fold inwards, transporting the pollen into the micropyle. *Larix occidentalis* Nutt. (Owens et al., 1994) and *Pseudotsuga menziesii* (Mirb.) Franco (Owens et al., 1991; Takaso and Owens, 1995) have non-saccate pollen and pollen entrapment happens in a manner similar to that in *Abies*. In *Micropeuce* (*Tsuga heterophylla* (Raf.) Sarg.) pollen germinate outside the ovule and produce an extended pollen tube which penetrates the micropyle (Owens and Molder 1984b). Several genera of Pinaceae have pollination mechanisms that are not well understood. *Cathaya*, *Keteleeria*, *Pseudolarix* and *Hesperoepuce* have saccate pollen but it is not known if a pollination drop is produced.

Pollen germinates after a variable amount of time within the ovule. Germination occurred within two days in white spruce (Dawkins and Owens, 1993) or it was delayed as long as two weeks (Owens and Molder, 1984a). *Picea* pollen tubes grow through the nucellus with no further delay after germination. In *Pinus contorta* Doug. germination was delayed until two months after pollination (Owens et al., 1981a). Pollen tubes became dormant and overwintered within the nucellus, and growth resumed in the following spring. Similar long delays in pollen germination occur in *Abies* (Owens and Molder, 1986), *Tsuga heterophylla* (Owens and Molder, 1984b), *Pseudotsuga menziesii* (Takaso et al., 1996), and *Larix decidua* Mill. (Barner and Christiansen, 1960). In these cases, pollen tubes grow through the nucellus without further delay and effect fertilization.

Delay between pollination and pollen germination coincides with maturation of the archegonia and germination in those genera probably requires a chemical signal of some sort from the ovule at the appropriate maturation stage (Said et al., 1991; Takaso et al.,

1996). Interactions between pollen and ovule have not been widely studied but are receiving attention recently (Chesnoy, 1993). Sweet and Lewis (1969) and Pettitt (1985) have described diffusible auxins and proteins, respectively, which are emitted from the pollen tube and which might affect development of the megagametophyte. In *Pseudotsuga menziesii*, a homogenate of megagametophyte tissue induced in vitro pollen tube formation while little or no response was observed when homogenate of nucellus or integument was added to the media (Takaso et al., 1996).

The pollen tube forms as an extension of the intine. In genera with saccate pollen, pollen tubes emerge at the leptolemma between the sacchi (Dawkins, 1990). In *Larix occidentalis* and *Pseudotsuga menziesii*, the exine split and was shed as the pollen elongated (Owens and Molder, 1979b; Owens and Morris, 1990). When elongating pollen contacted the nucellus, a pollen tube formed as an extension of the intine.

Development within pollen tubes seems similar for most genera of Pinaceae. Body cell and stalk cell move into the pollen tube. The body cell has invaginations with the tube cell which may be a feature of the mechanism by which cells move through the pollen tube (Bruns and Owens, 1989; Owens and Morris, 1990). Terasaka and Niitsu (1994) have characterized cytoskeletal elements in pollen tubes of *Pinus*. In generative cells, microtubules and F-actin networks were observed. The authors propose that actin-myosin contributes to extension growth and perhaps cell motility within the pollen tube, while microtubules play a part in determination of tube polarity. Body cell mitosis produces two male gametes (Singh, 1978). In *Picea glauca*, male gametes were formed within one day after pollen tube emergence from the leptolemma (Dawkins and Owens, 1993). In *Pinus*, body cell division occurs when the tube has progressed further through the nucellus after dormancy, and in *Pseudotsuga menziesii*, division occurs once the pollen tube reaches the archegonial chamber of the megagametophyte (Owens and Morris, 1991). Male gametes in

Pinaceae are free nuclei that are surrounded by a common dense cytoplasm which contains plastids and mitochondria within the body cell plasma membrane.

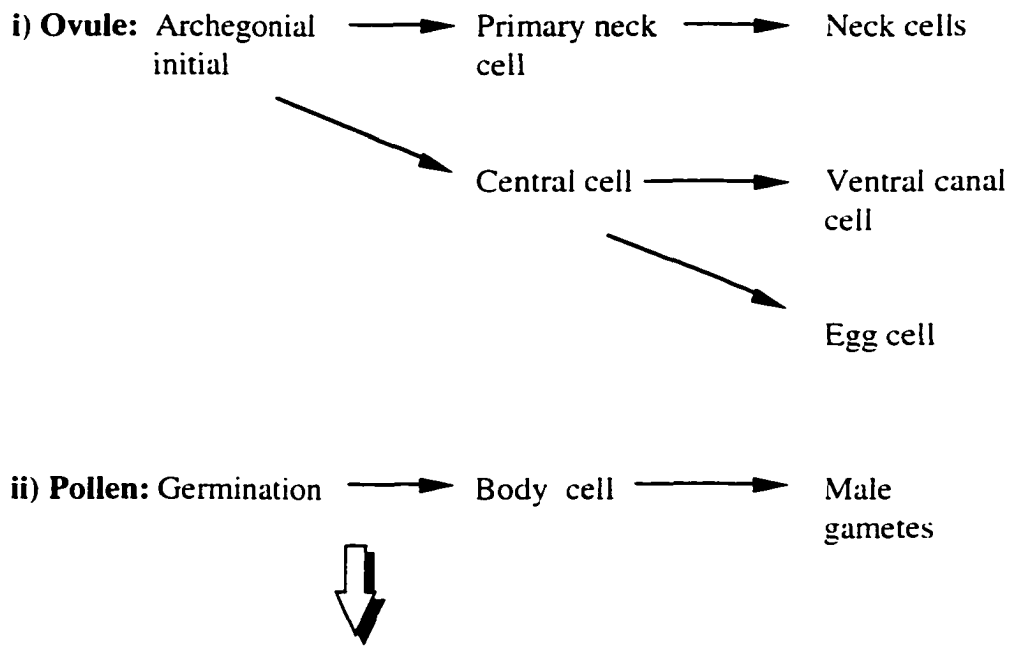
Megagametophyte development

Development of the megagametophyte of Pinaceae is described by Maheshwari and Singh (1967) and by Singh (1978). In general, megasporogenesis produces a tetrad of megaspores and the megagametophyte develops from the functional chalazal megaspore. The megasporangium forms the nucellus which surrounds the megagametophyte. Cells of the inner 1 to 3 layers of the nucellus around the megagametophyte are tapetal and by deteriorating contribute, at least, to the ectexine layer of the megaspore wall (Singh, 1978). Repeated mitosis without cell wall formation produces a coenocytic megagametophyte in which nuclei occupy the thin parietal cytoplasm. Continued mitosis, accompanied by cell wall formation results in a cellular megagametophyte.

Between 2 to 7 archegonial initial cells differentiate in the micropylar region of the megagametophyte. In interior spruce and *Pinus contorta*, 1 to 4 archegonia form (Owens and Molder, 1984a; Lill, 1976), and in *Pseudotsuga menziesii*, 4 to 6 archegonia form (Allen and Owens, 1972). A summary of the cell divisions that occur during archegonium development is presented in figure 4 (after, Owens and Blake, 1985). Chapter 6 of this dissertation is a detailed study of archegonium development in interior spruce.

Archegonium development and subsequent stages of sexual reproduction have been described for most pinaceous genera: *Abies* (Singh and Owens, 1981a, 1982; Owens and Molder, 1986), *Larix* (Owens and Molder, 1979b; Kosinski, 1986), *Picea* (Mergen et al., 1965; Owens and Molder, 1979a, 1980, 1984a; Singh and Owens, 1981b), *Pinus* (McWilliam and Mergen, 1959; Lill, 1976; Owens et al., 1982; Owens and Molder, 1984c), *Pseudotsuga* (Allen and Owens, 1972; Owens and Morris, 1990, 1991), and

Prezygotic development



Poszygotic development

Fertilization

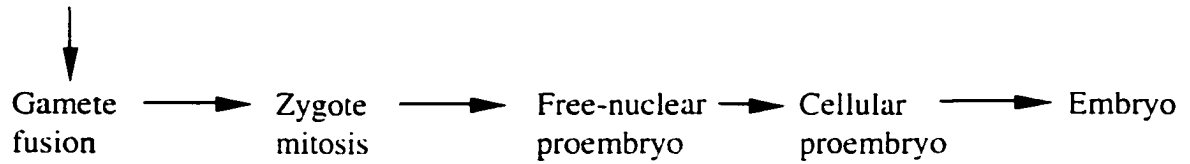


Figure 4. Stages of pre-, and postzygotic development studied in interior spruce

Tsuga (Stanlake and Owens, 1974; Owens and Molder, 1984c; Colangeli, 1989).

Archegonial initial mitosis is unequal and produces a large central cell and a small primary neck cell at the micropylar pole. The primary neck cell and its derivatives continue to divide and produce the archegonial neck which can be from 1 to 4 cell tiers in Pinaceae. Neck cells have thick primary cell walls and are the site of pollen tube penetration of the archegonium. Camefort (1965) described the early central cell of *Pinus nigra* Arnold. The cytoplasm of the rapidly enlarging cell was peripheral and the largest volume of the cell was composed of vacuoles which gave the cell its characteristic 'frothy' appearance. Each archegonium in Pinaceae is surrounded, except at its micropylar end, by small, angular cells of the megagametophyte that are called the archegonial jacket layer. Jacket layer cells are in close contact with the developing central cell via primary pit fields in the adjoining walls, and probably are the route by which most reserves are transported into the central cell. As the central cell develops, it enlarges to > 500 μm in length. Plastids elongate and encircle areas of central cell cytoplasm. Elaboration of the plastid membrane into concentric whorls or, by invagination, into separate compartments give them the characteristic appearance of large inclusions which measure 10 to 50 μm in diameter and occur in all pinaceous central and egg cells (Willemse, 1974). Plastids that have become large inclusions will be referred to from here on as modified plastids as this is more explicit terminology. Mitochondria of the central cell remain dispersed in the cytoplasm (Owens and Morris, 1990). Throughout central cell development, the large nucleus retains its position at the micropylar pole. Mitosis of the central cell produces the small ventral canal cell and a large egg cell. The ventral canal cell subtends the neck cells. No function is known for the ventral canal cell which usually is described as deteriorated at about the time of fertilization.

The egg cell is 500 to 600 μm long and 250 to 300 μm wide at maturity in most Pinaceae. As the nucleus migrates, by an unknown mechanism, to the central region of the

cell, the vacuolate appearance of the cytoplasm is diminished. Vacuoles are reduced in volume as the enlarging egg cell becomes more cytoplasmic and in the mature egg cell vacuoles are rare or absent. Modified plastids are concentrated in the peripheral and chalazal cytoplasm and the majority of the cell is composed of cytoplasm bound by small inclusions. In ultrastructural studies (eg. Chesnoy, 1987; Owens and Morris, 1990), small inclusions are seen to be areas of cytoplasm contained within a bounding membrane that in size appears similar to endoplasmic reticulum. Small inclusions vary in size from 5 μm in diameter to elongate forms that can be $> 50 \mu\text{m}$ in length. They do not usually contain organelles. Mitochondria of the egg cell migrate within the non-included cytoplasm to occupy a perinuclear zone around the large central egg nucleus. The perinuclear zone is 4 to 7 μm thick (Owens and Morris, 1991; Bruns, 1993) and includes vesicles, ribosomes, and microtubules as well as egg cell mitochondria. Perinuclear zone mitochondria of *Larix* (Camefort, 1967) were reportedly electron transparent and those of *Pseudotsuga menziesii* (Owens and Morris, 1990) were electron dense.

Fertilization and proembryo development

When egg cells mature, each archegonium can be fertilized by a pollen tube. Chapter 7 deals with fertilization and proembryo development in interior spruce. Pollen tubes penetrate the neck and ventral canal cells and rupture, releasing the male gametes and other tube contents into the egg cytoplasm, or into a receptive vacuole within the egg (Singh, 1978). A single male gamete nucleus moves towards the egg nucleus and fuses with it to form the zygote. Fusion occurs very quickly after pollen tube entrance into the egg cell. Many authors describe the difficulty of finding this stage in embedded specimens. As the male gamete nucleus moves towards it, a depression forms in the egg nucleus where fusion will occur and the egg nuclear membrane becomes highly invaginated (Owens and

Morris, 1991). Published transmission electron micrographs of the fusion nucleus are rare.

Development after gamete fusion is summarized in figure 4. The zygote nucleus undergoes mitosis shortly after fusion. Resultant proembryo nuclei are much reduced in volume and remain free in the archegonial cytoplasm. Nucleoplasm released from the zygote during mitosis surrounds each of the free nuclei and was called the neocytoplasm by Camefort (1958). At this stage, radical changes in the cytoplasm of the archegonium become apparent. Modified plastids begin to deteriorate and the small inclusions become round and vacuolate. Camefort (1969) speculated that the neocytoplasm which surrounds proembryo nuclei might protect them from the lytic effect of the archegonial cytoplasm which clearly begins to break down. Histochemical tests have shown production of acid phosphatases from within small inclusions in *Pinus nigra*. Synchronous mitosis of the nuclei result in four free proembryo nuclei, surrounded by neocytoplasm within the archegonial cytoplasm.

Free nuclei of the coenocytic proembryo migrate, while surrounded by neocytoplasm, to the chalazal end of the archegonium (Owens and Morris, 1991; Bruns, 1993) and settle into a single tier. Development of the proembryo continues until it comprises four tiers of four cells each in all Pinaceae except *Pseudotsuga* in which the mature proembryo has only three tiers (Allen and Owens, 1972; Owens and Morris, 1991). Singh's (1978) description of proembryo tier formation was derived from many sources and is characteristic. Synchronous mitosis of the free nuclei forms two tiers of four nuclei each prior to cell wall formation across the phragmoplasts between tiers. The chalazal tier is designated primary embryonal and the tier towards the micropylar pole, that remains open to the archegonial cytoplasm, is designated primary open. Cell walls form between nuclei within each tier and mitosis of the primary open tier produces the non-functional suspensor and open tiers. Mitosis of this tier is absent in *Pseudotsuga menziesii* (Owens

and Morris, 1991). As cell walls form between the non-functional suspensor and open tier nuclei, the primary embryonal tier divides to form the suspensor and embryonal tiers. The mature proembryo consists of, from the chalazal pole, embryonal, suspensor, non-functional suspensor, and open tiers. Owens and Molder (1984a) called these tiers, respectively, apical, suspensor, rosette, and open.

Rapid elongation and repeated divisions in the suspensor forces the embryonal tier into the nutritive prothallial tissue of the megagametophyte. Embryo and megagametophyte development beyond this point was described by Owens et al. (1993) for *Pseudotsuga menziesii* and by Krasowski and Owens (1993) for *Picea glauca*.

Cytoplasmic inheritance

Genomes of cytoplasmic organelles do not segregate in Mendelian fashion in higher plants. Rather, inheritance is usually uniparental, and in most angiosperms, maternal (Reboud and Zeyl, 1994). In contrast, chloroplast DNA (cpDNA) is inherited from the paternal parent and mitochondrial DNA (mtDNA) from the maternal parent in Pinaceae (Neale and Sederoff, 1989). There are two ways to determine the paternity of organelle genomes, molecular genetic analysis allows confirmation of paternity, and direct observation using anatomical techniques allows elucidation of the mechanism of inheritance. Neale and Sederoff (1989) used restriction fragment length polymorphisms to study organelle DNA in *Pinus taeda* L. and they found maternal inheritance of mtDNA and paternal inheritance of cpDNA. Organelles were inherited similarly in *Pseudotsuga menziesii* (Neale et al., 1986), and *Picea* (Stine et al., 1989). The only exception to strict paternal inheritance of cpDNA was reported in a hybrid *Larix* (Szmidt et al., 1987) which had a maternal restriction pattern. David and Keathley (1996) used a similar technique to demonstrate strict maternal inheritance of mtDNA in interspecific crosses of *Picea*. This is not always the case, however, as Wagner et al. (1991) found that 7 to 10% of crosses

between *Pinus contorta* and *Pinus banksiana* Lamb. produced progeny with paternal mtDNA restriction patterns. Reasons why this might be the case are seen in anatomical study of cytoplasmic inheritance.

Camefort was the first to describe cytoplasmic inheritance in ultrastructural studies of *Pinus* (1966) and *Larix* (1967). Chesnoy (1987) reviewed investigations of organelle inheritance. Plastids of the maternal cytoplasm distort and become modified prior to lysing with the archegonial cytoplasm after fertilization. Plastids of the body cell cytoplasm accompany the male gamete nuclei into the egg cytoplasm and they either surround the free nuclei of the proembryo or they remain in a cluster and disperse within the neocytoplasm once the free nuclei settle at the chalazal end. Inheritance of cpDNA, therefore, is observed to be paternal because of their distortion into modified plastids and subsequent deterioration. Owens and Morris (1991) found that a cluster of paternal organelles (both plastids and mitochondria) migrated with the proembryo free nuclei. They observed paternal mitochondria near the neocytoplasm of the proembryo and concluded that a fraction of the mitochondria inherited would be paternal in origin. Mitochondria seem to change in appearance although reports in the literature are not consistent on this point. The orthodox nature of mitochondria in the body cell cytoplasm, with well developed cristae, differentiates paternal mitochondria from maternal mitochondria which are often dark staining and distorted with electron translucent areas. By the time mitochondria occupy the neocytoplasm around proembryo nuclei at the chalazal pole, they appear orthodox and maternal and paternal ones are indistinguishable. Occasionally, modified plastids were trapped in the neocytoplasm of *Pseudotsuga menziesii* during cell wall formation. While the condition of DNA in modified plastids is unknown, the authors suggested that this could account for the small fraction of maternal cpDNA that is sometimes observed in heteroplasmic individual progeny (White, 1990). Bruns and Owens (1989) observed a similar pattern of cytoplasmic inheritance in *Pinus monticola* Dougl. and suggested a

possible low level of paternal mtDNA inheritance was possible. Cytoplasmic inheritance in interior spruce is discussed in Chapter 7.

Breeding system of *Picea* and incompatibility in conifers

Paternal inheritance of cpDNA and maternal inheritance of mtDNA allow population genetic studies of Pinaceae that would not be possible in angiosperm families. Assessment of population genetic patterns of variation in nuclear, chloroplast, and mitochondrial genomes allows assessment of the effects of evolutionary forces acting on genomes with different modes of inheritance. Furnier and Stine (1995) analyzed cpDNA and nuclear DNA variation in 22 populations of *Picea glauca* and found that a much higher proportion of the variability between populations was due to cpDNA variation than was due to nuclear DNA variation. Organelle genes were described as effectively haploid because of uniparental inheritance. The authors proposed that since the effective number of organelle genes is only half that of nuclear genes, the effect of genetic drift is much more pronounced in organelle genomes resulting in the observed pattern of variation. It is important, therefore, to assess the mode of inheritance and recognize the distinct patterns of variation between organelle and nuclear genomes when using them to assess the genetic structure of populations.

Much more work in population genetics has been devoted to assessment of the breeding system of Pinaceae. All conifers are self-fertile to some degree, although most suffer from high levels of inbreeding depression as estimated from seed losses after self-pollination. Fowler and Park (1983) found that the frequency of empty seed increased from 5 to 22 times when cross-, and self-pollinated *P. glauca* were compared and that inbreeding affected the form of viable progeny over a 17 year period. Park et al. (1984) estimated that inbreeding is kept at a low equilibrium level in natural populations of *P. glauca* (inbreeding coefficient $F=0.125$) by the deleterious effect of high numbers of lethal

recessive alleles in the population. However, Innes and Ringius (1990) have shown that *P. glauca* populations from eastern Newfoundland may have higher selfing rates than predicted (approximately 25%).

Many authors have described the deleterious effect of inbreeding on conifer seed production (Sarvas, 1968; Koski, 1973; Plym-Forshell, 1974; Woods and Heaman, 1989). Invariably, because some viable seed is produced from self-pollination, self-incompatibility (SI) is said not to occur (Mergen et al., 1965). Self-incompatibility occurs in many angiosperms. Gametophytic SI (Newbigin et al., 1993) causes arrest of pollen tubes in the style and sporophytic SI (Nasrallah and Nasrallah, 1993) prevents pollen germination on the stigma. A third category of SI is known as late-acting or ovular (Seavey and Bawa, 1986; Sage et al., 1994). In each case, fertilization does not occur. No study in conifers has reported failure of fertilization after self-pollination and this is explored further in Chapter 9.

Prezygotic losses have been estimated for a few conifers. Owens et al. (1991) studied seed production in *Pseudotsuga menziesii* and reported that prezygotic factors other than lack of pollen or very early ovule abortion resulted in loss of 11% of seed potential. Causes of prezygotic failure were not determined. Self-pollination of *Tsuga heterophylla* (Colangeli, 1989) resulted in a 12% increase in seed losses during prezygotic development. The implication is that incompatibility and late-acting SI may exist in Pinaceae. Physical manifestations of putative incompatibility mechanisms are only detectable by anatomical investigation of prezygotic ovules. Certain observations suggest that interactions between pollen and the nucellus (sporophytic) or the megagametophyte do occur in gymnosperms. Protein diffusion occurs from pollen tubes (Pettitt, 1985), and ovular secretions which induce pollen tube formation have been demonstrated in *Larix occidentalis* (Owens et al., 1994) and *Pseudotsuga menziesii* (Takaso et al., 1996). Certainly, prezygotic interactions between pollen and ovule should not be ruled out. Hard evidence of pollen and ovule

interaction comes from the crossability study of Hagman (1975) in which interspecific pollen arrested within the nucellus of *Pinus*. Steyn et al. (1996) have described rejection of incompatible male gametes by the egg cell of the cycad *Encephalartos villosus* Lem. which represents a type of prezygotic selection in a gymnosperm. Figure 5 shows the prezygotic stages which are described in Chapters 8 and 9.

Seed orchards and tree improvement

Genetic gain is most easily achieved if parent trees with desirable traits are sexually reproduced. Large amounts of genetically improved seed can be efficiently produced in seed orchards but production is significantly reduced by a number of factors. Lack of pollen, self-pollination, and contaminating pollen all reduce seed production or realized gain.

Low pollen production can be offset by using supplemental mass pollination (SMP, Askew, 1992; El-Kassaby et al., 1993; Eriksson et al., 1994). Table 3 presents unpublished results of an interior spruce SMP study carried out in 1991 and 1992. In 1991, natural pollen production was very low and SMP had a significant beneficial effect. In 1992, natural pollen was plentiful and SMP had no effect on pollination success. Overall, pollination success was low, which is why a study of the pollination mechanism was undertaken. If timed correctly, SMP can be effective. Supplemental pollen, used in an attempt to increase genetic gain, must occupy sites near the micropyle where it can compete for space with natural pollen and preferentially be taken into the ovule (Webber and Yeh, 1987). If SMP is left too late during the receptive period, pollen from non-selected, naturally pollinating trees may occupy the micropyle. Pollen management guides have been published by Webber (1987, 1991). Planned pollen application is required if panmixis is to be approached in the seed orchard. Genetic diversity of natural populations

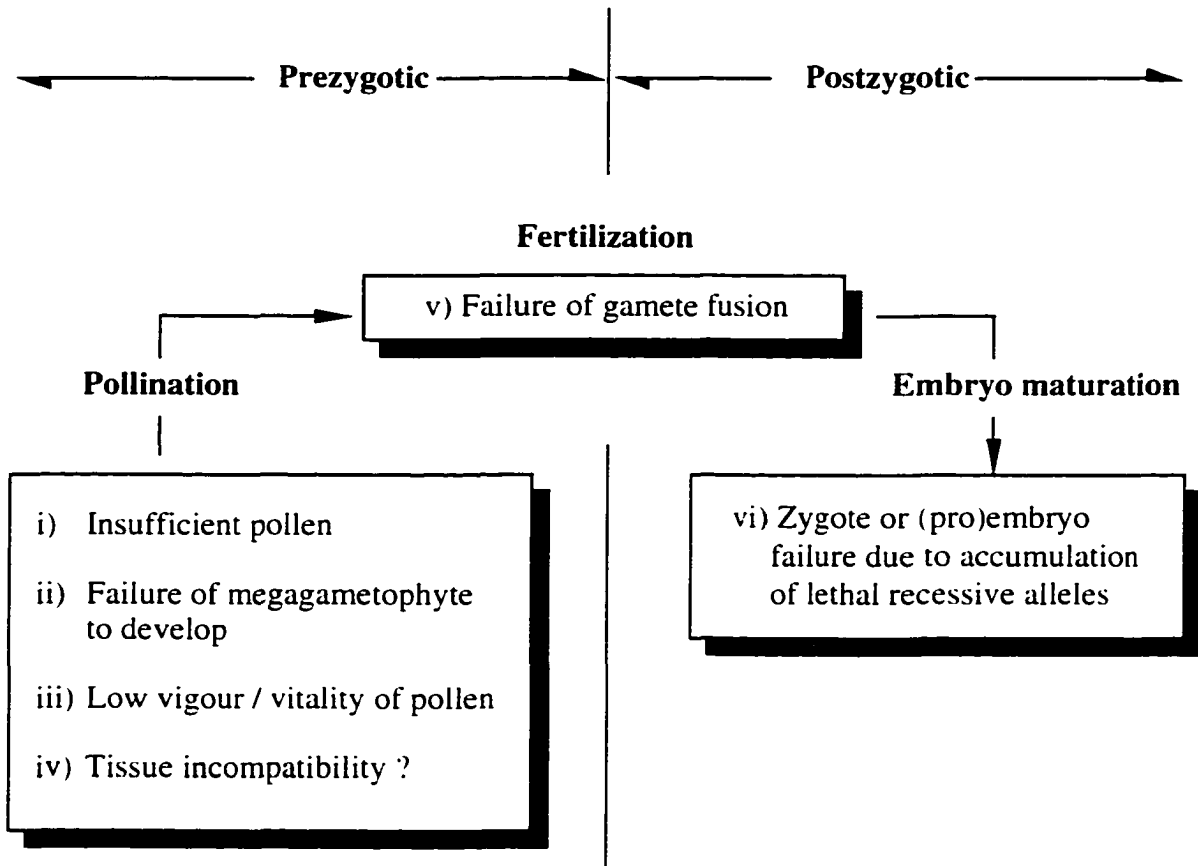


Figure 5. Potential reasons for failure of seeds to develop by sexual reproduction

Table 3. Pollination success in an interior spruce seed orchard with and without supplemental mass pollination (SMP) during seasons of low (1991) and high (1992) pollen production.

Pollination success (# pollen grains / ovule)			
1991		1992	
without SMP	with SMP	without SMP	with SMP
0.1±0.1 †	2.6±0.8	1.84±0.59	1.62±0.43

† significantly different ($p < 0.001$)

is well represented in seed orchard populations but effective paternity can be greatly reduced by unequal strobilus production or uneven pollen flow patterns in the orchard (El-Kassaby, 1992; O'Reilly et al., 1982). Self-pollination and resulting reduced seed production can be minimized by application of supplemental pollen which spatially, and perhaps genetically, competes with the self-pollen. Nakamura and Wheeler (1992) found significant differences in pollen parent success in *Pseudotsuga menziesii*. These differences were correlated with seed parent genotype and probably reflect seed losses from specific poor genetic combinations. Poor combining ability may result from common recessive alleles or it might be due to prezygotic factors which are, as yet, not described.

Chapter 3

Pollination mechanism of seed orchard interior spruce

Introduction:

Clonal seed orchards of coniferous species are usually established in areas that are warmer and drier than parent tree native habitats because cone-bud initiation is enhanced by increased temperature during vegetative shoot elongation (Ross, 1989). Extreme temperature or drought may however adversely affect the pollination mechanism. A feature of the pollination mechanism common to many conifer genera is the pollination drop (Doyle, 1945; Dogra, 1964; Singh, 1978; Owens and Blake, 1984; Tomlinson, 1994). In *Picea*, pollen grains enter receptive cones and stick to microdrops on the micropylar arms of the ovules. Pollen then moves into the ovule through interaction with the pollination drop which exudes from the micropylar canal (Sarvas, 1968; Owens and Molder, 1979a; Ho, 1984; Owens et al., 1987). Rainwater can transport pollen into the ovule of *Pinus taeda* L. prior to the time of pollination drop secretion (Greenwood, 1986; Brown and Bridgwater, 1987). Pollination involving rainwater rather than a pollination drop has not been reported in *Picea* but this aspect of the pollination mechanism is under investigation.

Seed cones of interior spruce (*Picea glauca* (Moench) Voss or *P. engelmannii* Parry and their hybrid in British Columbia) are receptive for pollination in the spring during the period when the ovuliferous scales elongate and reflex to reveal the ovules and the cone axis. The exact time of pollination drop secretion within the receptive period has not been determined for seed orchard trees although the period of maximum receptivity for pollination has been determined by correlational studies in which seed production was related to the time of controlled pollinations (Webber, 1991). Pollinations done when the

ovuliferous scales were fully reflexed resulted in the highest seed set so the presence of pollination drops at this stage was assumed.

Little is known about the occurrence or persistence of pollination drops in *Picea* growing under field-conditions as their in situ detection is difficult without destructive sampling of seed cones. In experiments using potted grafts of *P. engelmannii*, Owens et al. (1987) found that pollination drops were absent or small and did not persist when trees were moisture stressed to leaf water potentials of -1.2 to -2.2 MPa. Leaf water potentials this low could occur under the hot, dry field conditions common in the southern interior of British Columbia during spring-time. Our concerns were that successful pollination would not occur and that supplemental mass pollination could not be effective if pollination drops were absent or had withdrawn even though seed cones remained receptive in appearance. Low pollination success is a major factor in the low seed set which is commonly observed from open pollinated spruce (Nienstaedt and Teich, 1972; Owens and Molder, 1984a).

The general objective of this study was to compare seed cone external morphological stages (as described by Owens et al., (1987)) with the time of pollination drop secretion for interior spruce growing in a seed orchard. The results serve as a guide for correct timing of supplemental pollen application. Specific considerations were: i) to determine persistence of the various seed cone stages from pre-, through post-receptivity; ii) to correlate developmental changes in ovule integuments (micropylar arms) with the time of pollination drop secretion; iii) to determine the longevity of pollination drops, and; iv) to determine the time (if any) beyond pollination drop withdrawal during which ovuliferous scales remain reflexed even though seed cones are no longer receptive for pollination.

Materials and Methods

Clone selection and preparation

Interior spruce from the Quesnel lakes region of British Columbia were established by grafting in a clonal seed orchard at the Kalamalka Seed Centre near Vernon, British Columbia. Clones selected for the study were either eight or eleven years from grafting. Prior to bud burst, in late-April 1993, all ramets in the seed orchard were surveyed and one ramet from each of eight clones was chosen for study. Ramets with abundant seed cone buds distributed throughout their crowns were selected. Pollen-cone buds were removed from three branches of each ramet and paper pollination bags were placed, plastic window side down, over the seed cone buds to prevent pollination. At least 20 seed cone buds were enclosed by each bag. Three other branches were selected on each ramet and tagged but all cone buds were left in place and branches were left unbagged. Branch selection was made such that bagged and unbagged branches were distributed evenly around the crown of each ramet. Bud development was monitored daily until bud burst which occurred during the last few days of April for the majority of ramets selected.

Cone sampling procedure

Between May 2 and 13, 1993, cones were sampled once per day for two days then twice daily for the remaining ten days. Sampling times, with a few exceptions, were at 0600h and 1400h. At each sampling, two cones were collected from each ramet, one cone from within a pollination bag and one from an unbagged branch. Cones were selected from different branches in consecutive samples. Outliers, those cones which were far more advanced or retarded relative to the majority on the branch, were avoided in an attempt to minimize sampling of within-crown variation. Cones were placed individually into plastic

bags which contained a moistened paper towel and the bags were stored in an ice-cooled container for transport to the lab. Analysis began within 15 minutes of cone collection and generally required 4 to 6 hours for each set of 16 cones.

Cone and ovule analysis

Cone and ovule morphology were observed and scored using the quantitative index of development of Owens et al. (1987). Table 4 shows the six stages of development and the criterion used for scoring the developing cones. The most important criteria for estimating cone receptivity based on external morphology were the size and degree of reflexion of the ovuliferous scales. By visual estimation, cones were placed within one of the six developmental stages and when the stages persisted for several days were sub-categorized as early, middle or late within the stage. Throughout the study, representative cones were photographed. Ovules were sampled from dissected cones. The basal, mostly sterile, 5 mm of each cone was removed and discarded. Several days into the procedure, we realized that the presence or absence of pollination drops could be evaluated in situ by inverting cones and examining the ovules immediately distal to the cut surface without further initial dissection. This technique proved indispensable because pollination drops were usually destroyed when ovuliferous scales were removed from the cone axis for observation. Ovules were observed for pollination drops in undissected cones and then ovuliferous scales were removed from the cone axis for more detailed observation of the ovule integuments (micropylar arms) using the dissecting microscope. Several forms were possible for any pair of micropylar arms: they could be i) short or elongate, ii) closed or splayed, iii) turgid or withered, and iv) sticky microdrops could be present or absent. Presence or absence of pollen grains adhering to the micropylar arms was scored in unbagged cones. One ovule from each of 20 ovuliferous scales was sampled per cone.

Table 4. Stages of seed cone receptivity for pollination as described by Owens et al. (1987)

Cone stage	Ovuliferous scale development	Cone growth and receptivity
Stage 1	slightly longer than bracts but remaining appressed to cone axis	pre-receptive
Stage 2	much longer than bracts, beginning to reflex but not wide enough to admit pollen	elongating and broadening but pre-receptive
Stage 3	almost fully reflexed	elongating and becoming receptive
Stage 4	fully reflexed	elongation has ceased, maximal receptivity
Stage 5	beginning to close acropetally	becoming post-receptive
Stage 6	closed	post-receptive

This represented 10-15% of ovules per cone when the basal and distal generally sterile ovuliferous scales were omitted. During the study approximately 7500 ovules were observed.

Environmental measurements

Ambient air temperature was measured at each collection. Temperature measurements were corroborated by weather data supplied through the British Columbia Ministry of Forests Protection Branch. Data recorded hourly by Ministry of Forests monitoring stations included, temperature, relative humidity, wind speed and direction, and amount of precipitation. Heat sums were calculated as the number of hours during which the temperature remained above 10°C after ovuliferous scales began to elongate at bud burst.

Shoot water potential was measured periodically with a portable pressure bomb to compare the water status of unbagged branches with that of bagged branches.

Pollen cloud density was measured independently by British Columbia Ministry of Forests personnel using pollen monitoring stations located within the seed orchard.

Sample preparation for SEM

Several ovules sampled from each cone were fixed in Navashin's fixative (Berlyn and Miksche, 1976) with vacuum infiltration. At a later date, selected fixed ovuliferous scales were removed, dehydrated in a graded series of ethanol, critical point dried, gold-coated and mounted on aluminum stubs for viewing with a JEOL JSM 35U scanning electron microscope operating at 15 Kv.

Observations and results

Stages of seed cone receptivity

Seed cones of interior spruce developed through receptivity from May 2 through May 13, 1993. During the 12 day period, external morphology of the seed cones was as described by Owens et al. (1987) for potted Engelmann spruce grafts (Table 4). In general, cones progressed very rapidly from bud burst through the first three stages in which the cone axis and ovuliferous scales elongate. As the ovuliferous scales continued to elongate in late stage 3, they reflexed to allow pollen access to the ovules. Stage 4 of seed cone receptivity, in which ovuliferous scales remained maximally reflexed (fig. 6), tended to be a protracted stage prior to rapid acropetal cone closure in stage 5 of the post-receptive period (fig. 7). The mean number of days from bud burst to maximum receptivity was 4.8 ± 1.6 for all cones. Bagged and unbagged cones remained maximally receptive for 3.8 ± 1.5 days and 5.3 ± 1.6 days, respectively (table 5). The onset and duration of receptivity were also calculated in terms of heat sums. Maximum receptivity lasted 100 ± 31 hours above 10°C (table 5). Cone closure required 2.4 ± 1.4 days. When the stages of seed cone receptivity are plotted over time (fig. 14) roughly sigmoid curves result because of the persistence of stage 4. The data represented in fig. 14 were fitted with third order polynomial curves to highlight this trend. Cones of three of the eight clones studied did not develop in this fashion. Cones of clone 4752 (fig. 14d) persisted in a semi-closed early stage 5 condition for three days during which only the distal region was receptive. Cones of clones 4835 and 4777 (fig. 14 g and h) progressed rapidly from bud burst to cone closure and this is reflected in a more linear plot of the receptivity stages over time.

Table 5. Duration of seed-cone stages during the 12-day study period

Clone #	number of days per cone stage(s)			#hours >10°C during stage 4 *	
	pre-receptive	receptive		unbagged cones range	total
	all cones	bagged cones	unbagged cones		
	(stages 1-3)	(stage 4)	(stage 4)		
1879	4.0	5.2	5.5	36-133	97
1901	3.5	5.4	8.0	27-181	154
1920	4.5	3.1	6.0	45-167	122
1926	4.5	5.4	5.5	45-145	100
1944	4.0	4.2	6.5	36-157	121
4752	4.0	3.0	4.0	36-105	69
4777	8.5	2.0	3.5	113-193	80
4835	5.0	1.8	3.5	54-113	59
mean	4.8±1.6	3.8±1.5	5.3±1.6		100±31

* Hours, numbered from the onset of stage 1, during which the temperature was above 10°C while cones remained receptive.

Micropylar arm development

Each ovuliferous scale has two adaxial ovules located on either side of the point where the scale joins the cone axis (fig. 8). Ovules are inverted so that micropyles are in proximity with the cone axis and open downwards while the cone stands erect during pollination. The micropylar arms are extensions of the ovule integument. They become covered in sticky microdrops and capture windborne pollen. During bud burst, the micropylar arms were elongate and turgid and remained tightly together. They remained in this condition through stages 1-2 as the cone axis and ovuliferous scales began to elongate (fig. 9). While the micropylar arms remained tightly appressed the micropyle was sealed. As the ovuliferous scales began to reflex during stage 3, the micropylar arms began to spread apart. Micropylar arms spread directly apart or twist apart in a scissors-like manner, depending on space constraints within the cone. This results in opening of the micropyle (fig. 10). It was at this stage that pollen grains were first observed adhering to the inner surfaces and margins of the micropylar arms in unbagged cones. The presence of sticky microdrops on the inner surfaces of the micropylar arms was inferred at this stage because of the adherence of pollen and because these surfaces appeared shiny compared with other integumentary surfaces when examined by dissecting microscopy (Owens et al., 1987). While cones remained receptive during stage 4, the micropylar arms began to wither (fig. 11). Withering was usually first evident at the tips of the micropylar arms and progressed basally over the course of 3-4 days until they were completely desiccated (fig. 12). During this drying process, pollen continued to adhere to the micropylar arms and was therefore brought into closer proximity with the micropyle. As cones entered early stage 5 and began to close in their basal regions, the micropylar arms had often deteriorated to the point where only a rim of tissue remained around the micropyle. Micropylar arm withering proceeded

acropetally within a cone; when withering micropylar arms were observed in the basal part of a cone, those in the distal region very often were still splayed and turgid.

Pollination drops

Pollination drops were not observed until cones reached late stage 4 or early stage 5 and began to close. By this time, the micropylar arms had withered or completely deteriorated. The time of pollination drop appearance for both bagged and unbagged cones of each clone can be read from the graphic above each plot in fig. 14. First appearance of pollination drops was within the basal closing region of a cone. As the ovuliferous scales in the basal region of a cone began to bend upwards, closing the cone, their ovules and the ovules of ovuliferous scales in the immediately distal 2-3 whorls exuded pollination drops (fig. 13). Secretion of pollination drops proceeded acropetally, preceding ovuliferous scale closure along the cone axis. From the first sign of basal cone closure, complete cone closure usually required no more than one day and on occasion as little as 1 to 2 hours. In extreme cases, pollination drops were observed along the entire length of the cone axis in cones which were only closed slightly in their basal regions. These cones had been enclosed by pollination bags and closed within 1 to 2 hours of being picked whereas open cones without pollination drops remained open, or closed only slightly in their basal regions, after being picked. Pollination drops persisted within closed cones.

In all but clone 4777, pollination drops were observed much earlier in bagged cones and they persisted much longer than those within unbagged cones. Very often, pollination drops did not appear within unbagged cones until after cone closure and they were smaller than those found within bagged cones.

Environmental factors

Mean daily maximum temperature at Kalamalka Lake during the collection period was 21.2 ± 7.6 °C and the mean daily minimum temperature was 7.6 ± 4.7 °C (fig. 15). Temperatures of note include a low of -2.6 °C during the night of day five of collections and daytime highs surpassing 32 °C during the last three days of the collection period. The southernmost limit of the parent tree natural range is 230 km north of Kalamalka Lake. During the 12 day collection period the mean daily maximum temperature there was 18.8 ± 7.8 °C and the mean daily minimum temperature was 3.7 ± 4.6 °C. Sub-zero temperatures (low of -3.4 °C) were recorded in the parent tree natural range during the first three nights of the collection period.

The natural pollen cloud density (fig. 15) reached 4.7 pollen grains per mm^2 during day six of the collection period, decreased somewhat during the relatively windless days 7-10 and increased to a second peak of 3.8 pollen grains per mm^2 during a windy day 11.

During the hottest days of the collection period, shoot water potential measurements on unbagged branchlets showed them to be under no water stress relative to branchlets enclosed by pollination bags. Branchlet water potentials ranged from morning values of -0.25 MPa to afternoon values of -0.5 to -0.7 MPa. Relative humidity measurements were not made but condensation was observed within pollination bags even during the hottest afternoons.

Figures 6-13

- Figs. 6-7 Seed-cones of interior spruce.
- Fig. 6 Stage 4 cone in which ovuliferous scales (OS) are fully reflexed. Bar=0.5 cm.
- Fig. 7 Stage 5 cone which is beginning to close in basal region. Bracts (B) are visible in the distal cone region where ovuliferous scales remain reflexed. Bar=0.5 cm.
- Figs. 8-12 Scanning electron micrographs of ovules of interior spruce.
- Fig. 8 Two ovules (O) are located on the adaxial surface of each ovuliferous scale. Micropylar arms (MA) project into the space between the ovuliferous scale and the cone axis. Pollen (*) are sometimes preserved adhering to the micropylar arms during preparation for scanning electron microscopy. Bar=1.0 mm.
- Figs. 9-12 Changes in the micropylar arms during cone stage 4. Bars=0.5 mm.
- Fig. 9 Closed micropylar arms.
- Fig. 10 Splayed micropylar arms with adhering pollen. The arrowhead indicates the opening of the micropyle.
- Fig. 11 Withering micropylar arms.
- Fig. 12 Withered micropylar arms. In extreme cases, the micropylar arms deteriorate completely leaving a rim of tissue around the micropyle.
- Fig. 13 A stage 5 cone. The line (-PD-) indicates the region where pollination drops occurred relative to the closed region. As cone closure proceeds acropetally, pollination drops are secreted from distal ovules and persist within the closed region of the cone. Bar=0.5 cm.

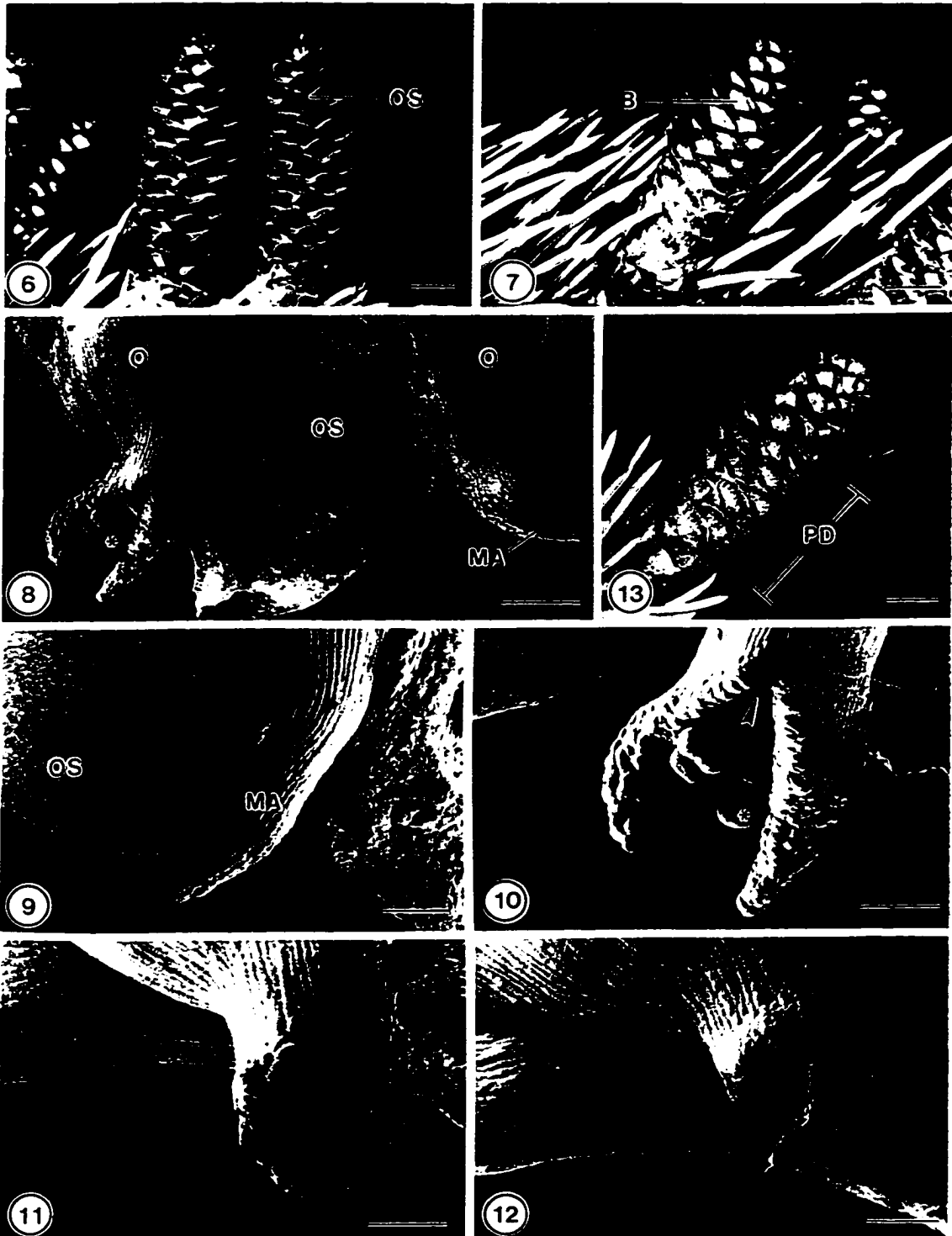
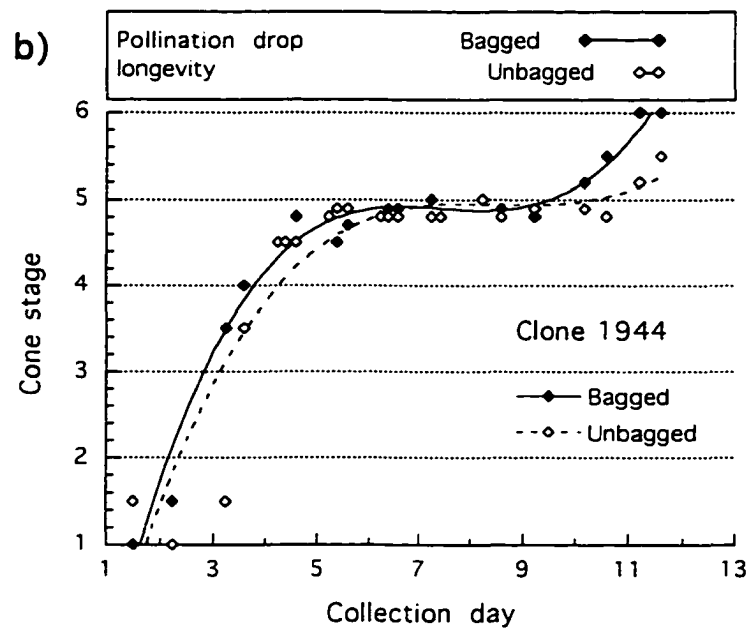
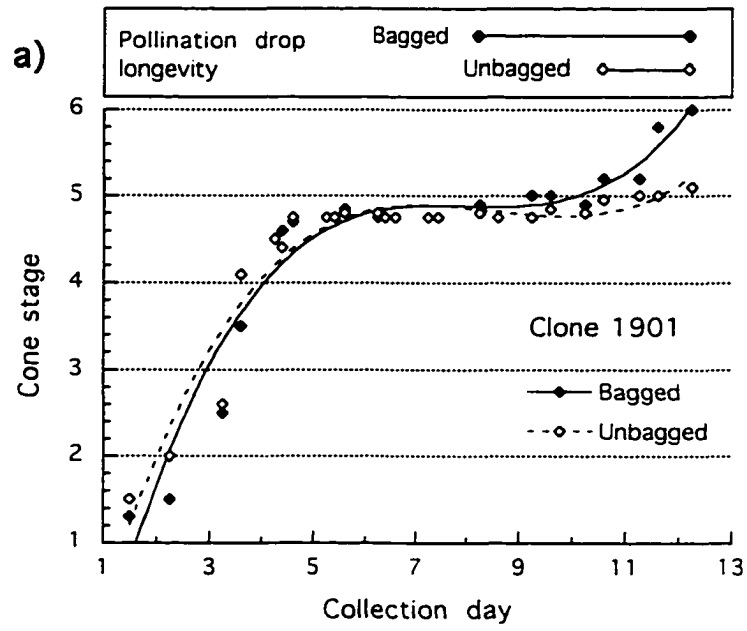
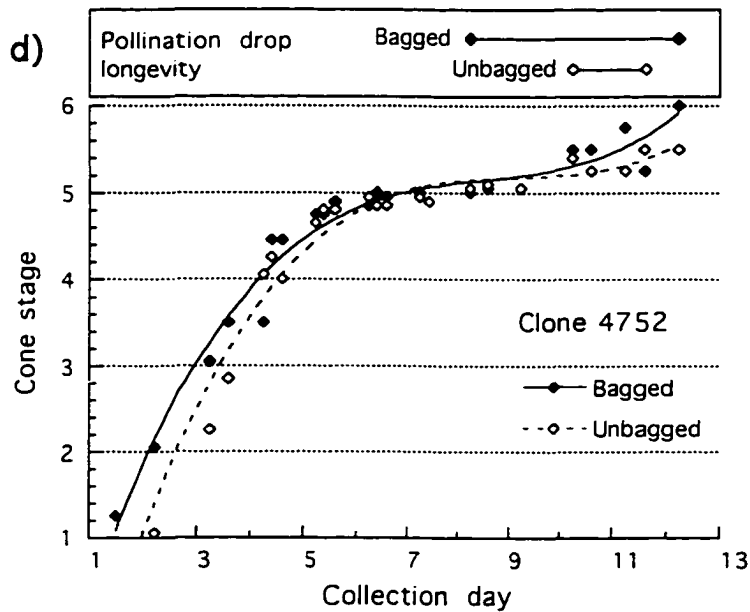
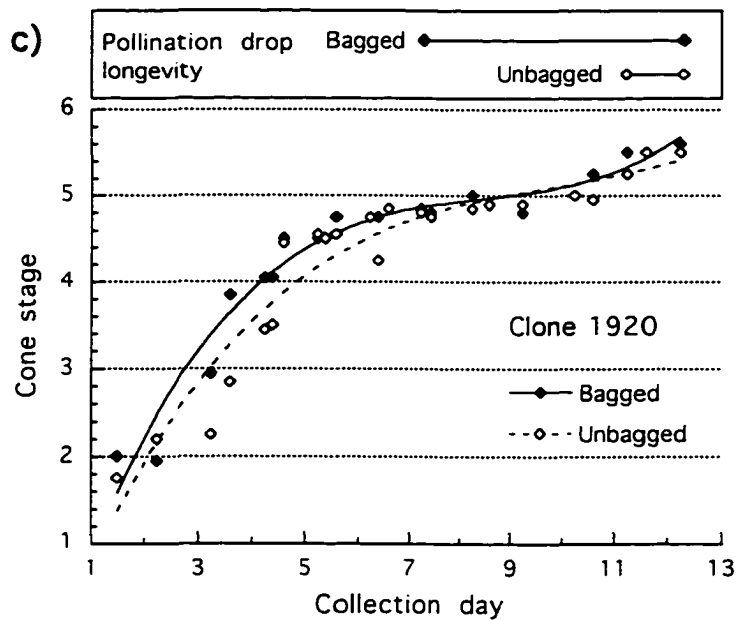
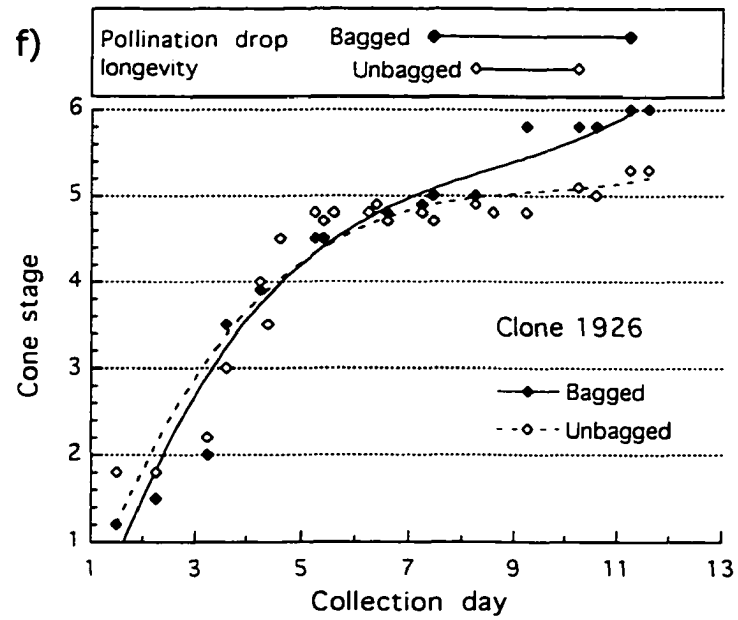
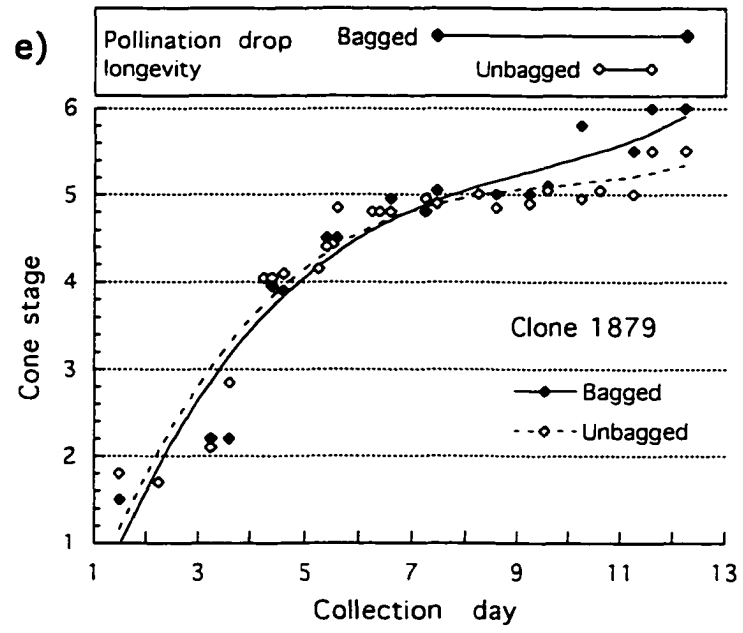


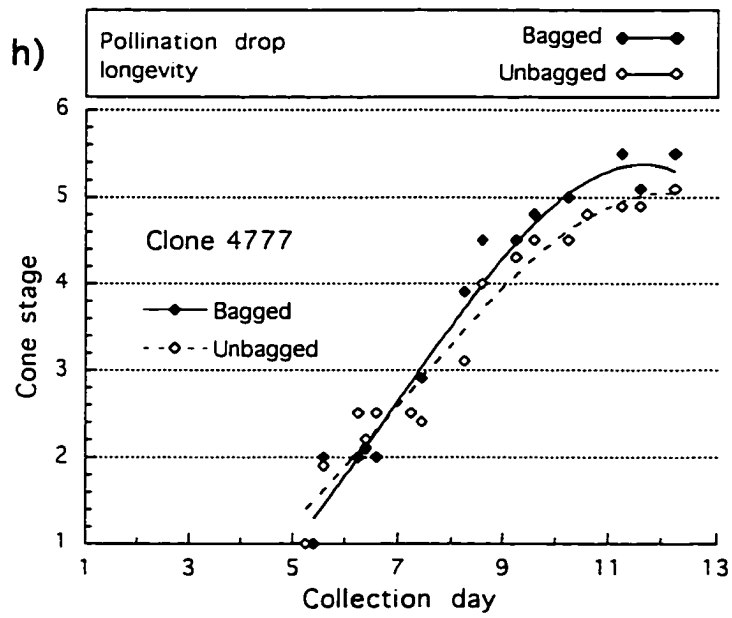
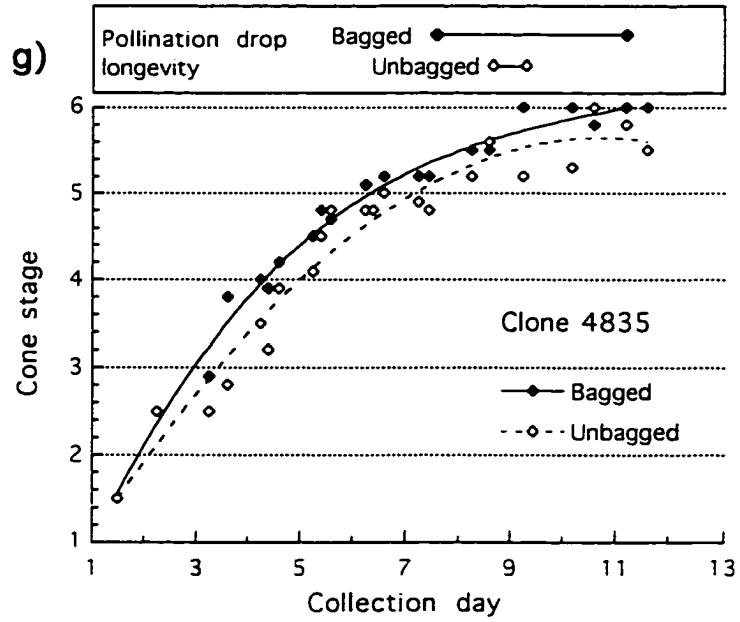
Figure 14

Fig. 14 a-h Seed-cone receptivity stages during the 12-day study period for each of eight clones. Solid lines represent cones enclosed in pollination bags and dotted lines represent unbagged cones. Cones remain open during stage 4 and begin to close at stage 5. The timing of pollination drop appearance and pollination drop longevity can be read from the graphic above each plot.









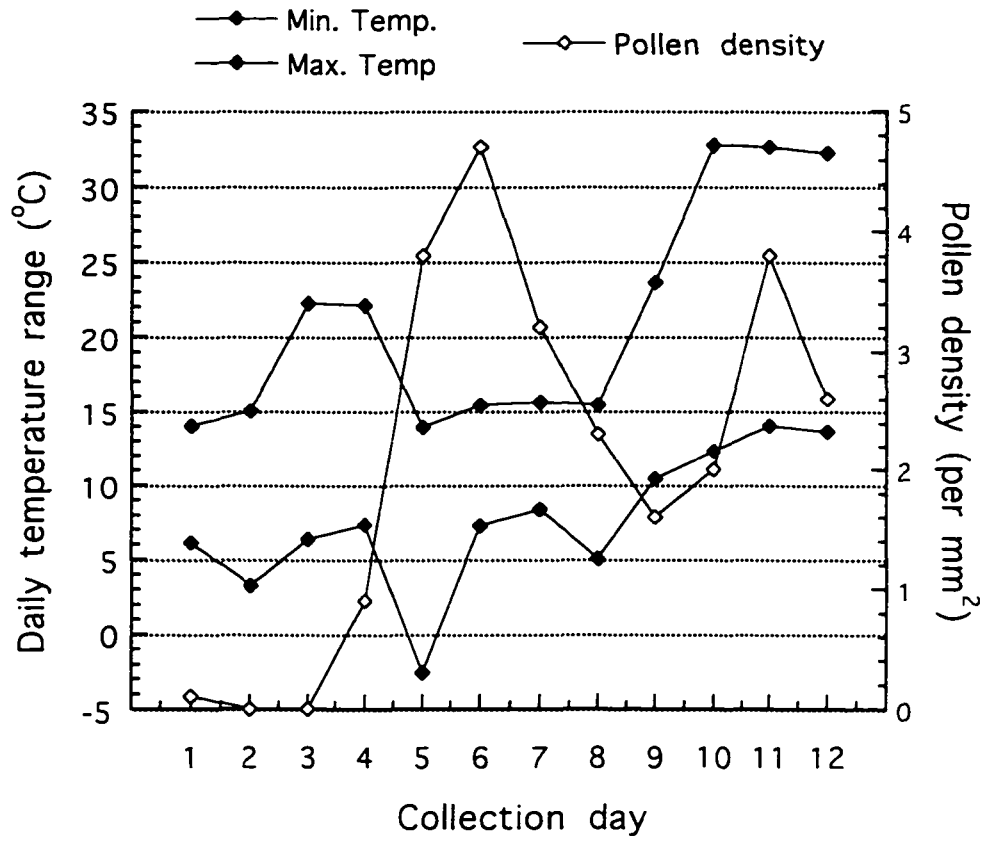


Figure 15. Temperature maximum and minimum and pollen cloud density at the Kalamalka Seed Centre during the 12-day study period, May 2 to 13, 1993.

Discussion

Seed cone receptivity

The pollination mechanism of seed orchard interior spruce has two distinct steps: i) seed cones open and windborne pollen is captured by the sticky microdrops on the micropylar arms, and; ii) at cone closure a pollination drop, which facilitates movement of pollen from the micropylar arms into the ovule, is secreted from each ovule. This entire process, from bud burst to cone closure, required 12 days. Seed cone external morphology followed a well documented developmental pattern during the receptive period (Owens et al., 1987; Webber, 1991). The period of maximum seed cone receptivity, which was called stage 4 by Owens et al. (1987) and the mid-stage of receptivity by Webber (1991), persisted for 3.8 ± 1.5 days in bagged cones and 5.3 ± 1.6 days in unbagged cones between approximately days 5 to 10 of the collection period. Unbagged cones required 100 ± 31 hours at greater than 10°C to pass through stage 4. During stage 4, ovuliferous scales became maximally reflexed and micropylar arms opened for pollen capture. As seed cones entered late stage 3 and early stage 4 the turgid micropylar arms splayed apart to occupy the space created around them by reflexing of the ovuliferous scales. Airborne pollen was captured on the sticky inner surfaces and margins of the micropylar arms. This mechanism of pollen capture on integumentary surfaces has been described for many species of *Picea* (Doyle and Kane, 1943; Owens and Molder, 1979a; Singh and Owens, 1981a; Owens and Blake, 1984; Owens et al., 1987), as well as other genera within the Pinaceae (Doyle, 1945; Dogra, 1964; Owens et al., 1981b; Takaso and Owens, 1995).

Prior to stage 4, seed cones were pre-receptive for 4.8 ± 1.6 days as they developed from bud burst to late stage 3. During pre-receptive stages the ovuliferous scales were not reflexed sufficiently to expose the micropylar arms for pollen capture. Cone closure (stage

5) required 2.4 ± 1.4 days. The duration of pre-, and post-receptive stages did not differ between bagged and unbagged cones. Seed cones of interior spruce appear to maximize the amount of time spent in pollen capture by remaining static in stage 4 for several days. Temperature affects the rate of seed cone development (Owens and Molder, 1979a; Brown and Bridgwater, 1987; Ross, 1989; Webber, 1991). Daily temperature maximums remained below 20°C for several days as the majority of clones became receptive and this may have slowed cone development through receptivity. Development through the receptive stage might be accelerated in a hotter season during which heat sum accumulation would be faster. Seed cones enclosed in bags began to close (early stage 5) prior to unbagged cones on the same tree in all clones studied. Owens and Blake (1984) also observed early closure of bagged cones relative to unbagged cones in *P. sitchensis* (Bong.) Carr. (Sitka spruce). This might result from higher temperatures observed inside the pollination bags at least during the hottest part of each afternoon. In our study, the majority of unbagged cones entered stage 5 and began to close at day 10 of collections, when daily maximum temperatures exceeded 30°C and nighttime temperatures also increased.

Two clones studied (4835 and 4777) had patterns of receptivity which departed from the commonly observed, persistent stage 4 pattern. Clone 4835 was the first clone in which seed cone buds burst and clone 4777 was very late. In both clones, unbagged cones remained at stage 4 for 3.5 days as opposed to 5.9 ± 1.3 days for all other clones. Cones of clone 4835 were in stage 4 at the time of the first pollen cloud peak, whereas those of clone 4777 passed through stage 4 during days 8 to 10 at a time when the pollen cloud density was relatively low.

Pollen cloud density was at a peak during the first three days of this receptive stage for most clones. A second peak in pollen cloud density which occurred 2 days after most clones had become post-receptive may have been due to a vagary of the environment. Windless conditions during days 8 to 10 of the collection period meant that although

microsporophyll dehiscence had occurred pollen-cones did not shed pollen until day 11 when winds provided sufficient branch agitation for pollen release.

Micropylar arm development

Micropylar arms withered and folded inwards as stage 4 progressed with the result that pollen grains adhering to them (unbagged cones) were brought closer to the micropyle. Withering of the micropylar arms has been associated with micropyle closure, after pollination drop secretion, once the receptive period has passed (Doyle and Kane, 1943; Owens and Blake, 1984). In our observations, micropylar arms withered completely prior to secretion of pollination drops. Withering and deterioration of the micropylar arms most likely result from continued secretion of the microdrops to which pollen adhere. Owens and Blake (1984) examined the ultrastructure of micropylar arms in Sitka spruce and found that the secretory cells completely autolyzed as the arms withered. Absorption of moisture by captured pollen may contribute to withering.

Pollination drop occurrence

Pollination drops are required during the second step of the pollination mechanism in *Picea* and have been well described by Owens et al. (1987), (also see Dogra, 1964 for a review of earlier literature). In laboratory experiments with potted Engelmann spruce grafts, Owens et al. (1987) were able to relate the external appearance of seed cones to stages of ovule development, time of pollination drop secretion and the period of maximum receptivity. We had difficulty achieving high pollination success in seed orchard interior spruce (unpublished data) using the same criteria for maximum receptivity. This led us to think that seed cones might develop differently under laboratory and field conditions. We suspected that hot, dry field conditions might adversely affect tree physiology either

resulting in reduced secretion or no secretion of pollination drops. This would reduce the efficiency of the second step of the pollination mechanism in which pollen is transferred from the micropylar arms into the ovule. The timing of pollination drop secretion had not previously been determined in seed orchard *Picea*. Doyle and Kane (1943) thought that in *Picea* this secretion could occur at any time once the ovule was mature. The state of ovule maturation cannot readily be determined in the field.

Our initial suspicion, that pollination drop secretion might be adversely affected by field conditions, was confirmed: pollination drops were not observed while the ovuliferous scales remained fully reflexed in stage 4. Doyle and Kane (1943) described pollination drop secretion in species of *Pinus* as a nighttime phenomenon but claimed that the pollination drop of *Picea* was not so precise; it could occur during daytime and was usually of larger volume than observed in *Pinus*. Owens et al. (1987) observed pollination drops by stage 4 in well watered, potted *Picea*. Observations of several ovules were made at 3:00 am on day seven of our study but no pollination drops were observed. We suspected that removing cones from the trees and transporting them to the lab, even enclosed in plastic bags, might cause the pollination drops to recede more rapidly than in cones left on the trees but this proved not to be the case.

Pollination drops were observed during day seven in the most advanced of the bagged cones which had reached stage 5 and began to close. Pollination drops were secreted initially within bagged cones which were beginning to close in their basal regions and drops did not recede upon removal of cones from branches. Pollination drops appeared in unbagged cones 1.3 ± 1.0 days after those in bagged cones. In unbagged cones, pollination drops were small and occurred only in the region of the cone which was closed or closing and they did not persist within the closed cone. Cone closure and pollination drop secretion seem intimately linked in this set of observations. Only as cones reached early stage 5 and began to close were pollination drops seen. Pollination drop re-

secretion from unpollinated ovules within still receptive cones has been described for *Pinus* (Lill and Sweet, 1977; Owens et al., 1981a; Brown and Bridgwater, 1987). Re-secretion of the pollination drop probably represents diurnal fluctuation in pollination drop volume driven by the interaction between continuous secretion and evaporation and might be observed when environmental conditions allow pollination drop secretion prior to cone closure. Water potential measurements more positive than -1.0 MPa show that even unbagged branchlets were under no significant moisture stress during the study period (Scholander et al., 1965). This suggests that pollination drops within unbagged cones were secreted at the same rate as those within bagged cones but were reduced in volume by greater evaporation.

Many of our observations suggest that the relative humidity is higher inside the pollination bags than outside: condensation was usually seen on the inner surface of the bags and the surface of ovuliferous scales, and when pollen was injected into the bags it adhered to these moist surfaces. Within bagged cones, pollination drops were always more voluminous and persisted longer. High humidity may contribute to pollination drop longevity by slowing evaporation. In bagged cones, pollination drops were sometimes present on all ovules even though cones were just beginning to close. Cones closed rapidly from this point.

Pollination drops occurred eventually in cones from all clones. Pollination drops were always secreted after micropylar arms had withered to some extent. Micropylar arms in bagged cones, in which pollination drops appeared earlier, were slightly withered and bent in towards the micropyle at the time of pollination drop secretion. This seems to be a normal occurrence in the pollination mechanism of interior spruce. In unbagged cones, the micropylar arms were often completely deteriorated by the time pollination drops were secreted. Higher humidity in the bags may have prevented complete desiccation of the micropylar arms as occurred in unbagged cones.

We emphasize that Owens et al. (1987) studied potted trees in a controlled environment and that we have studied seed orchard trees in a hot, dry environment. Both studies are of trees growing in opposite environmental extremes and neither set of results may reflect the natural pollination mechanism of interior spruce. Ovules of Sitka spruce secreted pollination drops while cones remained open in the equivalent of stage 4 (Owens and Blake, 1984). Sitka spruce grow in the relatively cool and moist coastal regions and it is probable that interior spruce trees growing under cooler, more humid conditions in the parent tree natural range would produce pollination drops, like Sitka spruce, while the cones are in stage 4. One function of pollination drops being secreted during stage 4 would be direct capture of windborne pollen as occurs in Cupressaceae and Taxodiaceae (Singh, 1978). This was observed for Sitka spruce (Owens and Blake, 1984) but was not a factor in our study of interior spruce. High humidity within bags may mimic conditions in the parent tree natural range. The hot, dry conditions, in which unbagged cones developed at Kalamalka Lake, caused withering of the micropylar arms to the point where their efficiency in pollen capture would have been reduced. Withered micropylar arms do not present a large surface area for pollen capture and would be incapable of continued secretion of microdrops to which pollen adhere. Both steps of the pollination mechanism of interior spruce; capture of pollen by the micropylar arms, and pollination drop secretion, are adversely affected by the hot, dry conditions at the Kalamalka Seed Centre. Excessively withered micropylar arms and ephemeral pollination drops potentially reduce pollination success and seed set in the seed orchard setting.

The two-step pollination mechanism of *Picea*, in which pollen are first captured by the microdrops on the micropylar arms over a number of days and then are taken into the ovule en masse by the pollination drop, ensures that pollen from many sources has a chance to contribute to the progeny of any ovule. This may not occur if pollination drops were secreted early in stage 4 and captured pollen just entering the cone, then immediately

receded, because not all clones shed pollen at the same time (O'Reilly et al., 1982). There is evidence that in conifer pollination, pollen that is first-on becomes first-in (Franklin, 1974; Owens and Blake, 1984; Ho, 1985) and effects most of the paternity within a cone (Webber and Yeh, 1987) or occupies most of the space within the ovule (Owens and Blake, 1984). As an ovuliferous scale remains reflexed, sites on the micropylar arms become occupied by captured pollen. When pollen is applied artificially (and abundantly) early on, the micropylar arms become fully occupied so that pollen added later is not captured (Franklin, 1974; Greenwood, 1986).

Supplemental mass pollination

Performing supplemental mass pollination (SMP) on a large scale is labour intensive and must be done at the busiest time of year. The danger is that pollen is being applied too early or too late and that great investment in collecting, storing and applying pollen will not show a return. If SMP is to be effective in seed orchards of spruce, pollen must be applied prior to excessive withering of the micropylar arms and prior to pollination drop withdrawal. Our observations show that clones of interior spruce from northern provenances produce pollination drops when established in a hotter, drier seed orchard setting. However, pollination drops are produced only as cones enter early stage 5 and begin to close. Timing of SMP must therefore be based on the condition of the micropylar arms which can quickly and easily be judged in the field with a 5 to 10x hand lens. Optimal time to pollinate will be when the ovuliferous scales are reflexed but while the micropylar arms remain turgid. If micropylar arms are withered, to the point where they no longer function in pollen capture, prior to SMP, then no increase in pollination success or seed set would be realized from SMP. Overhead misting has been used operationally to delay seed cone phenological development in conifer seed orchards (El-Kassaby et al., 1984). Misting has the effect of cooling the trees and retarding bud burst so that seed

cones become receptive synchronously and after the contaminating pollen cloud has passed. Misting used in the same fashion periodically during seed cone receptivity might reduce the accumulation of heat sums and prolong the period of maximum seed cone receptivity while slowing desiccation of the micropylar arms thereby facilitating use of SMP.

Seed yield can be increased by use of SMP in certain situations although reports exist of non-significant increases in seed production over naturally pollinated cones when operational SMP was used (Daniels, 1978; El-Kassaby et al., 1993; Bridgwater and Bramlett, 1982). Operational SMP in these cases was a one-time application of pollen during seasons of high pollen production. These same authors report success using SMP to increase seed yields in years when natural pollen cloud densities are low or in clones that become receptive non-synchronously with the pollen cloud peak (eg. our clones 4835 and 4777). Application of pollen should be made more than once during the receptive period because of natural variation in the timing of seed cone receptivity within and between trees. For maximum seed yield during seasons of low pollen cloud density, pollen application should be made as early as possible and repeatedly once seed cones open: i.e. reach late stage 3 or early stage 4 in our study. Clones that become receptive non-synchronously should be visited on an individual basis once they reach early stage 4. The genetic quality of seed produced is maximized when SMP is used within seed orchards to increase panmixis by reducing self-pollination or pollination by contaminating sources (El-Kassaby et al., 1993; Eriksson et al., 1994). When SMP is being used to increase the genetic quality of seed, pollen application should be made as soon as possible and repeatedly after spruce seed cones reach late stage 3 or early stage 4 so that applied pollen will occupy the turgid micropylar arms and exclude self and contaminating pollen.

Conclusion

The pollination mechanism of interior spruce seed orchard trees was adversely affected by higher temperature and lower moisture regimes than those of their geographical origin. Slight withering of micropylar arms is a normal feature of the pollination mechanism of interior spruce however, they wither excessively in unbagged cones compared to bagged cones in the seed orchard. Pollination drops are secreted later, and are smaller in unbagged cones. Desiccation of the micropylar arms in the low humidity seed orchard setting probably reduces their pollen capturing efficiency. Pollination drops, although small and short-lived, are produced at cone closure. Any pollen captured on the micropylar arms during cone stages 3 and 4 should be transported into the ovule. Supplemental mass pollination should be useful to increase seed yields in years when natural pollen cloud densities are low or to improve seed quality. We recommend that SMP be used early and repeatedly during the period of maximum receptivity (stage 4). Micropylar arms most likely capture pollen best prior to withering and may not be saturated by self-, or contaminating pollen early in stage 4.

Chapter 4

Pollen scavenging and rain involvement in the pollination mechanism of interior spruce

Introduction

In many conifer genera pollen moves into the ovule by means of a pollination drop. Excellent reviews of conifer pollination mechanisms have been published (Doyle, 1945; Dogra, 1964; Owens, 1993; Chesnoy, 1993; Tomlinson, 1994). Within Pinaceae, all genera have ovules that are inverted at the time of pollination. Most genera have saccate pollen (*Abies*, *Cathaya*, *Cedrus*, *Hesperopeuce*, *Keteleeria*, *Picea*, *Pinus*, *Pseudolarix*), others have non-saccate pollen (*Larix*, *Micropeuce*, *Pseudotsuga*). The role of sacchi (air bladders formed as lateral elaborations of the exine) has been debated. Historically, sacchi were thought to aid in pollen dispersal by wind or to reduce desiccation of the gametophytic tissue (Wodehouse, 1935). Doyle (1945) speculated that sacchi function as floatation devices required for pollination and pollen orientation on the nucellus by genera in which the micropyle is inverted at pollination. Tomlinson (1994) has emphasized the correlation between pollen morphology and the method of pollen capture and has confirmed floatation as the primary function of sacchi. It has been reported that pollen captured by a pollination drop either floats directly into the micropyle (Doyle, 1945; Tomlinson, 1991) or is entrained by the drop and enters the micropyle when the drop recedes (McWilliam, 1958; Owens, 1993; Tomlinson, 1994).

Saccate pollen and inverted ovules are considered basal (ancestral) conditions for Pinaceae (Doyle, 1945; Singh, 1978). From these considerations, the pollination drop as ancestral in Pinaceae has been inferred (Doyle, 1945) but no discussion of the selective forces which would have directed its evolution has been published.

In this paper we present new observations on the pollination drop mechanism of interior spruce (Engelmann spruce (*Picea engelmannii* Parry) or white spruce (*Picea glauca* (Moench) Voss) and their hybrid in British Columbia). In particular, we extend the observations of Runions et al. (1995) to show that interior spruce can 'scavenge' pollen from the bract and ovuliferous scale bases and seed cone axis in a manner analogous to some genera of Podocarpaceae (Tomlinson, 1991). Pollen floatation and pollen scavenging are demonstrated using physical models.

Our observations in the field and of experimental models suggest that rain plays a part in the pollination of interior spruce. This combined with various reports of rain involvement in the pollination of *Pinus* (Greenwood, 1986; Brown and Bridgwater, 1987), *Cedrus* (Takaso and Owens, 1995), and *Thuja* (Colangeli and Owens, 1990) suggests that the conifer pollination drop may not be ancestral but derived from a pollination mechanism in which rainwater was required for pollen delivery to the nucellus.

Materials and Methods

Observation of pollination drops

A detailed description of the procedure used for observation of pollination drops in interior spruce can be found in Runions et al. (1995). Trees studied were eight or eleven years from establishment as grafts in a clonal seed orchard at the Kalamalka Seed Centre near Vernon, British Columbia. One ramet from each of eight clones was chosen. During the natural pollination period, May 2 to 13, 1993, seed cones were sampled once per day for two days and twice daily for the remaining ten days. Sampling times, with a few exceptions, were at 0600h and 1400h. At each sampling, two seed cones were selected from each ramet. Cones were placed individually into plastic bags which contained a

moistened paper towel and the bags were stored in an ice-cooled container for transport to the lab. Analysis began within 15 minutes of cone collection.

Cones were sectioned transversally or longitudinally by hand and examined by dissecting microscope for presence of pollination drops. When pollination drops were observed, their size was recorded (small, medium or large) and the receptivity stage of the cone was noted (Owens et al., 1987). During this part of the procedure, approximately 7500 ovules were observed. Representative cones and pollination drops were photographed during this initial analysis. Whole cones and cone segments were fixed in Navashin's fixative (Berlyn and Miksche, 1976) with vacuum infiltration and preserved for later examination by light and scanning electron microscopy.

During the pollination period of 1994, seed cones and branches bearing seed cones were collected from potted grafts of interior spruce, supplied by the Ministry of Forests, Glyn Rd., Victoria, British Columbia. Samples were transported in ice-cooled plastic bags to the University of Victoria for observation and experimentation. When intact pollination drops were observed in dissected cones, they were photographed using a compound microscope with an obliquely placed light source.

Pollen floatation

Two interior spruce seed cones at the pollination drop stage were longitudinally sectioned by hand so that pollination drops remained intact. Cone sections were held vertically to mimic cone orientation at pollination and dry pollen was dusted over the exposed pollination drops. Saccate pollen of *Picea* was dusted onto one cone segment and non-saccate pollen of *Pseudotsuga* was dusted onto the other. The immediate result of contact between drop and pollen was recorded in each case.

Dry saccate pollen (*Picea*, *Pinus*, *Podocarpus*) and dry, non-saccate pollen (*Pseudotsuga*) was dusted onto the surface of a drop of water exuding from the openings

of two different water filled glass tubes. Pasteur pipettes (inner diameter = 1.2 mm) and 10 μ l pipettes (inner diameter = 0.5 mm) were used in various orientations from vertical to 45° above horizontal to simulate inverted micropyles which have an inner diameter of approximately 0.2 mm. A dissecting microscope placed on its side was used to monitor pollen from the time it contacted the drop.

For purposes of photography, a film of water approximately 1.0 mm thick was created between two microscope slides which had been sealed on three sides with dental wax. Saccate pollen was dusted onto the water at the exposed end of the slide and this end was sealed. Pollen floatation was observed by orienting the slides vertically. Photography of floating pollen was done in a dark room with the camera shutter held open while a flash unit was fired once per second. Pollen floatation speed was calculated by measuring change in position per time from photographic negatives ($n = 5$ observations / grain \times 100 grains).

Pollen scavenging

Inverted micropyles were simulated using water columns in glass tubes by equipping Pasteur pipettes with tissue paper wicks which protruded from the wide end. Pipettes with wicks were filled with distilled water. Before use, pipettes with wicks were placed in a beaker of water and placed under vacuum to remove air bubbles trapped in the tissue paper. A drop of water could be made to project from the narrow end of the pipette by careful addition of a small volume of water to the wick. The same drop could be made to recede into the pipette by touching the wick briefly with dry tissue paper.

Pipettes so equipped were clamped into place so that the opening was 3-5 mm above a piece of flat dental wax onto which dry pollen had been dusted. Pipettes were clamped at various angles from 45° to vertical above the plain of the dental wax. A large pollination drop was simulated by addition of several drops of water to the pipette wick.

The drop emerged from the pipette opening and made contact with the pollen covered wax surface while remaining continuous with the water column within the pipette. Over the next 20-30 minutes the drop was made to withdraw by periodically, briefly touching the wick with a dry piece of tissue paper. Two sets of experiments were conducted; one with saccate pollen of *Picea* and one with non-saccate pollen of *Pseudotsuga*. Each experiment was photographed at four stages, i) prior to drop exudation, ii) at drop contact with the wax surface, iii) during drop withdrawal, and iv) once continuity between the exuded portion of the drop and the water column within the glass tube had been broken.

Simulation of rain

A branch of interior spruce bearing eight seed cones which were at the pre-pollination drop stage with ovuliferous scales reflexed was clamped so that the cones remained erect, mimicking their orientation at pollination. Cones were artificially pollinated three times from different directions using a compressed air device to direct 0.3 ml amounts of pollen at the branch. Two control cones were protected and left unpollinated so that the degree of natural pollination, prior to branch collection, could be assessed. Once pollinated, three of the cones were enclosed by a plastic bag to keep them dry. The branch was sprayed with water in a fine mist from several directions at a distance of 30-50 cm. Misting was from at least 45° above the cones in an attempt to simulate a light rainfall (1 to 2 mm). After misting, the plastic bag enclosing the three dry cones was removed and the branch was allowed to dry for two hours. When dry, cones were carefully removed from the branch and sliced tangentially to reveal pairs of micropylar arms. Within cone pollen distribution was studied for three cases, i) unpollinated controls, ii) cones pollinated and protected from mist and, iii) cones pollinated and misted.

Staining of intact seed cones

Freshly collected branches of interior spruce bearing pre-pollination drop seed cones with reflexed ovuliferous scales were clamped so that cones were erect. A pipette was used to add 0.05% aqueous toluidine blue (CI 52040) to the ovuliferous scales in the basal one-quarter of several cones. After 30 minutes, ovuliferous scales in the distal part of the cones were examined for the presence of stain. The experiment was repeated using cones that had been stored for one year in Navashin's fixative (Berlyn and Miksche, 1976).

Freshly collected cones were submerged in 0.1% aqueous phosphine 3R (CI 46045) for five minutes, rinsed in tap water and tangentially sectioned for viewing by epifluorescence. Only hydrophilic surfaces, those free of wax or cuticle, stained by this procedure.

Sample preparation for SEM

Seed cones of interior spruce which had been fixed and stored in formalin-acetic acid-ethanol were dissected and ovules were dehydrated in a graded series of ethanol, critical point dried and gold coated. Specimens mounted on aluminum stubs were viewed with a JEOL JSM 35U scanning electron microscope operating at 15 kV.

Observations and results

Pollination drop in *Picea*

Seed orchard trees (1993) and those growing in pots (1994) produced pollination drops as seed cones began to close (cone stage 5, see: Owens et al., 1987) (fig. 16). Bract and ovuliferous scale insertion on the cone axis is spiral. Each ovuliferous scale bears two ovules which are inverted in the erect cone at the time of pollination. At cone closure, sealed spaces were formed within cones. Spaces were bordered by the bract and ovuliferous scale bases and occurred in a spiral around the cone axis (fig. 17). Two micropyles, one each from adjacent ovuliferous scales, projected downward into each space (fig. 18). By the time of cone closure, the micropylar arms had withered or deteriorated to the point where only a rim of tissue surrounded the micropyle opening. Ovules were inverted but micropyles were not always vertical in erect cones; they projected into the enclosed spaces at various angles from nearly horizontal to vertical. Most commonly, micropyles opened downwards at an angle between 45° and 90°. Pollination drops began to exude from micropyles while ovuliferous scales remained reflexed. Cones rapidly (< 1 day) closed completely and pollination drops became voluminous enough to swell beyond micropyle rims and contact the cone axis within enclosed spaces (fig. 19). After dissection, pollen was observed within ovules which had had pollination drops and distributed around micropylar openings but not within ovules which had not secreted pollination drops. When cones had been completely closed for more than 24 hours, remnants of pollination drops were observed as thin films of liquid within the enclosed spaces but drops continuous with a liquid column in the micropyle were no longer present. At the time of pollination drop secretion, tissue began to proliferate from bract bases into the enclosed spaces (fig. 20). Within 48 hours of cone closure, the tissue had begun to fill

these spaces (fig. 21). Four days beyond cone closure, intracone spaces had become completely filled with tissue (fig. 22).

Pollen floatation

Dry pollen of *Picea* dusted onto a natural pollination drop, which had been exposed by dissection, immediately entered the drop and floated, sacci upwards, to the highest point within the drop. When the micropyle was situated relatively vertically, opening above the drop, pollen entered the ovule or clumped at the opening. Orientation of pollen in the pollen chamber of the nucellus was generally with the sacci towards the nucellar surface (fig. 23).

Similar results were obtained when dry, saccate pollen of *Picea*, *Pinus* or *Podocarpus* was dusted onto the surface of an artificial drop exuded from a simulated micropyle (glass tube). Pollen floated, sacci upwards to the highest point within the drop and congregated. When the glass tube was held nearly vertically so that the exuded drop hung from the opening, pollen entered the tube and floated within the water column to its highest point. Pollen of *Picea* floated, sacci upwards, at 0.7 ± 0.3 mm / sec. (fig. 24). Dry, non-saccate pollen of *Pseudotsuga* sank within drops when dusted onto natural or artificial pollination drops or water in equivalent experiments. Inner diameter of glass tubes did not affect experimental results.

Pollen scavenging

The pollen scavenging experiment with saccate pollen of *Picea* is shown, viewed from above, in fig. 25. Pollen was dusted onto a flat piece of dental wax (fig. 25a). When the water contacted the wax surface pollen immediately floated, sacci upwards, through the drop to its highest point (fig. 25b). In this case, the pipette was oriented at 45° above the

plane of the wax surface and the highest point within the drop was above the opening of the pipette (i.e. the pipette projected into the drop). While the drop was receding, reduction in its volume brought its highest point down to the level of the pipette opening. As this happened, pollen began to float into the opening and upwards within the water column of the pipette (fig. 25c). Eventually, reduction in drop volume overcame cohesive forces between the part of the drop adhering to the wax surface and the water column of the pipette and the drop broke. A remnant drop on the wax surface contained a few pollen grains which had not floated into the pipette, however the majority of pollen which the drop had initially contacted moved to the highest point in the water column of the pipette. A large space, free of pollen, remained on the wax surface where the drop had touched initially (fig. 25d).

An equivalent experiment was done with non-saccate *Pseudotsuga* pollen (fig. 26). Pollen was dusted onto the dental wax surface (fig. 26a). When the simulated pollination drop made contact with the wax, pollen did not float but remained in place on the wax surface (fig. 26b). As the drop receded, pollen was captured by its surface tension and pulled along the wax surface (fig. 26c) but did not float or enter the pipette. All of the pollen originally in contact with the drop was left in the drop remnant, on the wax surface, once the drop had receded (fig. 26d).

Effect of rain

During the natural pollination period of interior spruce in 1993, pollen grains were routinely observed evenly distributed (not clumped) in the region of the micropyle and adhering to micropylar arms of ovules in seed cones which remained open and had not produced pollination drops. Following a brief rainfall on day seven of the pollination period we noticed a change in pollen distribution. Pollen had clumped (sometimes more than 20 grains) and tended to congregate at or in the opening of the micropyle. It was not

possible to determine by dissecting microscope in the field whether pollen had moved deep into the micropyle or into the pollen chamber of the nucellus.

Pre-pollination drop cones which were artificially pollinated had even distributions of pollen on micropylar arms and near the micropyles on the cone axis (fig. 27). Those cones which had been exposed to water spray and allowed to dry had pollen clumped in the micropyle (figs. 28-29). Control cones which were not artificially pollinated did not contain pollen.

Staining of intact seed cones

Aqueous toluidine blue moved upwards around the cone axis when it was applied dropwise to basal, reflexed ovuliferous scales in erect, pre-pollination drop seed cones of interior spruce. This wick effect was observed in whole cones in which the stain appeared at the margins of ovuliferous scales and bracts in the distal part of the cone over a 5 to 15 minute period after addition. Transverse cone sections taken distally had droplets of stain surrounding the micropylar arms after 15 minutes. Stain did not move upwards when the experiment was repeated at a later time with cones which had been preserved in fixative.

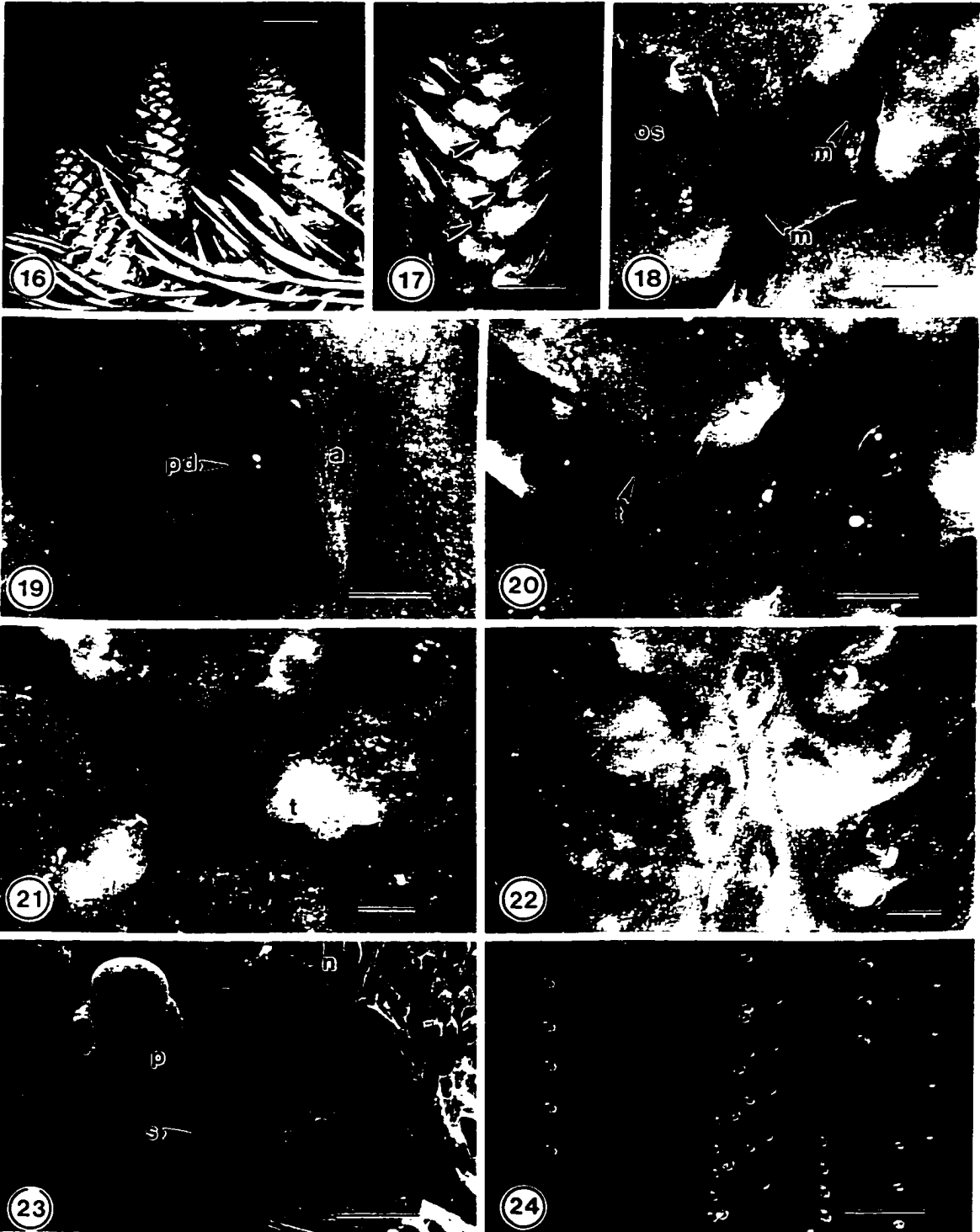
After submersion of intact, fresh cones in aqueous phosphine 3R, longitudinal sections were viewed by epi-fluorescence. Turgid micropylar arms and nucellar tissue stained in pre-pollination drop cones (fig. 30). Fig. 31 shows a pair of ovules on adjacent ovuliferous scales with micropyles projecting into the space that will be enclosed at cone closure. At pollination drop time, the micropylar arms have deteriorated to a rim of tissue which stains. Walls of the micropylar canal did not stain but the nucellus, with its concave pollen chamber, did (fig. 32).

Figures 16-24

- Fig. 16 Seed cones of interior spruce at the time of pollination. The cone on the left has ovuliferous scales fully reflexed and is receptive to pollen. The middle cone is beginning to close in its basal region and the cone on the right is closed and post-receptive. Bar= 1 cm.
- Figs. 17-22 Longitudinal, tangential and median hand-sections of seed cones.
- Fig. 17 A seed cone which is beginning to close in its basal region. Sealed spaces (arrowheads) form between the points of bract insertion on the cone axis once the cone closes. Bar= 5 mm.
- Fig. 18 An enlarged view of a space within a seed cone. At this point, the cone is receptive. Two micropyles (m), one each from ovules on adjacent ovuliferous scales (os), project into each cone space. Bar= 250 μ m.
- Fig. 19 At cone closure, a large pollination drop (pd) was secreted from each ovule into the cone space. Pollination drops are voluminous enough to contact the cone axis (a) and can merge with each other. Bar= 250 μ m.
- Fig. 20 At the time of pollination drop secretion, tissue (t) began to proliferate from bract bases into the enclosed cone spaces. Bar= 500 μ m.
- Fig. 21 Within 48 hours of cone closure, tissue (t) proliferation from the bract bases had begun to fill in the enclosed spaces. Bar= 250 μ m.
- Fig. 22 Tissue proliferation from the bract bases filled the enclosed cone spaces completely within four days of cone closure. (*) Sites of cone spaces filled by tissue. Bar= 100 μ m.

Fig. 23 Scanning electron micrograph of the pollen chamber formed in the distal end of the nucellus (n). The integument which formed the micropylar canal has been removed to reveal pollen (p) in the pollen chamber. Pollen were generally oriented with sacci (s) towards the nucellar surface. Bar= 50 μm .

Fig. 24 Time lapse photograph of pollen floating upwards in a water column. A flash was used once per second for five seconds to produce five images of each pollen grain. Pollen float, sacci upwards, at different speeds. Bar= 500 μm .



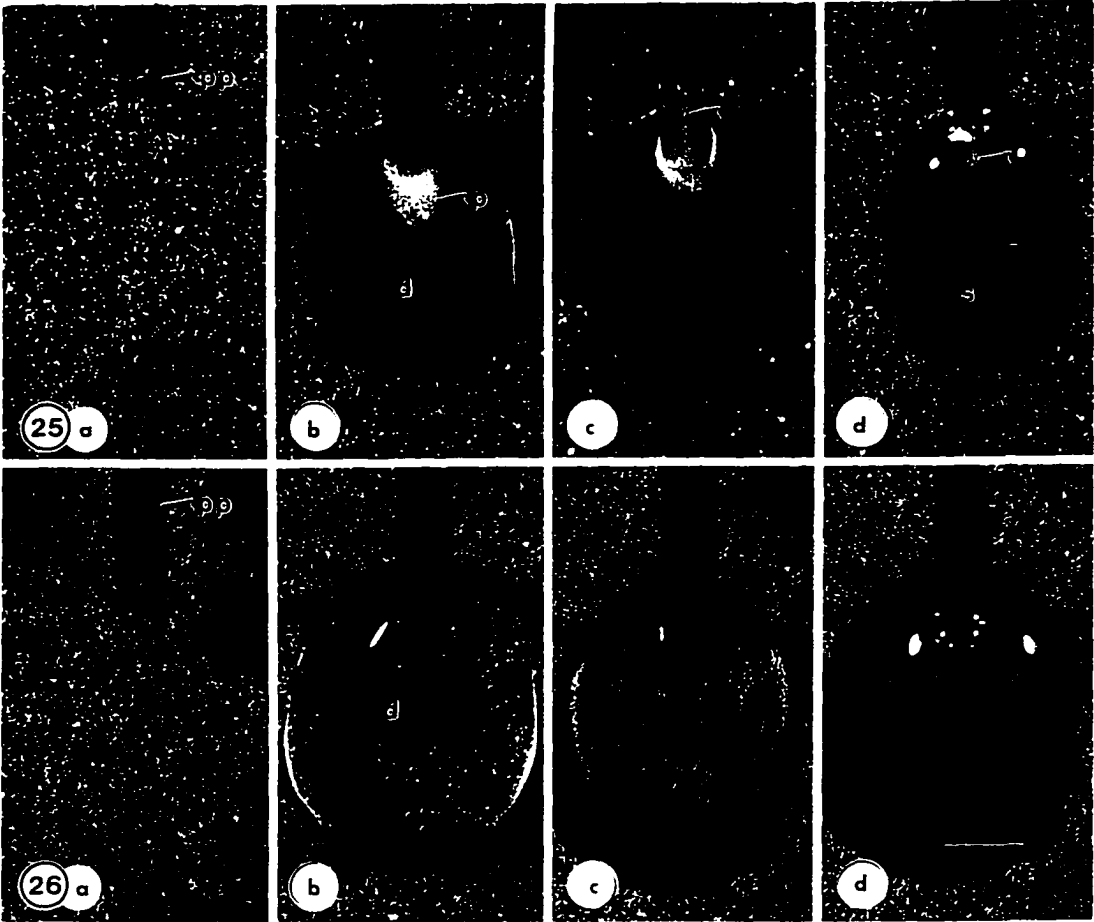
Figures 25-26

Figs. 25-26 Pollen scavenging experiment in which saccate and non-saccate pollen behaviour in water was compared. The experimental set-up is viewed from above in this series. A pasteur pipette was used to simulate a micropyle and positioned at a 45° angle 3 mm above the plain of a wax surface onto which pollen (dots) had been sprinkled. Bar= 2 mm.

Fig. 25 Saccate pollen of Picea.

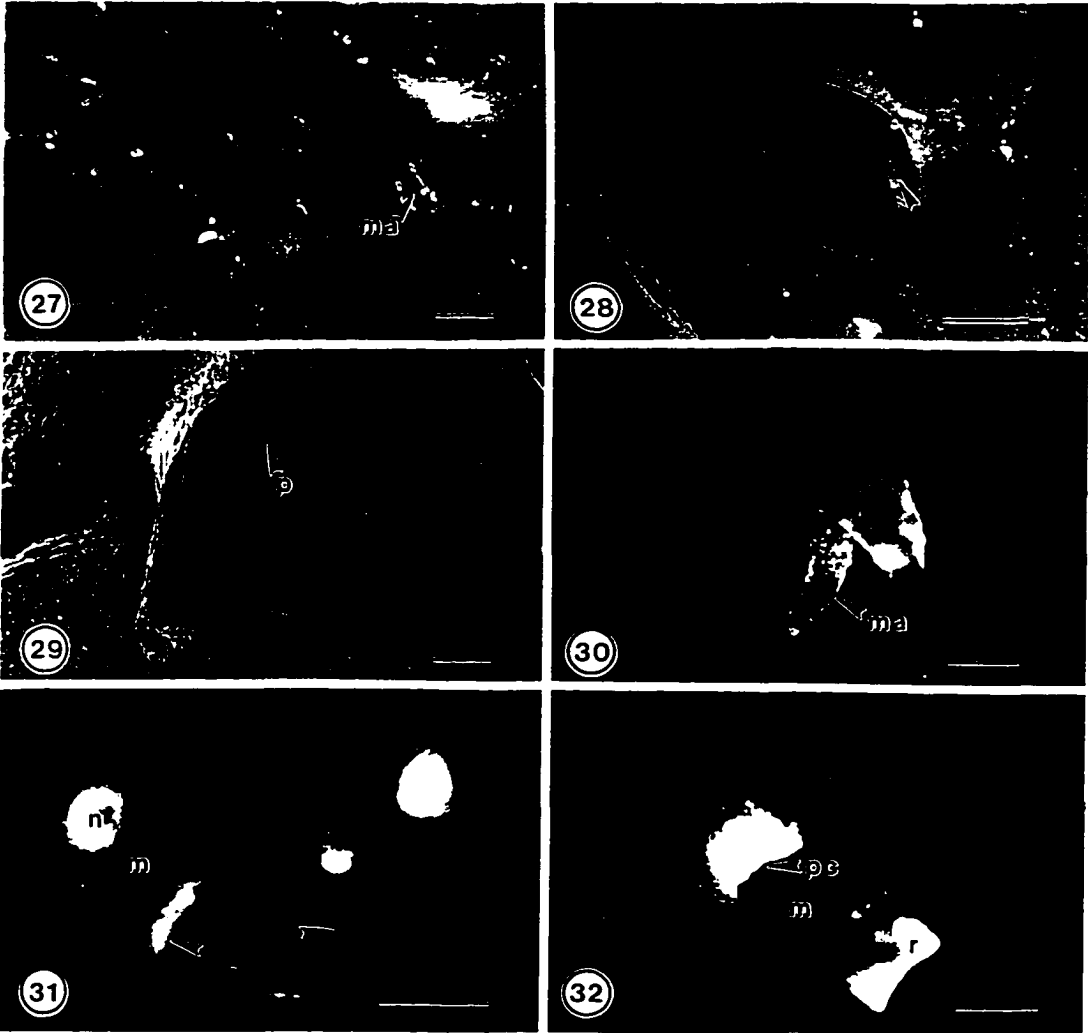
- a Prior to exudation of a simulated pollination drop. (pp) pasteur pipette.
- b Water was made to exude from the pipette and formed a drop (d) continuous between the wax surface and the water column within the pipette. The pipette opening was within the drop. Pollen (p) contacted by the drop floated within the drop to its highest point.
- c As the drop receded, its highest point came down to the level of the pipette opening and pollen floated into the water column and upwards within the pipette (arrowhead).
- d The drop receded to the point where continuity between the water on the wax surface and the water column was lost. Only a small proportion of the pollen originally contacted by the large drop remained in the drop remnant (arrowhead). The majority of pollen contacted by the drop had floated upwards within the pipette leaving a large area of the wax surface free of pollen (s).

- Fig. 26 Non-saccate pollen of Pseudotsuga.
- a Prior to exudation of a simulated pollination drop. (pp) pasteur pipette.
 - b Water was made to exude from the pipette and formed a drop (d) continuous between the wax surface and the water column within the pipette. Pollen did not float within the drop but remained on the wax surface.
 - c As the drop receded, pollen was pulled inwards but remained on the wax surface.
 - d The drop receded to the point where continuity between the water on the wax surface and the water column was lost. All pollen originally contacted by the drop remained within the drop remnant on the wax surface.



Figures 27-32

- Figs. 27-28 Longitudinal hand-sections through receptive (pre-pollination drop) seed cones which were artificially pollinated.
- Fig. 27 Pollen distribution is random on the micropylar arms (ma) when cones are protected from water misting after pollination. Bar= 500 μm .
- Fig. 28 When water was applied as a mist after pollination, pollen floated into the micropyle (arrowhead). Pollen are not distributed randomly on the micropylar arms as in fig. 27. Bar= 500 μm .
- Fig. 29 Scanning electron micrograph of pollen (p) clumped in the micropyle after application of water mist to a pollinated, receptive seed cone. Bar= 100 μm .
- Figs. 30-32 Fluorescence micrographs of ovular tissues stained with aqueous phosphine 3R to show hydrophilic surfaces of seed cones.
- Fig. 30 Micropylar arms (ma) are the only external cone surfaces which stain in receptive seed cones. All other surfaces are cuticularized and hydrophobic. Bar= 250 μm .
- Fig. 31 Two pairs of micropylar arms (arrowheads) project into each space within a receptive seed cone (see fig. 18). The stain has penetrated the length of the micropylar canal (m) to stain nucellar tissue (n). Bar= 500 μm .
- Fig. 32 At the time of pollination drop secretion, the micropylar arms have deteriorated to a rim of hydrophilic tissue surrounding the opening of the micropyle (r). Stain has travelled within the micropyle (m) to the nucellus which at this point has formed a concave pollen chamber (pc) (see fig. 23). The walls of the micropyle are hydrophobic and do not stain. Bar= 250 μm .



Discussion

Pollination drop and pollen scavenging

Development of the seed cone and ovules of interior spruce during pollination was described earlier (Runions et al., 1995). In seed orchard trees and potted grafts, pollination drops are secreted as the ovuliferous scales in the basal part of the seed cone begin to close. Within the seed cone, micropyles open into spaces abaxial to the ovuliferous scale on which they occur. Once the ovuliferous scales close, spaces within a cone form a spiral series of chambers in which pollination drops persist. Ovuliferous scales have waxy surfaces and exude resin at their margins. At cone closure the ovuliferous scale margins meet and stick together sealing these spaces against desiccation. Previously, the pollination drop of *Picea* was described as a drop supported by the micropylar arms (Owens and Blake, 1984; Owens et al., 1987). We have shown that the pollination drop can become much larger than this and can contact surfaces adjacent to the micropylar arms and other drops within the enclosed spaces. Observation of pollination drops has traditionally been facilitated by removal of ovuliferous scales from the cone axis. However, this procedure usually destroys the larger pollination drops which are readily observed when the cones are carefully hand sectioned tangentially.

Secretion of pollination drops is a continual process balanced by evaporation. Cycles of secretion and recession of pollination drops in unpollinated ovules of *Pinus* are reported (McWilliam 1958; Lill and Sweet, 1977; Owens et al., 1981a; Brown and Bridgwater, 1987). The same authors however describe pollination drop persistence under high humidity. When pollen is introduced into a pollination drop, the drop rapidly recedes while unpollinated drops persist (Doyle, 1945; Owens et al., 1987). Pollen entering a pollination drop may effect an immediate end of continuous drop secretion (by an unknown signal) causing rapid drop evaporation in a dry atmosphere, e.g. under a microscope lamp.

However, contact between pollen and the pollination drop of interior spruce did not result in rapid withdrawal of the drop in closed, intact cones. Dissection of ovules revealed pollen on the nucellus two days after cones had closed but pollination drop remnants remained in the enclosed spaces which were becoming filled with the tissue proliferating from bract bases.

Tomlinson et al. (1991) describe pollen scavenging by different genera of Podocarpaceae. In many genera large pollination drops are secreted from inverted ovules and can spread over adjacent surfaces of the bract and cone axis. Pollen present on these surfaces may be captured and float upward into the micropyle. Pollination is thus extended in time and space, i.e. pollen need not land directly on the pollination drop. In *Pinus* and *Picea*, the receptive period is extended by adherence of pollen to the micropylar arms prior to secretion of the pollination drop (Doyle and O'Leary, 1935; Owens et al., 1981a; Owens et al., 1987; Runions et al., 1995). Pollen adherence to integumentary surfaces prior to pollination drop secretion was described by Tomlinson (1991) as constituting a minimal form of pollen scavenging by members of Pinaceae. Our observations of *Picea* now show extensive pollen scavenging. Pollination drops were large enough to collect pollen from surfaces other than the micropylar arms. Reduced evaporation within the enclosed cone spaces resulted in formation of very large pollination drops and pollen need only have been near the micropylar arms on the cone axis to be captured.

Our pollen floatation experiments conducted with artificial micropyles confirm Lill and Sweet's (1977) observation that saccate pollen enters a water drop or column and rapidly floats to the highest point where it remains completely submersed. Pollen floatation speeds measured in our experiment indicated that no more than two seconds would be required after contact with the pollination drop for pollen of *Picea* to float to the nucellus. Pollen of *Podocarpus andinus* Poepp. ex Endl. (Podocarpaceae) behaved in floatation as did the pollen of *Picea*. This is in contradiction with Tomlinson (1994), in which saccate

pollen of *Dacrycarpus* (Podocarpaceae) is depicted floating, sacci outwards, on the surface of a pollination drop without entering it. In the latter case, pollen does not float upwards within the drop: recession of the drop would be required to transport pollen to the nucellus. This conflict calls for a reexamination of pollen-pollination drop interaction in Podocarpaceae before a generalization regarding pollen floatation can be made. Our results confirm Doyle's findings (1945) that pollen floatation and orientation on the nucellus are the most important functions of sacci. Pollination drop recession has a secondary function in pollen scavenging, however. Micropyles are not usually vertical and the high point within a large pollination drop often is above the micropylar opening. In the pollen scavenging experiment, pollen floating in a simulated pollination drop above the pipette opening only floated into the pipette once the water had receded somewhat bringing the highest point within the exposed drop down to the level of the opening. Surface tension during slow drop recession was sufficient to maintain continuity between the secreted portion of the drop and the water column in the pipette at least until pollen entered the pipette. Very few saccate pollen grains remained floating in the drop remnant after drop recession. Pollen stranded outside the pipette was prevented from entering because they adhered to the outer glass wall of the tube. Pollen capture is an imperfect process in situ as well. Individual pollen grains or clumps are often observed adhering to the micropylar arms after pollination drop secretion and recession in closed cones. This problem should be minimized because selective pressures would tend to optimize pollen shape and size for efficient capture by a pollination drop. As well as being floatation devices, sacci may increase adhesion between the surface of a receding pollination drop and the pollen. Adhesion between pollen and the pollination drop increases as does surface area of the pollen in grains of equal density (capillary adhesion; Vogel, 1988) and smooth contours would tend to reduce the drag produced by angular surfaces. Surface area to volume ratio in *Dacrycarpus* pollen might be high enough to produce adhesion sufficient to hold it within

the pollination drop surface as reported by Tomlinson (1994). Sacci size can not increase indefinitely. It is constrained by spatial and weight restrictions as pollen must not increase in size unreasonably or several grains at a time would not fit into a micropyle and the resultant increase in mass would adversely affect wind pollination.

Non-saccate pollen did not float upwards within the simulated pollination drop in the pollen scavenging experiments but remained in place on the wax surface. Non-saccate pollen were pulled up in an inclined, inverted tube, in the receding, artificial pollination drop only if they were placed directly into the meniscus within the tube. The pollination mechanism of Podocarpaceae, which effectively selects saccate pollen over non-saccate pollen and non-floating contaminants, has been called an exclusion mechanism by Tomlinson (1994). However, Lill and Sweet (1977) found non-saccate pollen in *Pinus* ovules. Our experimental result suggests that non-saccate pollen would only be taken into an inverted ovule if it landed directly on a receding pollination drop. Non-saccate pollen adhering to the micropylar arms of *Picea* prior to pollination drop secretion would be washed to the bottom of the enclosed space and remain in the drop remnant as the drop receded. The pollination mechanism of *Picea* functions as an exclusion mechanism in this case. McWilliam (1958) reported that *Pinus* pollen occurred on the nucellus with no particular orientation. We found by careful dissection of *Picea* cones that pollen grains were roughly oriented with sacci towards the nucellus. Saccate pollen grains commonly germinate in their proximal hemisphere i.e. nearer to the sacci (unpublished results). Physical constraints like crowding and clumping during floatation meant that pollen was not always oriented in this manner but it seems that selection has favoured a mechanism that orients pollen within the ovule for germination towards the megagametophyte in the absence of physical constraint.

Within four days of cone closure in *Picea*, the spaces that micropyles projected into became filled by a proliferation of parenchymatous tissue from bract bases. This tissue

may function to seal the micropyle and prevent desiccation and predation of ovular tissues during fertilization and seed maturation.

Effect of rain in pollination

Rain has been reported to aid in pollination in *Pinus* (Greenwood, 1986; Brown and Bridgwater, 1987) and *Cedrus* (Takaso and Owens, 1995). In both genera, rainwater floats saccate pollen into inverted ovules. Pollen distribution on the micropylar arms of seed-orchard interior spruce was also affected by rain. Prior to rain, pollen was distributed evenly on the micropylar arms, adhering to microdroplets (Owens et al., 1987), and clumping was seldom observed. After a brief rainfall, pollen had congregated into clumps, sometimes of more than 20 grains, and was often found filling the micropylar opening. However, we were unable to determine in this case if pollen had floated the length of the micropyle to the nucellus because we had insufficient numbers of pre-pollination drop cones to study. It was evident that rain or simulated rain assisted in the pollen scavenging process by collecting pollen and moving it close to the micropyle.

Aqueous toluidine blue was used to simulate the movement of water within fresh receptive seed cones of interior spruce. Bract and ovuliferous scale bases are arranged in a tight spiral around the cone axis and water or aqueous solutions applied drop-wise to the base of a receptive cone tends to accumulate rather than drain away. The stain moved upwards by capillarity around the cone axis (where the hydrophilic micropylar arms are) and spread to ovuliferous scale margins at the distal end of freshly collected cones. Rainwater entering an erect seed cone may move upwards in the same manner. A relatively large volume of stain (approx. 3 ml) was added to the basal part of the cone before movement to the tip was observed. Aqueous stains did not move upwards within cones that had been preserved in fixative. Fixatives which contain ethanol remove the waxy coating from cone surfaces. The cuticularized hydrophobic surfaces within fresh cones

channel water to non-cuticularized, hydrophilic surfaces. Aqueous phosphine 3R, a fluorochrome which colours neutral lipids and does not stain wax or cuticle (Clark, 1981), coloured only the micropylar arms and nucellar tissue in freshly collected cones. These non-cuticularized surfaces are hydrophilic and rainwater should wet them. This occurs in *Pinus* (Greenwood, 1986) and possibly in *Picea* where nucellar tissue was stained. Stain traveled within the micropylar canal and stained nucellar tissues but not the inner surface of the integument which is cuticularized. Micropylar canals are also cuticularized in *Cedrus* (Takaso and Owens, 1995) and *Pseudotsuga* (Takaso and Owens, 1994). Functionally, the hydrophobicity of the micropylar canal may be required to establish a water column between the micropylar arms and the nucellus when water is applied externally and thereby increase the efficiency of this pollen scavenging mechanism. Overhead water spraying is used in certain seed orchards because the cooling effect delays development of reproductive buds in the spring (Fashler and El-Kassaby, 1987). Pollen from contaminating sources is thus shed prior to the receptive period of these artificially cooled seed cones. Reduction in genetic quality of seed lots produced is therefore minimized. Runions et al. (1995) described accelerated development of seed cones, through the receptive stages, in hot, dry conditions. Occasional overhead misting was suggested as a means of prolonging the receptive period by cooling trees once seed cones had opened. Based on our observations, water applied as a light, periodic mist should perform the dual role of slowing development of seed cones and simulating rain in the pollen scavenging mechanism of interior spruce. Particularly in hot, dry years, overhead misting should help increase the efficiency of pollen capture by the ovules of *Picea* and *Pinus*.

The pollination drop in conifer ancestors

Doyle (1945) described ancestral conifers as possessing inverted ovules (at pollination) and saccate pollen. From this description he inferred a pollination drop mechanism as basal within Coniferales. This reasoning has been supported by contemporary authors (Singh, 1978; Taylor, 1988). Observations of rain involvement in the pollination mechanisms of some modern conifers (Greenwood, 1986; Brown and Bridgwater, 1987; Colangeli and Owens, 1990; Takaso and Owens, 1995) suggest that rainwater may have been a component of ancestral conifer pollination mechanisms.

Water is required in the free-sporing reproduction of gymnosperm ancestors such as Progymnospermophyta (Gifford and Foster, 1989). Spores germinate and gametes are motile only in an aqueous environment. The earliest radiations of conifers began in the Middle Pennsylvanian (Mapes and Rothwell, 1991). Moisture would still have been required for pollen germination and gamete survival and it is conceivable that rainwater played this role. Selective pressure for an internally secreted pollination drop would have been due to the combination of dry habitats of the early conifers (Mapes et al., 1989) and the requirement for moisture in pollen germination. If voluminous, the pollination drop would also have scavenged pollen from integumentary surfaces in a manner analogous to that shown for rain. Therefore, the pollination drop as a feature of the pollination mechanism in ancestral conifers should not be stated unequivocally. At the very least, rainwater and pollination drops could have worked in concert, as they do in the modern conifers, to scavenge and deliver pollen into the ovule.

Chapter 5

Pollination of *Picea orientalis*: saccus morphology governs pollen buoyancy

Introduction

Pollen of many conifers including all spruce (*Picea*) species have sacci, the structures commonly referred to as 'wings'. Sacci are generally misinterpreted as structures which aid in wind dispersal or pollen orientation on the nucellus (Proctor et al., 1996). These may be exaptations as defined by Gould and Vrba (1982), however most evidence supports the conclusion that sacci function primarily as floatation devices and should more aptly be called 'bladders.'. The case has been made that by retaining air, sacci make pollen buoyant to increase the efficiency of pollination (Doyle and O'Leary, 1935; Doyle, 1945; Sporne, 1965; Tomlinson, 1994; Runions and Owens, 1996). We provide evidence of this role for sacci by examining the pollination mechanism of oriental spruce (*Picea orientalis* (L.) Link) in which the saccate pollen sink, as an exception to the rule, and in contrast with the pollen of all other spruce species that floats.

In general, a suite of pollination characteristics evolve in concert. Conifers with floating, saccate pollen have ovules which open downwards during the pollination period (Singh, 1978). A pollination drop secreted by the ovule exudes from the micropyle. Wind blown pollen adheres to surfaces near the micropyle and, when contacted by the pollination drop, floats upwards into the ovule (Runions and Owens, 1996). In oriental spruce, ovule orientation and pollen buoyancy remain correlated characters but these characters differ significantly from those in other spruce species. Because the seed cone is pendant at receptivity, ovules open upwards during pollination drop secretion. Pollen have sacci and would be expected to remain floating in the pollination drop. In fact, pollen floats briefly

and then sinks into the upright ovule (Doyle, 1945). Pollination in this exceptional species has called into question the established, conserved connection between pollen floatation and ovule orientation and made necessary an evaluation of saccus function in pollen floatation.

Because the physical attributes that differentiate saccate floating from saccate sinking were unknown, we used a variety of anatomical techniques to compare the sinking pollen of oriental spruce with the floating pollen of white spruce (*P. glauca* (Moench) Voss). Our hypothesis was that the wall layer of the saccus, the exine, must be anatomically different, or must function differently during pollen hydration between the species. The conventional method used to prepare pollen samples for transmission electron microscopy (TEM) is dehydrating and is not practical for examination of the saccus exine in a hydrated state. A system which enabled examination of hydrated pollen wall layers was adapted from Kurmann (1990). By this technique, ultrathin sections of pollen exine in hydrated condition were obtained. Hydration of pollen was studied by confocal laser scanning microscopy (CLSM) and found to be a dynamic process. In oriental spruce, pollen hydration results in a reduction in buoyancy. Loss of buoyancy is correlated in our observations with hydrated exine character and determines saccate floating or sinking.

Materials and methods

Pollen collection and storage

Several oriental spruce trees are in the rhododendron gardens at the University of Victoria. Pollen cones were collected as they were beginning to shed pollen on May 6 and 11, 1996. Pollen was dried at room temperature for two days and stored in the refrigerator at 4°C in small capped vials. Pollen of white spruce had been collected at the Kalamalka Seed Centre in Vernon, British Columbia during the previous growing season and stored in the freezer at -5°C.

Pollen hydration

Observation of pollen hydration was generally done by dissecting microscope. An artists #1 paintbrush was dipped into dry pollen and the pollen was subsequently dusted onto a drop of water on a microscope slide.

Preparation of samples for scanning electron microscopy (SEM)

Pollen specimens (which are dehydrated so that critical point drying is not required) were prepared for SEM observation by dusting them onto sticky-tape coated aluminum stubs and gold coating. Specimens were viewed with a JEOL JSM 35U SEM operated at 15 kV.

Preparation of pollen samples in hydrated state for light, and transmission electron microscopy (TEM)

To prepare hydrated pollen samples for light and transmission electron microscopy, freeze-fixation / freeze substitution was used. This procedure avoids the dehydrating steps required in conventional fixation for Spurr's resin embedment of tissues. Pollen was hydrated in a 0.3% agar solution at 35°C for 10 min. Silver wire loops were coated with 0.6% formvar film and dipped twice into the hydrated-pollen and agar mixture. Coated loops were then plunged rapidly into liquid propane at -190°C in a Reichert KF80 immersion cryofixation system. Loops with frozen pollen were transferred to a Reichert CSauto cryosubstitution apparatus and freeze substituted at -90°C in a mixture of dry acetone with 1.5 % osmium tetroxide for 70 hours. Freeze substituted samples were warmed at a rate of 5°C per hour to 15°C, infiltrated with Spurr's resin for 48 hours and polymerized at 60°C for 18 hours.

For light microscopy, sections, between 0.5 and 1.0 μm thick, of pollen embedded in Spurr's resin were cut with a Reichert Ultracut E microtome and stained with toluidine blue (C.I. 52040) at pH 11.1 (O'Brien and McCully, 1981). Stained sections were mounted in distilled water, coverslipped and observed with a Leitz Labrolux S microscope and camera system. Hydration of pollen was studied by fluorescence microscopy. To do this, pollen was hydrated in 0.01% aqueous calcofluor white M2R (C.I. 40622) and observed with a Leitz Orthoplan microscope equipped with a Leitz BP 350-460 excitation filter block G.

For TEM, sections of the Spurr's resin embedded pollen were cut at 65 nm and collected on formvar coated, 75 mesh copper grids. Grids were stained with uranyl acetate and lead citrate. Observation of stained sections was with an Hitachi H-7000 TEM operated at 75 kV.

Confocal microscopy: image acquisition

Pollen hydrated in various fluorochrome solutions (see next section) was studied using a Zeiss LSM 410 confocal microscope equipped with krypton and argon laser excitation at wavelengths of 488, 568, and 647 nm. Two types of image were recorded, surface projections and extended depth of focus sections. In each case, a stained pollen grain was first scanned in incremental steps along the z-axis to produce a stack of optical sections (z-sections) representing different depths within it. Each optical section was created as an 8 second scan with 4x line averaging (32 sec.). For surface projections, which resemble SEM images, 30 x 0.5 μm z-sections were projected with maximum overlay, i.e. each z-section blocks the part of the adjacent image that it overlays to produce a representation of surface features only. For extended depth of focus sections, 4 x 0.5 μm median z-sections were composited to produce an image in which fluorescent emission from above and below the section plane was eliminated.

Confocal microscopy: staining

For surface projections, pollen were hydrated in an aqueous 0.01% phosphine 3R (C.I. 46045) solution for > 5 min. To prevent pollen from moving during microscopy, the staining solution was mixed dropwise with Farrant's medium (BDH), a viscous, water soluble mounting medium containing glycerin and gum arabic. For extended depth of focus sections, pollen were hydrated in a solution containing equal parts of, i) $1.0 \times 10^{-4}\%$ rhodamine B (C.I. 45170) in 0.05M phosphate buffer at pH 5.8, and ii) $1.0 \times 10^{-1}\%$ fluorescein diacetate stock (Sigma) in 0.05M phosphate buffer at pH 5.8. The stock solution of fluorescein diacetate was made by dissolving 2.0 mg per ml in acetone. Pollen hydrate fully in approximately one minute in this solution. To extend the hydration time for confocal microscopy, and to prevent pollen from moving during microscopy, the staining solution was mixed dropwise with Farrant's medium as described above. The resulting solution has higher osmotic potential than the stain solution and caused plasmolysis of the stained pollen grains which rehydrated again completely over the next 15 min.

Observations and results

Seed cone orientation and the pollination mechanism of oriental spruce

When receptive for pollination, seed cones of oriental spruce appeared similar to seed cones of other species of *Picea* except that they were pendant rather than upright on the branch (fig. 33). Bracts and ovuliferous scales reflexed in a manner that opened the cones, revealing the micropylar arms (extensions of the ovule integument around the opening of the micropyle, see: Runions et al., 1995). Wind blown pollen adhered to the micropylar arms prior to cone closure and pollination drop exudation. As the cones began

to close, pollination drops were secreted from the upright ovules (fig. 34). Through interaction with the pollination drop, pollen moved into the ovule. Pollen applied to an exposed pollination drop in a experimental set up floated for approximately one min. within the pollination drop and then sank into the micropyle. Figure 35 contrasts the pollination mechanisms of oriental spruce and white spruce.

Comparing pollen morphology and anatomy between oriental and white spruce

Morphologically, pollen of the two species appeared similar but functionally they were quite different. When pollen of white spruce (and all other saccate pollen species which have been tested) was added to a drop of water, it continued to float for as long as it was observed (> 3 days). In contrast, pollen of oriental spruce floats for 1-3 min. and then sinks. Small bubbles were seen in the sacci of oriental spruce pollen when it was added to the water, but these shrank and quickly disappeared (fig. 36).

When dry pollen were compared by SEM, they appeared similar (fig. 37) although pollen of oriental spruce were slightly smaller. Even at high magnification (fig. 38), no differences which could be interpreted as having functional significance were observed although the sculptured pattern of exines were different.

Staining properties of pollen exines were compared by confocal microscopy and, again, no interpretable differences were observed (fig. 39). In each case, the exine was stained by phosphine 3R, which is specific for neutral lipids, in its proximal region and sacci. An approximately T-shaped region between the sacci remained relatively unstained, indicative of a lower neutral lipid content.

Hydrated pollen grains appeared similar in anatomical detail when examined by light microscopy (fig. 40). The large tube cell was bounded by the intine wall layer and was filled with small vacuoles. Body and stalk cells were bounded by primary cell walls which appeared continuous with the intine at the proximal pole. Remnants of prothallial

cells were embedded in the intine adjacent to the site of stalk cell attachment. In each species, exine stained similarly with toluidine blue and were continuous around the pollen, although very thin at the distal pole. Sacci appeared similar between species. In relative terms, the sacci of oriental spruce might be smaller than those of white spruce but the difference is slight.

Hydrated saccus exine appeared different when the two types of pollen were compared in TEM micrographs (fig. 41). The sacci of white spruce pollen consist only of an homogenous appearing ektexine approximately 0.25 μm thick. Inward projections of the ektexine form a reticulate network when viewed in cross section (fig. 41a). Enclosed and partly enclosed spaces, which vary from 0.25 μm to 5.0 μm across, were formed by this reticulate network. In contrast, the ektexine of oriental spruce pollen sacci was thinner and porous (fig. 41b). Elaborations of the ektexine formed inward projections but enclosed spaces, capable of trapping air, were very uncommon.

Hydration of oriental spruce pollen

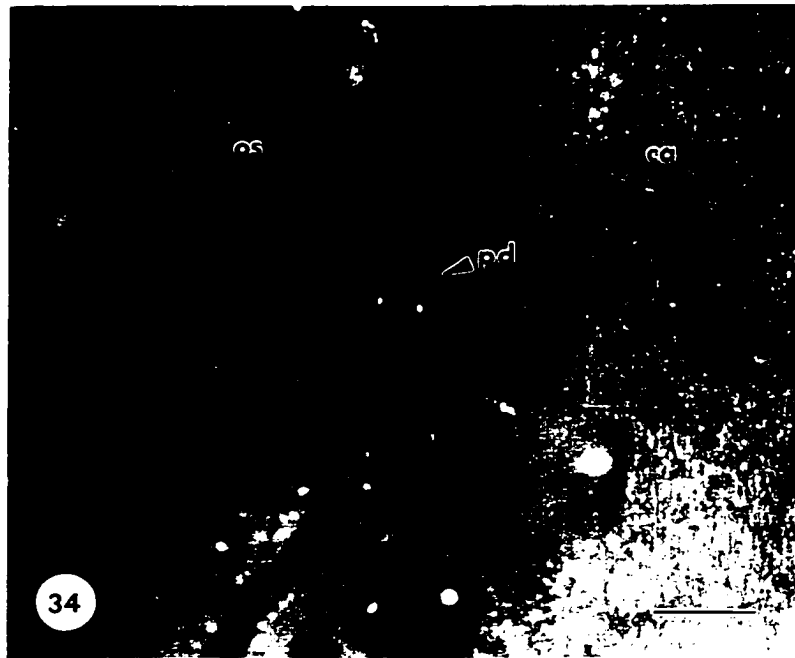
Fluorescence and confocal microscopy were used to monitor the course of hydration of oriental spruce pollen. When pollen was hydrated in calcofluor white M2R solution as a test for cellulose, strong staining occurred in the T-shaped region which was described in the previous section as unstained by phosphine 3R (fig. 42). Higher magnification revealed that the staining was in the cellulosic intine underlying the exine. The exine is porous in this region. Eventually, the exine split in the distal region between sacci and the intine was visible (fig. 43). When pollen were left in the calcofluor white M2R staining solution for > 24 hours, they germinated at low level and the pollen tube wall, which is cellulosic and an extension of the intine, stained (fig. 44).

Pollen hydration resulted in rapid swelling of the tube cell and surrounding intine. The dynamics of this process were observed by confocal microscopy. Exine layers of the

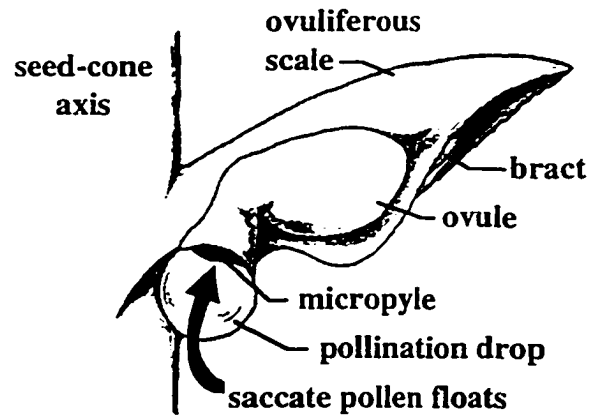
pollen stained with rhodamine B, a fluorochrome incapable of penetrating the plasmalemma. Fluorescein diacetate was used as a vital stain within the cytoplasm at the same time. This stain crosses the plasmalemma and becomes fluorescent if esterases cleave the acetate. Fluorescence indicates, therefore, that the pollen being studied is alive. Rehydrating pollen grains were scanned with the CLSM to produce median optical sections periodically during a 15 min. period following immersion in staining solution. In aqueous solutions with low osmotic potential, full hydration of pollen required only about one minute. The osmotic potential of the medium was increased by addition of Farrant's medium to slow hydration for the purposes of confocal microscopy. As well as preventing pollen from moving during confocal microscopy, Farrant's medium seemed to prevent quenching of the fluorochrome. Farrant's medium was not toxic to pollen even after three hours of immersion as indicated by fluorescein diacetate staining. Figure 45 shows median sections of the same pollen grain of oriental spruce at 1, 8, and 15 min. during hydration. Exine layers including the ectexine of the sacchi fluoresced red and the cytoplasm of the three cells within the pollen body fluoresced green. Esterase activity was concentrated around the nuclei of the stalk, body, and tube cells. Cytoplasm within the pollen grain was predominately that of the tube cell (largest nucleus) which surrounds the smaller stalk and body cells. As pollen hydrated, the tube cell swelled to occupy most of the saccate air space (arrowheads in fig. 45).

Figures 33-34

- Fig. 33 A receptive seed cone of oriental spruce. At receptivity, seed cones are pendant rather than upright like those of other *Picea*. Ovuliferous scales reflex so that windborne pollen can enter the cone and stick to the micropylar arms near the micropyle of the ovule. Bar=2cm.
- Fig. 34 Near the end of the receptive period, pollination drops (pd) are secreted from each ovule. Off-median longitudinal hand-section of a receptive seed cone. ca - seed cone axis, os - ovuliferous scale. Bar=0.5mm.



a) **Interior spruce
ovules inverted**



b) ***Picea orientalis* (oriental spruce)
ovules upright**

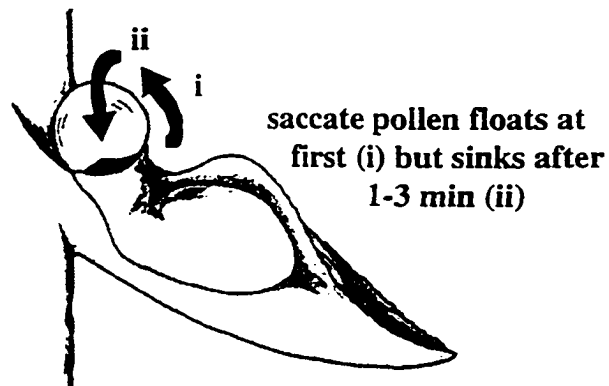
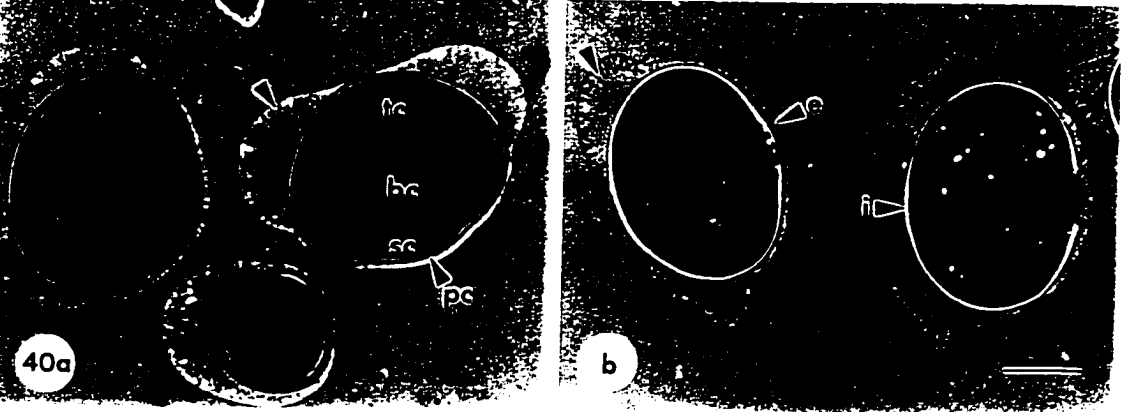
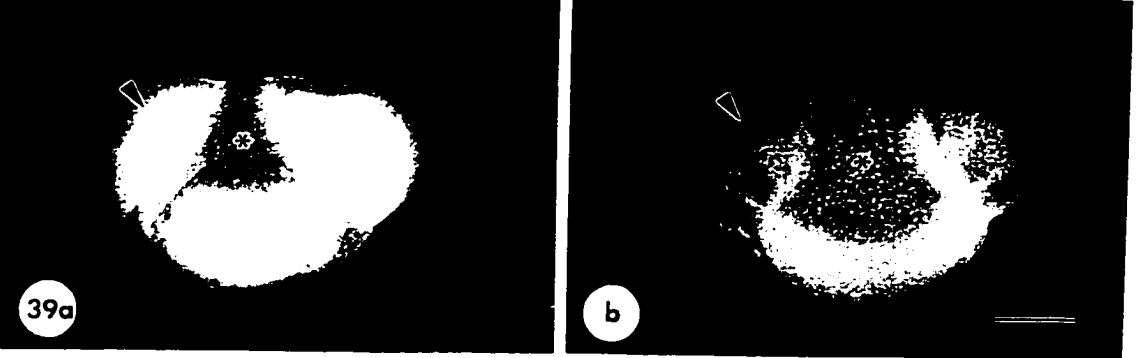
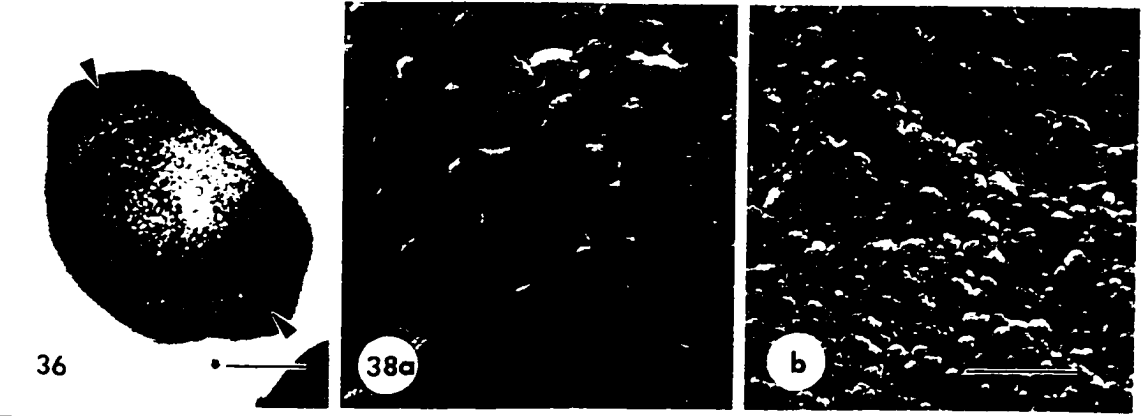
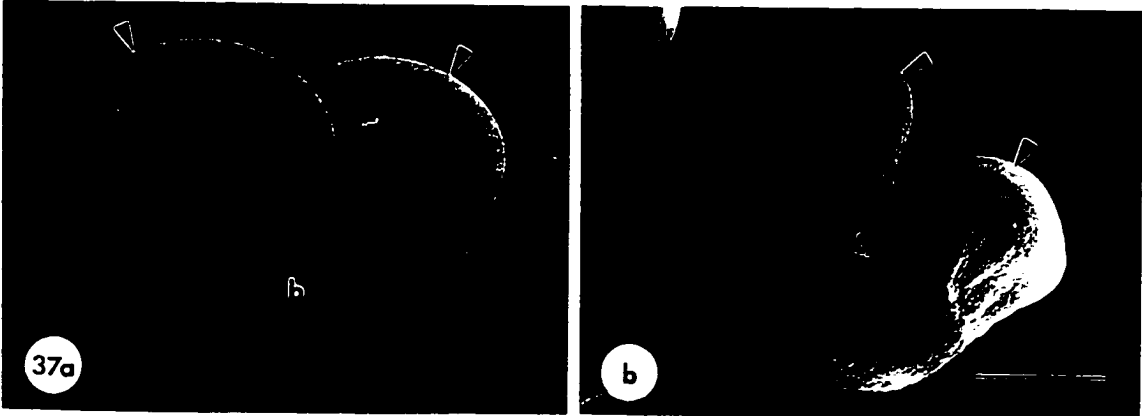


Figure 35. Ovules of interior spruce (a) are inverted at pollination. The saccate pollen floats into the micropyle in the pollination drop. Oriental spruce pollen is saccate as well but it floats only briefly before sinking into the ovule which is erect at pollination (b).

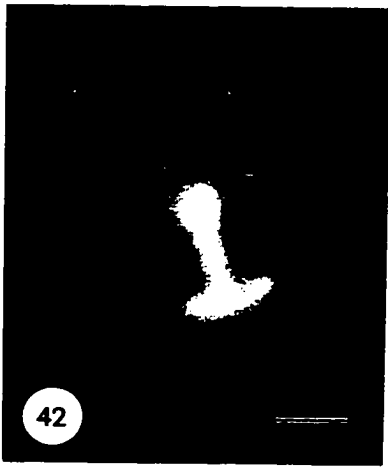
Figures 36-40

- Fig. 36 When added to water, the pollen of oriental spruce sinks. After pollen sinking, small air bubbles (arrowheads) remain visible in the sacci for a short time. LM. Bar=25 μ m.
- Figs. 37-40 Comparisons between a) white spruce pollen, and b) oriental spruce pollen.
- Fig. 37 Dry pollen. Sacci (arrowheads) appear slightly larger in white spruce. b - pollen body. SEM. Bar=25 μ m.
- Fig. 38 High magnification of the surface of the exine in the saccate region of each species. White spruce saccus exine is not as finely sculptured as that of oriental spruce although they appear similar. SEM. Bar=2 μ m.
- Fig. 39 Surface features of each pollen vary when stained with phoshine 3R. Surface of the sacci of white spruce stained to a greater degree than oriental spruce (arrowheads). In each type of pollen, the distal region between sacci was relatively unstained (*). Confocal surface projection. Bar=25 μ m.
- Fig. 40 Pollen of each species appeared similar after freeze-fixation / freeze-substitution and thin sectioning. arrowheads - sacci, tc - tube cell, bc - body cell, sc - stalk cell, pc - prothallial cells, e - exine, i - intine. LM. Bar=25 μ m.



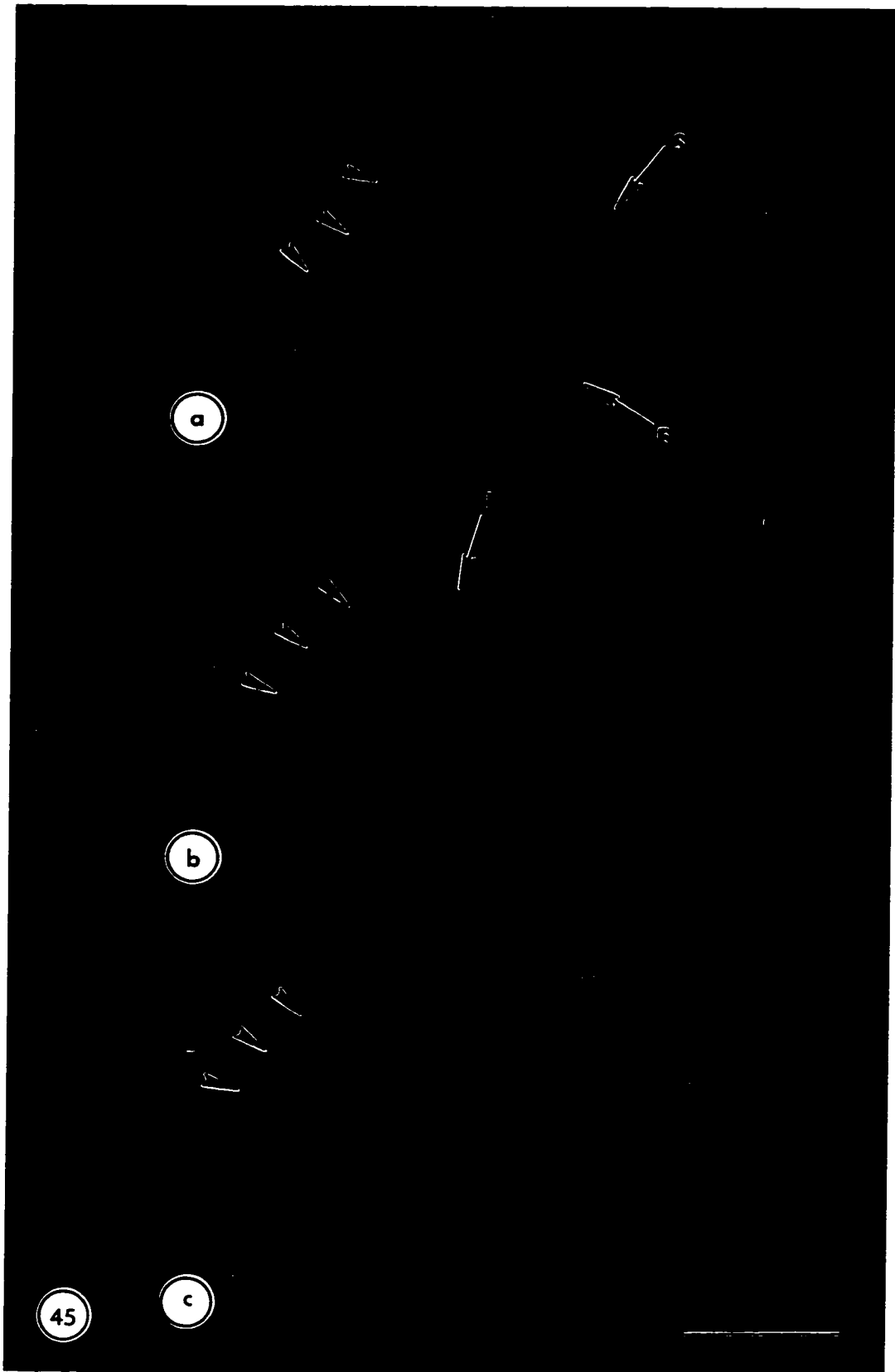
Figures 41-44

- Fig. 41 At the ultrastructural level, differences were evident in exine (e) structure of the sacci of hydrated pollen. Sacci of white spruce pollen (a) is not as porous as sacci of oriental spruce pollen (arrowheads in b). In white spruce pollen, projections within the ectexine form enclosed or partly enclosed air spaces (*) which are uncommon in oriental spruce pollen. TEM. Bar=1 μ m.
- Figs. 42-44 Hydration and germination of oriental spruce pollen.
- Fig. 42 Calcofluor white stains the distal region between sacci that appeared relatively unstained with phosphine 3R (see fig. 39). Close examination revealed that stain did not enter the symplast but concentrated in the intine at the site of water entry during pollen hydration. Bar=25 μ m.
- Fig. 43 At germination, the exine splits and the intine (i) is visible at the site where the pollen tube will emerge. Confocal surface projection. Bar=25 μ m.
- Fig. 44 Pollen tube (pt) stains with calcofluor white after germination. Bar=50 μ m.



Figures 45

Fig. 45 Confocal laser scanning microscopy was used to study the process of hydration in oriental spruce pollen. Swelling of the pollen body (arrowheads) forces air out of the sacci (S). (a) 1 min. after start of hydration. (b) 8 min. after start of hydration. (c) 15 min. after start of hydration. At 15 min. the pollen is fully hydrated (in situ hydration requires only 30 sec. to 1 min. the process was slowed here by using a high osmotic potential hydration solution). Fluorescein diacetate staining indicates esterase activity in the living cells (green) and rhodamine B stains the exine (E - red). Confocal extended depth of focus section. I - intine. Bar=25 μ m.



Discussion

Pollination mechanism of oriental spruce

Sacci of oriental spruce pollen differ in functional morphology from those of white spruce pollen (Runions and Owens, 1996), but they still function to provide a brief period of floatation. Pollen contacted by the pollination drop of this species float to a position above the opening of the micropyle before sinking into the ovule. Routine anatomical investigations of pollen from different species of *Picea* gave no indication why pollen of oriental spruce should sink while that of other species remained floating. Investigation of pollen by SEM, and by surface staining properties did not allow description of significant differences between species. The exine of pollen of each species was strongly stained by phosphine 3R over the sacci and in the proximal region. Phosphine 3R stains neutral lipids and thus indicates that although the pollen are buoyant (at least briefly, in the case of oriental spruce), the pollen surface is hydrophilic and wettable.

Our hypothesis, that anatomical differences must be a factor governing pollen behaviour in water, was correct, but the differences were only visible when hydrated pollen were compared by TEM. Freeze-fixation / freeze-substitution proved invaluable as the hydrated pollen were not, therefore, subject to the dehydration that routinely results in tissue shrinkage and artifacts when standard embedment protocols are used. Sacci of oriental spruce pollen can be considered porous when compared to the relatively non-porous, air-trapping sacci of white spruce. The exine layer which forms the sacci is the ektexine as defined by Kurmann (1990). Deposition of the ektexine in her study of *Tsuga canadensis* (L.) Carr. pollen was mediated by orientation of microfibrils in the microspore surface coating. If orientation of microfibrils can be considered the precursor to ektexine pattern formation there is a possible genetic basis for species differences that might not be

well defined if the ectexine was randomly deposited. This allows for evolution of these characteristics.

Existence of holes in the sacci of oriental spruce pollen suggested the reason why this saccate pollen should sink but the reason why air is displaced when pollen are added to water remained unclear. These holes are of very small diameter and, at least initially, air is retained within the sacci. The confocal microscope allowed visualization of details of pollen hydration. Pollen tube cell and intine expansion into the saccate space occurs rapidly once pollen are added to water. Rapid reduction in saccus volume necessarily results in displacement of air from the sacci, otherwise the pressure and temperature increase associated with gas compression would seem detrimental. White spruce pollen retain enough air within the reticulate network of ectexine extensions and enclosed spaces in the sacci to remain buoyant while air is not trapped in the porous ectexine of oriental spruce pollen.

Water enters through the distal pole of the pollen in a T-shaped area between the sacci. In dehydrated pollen, the sacci close together to hide this flexible region of exine, the leptolemma, which, upon hydration, becomes the site of pollen tube emergence. During the course of hydration in calcofluor white M2R, the fluorochrome stained the intine most intensely in this T-shaped region. As water entered the pollen, stain molecules were excluded and accumulated at the plasmalemma within the intine at the site of water entry. Canny (1990) described this sort of stain accumulation, in sumps, as water enters the symplast of a cell. Prolonged staining of pollen by calcofluor white M2R did not inhibit germination and, in germinants, the staining was clearly localized to the intine and pollen tube. In this case, the bright fluorescence in the region between sacci highlights the movement of water into the tube cell, subsequent inflation of the tube cell and intine reduce the saccate volume, and the porosity of the ectexine determines the sinking or floating nature of the pollen.

Sacci function and evolution of pollination mechanisms in Pinaceae

Characters integrated in pinaceous pollination mechanisms include, i) orientation of the seed cone and, therefore, ovules at the time of pollination, ii) pollen with or without sacci, and iii) a pollination drop involved in pollination or not. Evolutionary change in one of these characters requires compensatory change in the others if the pollination mechanism is to function efficiently. Since all genera within Pinaceae which secrete a pollination drop, except oriental spruce, have inverted ovules and floating pollen, we consider this character set to be the ancestral condition (Doyle, 1945; Mapes, 1987; Osborn and Taylor, 1994). In oriental spruce, the relationship between ovule orientation and pollen floatation has changed in a manner that supports the role of sacci in pollen floatation. Change in ovule orientation has been effected by a change in seed cone orientation at the time of pollination. Ovules are inverted when seed cones are upright on the branch and they are upright when seed cones are pendant. The importance of ovule orientation in the pollination mechanism is highlighted by the fact that seed cone stalks bend to orient cones vertically before they become receptive to pollen.

Brief floatation of oriental spruce pollen may be adaptive and retained, or might represent an intermediate step towards complete loss of sacci function. Pollen scavenging, in which pollen that land on distal seed cone structures float into the micropyle upon contact with a large pollination drop, has been described for other conifer species (Tomlinson et al., 1991; Runions and Owens, 1996). Retention of sacci in oriental spruce may confer a selective advantage by allowing pollen floatation from the point of pollen capture on the seed cone to the micropyle, thus extending the possibility of pollination temporally and spatially. At the same time, saccate pollen of sympatric conifer species, because it floats, would be excluded from the micropyle during the time that sinking pollen of oriental spruce occupied the site of germination on the nucellus within the ovule. Pollen selection mechanisms which discriminate pollen types based on floatation have been termed

exclusion mechanisms by Tomlinson (1991). Conifer species from families other than Pinaceae (eg. Cupressaceae) that have upright ovules which secrete pollination drops have non-saccate pollen. This pollen lands in the pollination drop and sinks immediately into the ovule (Tison, 1911; Owens and Molder, 1980b). In these species, because pollen land directly in the pollination drop, there is no requirement for pollen floatation.

Diversifying selection has resulted in two pollination mechanisms within *Picea*. Intermediate ovule orientations and within species variation in pollen buoyancy are unknown although evolution of this phenotypic gap has, no doubt, proceeded through a series of intermediate steps (Maynard Smith et al., 1985). How the shift from the normal situation of inverted ovules and floating pollen occurred can only be speculated upon. In one scenario, the erect position of seed cones was lost in an ancestral oriental spruce but the loss was not completely maladaptive. Buoyant, saccate pollen floating within pollination drops would have been taken into the more or less upright ovules as the pollination drops receded. Genetic control of the upright seed cone position, once lost, would be unlikely to have been regained and selection on sinking pollen and inverted seed cone position to increase pollination efficiency would have driven the concerted evolution of these traits.

Loss of the erect character of seed cones at pollination would be maladaptive in large populations or where sympatric species created competition. Stabilizing selection (Charlesworth et al., 1982) would tend to maintain the basal condition in ancestral populations under adaptive constraint due to competition. The proposed scenario requires a reduction in selective pressure as might occur if a founder species was relatively isolated and in a small population (Jernigan et al., 1994). Modern oriental spruce is native to the Caucasus mountains of North Eastern Turkey, and Georgia (Davis, 1965) where it is isolated from other spruce species. Isolation has allowed not only evolutionary change in the pollination mechanism but in vegetative characters as well. Oriental spruce is distinct from other spruces in leaf form. Despite its desirable appearance (Dallimore and Jackson,

1974), hybrids with other *Picea* are unknown. Many *Picea* species hybridize readily and the lack of oriental spruce hybrids might reflect not genetic incompatibility but an unrecognized incompatibility in pollination mechanisms. Hybridization of oriental spruce with other spruce species was attempted without success (Wright, 1955), and apparently with success but the authors claim that these progeny were unverified and could have been the result of self-fertilization (Mergen et al., 1965).

Several genera have arisen within Pinaceae since establishment of the pines (*Pinus*) and spruces (Chase et al., 1993; Hart, 1987; Price et al., 1987). These more modern genera, eg. *Pseudotsuga*, have diverged in pollination mechanism but in a manner different from oriental spruce. In these cases, evolutionary loss of the exuded pollination drop means that pollen are not required to float and sacci have been lost or are vestigial and do not function (Owens et al., 1981b). In *Abies*, no pollination drop is exuded by the ovule but the pollen are saccate and float. This seemingly contradictory situation might be explained by field observations (unpublished) which suggest that atmospheric moisture in the form of condensation or rain can fill the micropyle and cause pollen to float into the ovule. The possibility that *Abies*, and to a lesser extent even species with pollination drops, utilize rainwater in pollination is under investigation. Whatever the factors governing speciation in each case, retention of sacci correlates with the requirement for pollen that float, even if only briefly as observed in oriental spruce, as a component of the pollination mechanism.

Chapter 6

Sexual reproduction of interior spruce: pollen germination to archegonial maturation

Introduction

Studies of many conifers indicate that embryos can fail to form even after successful pollination. Seed losses due to observable factors such as lack of pollination and embryo abortion do not account for total seed lost. Prezygotic seed losses, i.e. failure of fertilization for any reason after successful pollination, are quantified simply as the difference between total seed loss and loss due to embryo abortion. Reasons for prezygotic failure are historically not explicitly stated as full developmental studies of resin embedded tissues are required for description of prefertilization events within the ovule. Owens et al. (1991) did a study of ovule development in Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.) and reported that 11% of seed losses were due to prezygotic factors other than lack of pollen or prematurely aborted ovules.

Departures from the normal course of sexual reproduction which result in prezygotic failure can be categorized as due to: i) abnormal anatomical development within the ovule, or ii) incompatibility between maternal and paternal tissues or germ units. Incompatibility issues are becoming more widely recognized in conifers. Owens et al. (1994) suggested that interaction may occur between conifer pollen and the receptive surface (nucellus or integument), and Takaso and Owens (1994, 1996) have described secretions from the ovule of Douglas fir which affect pollen germination.

Self-incompatibility, as it exists in many angiosperms (Newbigin et al., 1993; Nasrallah and Nasrallah, 1993), is generally described as non-existent in conifers. Reduction in seed set after self-pollination in conifers is generally attributed to genetic load

(Koski, 1973) and conifers are described as carrying a heavy genetic load of lethal or sublethal alleles (Namkoong and Bishir, 1987; Savolainen et al., 1992). To estimate genetic load in those studies, the authors assumed that fertilization occurred prior to seed failure. Assumptions of this sort should not be made prior to detailed developmental study during fertilization.

To make possible a description of prezygotic factors affecting seed set in interior spruce (white spruce (*Picea glauca* (Moench) Voss), or Engelmann spruce (*P. engelmannii* Parry), or their hybrid in British Columbia), a detailed anatomical study was first made of the period from pollen germination until early embryo formation. This chapter and Chapter 7 describe the results of the anatomical study as a prelude to Chapter 8, in which prezygotic failures due to developmental abnormalities are described. Chapter 9 addresses self-incompatibility issues which result in prezygotic failure.

Normal prezygotic development of white spruce (Mergen et al., 1965; Owens and Molder, 1979a), sitka spruce (*P. sitchensis* (Bong.) Carr.; Owens and Molder, 1980a), Engelmann spruce (Singh and Owens, 1981b), and interior spruce (Owens and Molder, 1984a) have been made utilizing paraffin embedded tissues. This study extends those observations which were relatively consistent within *Picea*. Some observations on pollen tube growth and development are included in Chapter 6 although a more detailed account is given in Dawkins and Owens (1993). The bulk of this chapter describes the nucellus, archegonial development, and provides an overview of the mature archegonium.

Materials and methods

Tree selection and pollination

Trees used in this study had been established as grafts in a clonal seed orchard at the Kalamalka Seed Centre near Vernon, British Columbia in 1982. In April 1992, two ramets from each of eight clones (QL orchard clones 1830, 1879, 4700, 4709, 4757, 4768, 4777, and 4835) were used in a survey of development during fertilization. Seed cones were collected from these trees and ovules were embedded in paraffin. In April 1993, individual ramets of three clones (QL orchard clones 1926, 1944, and 4752) were used for a more detailed study of fertilization. Seed cones were collected from these trees and ovules were embedded in resin.

Each clone had an abundant supply of developing pollen cones, and seed-cone buds. In 1992, seed cones were left open to natural pollination. In 1993, pollen-cone buds were removed and isolation bags placed over branches bearing seed-cone buds prior to the pollination period. Fresh pollen was collected from 12 clones which were not otherwise included in the study, and mixed. Pollen was tested to ensure that it was between 5-10% moisture content prior to artificial pollinations. Pollinations on May 6 and 7 were accomplished by injecting 0.5 ml aliquots of pollen into each bag daily during the period when seed cones appeared maximally receptive. Once seed cones had become postreceptive, the paper bags were replaced with mesh bags which excluded insects.

Anatomical study of fertilization period

In 1992, the natural pollination period extended from April 18 to May 6. Postreceptive seed cones were collected from trees on May 12, 19, 27, and June 2, and placed into Navashin's fixative (Sass, 1958) for storage. Twenty ovules per clone per

collection which had been embedded in paraffin were sectioned for a total survey of 640 ovules.

In 1993, beginning one week after the final pollination, ovules were collected and prepared for the developmental study. Every two days for 18 days, two seed cones were collected per treatment per tree. Median longitudinal sections less than 1 mm in thickness were cut from ten ovules from the centre region of each cone, fixed in phosphate buffered glutaraldehyde, post-fixed in OsO_4 , dehydrated and embedded in Spurr's resin. Fifty-five ovules were sectioned. In each study, ovules were serially sectioned at 0.8 μm with a Riechert Ultracut E microtome, and stained with toluidine blue (CI 52040) at pH 11.0 for light microscopy (O'Brien and McCully, 1981). For transmission electron microscopy, sections were cut at 0.06 μm , picked up on 300-mesh or 75-mesh copper grids, stained with uranyl acetate and lead citrate and examined with an Hitachi TEM. All of the developmental stages described occurred during the eight day period from 13 to 20 days after the final pollination.

Observations and results

Pollen tube

Pollen within the pollen chamber in the apex of the nucellus germinated in fewer than 13 days after pollen application. Pollen tubes emerged from the distal portion of the grain between the sacchi. The exine ruptured and pollen tubes grew through the nucellus towards the megagametophyte. Within a day after pollen tube penetration into the nucellus, the body cell underwent mitosis to produce two male gamete nuclei (fig. 46). At low magnification, phragmaplast microtubule bundles and the forming cell plate were apparent but in the advanced pollen tube no evidence was seen of a cell wall separating the male gametes (fig. 47). No consistent differences in appearance were observed between the

male gamete nuclei. In some cases they looked identical and in others they differed in size or in nucleoplasmic staining properties. The common cytoplasm around the gamete nuclei was densely packed with plastids (proplastids) and mitochondria (fig. 48). Plastids were ovoid or elongate with a darkly staining stroma and a sparse thylakoid membrane system. Mitochondria were spherical with well defined inner, and outer membranes and cristae. The stalk cell retained its thin cell wall as it migrated along with the male gametes in the tube cell.

Nucellus

Cells in the central micropylar region of the nucellus were thin walled, had few vacuoles, and contained amyloplasts with abundant starch grains (fig. 49). The peripheral 1 to 3 layers of nucellar cells in the micropylar region had an accumulation of lipids between their cell walls and plasmalemmas. This accumulation, which stained positively for lipids with toluidine blue (Yeung, 1990), was greatest inside the outer tangential cell walls but occurred, as well, in radial and inner tangential cell walls (fig. 50). Lipid bodies varied in size and the smallest ones impregnated the inner layer of the cell wall (fig. 51). The site of lipid production was either within these nucellar cells or in the adjacent cells of the inner layer of the seed coat which appeared deteriorated, suggestive of secretion earlier in development. Cytoplasm of nucellar cells in the central region was heavily stained by toluidine blue (fig. 52). Staining was most intense in the region near a pollen tube. This staining was less in unpollinated ovules.

Megagametophyte

At pollination, the megagametophyte was cellular. Prothallial cell walls were very thin and the cells contained only a thin peripheral cytoplasm. Cells, thought to be tapetal, in the inner 2 to 3 layers of the nucellus surrounding the megagametophyte, began to breakdown as the megagametophyte began to enlarge (fig. 53). Lipids liberated by this breakdown accumulated on the megaspore wall around the megagametophyte (fig.54). The megaspore wall was thick and elaborated into short, outward surface projections around flanking and chalazal regions of the megagametophyte, and very thin around the micropylar region. Prothallial cells of the megagametophyte did not have characteristics of secretory cells and did not appear to contribute to formation of the megaspore wall.

Prior to pollen germination, the archegonial initial cells had formed in the micropylar end of the megagametophyte and each had undergone mitosis to produce a central and primary neck cell (fig. 55). Each megagametophyte had 2 to 3 archegonia. The central cell contained many large vacuoles and its nucleus remained at the micropylar pole. At this stage pollen germinated and grew through the nucellus over a 3 to 5d period.

Central cell mitosis produced the egg and ventral canal cell by approximately the time pollen tubes had grown halfway through the nucellus to the megagametophyte. The central cell nucleus remained in position at the micropylar pole of the cell and isolated a small, lens-shaped region of cytoplasm which became the ventral canal cell cytoplasm (fig. 56). The central cell remained vacuolate during mitosis. A thin cell wall formed between the ventral canal cell and the egg cell.

Small, densely cytoplasmic cells of the archegonial jacket surrounding each archegonium underwent synchronous mitosis during central-cell division. Doubling of the number of archegonial jacket cells accommodated rapid enlargement of the egg cell as it matured. Archegonial jacket cells had thin primary cell walls with primary pit fields in their inner tangential walls, adjacent to the egg cell (fig. 57). Usually each archegonium was

completely surrounded by an archegonial jacket layer. In a few cases, closely appressed egg cells shared a single layer of archegonial jacket cells or the jacket layer was absent between egg cells (see figs. 67 and 88).

Mitosis of the primary neck cell and its progeny proceeded immediately after archegonial initial division and resulted in a single tier of four neck cells. As the neck cells divided to form several tiers, they, and the prothallial cells which surrounded them in several layers, became metabolically very active as indicated by fluorescein diacetate staining (fig. 58). Figure 59 shows mitosis of the neck cells which were surrounded by heavily staining adjacent cells. These adjacent cells were continuous with the archegonial jacket cells but were 3 to 4 layers thick around each neck. Also at that time, nucellar cells in several layers near each neck deteriorated so that a depression, the archegonial chamber, formed. The megaspore wall remained intact between the nucellus and neck cells. At maturity, there were 3 to 4 tiers of four cells each so that the ventral canal cell and egg cell subtending the neck were deep within the tissue of the megagametophyte (fig. 60). Mature neck cells had very thick, primary cell walls. Immediately upon maturation, the neck cells began to deteriorate and separate somewhat in their middle lamellar region while surrounding cells remained intact with no sign of cytoplasmic deterioration. Deterioration of neck cells was observed in pollinated and unpollinated ovules. As deterioration proceeded, large lipid bodies accumulated between the neck cells and the megaspore wall, in the archegonial chambers (figs. 61-62). Origin of the lipids in these accumulations was not determined. Cells surrounding the neck cells were more secretory in appearance. They had undulating plasma membranes and abundant dictyosomes. Deteriorating neck cells and the healthy-appearing surrounding cells contained lipid bodies and lipids were visible in the lamellar space between their cell walls (fig. 63-64). Neck cells had deteriorated to the extent that organelles were not intact and lipid secretion may have been the cause or effect of this deterioration.

Mature archegonium

Egg cells became mature just before pollen tubes reached the neck (fig. 65). Egg cells were very large, ovoid in longitudinal section and 250 x 500 μm . The egg nucleus was spherical and approximately 70 to 80 μm in diameter. Nucleoplasm was homogenous and contained several nucleoli. By the time pollen tubes contacted the megaspore wall, the egg nucleus had migrated into a central position (fig. 66). Egg cell cytoplasm increased in volume after central cell division and most of the large vacuoles disappeared. Cytoplasm of the mature egg cell had, at most, 1 to 2 small vacuoles.

Egg cell cytoplasm is generally described as containing large and small inclusions. In figure 67, the modified plastids are the regions of cytoplasm surrounded by darkly staining membranes. Formation of modified plastids began at central cell division as all of the plastids in the cell became elongate and deformed in a manner which encompassed areas of cytoplasm (figs. 67-68). Few, if any, organelles were included in the modified plastids. Modified plastids were concentrated towards the chalazal end and periphery of the mature egg cell (fig. 69). They became very robust with concentric systems of membranes which compartmentalized areas of cytoplasm (fig. 70). Formation of modified plastids was not restricted to the egg cell, plastids in all cells of the megagametophyte (with the possible exception of the prothallial cells) deformed in the same fashion (fig. 71). Plastids in sporophytic tissue did not deform.

Small inclusions are more difficult to describe. They are the smaller, spherical or elongate regions of cytoplasm which are conspicuous because they are slightly more dense than surrounding cytoplasm and contain no organelles (fig. 70). They were bound by a single, thin membrane (fig. 70). The pathway of egg nucleus migration (and at a later stage, of male gamete nucleus movement) through the cell cytoplasm was visible in the pattern of small inclusions in its wake (fig. 69). Mitochondria of the egg cell were excluded from small inclusions (fig. 72). A large portion of egg cell mitochondria were

concentrated into many small groups in a perinuclear zone around the egg cell nucleus (fig. 73). Not all mitochondria moved into the perinuclear zone, many were stranded at a distance from the nucleus as small inclusions enlarged and sealed off the perinuclear cytoplasm (fig. 74). Upon formation of the perinuclear zone, the egg nuclear membrane became convoluted (fig. 75). At this point, the egg cell was mature and ready to be fertilized.

Figures 46-54

Figs. 46-48 Pollen tube. (nu - nucellus)

Fig. 46 Body cell division occurred shortly after pollen germination to form the male gametes (σ^i and σ^{ii}). A cell plate appears to form across the phragmoplast (arrowhead). DIC. Bar=50 μ m.

Fig. 47 Pollen tube (pt) has grown through the nucellus. Male gamete nuclei (*) differ in staining intensity. LM. Bar=10 μ m.

Fig. 48 Body cell cytoplasm surrounding one of the male gamete nuclei (σ) contains plastids (p) and mitochondria (m). TEM. Bar=2.5 μ m.

Figs. 49-52 Details of the nucellus (nu).

Fig. 49 Cells in the central part of the apical nucellus that the pollen tube grows through are small and angular with thin primary cell walls. Plastids contain abundant starch. LM. Bar=4 μ m.

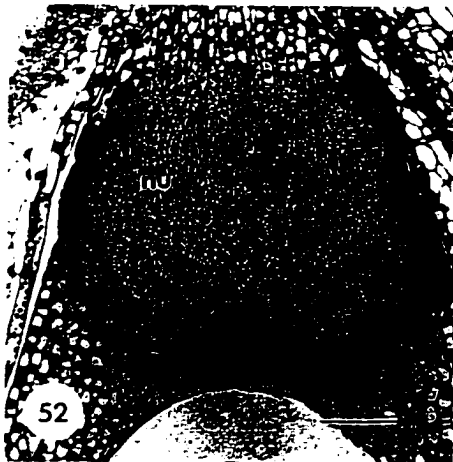
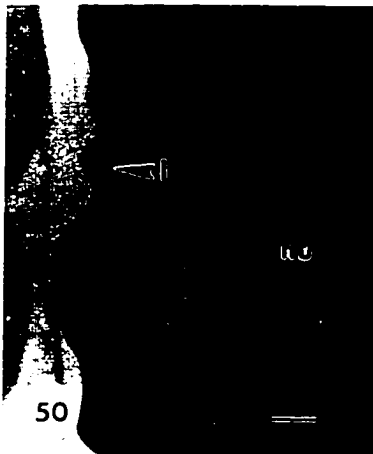
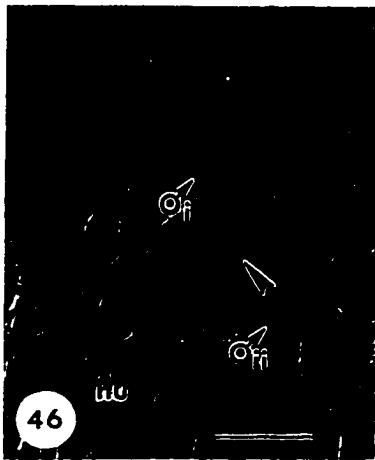
Fig. 50 The outer 1 to 2 layers of nucellar cells accumulate lipids (l) between plasmalemma and cell wall. Lipid deposits are greatest in the outer tangential walls. LM. Bar=10 μ m.

Fig. 51 Lipid (l) deposits in cells of the outer layer of the nucellus occur between plasmalemma (arrowhead) and the cell wall (cw). TEM. Bar=0.5 μ m.

Fig. 52 After the pollination period, cells in the central apical region of the nucellus (dark staining region) become metabolically more active than surrounding cells as indicated by intense staining with toluidine blue. This type of staining is not as intense in unpollinated ovules. LM. Bar=200 μ m.

Fig. 53 The inner 1 to 3 layers of nucellar cells surrounding the megagametophyte (mg) are tapetal. Tapetal (t) deterioration occurs as the archegonia mature within the megagametophyte. LM. Bar=20 μ m.

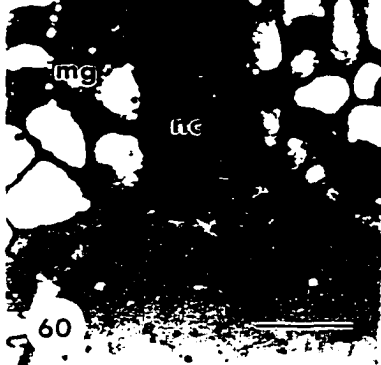
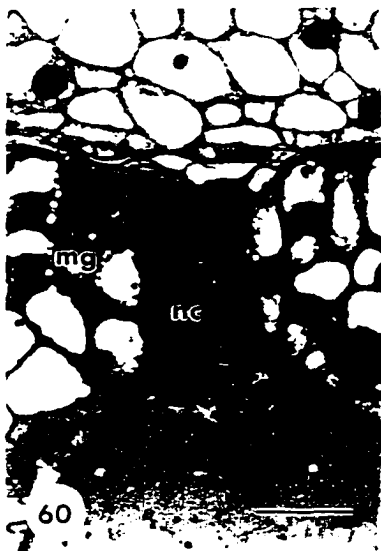
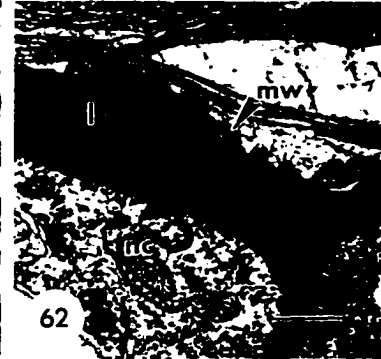
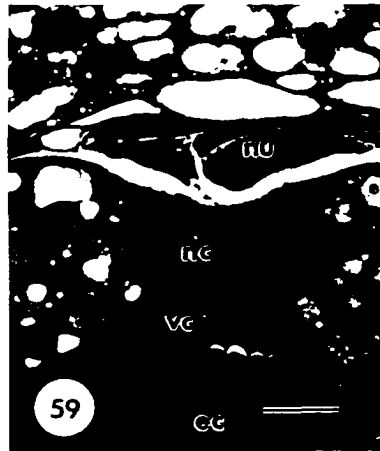
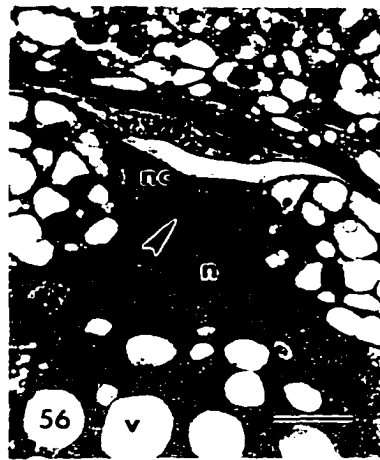
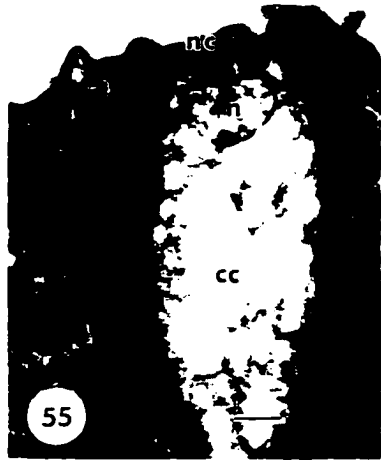
Fig. 54 Tapetal cells are secretory in nature and as they deteriorate, the ectexine of the megaspore wall (mw) is deposited around the megagametophyte. TEM. Bar=2 μ m.



Figures 55-64

- Fig. 55 Mitosis of the archegonial initial produces a vacuolate central cell (cc) with a large, nucleus (n) and a smaller primary neck cell. Mitosis of the primary neck cell gives rise to a single tier of neck cells (nc). LM. Bar=25 μ m.
- Fig. 56 Prior to central cell division, the large prophase central cell nucleus remains at the micropylar pole of the cell isolating a small region of cytoplasm (arrowhead) that forms the ventral canal cell cytoplasm. v - vacuole. LM. Bar=25 μ m.
- Fig. 57 Archegonial jacket cells (aj) are small and angular and completely surround the central cells and early egg cells (ec). TEM. Bar=5 μ m.
- Figs. 58-64 Neck cells (nc).
- Fig. 58 Neck cells, and surrounding cells, become very metabolically active as they mature, as indicated by fluorescein diacetate staining. Confocal. Bar=20 μ m.
- Fig 59 Central cell division produces the egg cell (ec) and the smaller ventral canal cell (vc). Continued mitosis of the neck cells produces a second tier. Cells of the inner layer of the nucellus (nu) deteriorate above each neck forming a depression. LM. Bar=20 μ m.
- Fig. 60 The mature archegonium has a neck comprised of 3 to 4 tiers of cells. Proliferation of cells in the apical region of the megagametophyte (mg) surrounding each neck results in mature archegonium becoming embedded deeply in the megagametophyte. LM. Bar=50 μ m.
- Fig. 61 Mature neck cells have thick primary cell walls and the middle lamellae sometimes deteriorates. Lipid deposits (l) accumulate in the depression between each neck and the nucellus prior to fertilization. LM. Bar=25 μ m.

- Fig. 62 Lipid deposits occur in the depression between neck cells and the megaspore wall (mw). TEM. Bar=1 μ m.
- Fig. 63 Neck cells deteriorate but adjacent cells of the megagametophyte, that may represent an extension of the archegonial jacket (aj), appear healthy and secretory with abundant lipid bodies, plastids, mitochondria, and dictyosomes. TEM. Bar=2 μ m.
- Fig. 64 Lipids are abundant in the cell walls between neck cells and the adjacent cells of the megagametophyte. Lipids occur in the cell walls between most cells in the neck. TEM. Bar=1 μ m.

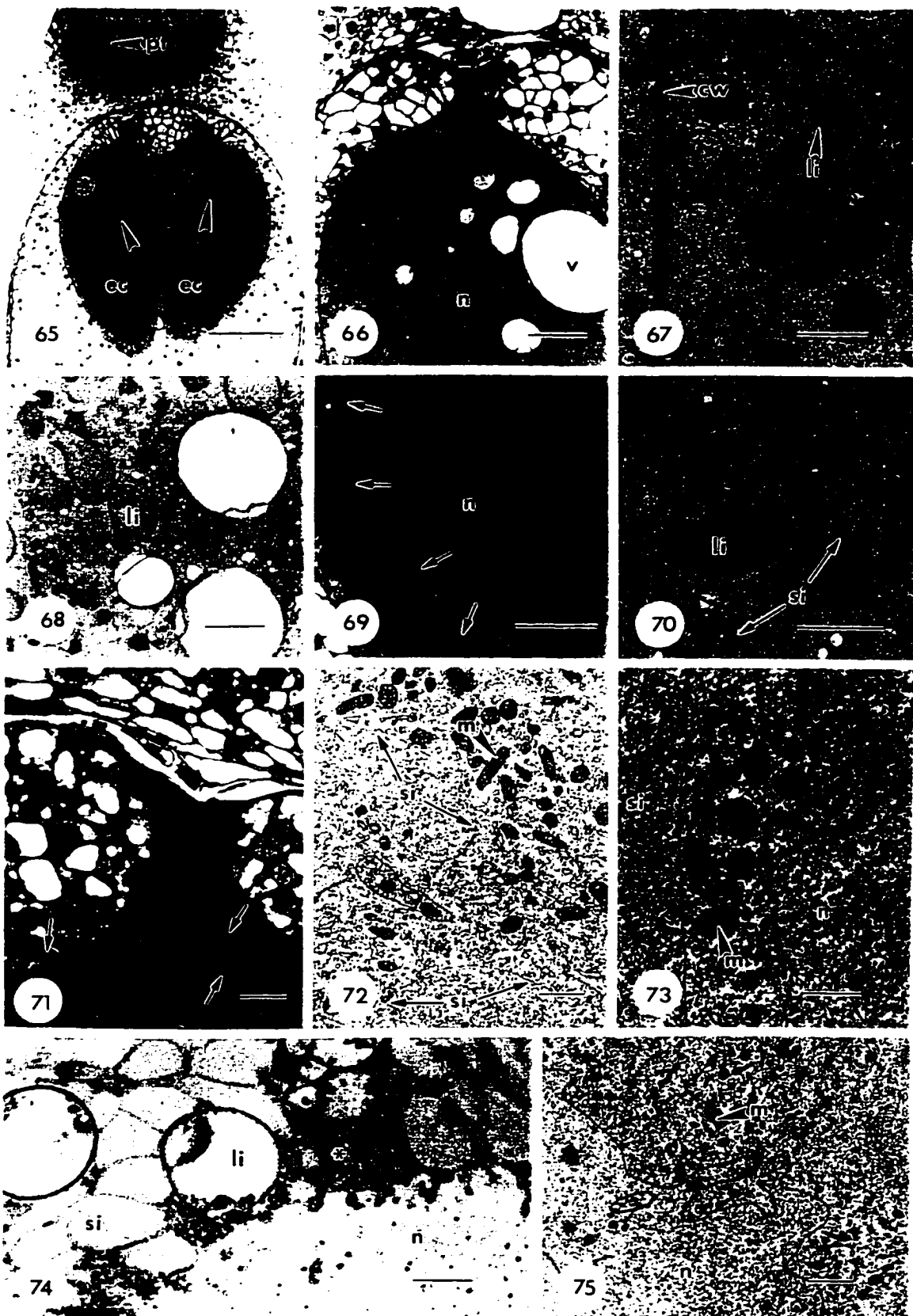


Figures 65-75

- Fig. 65 Egg cells (ec) mature as pollen tubes (pt) grow through the nucellus. Egg nuclei (arrowheads) migrate into the central cytoplasm. LM. Bar=200 μ m.
- Fig. 66 The nucleus (n) of the mature egg is 70 to 80 μ m in diameter. Most of the vacuoles (v) which characterized the central cell disappear during egg cell maturation. LM. Bar=50 μ m.
- Figs. 67-68 Modified plastids (li) form (in the early egg cell, fig. 67 or in the central cell, fig. 68) by elongation of plastids that engulf cytoplasm that is usually free of organelles. Egg cells enlarge to such an extent that the archegonial jacket layers between them are sometimes lost and the egg cell walls (cw) come into contact. Fig. 67, TEM. Bar=10 μ m. Fig. 68, LM. Bar=5 μ m.
- Fig. 69 Modified plastids (arrows) are most concentrated towards the egg cell periphery and chalazal end while the egg nucleus occupies the central cytoplasm. LM. Bar=50 μ m.
- Fig. 70 Modified plastids (li) of the mature egg cell are highly convoluted with many intra-inclusion compartments. Small inclusions (si) are areas of homogenous cytoplasm, bound by only a single thin membrane. TEM. Bar=5 μ m.
- Fig. 71 Modified plastids (arrows) form by plastid distortion in the ventral canal cell and archegonial jacket cells as well as the egg cell. LM. Bar=25 μ m.
- Fig. 72 Mitochondria (m) of the egg cell occupy the cytoplasm not bound by small inclusions (si). TEM. Bar=1 μ m.
- Fig. 73 Many of the mitochondria of the egg cell cluster in the perinuclear zone near the egg nucleus. TEM. Bar=1 μ m.

Fig. 74 In the mature egg cell immediately prior to fertilization, small inclusions completely surround the egg nucleus and barricade the perinuclear zone that includes large clusters of egg cell mitochondria (*). Occasionally, Modified plastids are trapped near the egg nucleus. LM. Bar=5 μ m.

Fig. 75 In the mature egg cell immediately prior to fertilization, the egg nucleus becomes very invaginated. Mitochondria change in appearance. Some became elongate and electron dense, while others become electron translucent. TEM. Bar=2.5 μ m.



Discussion

Many stages of development in the ovule of interior spruce were the same as those published by other investigators of *Picea* (Mergen et al., 1965; Owens and Molder, 1979a; Dawkins and Owens, 1993 for white spruce; Singh and Owens, 1981b for Engelmann spruce; and Owens and Molder, 1984a for interior spruce). Certain features of ovule development were observed here for the first time, or at higher resolution than in previous studies. The results reported in this chapter represent an overview of normal development from the time of pollen germination until archegonium maturation. Where results correspond with those of previous investigators this is noted.

In interior spruce, pollen germination and pollen tube growth in the nucellus were coincident with archegonium development so that pollen tubes arrived at the megagametophyte as egg cells became mature.

Pollen tube

Dawkins and Owens (1993) present the most detailed account of pollen germination and tube growth through the nucellus in *Picea*. In interior spruce, pollen germinated within 13 days after pollination. Body cell division occurred once the pollen tube had penetrated a short way into the nucellus. This was observed in Engelmann spruce by Singh and Owens (1981a) and is unusual as, in conifers, male gametes generally form later, in the vicinity of the megagametophyte (Owens and Molder, 1977). Pollen of white spruce remained in the pollen chamber at the tip of the nucellus for 2 to 3 weeks prior to germination in an earlier study (Owens and Molder, 1979a) but for only two days in the study of Dawkins and Owens (1993). Time to germination may depend on environmental cues. Germination required 2 to 3 weeks when trees were studied in their natural, cooler range. Culture of trees in the hotter and more arid environment of the seed orchard in this study meant that

warmer than natural temperatures prevailed during pollination. Reports of pollen germination in hours or a few days are from potted trees kept at room temperature (Dawkins and Owens, 1993) or when pollen is cultured in vitro at room temperature (Webber, 1991) or warmer (28°C, deWin et al., 1996). Small branches in the pollen tube may serve an anchoring or haustorial function (Johri, 1992) but a single main tube always formed and grew directly towards the megagametophyte. Thin pollen tube processes near the tip which penetrated around nucellar cells were branch-like but reduced in size as the nucellar cells deteriorated and the tube expanded into the vacated space. In culture, 10 to 50% of the pollen tubes of scots pine (*Pinus sylvestris* L.) ramified (deWin et al., 1996) which suggests that they may be chemotactic and that they branch when no directional signal is received from the megagametophyte. Takaso et al. (1996) describe the response elicited in pollen tubes of *Pseudotsuga menziesii* when a homogenate of the megagametophyte was supplied in the culture medium. Homogenate of the megagametophyte of interior spruce may reduce in vitro branching of pollen tubes but this has yet to be tested.

Male gametes of interior spruce were as described in Dawkins and Owens (1993). Mitochondria of the body cell cytoplasm surrounded the male gamete nuclei and were distinguishable from those within the archegonium because they possessed better developed cristae. Plastids surrounding the male gametes were conventional in appearance and clearly distinguishable from those of nucellar cells or the incipient modified plastids in the central cell.

Nucellus

Cells in the central column of the apical region of the nucellus contained abundant starch which was hydrolyzed in cells proximal to the growing pollen tube. Cytoplasm of all cells in the central nucellar apex stained strongly with toluidine blue during pollen tube growth. Red or purplish staining of the type observed is characteristic of increased levels of RNA, polycarboxylic acid, and related compounds of cellular metabolism (O'Brien and McCully, 1981). Nucellar cells seem to increase their metabolism and begin starch hydrolysis and production of compounds necessary for pollen tube growth throughout the apical region, but heightened metabolism does not require physical contact with the growing pollen tube. Nucellar cells were not, however, observed to be apoptotic (Bell, 1996; Havel and Durzan, 1996) in that they did not spontaneously deteriorate and die prior to physical contact with the pollen tube. Pollen tube growth was intercellular in the nucellus. Deterioration of nucellar cells (and cells of the megagametophyte, see chapters 7 and 8) in contact with the pollen tube tip suggests that substances released from the tube mediate cell wall or middle lamella dissolution. Pettitt (1985) determined that many proteins with hydrolytic properties were released from the pollen tube tip of related conifers.

A layer 2 to 3 cells thick at the margin of the nucellar apex had abundant lipid accumulation between the plasmalemma and cell wall. These lipids impregnated the cell walls and may have a waterproofing function. The nucellar apex is a small, exposed tissue which must remain hydrated during development and for pollen germination and growth. Particularly when the micropyle is open before pollination, the nucellus would be susceptible to desiccation. Lipids in the outer cells walls may slow evaporation. Tillman-Sutela et al. (1996) described lipids in the collapsed nucellar layers of mature seeds of scots pine and ascribe to them a function in regulating germination. Perhaps the origin of the

lipids they describe is in the nucellar apex prior to pollination but the lipids observed in interior spruce do not extend into the chalazal region.

Megagametophyte

By the time pollen germinated, the megagametophyte was cellular and at least at the stage of archegonial initial division. Development during the following 5 to 7 day period resulted in archegonia which were considered mature and ready for fertilization.

Megaspore wall

Nucellar cells in the inner 1 to 3 layers surrounding the megagametophyte had a tapetal function. Degeneration of these cells resulted in release of a lipidic substance which had the same staining properties as the megaspore wall. In the early cellular megagametophyte, the megaspore wall is composed of an inner layer that is pectic cellulosic in composition and an outer layer which stains positively for lipids (Favre-Duchartre, 1956). These layers were dubbed intine and exine respectively, and described as functionally similar to the same layers in pollen (see Kurmann, 1990). The origin of the intine layer, whether gametophytic as described for *Ephedra* by Moussel and Moussel (1973), or from the nucellar tapetum as described for several gymnosperms by Pettitt (1966), was not determined here as it was in place prior to the first specimen collection. Deposition of material which was liberated from deteriorating tapetal cells did result in thickening and elaboration of the ectexine layer of the megaspore wall in interior spruce so the wall can be described as, at least partly, sporophytic in origin. In the fully developed megagametophyte of interior spruce, the megaspore wall was thickest in the chalazal region and thinnest at the site of pollen tube penetration near the neck cells of the archegonium.

Cell divisions in the archegonia

Development of archegonia occurred by a series of cell divisions (Singh, 1978; Singh and Owens, 1981a) common to all genera of Pinaceae. Megagametophytes of interior spruce had 2 to 3 archegonia. Uneven division of an archegonial initial produced a large central cell and a small, distally placed primary neck cell. Uneven division of the central cell produced a large egg cell and a small ventral canal cell which subtended the archegonial neck. Pollen was not required for normal megagametophyte or archegonial development and is not required as a signal for initiation of megagametophyte development in most conifers (Owens and Blake, 1985).

Archegonial initials were vacuolate. Rapid enlargement of the central cell was accompanied by a reduction in vacuole size and number, and the mature egg cell was almost completely filled with cytoplasm. There is approximately an order of magnitude difference in length between the archegonial initial and egg cell (40 μm to 400 μm). This represents a change in volume which approaches three orders of magnitude in a very short time. Vacuoles contain solutions at high osmotic potential (Salisbury and Ross, 1985) and exert the pressure required to keep the developing cell turgid while it is being partitioned with cytoplasmic components.

Asymmetric mitosis of the central cell was preceded by isolation of a small area of cytoplasm between the nucleus and neck cells. A similar situation was observed in *Abies grandis* Lindley (Singh and Owens, 1982). This area of cytoplasm was in position to form the ventral canal cell cytoplasm after central cell mitosis. The mechanism, which is probably cytoskeletal, by which the central cell nucleus is held in place at the micropylar pole was not observed. Upon division, a cell wall formed between the ventral canal cell and egg cell before the egg nucleus began to migrate into the central region.

Neck cells and the archegonial jacket layer

Primary neck cell division and subsequent neck cell mitosis resulted in a 3 to 4 tiered neck. Reports of two neck cell tiers in Engelmann spruce (Owens and Molder, 1984a) and three tiers in white spruce (Owens and Molder, 1979a) probably represent natural variation in planes of cell division. Very often the actual number of neck cell tiers was impossible to determine as the cells were not symmetrically oriented.

Archegonial jacket cells differentiated from prothallial cells surrounding the archegonial initial. They were densely cytoplasmic and divided synchronously during central cell division. The increase in archegonial jacket cell number compensated for size increase in the egg cell. The structural role that must be played by archegonial jacket cells in support of the large central and egg cells has not been mentioned in the literature. These cells are small and the resultant complex of radially oriented, primary cell walls may provide support for the large, thin walled central and egg cells while still allowing flexibility for their expansion. Individual archegonia were entirely enclosed by an archegonial jacket layer as a rule, however, rapid enlargement of the egg cell periodically resulted in separation of jacket cells and direct contact between neighboring egg cells as reported by Maheshwari and Singh (1967). The archegonial jacket did not enclose the neck of the archegonium but did proliferate into a 2 to 3 cell layer thick zone around the mature neck cells. Singh (1978) cited the work of Maugini and Fiordi (1970) who reported that storage products, starch and protein, of the megagametophyte cells are solublized and translocated into the central and egg cells through the archegonial jacket cells. This was not verified in interior spruce, but very thin, primary pit fields formed between the archegonial jacket cells and the egg cell. No breakdown of cell walls between the archegonial jacket and the egg cell, as reported in *Pinus* (Singh, 1978), was observed, nor has this breakdown been reported for any other taxa in more recent literature (Owens and Morris, 1990). Such reports in older literature may have resulted from poor fixation or processing

of tissue. Archegonial jacket cells did, however, become vacuolate and appeared secretory in nature during egg-cell maturation.

As the egg cell neared maturity, lipid secretions from the neck cells and adjacent archegonial jacket cells accumulated between the neck and megaspore wall in the archegonial chamber. This secretion could not be attributed to one cell type as lipid bodies were observed within the cell walls between neck cells and archegonial jacket cells, and even in the cell walls between neck cells and the ventral canal cell. Neck cell cytoplasm deteriorated prior to pollen tube arrival. Singh (1978) noted this deterioration in *Cephalotaxus* and attributed it to secretion contributing to production of the very thick, primary cell walls as observed here in interior spruce. Archegonial jacket cells surrounding the neck remained densely cytoplasmic and secretory with many lipid bodies and dictyosomes. Fluorescein diacetate staining of the megagametophyte at this stage indicated esterase activity in all of the archegonial jacket cells but the highest intensity was in the neck and surrounding cells. Lipid accumulation in the archegonial chamber occurred at exactly the site to which pollen tubes must grow (Chapter 7). Takaso and Owens (1996) described similar lipid-like secretions in the walls of prothallial cells in the apical portion of the megagametophyte of *Pseudotsuga menziesii* before fertilization.

A central question in the study of conifer reproductive biology is, how do the pollen tubes find the neck? Mascarenhas (1993) discussed chemotropism of the angiosperm pollen tube. Pollen tubes of pearl millet (*Pennisetum glaucum*) were chemotropic to a variety of chemicals including: glucose, calcium, and low molecular weight proteins (Reger et al., 1992). This list does not include lipids but it is not exhaustive and a positive staining reaction for lipid does not completely characterize the secretion in the archegonial chamber of interior spruce ovules.

Mature archegonium - cytoplasmic inclusions and the perinuclear zone

Archegonia were considered mature when the egg nucleus had migrated to the middle of the cell and was surrounded by a perinuclear zone containing clusters of maternal mitochondria. At this stage, vacuolization of the egg cytoplasm was minimal and large and small inclusions were prominent features.

Chesnoy and Thomas (1971) reviewed large inclusion form and function as interpreted by earlier workers. They cite the pioneering electron microscope studies of Camefort (eg. Camefort, 1959) as the first to give evidence of large inclusion formation as it is now known to occur. Modified plastids formed in interior spruce when plastids of the central cell elongated and deformed to encompass regions of cytoplasm. Deformation of plastids continued during egg cell maturation. Plastids in the archegonial jacket cells and ventral canal cell deformed into modified plastids at the same time, while those in nucellar cells did not.

Interpretation of small inclusion formation is more difficult. They are easily seen in the light microscope because of what they do not contain. They are regions of cytoplasm devoid of organelles and bound by a simple membrane. Cytoplasm within small inclusions was sometimes more densely staining than surrounding, non-included cytoplasm. The cytoplasmic fraction not bound by small inclusions contained all of the maternal mitochondria and so appeared granular by comparison. Earlier reports show vacuolization within the bounding membrane of small inclusions during egg cell maturation (Singh, 1978). This was not observed in interior spruce. Fixation and dehydration of cytoplasm can result in shrinkage, and apparent vacuolization may have been artifactual. I will extend this reasoning to propose a function for small inclusions.

Nuclei and mitochondria move within the egg cell by a mechanism that is not understood. Small inclusions are positioned to mediate or, at least, regulate movement of nuclei and organelles. Mersey and McCully (1978) described vesicularization of a

pleiomorphic canalicular system within petiolar hair cells of tomato (*Lycopersicon esculentum*) after chemical fixation. Endogenous membrane systems exist within many plant cell types and organelles and nuclei have been observed to move along them in actively streaming cytoplasm. Addition of fixatives very quickly results in a change in appearance of the membrane systems. Extended membranes round up into smaller vesicles (see figs. 9 to 12 of Mersey and McCully, 1978). Admittedly, the vesicles resulting from fixation of petiolar hair cells were much smaller than many of the small inclusions in interior spruce but they appear analogous.

How the organelles and nuclei might move along the small inclusion membrane system is open to conjecture. Cytoskeletal elements are most likely involved but were rarely observed within the egg cell cytoplasm because the fixation used was inappropriate for their preservation. Individual microtubules were preserved in the cytoplasmic fraction not contained in small inclusions. Microtubules were oriented with long axes roughly towards the central nucleus, in fact, they made good pointers when trying to find the nucleus by high magnification TEM. Actin-myosin mediated movement of organelles and male gametes has been demonstrated in angiosperm pollen tubes (Heslop-Harrison and Heslop-Harrison, 1989) and postulated but not conclusively demonstrated in angiosperm egg cells (Russell, 1993; Huang and Russell, 1994). Terasaka and Niitsu (1994) have demonstrated filaments of F-actin and myosin-coated cytoplasmic components in the pollen tubes of *Pinus sylvestris* L.. This actin-myosin association may extend into the egg cell of pinaceous species.

Small inclusions also seemed to play a role in formation of the perinuclear zone. They partitioned the egg cell cytoplasm in such a way that the non-included cytoplasm was relatively small in volume. Small inclusions increased in number towards the periphery of the egg cell as it matured and may simply have squeezed the cytoplasmic fraction containing mitochondria into the centre. Certainly, when the immature egg nucleus had recently

migrated to the centre of the cell, elongate small inclusions filled the area in its wake so that non-included cytoplasm was displaced towards the nucleus.

Questions of small inclusion form and function may be resolvable if the egg cell cytoplasm can be studied in the absence of artifacts induced by chemical fixation. This is true in any case where deduction of cell function is attempted by interpretation of chemically fixed structures. Freeze-fixation, if possible in a cell so large, or confocal microscopy after staining with fluorescent vital stains may help resolve some of these issues.

At maturity, the egg cell had a large centrally located nucleus surrounded by clusters of maternal mitochondria. Small inclusions partitioned the egg cell cytoplasm in such a way that modified plastids were held towards the periphery where they had formed. The perinuclear zone, which contained clusters of maternal mitochondria, was entirely surrounded by small inclusions and spatially isolated from other small regions of non-included cytoplasm which contained maternal mitochondria. Formation of the small inclusion bound perinuclear zone and the undulating appearance of the egg nuclear membrane were good indices of egg cell maturation and competence for fertilization.

Chapter 7

Sexual reproduction of interior spruce: fertilization to early embryo formation

Introduction

Fertilization occurs soon after megagametophyte maturation in conifers. Only a few studies have used other than paraffin embedded tissues to study fertilization. The earliest TEM studies of conifer megagametophytes were done by Camefort (eg. 1959), Chesnoy (1967), and Willemse (1968). Camefort, in particular, produced many papers describing the nature of the egg cell cytoplasm, mechanisms of cytoplasmic inheritance, and formation of proembryos in *Pinus* and *Larix*. More recently, cytoplasmic inheritance and proembryo development have been studied in *Pseudotsuga* (Owens et al., 1991; Owens and Morris, 1991), *Larix* (Owens et al., 1994), and in the Araucariaceae (*Agathis*, Owens et al., 1995).

The fertilization period covered here for interior spruce (white spruce (*Picea glauca* (Moench) Voss), or Engelmann spruce (*P. engelmannii* Parry), or their hybrid in British Columbia) includes development from the time pollen tubes contact the mature archegonium until the early embryos are formed. Gamete fusion forms the zygote so all stages prior to fusion are considered prezygotic. Postzygotic development from zygote mitosis through proembryo formation is described in the interests of completeness. Neither the mechanism of cytoplasmic inheritance, or proembryo formation in *Picea* have been subjects of ultrastructural study. Krasowski and Owens (1993) detailed postfertilization development of the megagametophyte and embryo of white spruce. This chapter concludes with a special case description of an anomalous fertilization which nonetheless resulted in a healthy appearing proembryo, and leads into chapter eight in which anomalies resulting in seed losses are described.

Materials and methods

As described in chapter six. Fertilization events described were from samples collected between May 23-27, 1993, 16 to 20 days after pollination.

Observations and results

Fertilization

By 15 to 16 days after pollination, pollen tubes had reached the megagametophyte. Pollen tubes grew through the nucellus until they contacted the megaspore wall and then spread over the apex of the megagametophyte until a neck was encountered. A thin pollen tube process then extended through the megaspore wall and between the neck cells (fig. 76). Neck cells contained deteriorated cytoplasm by this stage and pollen tube penetration between them occurred via separation of their middle lamellae. Each archegonium was penetrated by only one pollen tube. The pollen-tube tip grew into the ventral canal cell. Dissolution of the tube tip and part of the common cell wall between ventral canal cell and egg cell happened concurrently. The tube tip was much smaller in diameter than the male gamete nuclei. Growth proceeded through an opening in the ventral canal cell wall then the pollen-tube wall ruptured and extended into the egg cell cytoplasm (fig. 77). Pollen tube contents were released into the egg cell cytoplasm. Starch grains that had moved into the egg from the tube cell cytoplasm were a reliable signal that fertilization had occurred as they did not occur in the unfertilized egg cytoplasm. A dense deposit, which stained positively for polysaccharides, was deposited around the remnants of the pollen tube and the ruptured ventral canal cell wall within the egg cell cytoplasm (figs. 77-79). This deposit was located so that the site of pollen tube entry into the egg cell was effectively sealed after fertilization. Many small vacuoles appeared in the region of common cytoplasm in the micropylar end of

the egg. These may have formed *de novo*, but they appeared similar to the vacuoles of the pollen tube cell cytoplasm and likely moved into the egg cytoplasm with the other pollen tube contents. In either case, vacuolation in the micropylar cytoplasm of the egg cell was the earliest sign of the radical change in egg cell appearance which accompanied fertilization.

Male gamete nuclei were the first pollen tube component to move into the egg cell cytoplasm and, in most cases, no large vacuole formed at the site of male gamete entry until after they had moved farther into the cytoplasm (fig. 77). Coalescence of smaller vacuoles, on occasion, lead to formation of a larger vacuole in this region after passage of the male gametes. Male gamete nuclei became spherical after passage into the egg cell and were approximately 50 μm in diameter. The leading male gamete nucleus, had an extension towards the egg nucleus (fig. 77). This elongate form was characteristic of the leading gamete as it moved towards the egg nucleus. Within 24 hours (and perhaps much less), the leading male gamete nucleus moved to the egg nucleus in the central part of the egg cell cytoplasm and gamete fusion occurred (figs. 80-81, 83). Paternal plastids which moved into the egg cytoplasm retained the orthodox appearance they had while in the growing pollen tube (fig. 82). Paternal mitochondria were larger and rounder than maternal ones which were distorted and contained electron dense and translucent areas. Supernumerary nuclei and other pollen tube contents, including the trailing male gamete nucleus, were confined to the site of entry or the peripheral egg cytoplasm (fig. 81).

As nuclear fusion proceeded, supernumerary nuclei were enveloped by membranes and eventually deteriorated. Small inclusions continued to surround the fusion nucleus except where they had been displaced by passage of the male gamete (fig. 80). A darkly staining region was observed in the peripheral nucleoplasm of the male gamete nucleus just prior to, and during fusion (fig. 83). Contact between the male gamete nucleus and egg nucleus resulted in formation of a depression in the female into which the smaller male

settled. Nuclear membranes in the contact zone broke down to allow fusion of nucleoplasm (fig. 83). A neocytoplasm containing maternal and paternal organelles, as defined for some other genera of Pinaceae (Camefort, 1966), was not well defined at the zygote stage. Maternal mitochondria continued to surround the fusion nucleus in several small groups as they had done in the perinuclear zone surrounding the egg nucleus. Paternal plastids and mitochondria remained in small clusters at a distance from the fusion nucleus (fig. 83).

First mitosis of the zygote occurred shortly after gamete fusion. The zygote nuclear membrane disappeared and chromosomes condensed within an organelle free zone of nucleoplasm (fig. 84). During zygote mitosis the neocytoplasm became distinct. By telophase, plastids and mitochondria surrounded each of the proembryo nuclei. Plastids were paternal in origin and all mitochondria appeared to be the maternal form. Mitochondria were numerous around each nucleus, but plastids were sparsely distributed. The second mitosis, which resulted in a proembryo with four free nuclei, proceeded immediately, and synchronously, within the neocytoplasm in the center of the archegonium. Microtubule arrays of the spindle fibres were evident during mitosis (fig. 85). These were the only observation of microtubule bundles during fertilization and proembryo development. Single, or small groups of 2 to 3 microtubules, were seen occasionally during archegonial development and migration of various nuclei. These occurred in the egg cell cytoplasm outside of the small inclusions, and around gamete and proembryo nuclei.

Coincident with gamete fusion, the cytoplasm surrounding the zygotic neocytoplasm underwent a significant change in appearance. This change proceeded in a wave from the chalazal end as the archegonial cytoplasm lysed (fig. 86). As a result, fertilized and unfertilized archegonia became distinct in appearance (fig. 87). Change in the nature of small inclusions accounted for most of the contrast. In fertilized eggs, small

inclusions became spherical and the cytoplasm within was reduced in volume and became more heavily staining. Reduction of the cytoplasmic volume resulted in vacuole formation within the small inclusions. Modified plastids within the fertilized egg, and within the archegonial jacket cells, became highly convoluted and deteriorated (fig. 88).

One special case of fertilization observed is worthy of description. Two normally developed, mature archegonia were within an ovule which had been pollinated by a single pollen grain. The pollen tube grew into the neck of one archegonium, out through the side of the neck, and through the prothallial cells of the megagametophyte towards the other archegonium. Upon encountering the archegonial jacket cells of the second archegonium, the pollen tube turned and grew through them acropetally around the archegonium into proximity with the ventral canal cell. Fertilization was effected by pollen tube penetration through the egg cell wall near the ventral canal cell (fig. 89), and a normal appearing proembryo was produced.

Proembryo and early embryo

The four free nuclei of the proembryo, approximately 40 μm in diameter each, migrated towards the chalazal end of the archegonium (fig. 90). As they migrated, the nuclei did not necessarily remain near each other. Each nucleus was surrounded by a separate zone of neocytoplasm (fig. 91) that contained numerous mitochondria and some plastids. Inclusions of the archegonium were completely excluded from neocytoplasmic zones. As the nuclei moved closer together in the tapering chalazal end of the archegonium, neocytoplasms mixed and the nuclei became irregular in shape (fig. 92). The neocytoplasm at this stage contained many small lipid bodies. Once settled into a single tier, the proembryo nuclei became spherical and were surrounded by neocytoplasm which was densely packed with mitochondria and contained sparsely distributed plastids (fig. 93).

Rough endoplasmic reticulum was plentiful around the proembryo nuclei (fig. 94) and areas of cytoplasm at the periphery of the neocytoplasm became vacuolate.

All four free nuclei settled into one tier before undergoing synchronous mitosis (fig. 95) to produce a two tier proembryo with eight nuclei. Prior to this mitosis, all plastids had migrated into a chalazal position relative to the nuclei so that they were included in only the chalazal-most tier of cells once cell wall formation occurred. Cross walls formed first between the nuclei in each tier (fig. 96). Longitudinal walls then formed between the nuclei within each tier. Cell walls generally only formed between nuclei, so the tier of cells at the micropylar end remained open to the archegonial cytoplasm. Subsequent mitosis were asynchronous between tiers. The primary open tier divided and cell walls formed (fig. 97) before mitosis started in the primary embryonal tier at the chalazal end (fig. 98). First sign of cell wall synthesis was an accumulation of membranes and small vesicles at the cell plate (fig. 99). At a slightly later stage, thin cell walls and plasmalemma formed (fig. 100). Cytoplasm of cells in the embryonal tier was dense with ribosomes, endoplasmic reticulum, dictyosomes, lipid bodies, and protein crystals in the vicinity of the forming cell wall. Some mitochondria, which had remained spherical until this stage, elongated during cell wall synthesis. All proembryonic cells were fully surrounded by cell walls except those in the open tier. This rule was sometimes violated as partial and complete cross walls were occasionally observed to separate the open tier cells from the archegonium cytoplasm (fig. 101).

A fully formed proembryo of interior spruce consists of four tiers of four cells each (fig. 102). In order from the chalazal end these are called, i) embryonal tier, ii) suspensor tier, iii) non-functional suspensor tier, and iv) open tier. Embryonal cells were small, and densely cytoplasmic with small nuclei. Cells were larger, with larger nuclei, and more vacuolate towards the open tier. Composition of cytoplasm was visibly different between embryonal and suspensor tier cells (fig. 103). Cells in the embryonal tier were densely

cytoplasmic with some small vacuoles and plastids, while cells of the suspensor tier were very vacuolate and lacked plastids. Deterioration of cytoplasm in peripheral regions of embryonal tier cells adjacent to the suspensor tier was coincident with the appearance of lipid bodies and protein crystals near the site of breakdown (fig. 104). In the mature proembryo, a very thin cell wall fully enclosed the embryonal tier cells and could be seen as distinct from the common wall between the former egg, and archegonial jacket cells in the chalazal-most region where the embryo would penetrate into the megagametophyte (fig. 105. also see fig. 93).

Early embryo penetration of the megagametophyte was due to elongation of the suspensor tier cells (fig. 106). Non-functional suspensor tier and open tier cells of the proembryo did not divide or elongate.

Figures 76-82

Figs. 76-78 Pollen tube (pt) penetration of the egg cell (ec).

Fig. 76 Pollen tubes grow to the megaspore wall. They grow directly to a neck or turn and grow over the apex of the megagametophyte until a neck is encountered (dashed line indicates pollen tube pathway into egg). LM. Bar=50 μ m.

Fig. 77 Pollen tubes push between the neck cells into the ventral canal cell which deteriorates. Usually the pollen tube extends into the egg cytoplasm and then the male gametes are released. The second male gamete (σ^7 ii), which will not effect fertilization remains in the apical part of the egg. LM. Bar=25 μ m.

Fig. 78 After pollen tube penetration of the egg cell, a thick polysaccharide coating, that has staining properties similar to primary cell wall, accumulates around the remnants of the pollen tube and ventral canal cell wall (arrowheads). LM. Bar=25 μ m.

Fig. 79 As in fig. 78. The polysaccharide coating (arrowheads) on the post-fertilization ventral canal cell wall (cw) becomes very thick and appears to plug the rupture caused by pollen tube entry. aj - archegonial jacket. TEM. Bar=2 μ m.

Fig. 80 The leading male gamete nucleus (σ^7) fuses with the egg nucleus (Q). The pathway of the male gamete can be seen because small inclusions have been displaced (above and to the right of the male gamete nucleus). LM. Bar=20 μ m.

Fig. 81 The trailing male gamete nucleus (σ^7) remains in the apical archegonial cytoplasm. DIC. Bar=25 μ m.

Fig. 82 Pollen body cell organelles accompany male gamete nuclei into the egg cell. Paternal plastids (p) are elongate and electron dense. TEM. Bar=1 μ m.

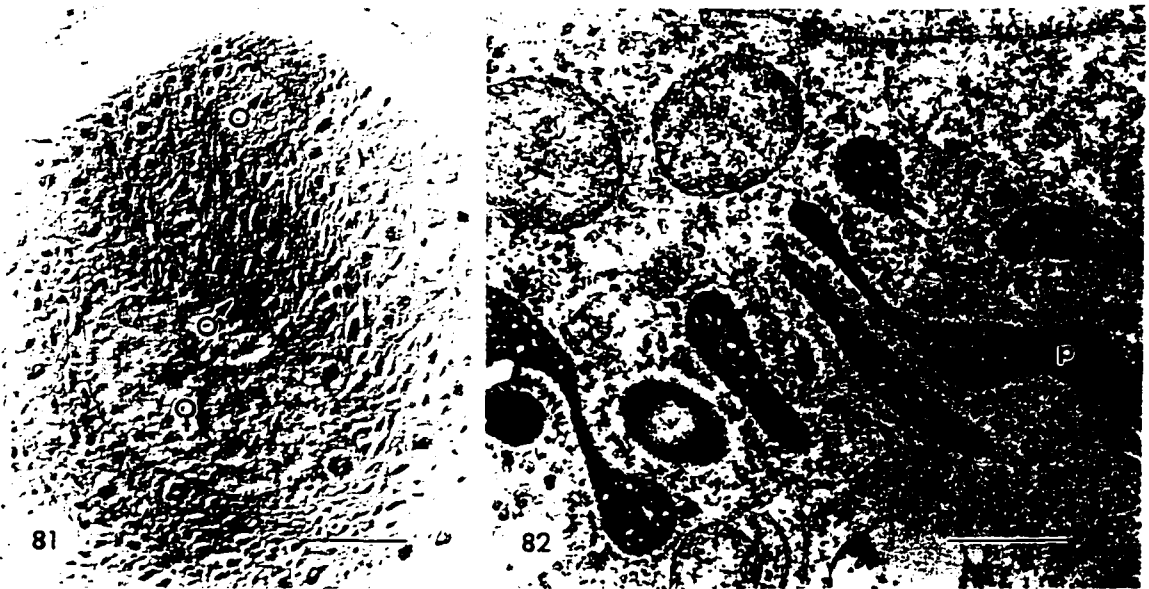
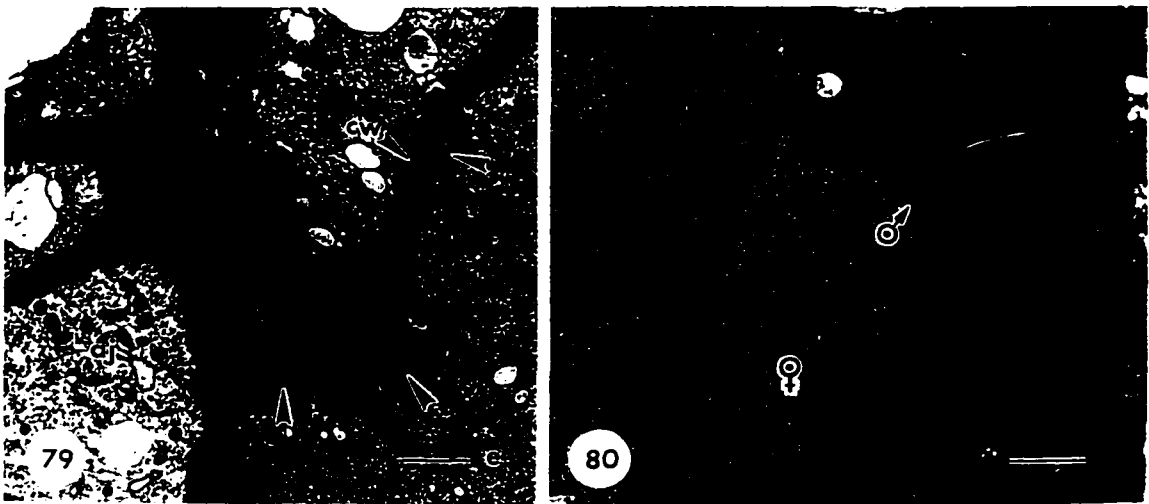
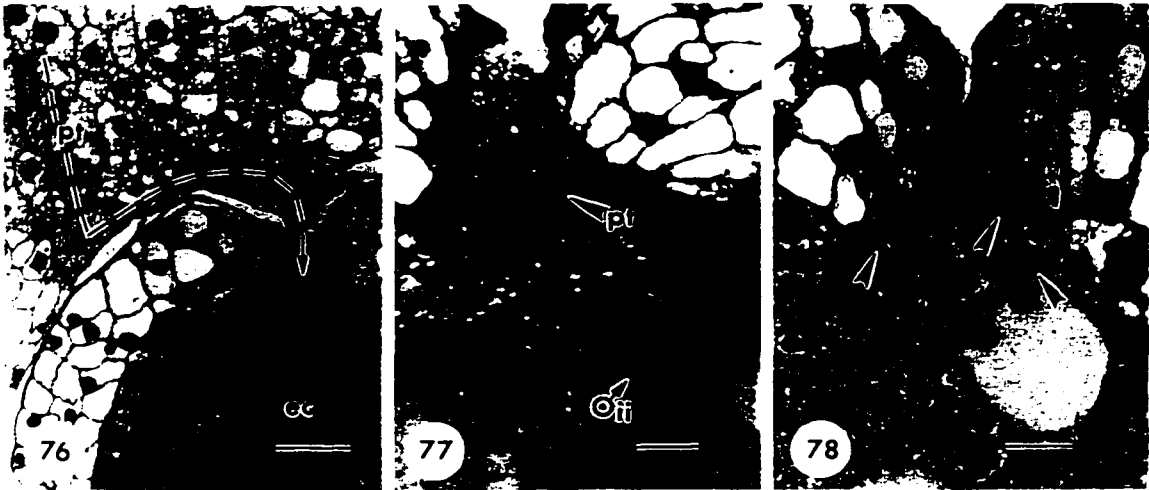
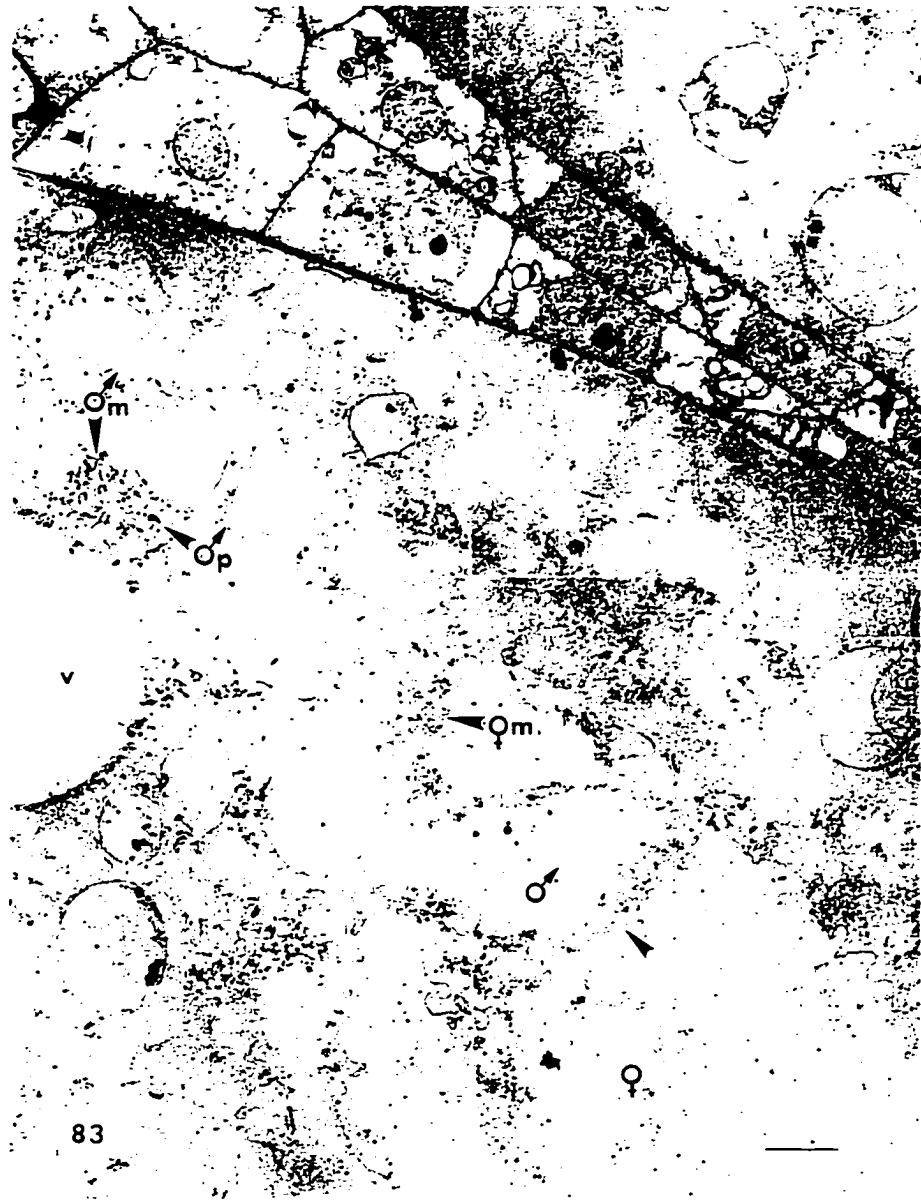


Figure 83

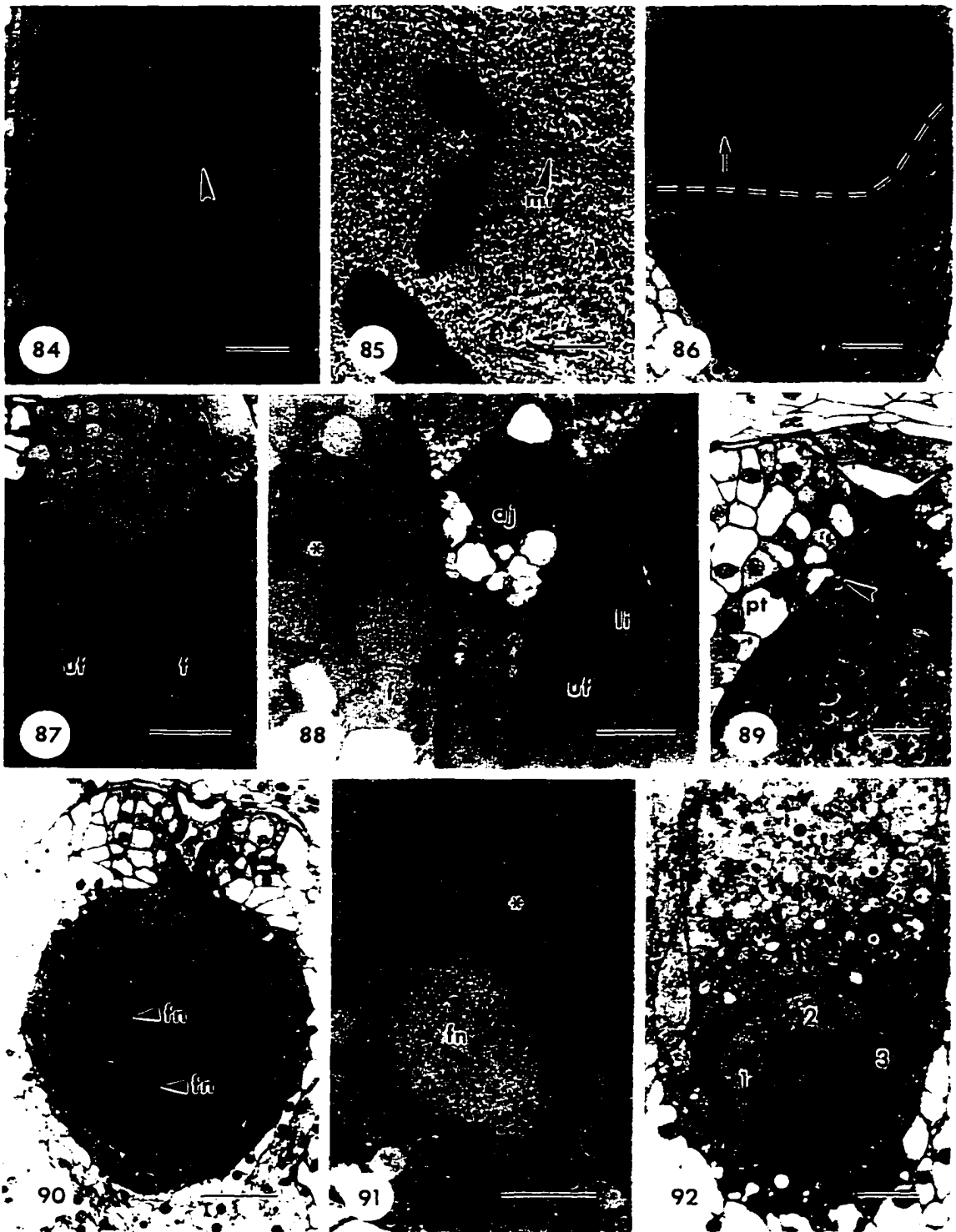
Fig. 83 As in fig. 82. Nuclear membranes between the male gamete nucleus (σ) and the egg nucleus (φ) fuse (arrowhead) and channels of nucleoplasm span the gaps that form. Maternal mitochondria remain in several discrete clusters (φ m) around the fusion nucleus. Paternal organelles lag far behind the fertilizing male gamete nucleus. Paternal mitochondria (σ m) are clustered into large groups and are clearly distinguishable from the maternal mitochondria. Paternal plastids (σ p) are rarely seen. They were isolated like the one shown here or occur in groups of 5 to 10. TEM. Bar=10 μ m.



Figures 84-92

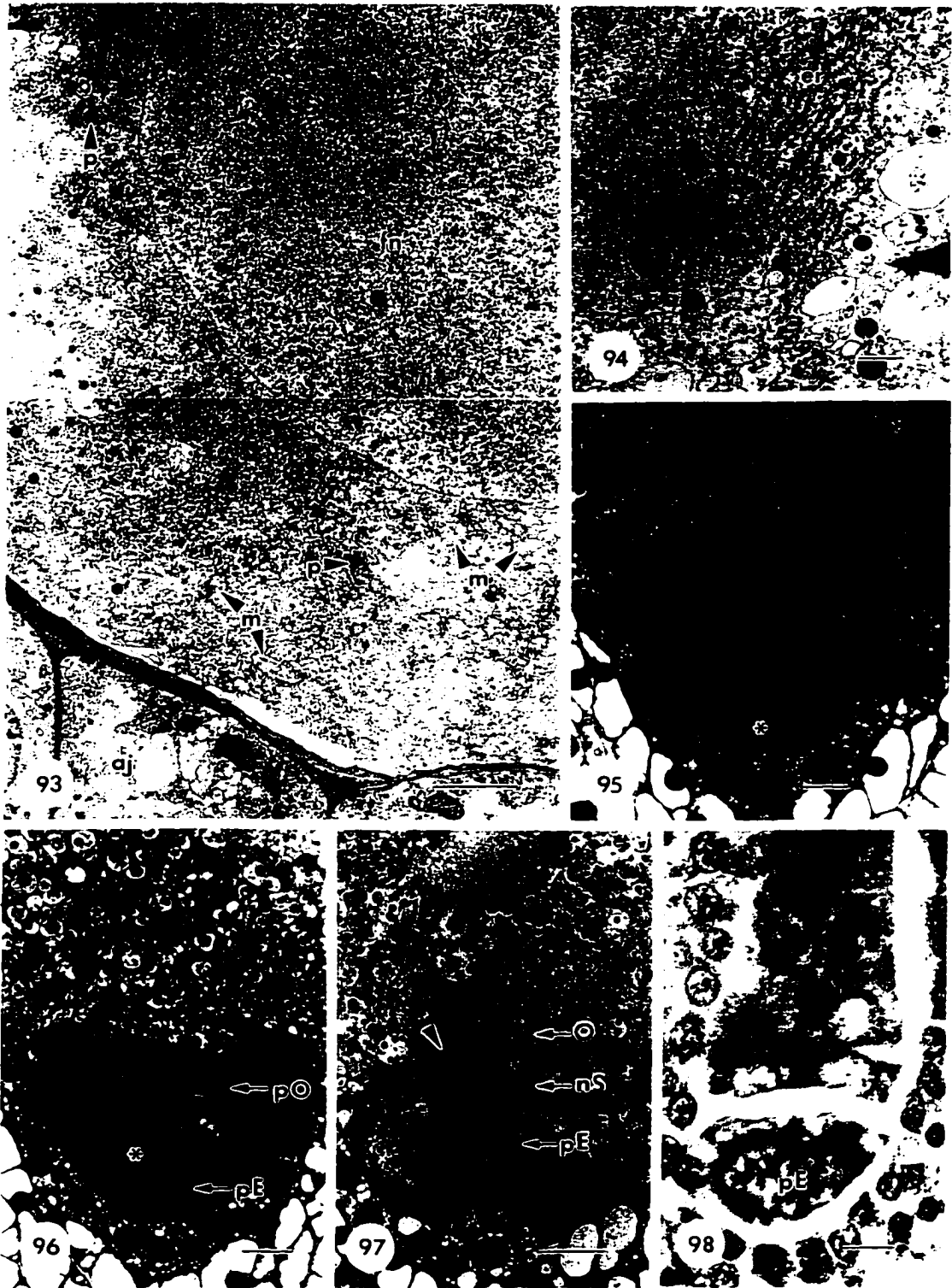
- Fig. 84 Mitosis of the zygote. Metaphase of nuclear division (arrowhead) occurs in the central part of the archegonial cytoplasm. LM. Bar=50 μ m.
- Fig. 85 Metaphase chromosomes during zygote mitosis. Chromosomes are surrounded by an homogenous appearing nucleoplasm which contains microtubule bundles (mt) of the spindle apparatus. TEM. Bar=1 μ m.
- Fig. 86 After fertilization, a radical change takes place in the appearance of the archegonial cytoplasm. Small inclusions become round and vacuolate. The change occurs in a wave from the chalazal end (arrow - direction of wave, dashed line - wavefront). This archegonium is at a more advanced stage (free nuclear proembryo) than the one in fig. 84 but the cytoplasmic change has not progressed to the same extent (compare fig. 84). LM. Bar=50 μ m.
- Fig. 87 The cytoplasmic change characteristic of a fertilized archegonium (f) makes it easily distinguishable from an unfertilized egg cell (uf). LM. Bar=100 μ m.
- Fig. 88 Modified plastids (li) remain intact in an unfertilized egg cell (uf) while those in an adjacent, fertilized egg (f) deteriorate. Remnants of deteriorated modified plastids remain in the cytoplasm (*). aj - archegonial jacket. LM. Bar=10 μ m.
- Fig. 89 An anomalous case of fertilization. The pollen tube (pt) diverted from the neck of an adjacent archegonium and grew through the prothallial cells of the megagametophyte until it contacted the archegonium. It turned and grew towards the apex of the archegonia but ultimately fertilized the egg through the side (arrowhead) and a normal appearing proembryo formed. LM. Bar=50 μ m.

- Fig. 90 The coenocytic proembryo with four nuclei, the two visible in this section (fn) are migrating to the chalazal pole of the archegonium. LM. Bar=100 μ m.
- Fig. 91 Each free nucleus (fn) of the proembryo is surrounded by neocytoplasm composed of a thin layer devoid of organelles immediately outside of the nuclear membrane and a thicker layer packed with organelles (*). LM. Bar=25 μ m.
- Fig. 92 As the free nuclei of the proembryo near the chalazal pole of the archegonium, they become distorted and their neocytoplasm merge. Eventually, the free nuclei settle into a single tier. The darkly stained dots visible within the neocytoplasm are lipid bodies which are produced in abundance at this stage. LM. Bar=50 μ m.



Figures 93-98

- Fig. 93 Free nuclei (fn) of the proembryo become spherical after settling into a single tier at the chalazal pole of the archegonium. The neocytoplasm loses the thin zone which had been free of organelles and, particularly on the chalazal side of the nuclei, mitochondria (m) congregate against the nuclear membrane. Plastids (p) were sparsely distributed around the nuclei. aj - archegonial jacket. TEM. Bar=5 μ m.
- Fig. 94 Rough endoplasmic reticulum (er) occurs in abundance around the free nuclei of the proembryo once they settle into a single tier. TEM. Bar=1 μ m.
- Figs. 95-98 Cell divisions and cell wall formation in the proembryo. LM. Bars=50 μ m.
- Fig. 95 Synchronous mitoses occur in all four proembryo nuclei. All plastids which surrounded the free nuclei move to the chalazal pole (*) prior to mitosis.
- Fig. 96 Cell walls form between products of the previous mitosis resulting in a 2-tiered proembryo with four nuclei in each tier. All of the proembryo plastids (*) remain in the primary embryonal tier (pE) and mitochondria segregate into both tiers. The primary open tier (pO) remains open to the archegonial cytoplasm.
- Fig. 97 After cell walls form between cells within each tier, the primary open tier cells divide synchronously to form the non-functional suspensor tier (nS) and the open tier (O). The primary embryonal tier (pE) did not divide. arrowhead - cell plate formation.
- Fig. 98 Mitosis in the primary embryonal tier (pE), to produce the embryonal and suspensor tiers, occurs once cell walls have fully formed in the two other tiers.



Figures 99-106

Figs. 99-100 Cell wall (cw) formation within the embryonal tier. TEM. Bars=1 μ m.

Fig. 99 The first indication of cell wall formation in the embryonal tier is an accumulation of membranes and coated vesicles (arrowheads) across the phragmoplast.

Fig. 100 As the cell wall forms, storage product metabolism is visible. Lipid bodies (lb) arise in the cytoplasm and move through the plasmalemma to lie against the forming cell wall. Protein crystals (pc), elongate mitochondria, dictyosomes, and endoplasmic reticulum are all concentrated near the site of cell wall formation.

Fig. 101 Occasionally cell walls (arrowhead) form to enclose, or partly enclose cells in the open tier (O) of the proembryo. Bar=25 μ m.

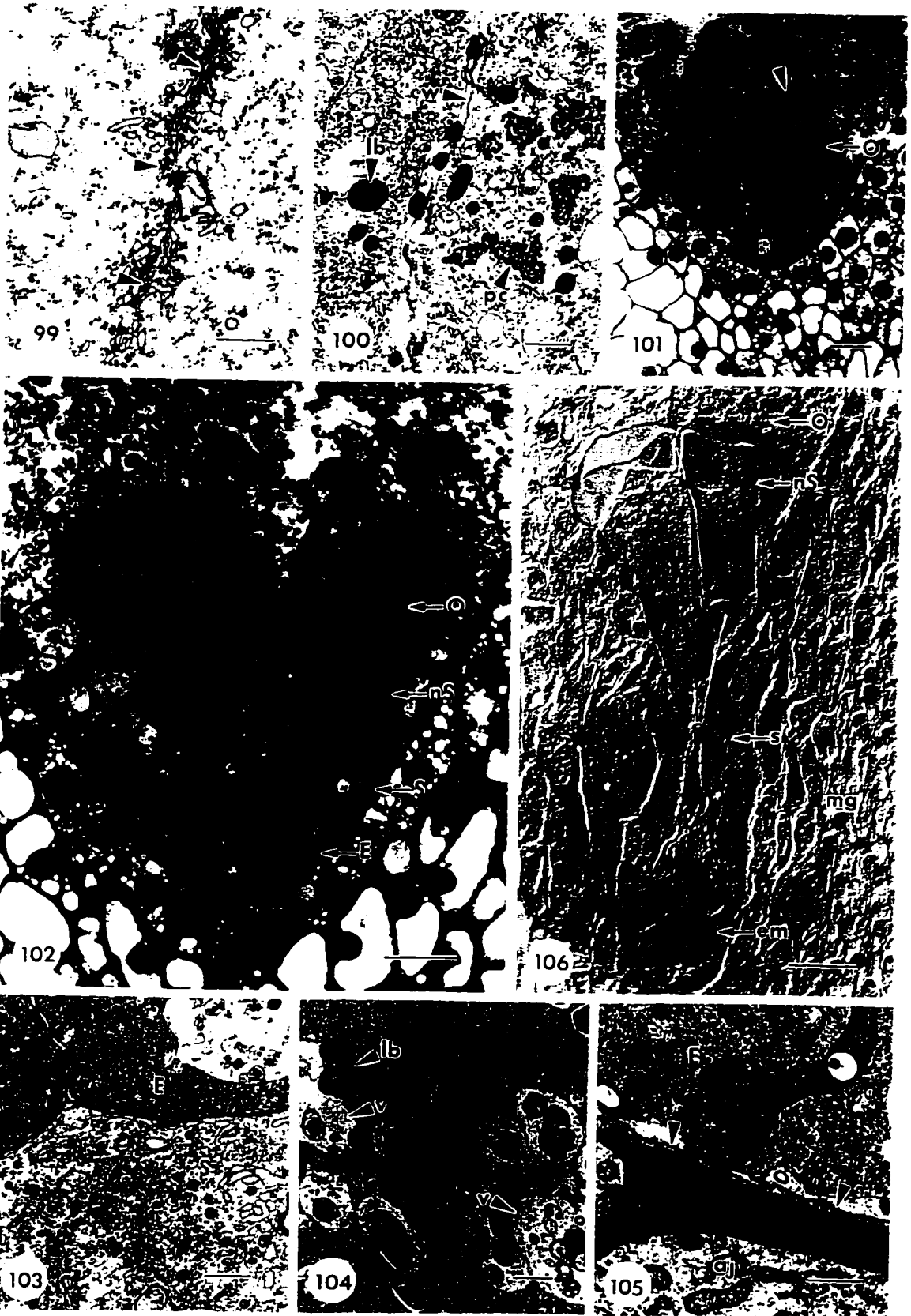
Fig. 102 The mature proembryo consists of four tiers of four cells each. E - embryonal tier, S - suspensor tier, nS - non-functional suspensor tier, O - open tier. Nuclei are smallest in the embryonal tier and are progressively larger through the tiers towards the micropylar end. Bar=25 μ m.

Fig. 103 Embryonal tier cells contain dense cytoplasm relative to cells in the suspensor tier. Large clusters of mitochondria are scattered throughout suspensor tier cells. TEM. Bar=2 μ m.

Fig. 104 After cell wall formation in the embryonal tier, large vacuoles appear in the cytoplasm towards the micropylar end. Vacuole formation coincides with formation of lipid bodies. TEM. Bar=1 μ m.

Fig. 105 Prior to embryo penetration of the megagametophyte, the thin cell walls (arrowheads) of the embryonal tier cells separate from the thicker wall of the archegonium at the chalazal pole. The plasmalemma undulates and lipid-like secretions are observed in the space between the cell wall and plasmalemma. TEM. Bar=1 μ m.

Fig. 106 Suspensor cells (s) elongate and push the early embryo (e) into the megagametophyte (mg). No elongation occurs in the non-functional suspensor tier or the open tier of the proembryo. DIC. Bar=50 μ m.



Discussion

Pollen tube growth through the nucellus of interior spruce coincided with maturation of the archegonia so that no delay was observed between the time pollen germinated and fertilization occurred. Pollination to fertilization required 15-20 days in this study. Time from pollination to fertilization is variable among genera of Pinaceae. No delay occurs in *Picea* (Owens and Molder, 1979a; Singh and Owens, 1981a), whereas genera such as *Pseudotsuga*, *Larix*, *Tsuga*, and *Abies* delay pollen germination for several weeks after pollination during maturation of the megagametophyte (eg. Takaso and Owens, 1994). In *Pinus*, winter dormancy occurs between pollen germination and fertilization (Owens et al., 1982). Seed development from the time of fertilization is roughly the same for all temperate Pinaceae (Singh, 1978).

Pollen tube penetration and the ventral canal cell

Pollen tubes grew with little or no deviation through the nucellus until they contacted the megaspore wall. Tubes then ballooned out over the apex of the megagametophyte until an archegonial chamber was encountered above the neck of an archegonium. If a chemical or physical signal exists which causes pollen tube growth towards an archegonium (eg. the lipid accumulation in the archegonial chamber which was described in Chapter 6) it does not cause angular growth of the tube within the nucellus directly to an archegonial neck. By spreading over the megagametophyte apex, pollen tubes seemed to encounter archegonia at random. A similar description of pollen tubes splaying out over the archegonial chamber of Engelmann spruce was provided by Singh and Owens (1981a). Neck cell middle lamellae lysed as the cells deteriorated and pollen tubes pushed between them. Deterioration of the neck cells before pollen tube arrival probably reduces their turgidity and makes a good conduit for the pollen tube. Rupture of

the pollen tube occurred as it grew into the ventral canal cell and, in some cases, the ruptured pollen tube projected well into the micropylar part of the egg cell cytoplasm. A substance deposited at the site of pollen tube entry into the egg cell had the same staining characteristics as the primary cell wall, with toluidine blue. This substance was probably pectinaceous, or some other α (1-4) linked polysaccharide (O'Brien and McCully, 1981). At high magnification, no fibrils were observed in the secretion and it may not be cellulosic. Functionally, the substance formed a plug at the site of pollen tube entry and may have prevented penetration by other pollen tubes.

Fertilization and cytoplasmic inheritance

Literature on conifer reproduction usually describes a large vacuole, the receptive vacuole, forming in the egg cell cytoplasm and into which the male gametes are delivered. In interior spruce, male gamete nuclei and surrounding body cell cytoplasm were delivered into egg cell cytoplasm and vacuolation occurred after gamete delivery as the vacuolate contents of the pollen tube cell entered the egg cell.

Both male gamete nuclei entered the egg cell and the leading one moved directly towards the egg nucleus, while the trailing one remained in the micropylar, or peripheral region of the egg. Within Pinaceae, paternal plastids and some mitochondria usually accompany the fertilizing male gamete nucleus or follow it closely in a large cluster (Camefort, 1968; Chesnoy, 1987; Bruns and Owens, 1989; Owens and Morris, 1991; Mogensen, 1996). In the two cases where gamete fusion was observed by TEM in this study, paternal plastids and mitochondria were observed clustered but trailing the fertilizing male gamete nucleus at some distance. Paternal plastids eventually moved into the neocytoplasm of the proembryo. Paternal mitochondria were distinguishable from maternal mitochondria and all mitochondria in the neocytoplasm had maternal morphology. Camefort (1966) did not find paternal mitochondria in the neocytoplasm of *Pinus*. Results

of genetic probe based studies (Sutton et al., 1991; David and Keathley, 1996) indicate that mitochondrial inheritance is strictly maternal in *Picea*. The observations of this study support the findings of the molecular genetic studies, and indicate that *Picea* differs from *Pinus* (Bruns and Owens, 1989; Wagner et al., 1991), and *Pseudotsuga* (Owens and Morris, 1991) in which a small percentage of mitochondria may be paternally inherited.

Cytoplasmic inheritance in Pinaceae differs from that in the majority of angiosperms in which organelles are maternally inherited. The reason why organelles should be uniparental in origin is unclear (Reboud and Zeyl, 1994; Mogensen, 1996). Perhaps uniparental inheritance provides a selective advantage that would be lost if incompatible organelle genomes co-occurred. As illustration, recombination between mitochondrial genomes can occur (Birky et al., 1982) and may result in disruption of selectively advantageous gene linkages, with subsequent reduced fitness of progeny. Many similar lines of reasoning are presented (Reboud and Zeyl, 1994) but none convincingly explain the non-Mendelian mode of organelle inheritance in seed plants. In the most recent review of organelle inheritance, Mogensen (1996) says, "Perhaps it really does not matter which parental cytoplasm is eliminated, as long as only one is inherited." Cato and Richardson (1996) describe a rapid, polymerase chain reaction-based assay to detect polymorphism at chloroplast simple sequence repeat loci. Tools such as this, combined with the observation of Furnier and Stine (1995), that chloroplast and nuclear genomes evolve at different rates within and between populations based on mode of inheritance, will facilitate the study of gene flow patterns for phylogenetic analysis. Determination of the sorting mechanism by which paternal plastids move into the proembryo neocytoplasm while paternal mitochondria are excluded should be a major priority because it would help elucidate the general mechanism of nuclear and organelle movement. Possibilities are that the plastids are recognized and moved by an intracellular transport system, or simply that all organelles are moved but that paternal mitochondria deteriorate at about the time of gamete fusion.

The leading male gamete nucleus became elongate towards the highly invaginated egg nucleus. At contact, and during gamete fusion, the male gamete nucleus had a peripheral band of higher staining density in its nucleoplasm. Density increase in the nucleoplasm appeared to be caused by concentration of ribosomes, possibly indicative of increased transcription during fusion.

Mitosis of the zygote probably occurred within 24 hours of gamete fusion as estimated by the infrequency with which this stage was observed in embedded samples. By the time chromosomes had condensed at the metaphase plate in the zygote, the perinuclear zone with maternal mitochondria and paternal plastids was well defined and bordered by small inclusions. Paternal plastids did not remain in a discrete cluster as described in *Pseudotsuga menziesii* (Owens and Morris, 1991) but surrounded the mitotic nucleus at low density. A second, synchronous mitosis of the daughter cells then occurred resulting in four free nuclei of the coenocytic proembryo within the central region of the archegonium cytoplasm.

A radical transformation, which completely altered the appearance of the archegonium cytoplasm, occurred after fertilization. This transformation proceeded in a wave from the chalazal end. Owens and Morris (1991) describe the same change occurring from the micropylar end in *P. menziesii*. Cytoplasmic areas within the small inclusions shrank, and small vacuoles appeared throughout the archegonium cytoplasm while modified plastids disintegrated. In this lysing process maternal plastids were entirely eliminated by the time the free nuclear proembryo formed.

Fertilization: an anomalous special case

In one ovule, a pollen tube penetrated the neck of one archegonium before diverting and successfully fertilizing an adjacent archegonium through the side of the egg cell near the ventral canal cell. It is difficult to draw conclusions from an isolated observation, but in any case, this was an interesting occurrence. Willson and Burley (1983) and Haig (1992) say that mate choice by gametes or maternal sporophytes would have beneficial genetic consequences for offspring if it occurs. It is difficult to imagine, however, how a pollen tube diverting from one egg cell to another within an archegonium can represent mate evaluation when egg cells are genetically identical. More likely, a signal exists from archegonium to pollen tube, eg. a Ca^{2+} gradient (Steer and Steer, 1989), or some other ovular secretion (Takaso et al., 1996) which is indicative of egg cell maturation and competence for fertilization. In the absence of such a signal, a pollen tube might divert to an adjacent egg. In Chapter 8, on anomalies, the ventral canal cell will be proposed as a possible origin of such a signal. Interestingly, the diverted pollen tube grew through cells of the megagametophyte, the archegonial jacket and egg cell wall to effect fertilization. Cell walls of these cells were destroyed, unlike cells of the nucellus or neck cells which are pushed aside as they separate at the middle lamellae. Enzymes in the emission from the pollen tube tip (Pettitt, 1985) are therefore cytotoxic to most cells of the megagametophyte.

Proembryo and early embryo

Migration of the four free nuclei of the coenocytic proembryo to the chalazal end of the archegonium required 1 to 2 days. If nuclei were close together they shared a common neocyttoplasm, otherwise, each nucleus was surrounded by its own neocyttoplasm. A large body of literature exists on proembryo development and terminology in conifers (see, Singh, 1978). In general within Pinaceae, a mature proembryo consists of four tiers of

four nuclei each. Descriptions of proembryogeny in Pinaceae are provided by Buchholz (1929), Chowdhury (1962), Doyle (1963), Dogra (1967), Mehra and Dogra (1975), and Singh (1978). I will, for the most part, follow Singh's (1978) description of proembryogeny as my observations in interior spruce closely match his description.

As during migration of the egg nucleus, no bundles of microtubules were observed near the proembryo free nuclei. When observed, single microtubules within the neocytoplasm were aligned in the direction of nuclear migration. Existence of cytoskeletal based transport systems, like the actin-myosin based system in the embryo sac of tobacco (*Nicotianum*, Huang and Russell, 1994), cannot be ruled out. Examination of individual ultrathin sections makes description of overall three dimensional structure difficult. Microtubule or microfilament arrays, if they exist in the conifer egg cell or neocytoplasm would best be studied by confocal microscopy or by reconstruction from serial ultrathin sections using the powerful software now available (see Mogensen, 1996).

As the four free nuclei neared the chalazal end of the archegonium, they distorted and elongated at their chalazal ends. This distortion did not persist, the nuclei rounded up prior to synchronous mitosis. Neocytoplasm was metabolically active prior to mitosis as indicated by lipid body accumulation and a large amount of rough endoplasmic reticulum surrounding each nucleus. Round to elongate mitochondria filled the zone of neocytoplasm in which metabolic activity was highest, but a thin zone of neocytoplasm devoid of mitochondria, described also by Bruns (1993) in *Pinus*, remained immediately outside each nuclear membrane. Plastids were far fewer in number than mitochondria and prior to mitosis they migrated into the chalazal-most neocytoplasm so that it became polarized. Huang and Russell (1994) describe synergid and central cell cytoplasm with polarized organelle distributions of this sort in tobacco. In that study, microfilaments and microtubules were associated with organelle surfaces and may have been involved in establishment of cytoplasmic polarity. Actin fibres are difficult to preserve by chemical

fixation for conventional TEM. Immunolabelling of rapidly frozen / freeze substituted tissue with anti-actin, or staining with rhodamine-phalloiden may reveal a microfilamentous network associated with organelle movement in the conifer proembryo.

Synchronous mitosis of the four free nuclei resulted in eight free nuclei organized in two tiers of four. Cell wall formation between the daughter cells resulted in an enclosed primary embryonal tier at the chalazal end and a primary open tier (Singh's (1978) primary upper tier). Cell walls then formed between cells in each tier. Primary embryonal tier cells were densely cytoplasmic and contained all of the plastids of the proembryo in their chalazal cytoplasm. Cell wall formation was evident first across the phragmoplast between nuclei and later around the cell periphery. Primary open tier cells remained open because cell wall formation between nuclei was interrupted when walls extended into the archegonium cytoplasm. Large and small inclusions of the archegonium came into contact with the primary open tier cells but remained separated from cells in the primary embryonal tier.

Mitosis of the primary open tier resulted in a three tiered proembryo and as cell walls were forming between those daughter cells, mitosis occurred in the primary embryonal tier. The four tiers of the mature proembryo, named from the chalazal end, are. i) embryonal tier, ii) suspensor tier, iii) non-functional suspensor tier, and iv) open tier. Occasionally the open tier cells appeared enclosed by cell wall although the completeness of closure was not ascertained. A thin, dense cytoplasm surrounded the nuclei in all tiers although the three micropylar-most tiers were very vacuolate. Embryonal tier cells were densely cytoplasmic at first but, as cell wall production continued, vacuoles formed in areas adjacent to the walls. Lipid bodies and protein crystals were abundant in the cytoplasm near sites of cell wall synthesis, and vacuolation probably represented areas of cytoplasm which had been catabolized for wall components.

Open tier and non-functional suspensor tier cells seemed to have no function in the mature proembryo other than to isolate the suspensor and embryonal cells from the rapidly lysing cytoplasm of the archegonium. Division of the primary open tier to form the two apparently non-functional tiers does not occur in *Pseudotsuga* (Allen, 1943). Various phylogenetic analyses, eg. Hart (1987), place *Pseudotsuga* later than *Picea*. Loss of the mitosis which produces the two apparently non-functional tiers without loss of proembryo viability probably reflects their non-functionality even at an earlier evolutionary stage.

Suspensor tier cells were vacuolate, which is characteristic of cells undergoing rapid elongation. No delay occurred between proembryo maturation and elongation of suspensor cells which forced the embryonal tier cells into the centre of the megagametophyte. Secondary division and elongation of suspensor tier cells forced the early embryo quickly into the central part of the megagametophyte while subsequent divisions in the embryo tier produced a compact mass of embryonal cells.

Simple polyembryony in interior spruce was not described in this study but, when multiple archegonia were fertilized, the first embryo into the megagametophyte seemed to grow rapidly while others remained small or deteriorated. Barring lethal allele combinations then, success may be dependent on speed of proembryo development and embryo penetration into the megagametophyte. Embryo competition in this sense is probably very real (Haig, 1992) and might have selected for rapid development through fertilization in Pinaceae. In interior spruce, embryo penetration into the megagametophyte occurred as little as 3 to 4 days after pollen tube penetration of the neck cells.

Chapter 8

Sexual reproduction of interior spruce: abnormalities leading to failure

Introduction

Many factors can adversely affect ovules and seed during development (Owens et al., 1991). In a related study of interior spruce, viable seeds were produced in only 35.0 ± 20.4 to $50.5 \pm 20.0\%$ of ovules (see table 7, Chapter 9). At least 50 percent of potential seeds did not develop to maturity after controlled cross-pollination. Loss of such a high number of ovules seriously affects reforestation programs which require millions of seedlings annually. Lack of pollination in interior spruce is a problem and was addressed by Runions and Owens (1996). During the studies of normal ovule development during fertilization, many abnormalities of development, which resulted in failures of fertilization were observed. These are presented here and an attempt has been made to quantify the seed lost due to prezygotic failure. In addition, by their effect on subsequent development, some of the abnormalities observed have provided insight to processes within the ovule. In particular, the function of the ventral canal cell has never been understood and it is generally disregarded (Singh, 1978). Abnormal ventral canal cell formation and correlated aberrant pollen tube behaviour in this study provide evidence for the role of the ventral canal cell in fertilization.

Materials and methods

As described in chapter six. Abnormalities described were from samples collected between May 20 to 27, 1993, 13 to 20 days after pollination.

Observations and results

Failure of interior spruce to produce healthy appearing embryos for any reason, including lack of pollen, were common and can be broadly categorized as occurring during prezygotic or postzygotic stages. Prezygotic failures precluded fertilization. Estimation of losses for reasons described below are given in table 6.

Prezygotic failure

Pollen germination and growth was independent of the state of the megagametophyte. Complete abortion of the cellular megagametophyte was detectable by examination of the remnant tissue. Earlier loss of the megagametophyte, due to failure of megasporogenesis, or the megaspore, could only be inferred from observations of ovules that appeared 'empty.' Empty ovules were common. When the megagametophyte failed to form, the nucellus was shrunken and contained little starch. Nucellar cells did contain starch if the megagametophyte had aborted after cellularization. Under these circumstances, pollen tubes penetrated the nucellus and grew towards the remnant megagametophyte (fig. 107). Figure 108 shows an ovule in which the megagametophyte had aborted at the early cellular stage. One large cell in the apical region of the remnant might be an archegonial initial.

Less common was the situation in which prothallial cells of the megagametophyte appeared well formed but archegonia had aborted at an early stage (fig. 109). This was generally an all-or-none scenario; only on one occasion were two healthy, and one aborting archegonia observed within an ovule. When all archegonia had aborted, cells in the central column of the nucellar apex contained dense cytoplasm and starch, indicative of normal

Table 6: Prezygotic abnormalities observed

Type of prezygotic abnormality	Number of abnormalities observed (/ 55 ovules sectioned)	
	Abnormalities which precluded fertilization in the ovule	Abnormalities in individual archegonia
Pollen tube early	4	-
Ventral canal cell misplaced	1	6
Deformed nucellus	3	-
Failure of gamete fusion	-	1*
Total	8	7

* unconfirmed observation

development. In the least common abortion type, archegonia appeared virtually mature, with well formed neck and ventral canal cells, an archegonial jacket layer, and a centrally located egg nucleus (fig. 110). Despite the presence of germinated pollen, these archegonia began to deteriorate, first at the peripheral region of the egg cell where its cytoplasm was in contact with the archegonial jacket cells.

Otherwise healthy archegonia deteriorated because of lack of pollen but these persisted in healthy-looking condition until at least 15 days after pollination, when adjacent eggs, or eggs in other ovules, had been fertilized. The initial stage of archegonium abortion due to lack of pollen included dissolution of the egg nuclear membrane and modified plastids so that the egg cell cytoplasm contained only small inclusions surrounding their granular remains (fig. 111). One of the controlled pollination experiments utilized pollen which had been stored for five years but which should still have been vigorous enough to affect fertilization (Webber, 1991), however a high proportion of this pollen was dead. In many instances, all pollen in an ovule was dead (fig. 112) which resulted in archegonia abortion as described for absence of pollen.

In a few ovules of one of the three clones studied (clone 1944), pollen tubes penetrated to the megagametophyte prior to egg cell maturation. When pollen tubes arrived at the megagametophyte while archegonia were still at the central cell stage, the male gametes aborted (fig. 113).

A unusual and infrequently occurring abnormality observed only in clone 1944 was an incomplete nucellus. The nucellus did not completely enclose the megagametophyte which was therefore in contact with the inner layer of the seed coat along one side (fig. 114). All ovular cell types appeared normal but the archegonia failed to develop beyond the central cell stage. Pollen germinated and grew through the nucellus in one of three ovules observed with this abnormality. Absence of the nucellus, and therefore of the tapetal layer,

along one side of the megagametophyte resulted in a much reduced megaspore wall in that region relative to regions in contact with the nucellus (fig. 115).

The most surprising abnormality in archegonial development was the misplaced ventral canal cell. Several examples of this situation were observed, and it happened in all three clones studied. Two types of this abnormality occurred. In the first, the ventral canal cell was displaced to one side of the archegonium rather than being situated in contact with the neck cells. It remained small and enclosed by a cell wall (fig. 116). In the second type, the ventral canal cell nucleus was not restrained proximal to the neck but migrated into the egg cell cytoplasm, resulting in a bi-nucleate appearing egg cell. These ventral canal cell nuclei were large, each had a perinuclear zone, and they were indistinguishable from the nearby egg nucleus. A cell wall formed in the middle region of the egg cell cytoplasm between the nuclei but never was long enough to traverse the entire width (fig. 117 and 119). When the ventral canal cell was displaced to one side of the archegonium, pollen tubes did not grow through the neck cells but grew through the prothallial, and archegonial jacket cells towards the location of the ventral canal cell. Cell walls in the proximity of the pollen tube tip broke down and deposits of carbohydrates lined the egg cell and ventral canal cell common wall. The pollen tube appeared to penetrate the egg cell and the ventral canal cell on occasion but fusion between a male gamete nucleus and the egg nucleus was never observed when the ventral canal cell was displaced to one side of an archegonium.

Postzygotic failure

Postzygotic failure of the zygote, proembryo, or embryo were not categorized in this study but several examples are included to provide contrast with the prezygotic types of failure described.

An indeterminate situation is illustrated in figure 118 in which two shrunken nuclei were observed within the egg cell cytoplasm. Pollen tube contents in the surrounding

cytoplasm indicate that fertilization has occurred but the cytoplasm shows no evidence of the change associated with fertilization (see Chapter 7). The two nuclei may be the male and female gametes, in which case gamete fusion did not occur, or they may represent failure after the first zygotic mitosis. Unresolvable observations like this were made in only two ovules.

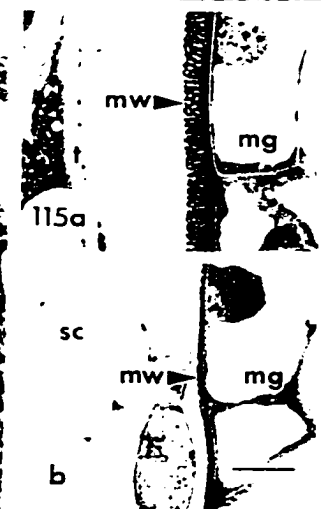
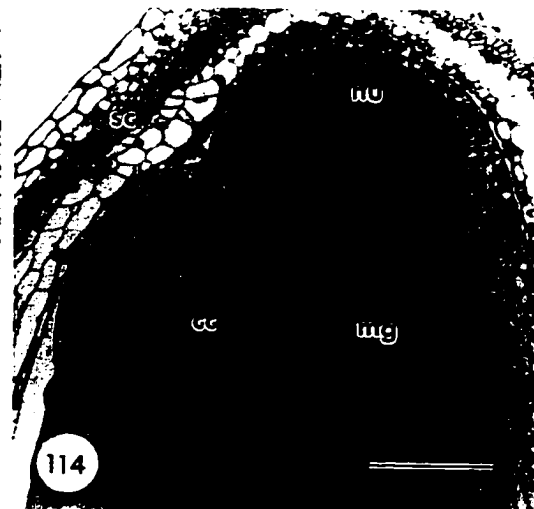
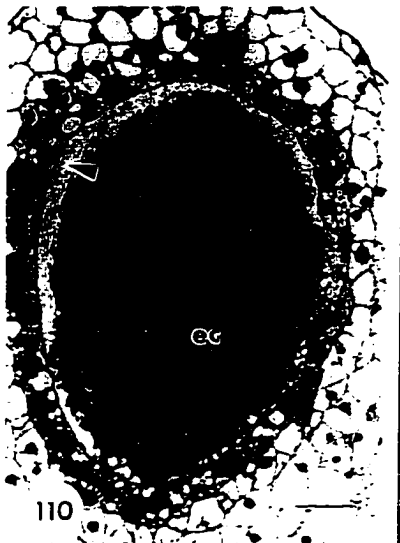
Proembryo development to the cell wall formation stage usually proceeded normally. In one interesting case, a large, free ventral canal cell nucleus did not prevent fertilization (fig. 119). The ventral canal cell nucleus remained in the micropylar half of the egg cytoplasm and fertilization occurred normally through the neck. Zygote mitosis proceeded and a free-nucleate proembryo was formed, however, the nuclei distorted and appeared to be aborting (fig. 120). From the stage of cell wall formation in the proembryo until emergence of the early embryo into the prothallial cells, many abortions occurred in the embryonal tier, or embryonal mass of cells (fig. 121).

Figures 107-115

- Fig. 107 Pollen germinated, and pollen tubes grew through the nucellus even if the megagametophyte had aborted. In this case, the pollen tube turned within the nucellus (dashed line) and the tube tip, with male gametes (σ^7), grew to the micropylar part of the aborted megagametophyte. LM. Bar=50 μ m.
- Fig. 108 Early abortion of megagametophytes occurred frequently. In an aborted megagametophyte (mg), a large cell in the apex (ai) retained cytoplasm and a nucleus and may have been one of the archegonial initials. LM. Bar=250 μ m.
- Fig. 109 Only infrequently did all archegonia (a) abort within an otherwise healthy appearing megagametophyte (mg). LM. Bar=50 μ m.
- Fig. 110 Abortion of the early egg cell (ec) was also uncommon. Egg cell cytoplasm first became vacuolate where it was adjacent to archegonial jacket cells, and then it deteriorated so that it appeared shrunken (arrowhead). LM. Bar=50 μ m.
- Fig. 111 In unpollinated ovules, egg cells began to deteriorate approximately 15 days after the pollination period. The egg cell nucleus, ventral canal cell (arrowhead), and modified plastids deteriorated before any other signs of abortion were observed in the cytoplasm. LM. Bar=50 μ m.
- Fig. 112 Many pollen grains (po) were dead and occupied the pollen chamber, therefore competing with viable pollen for space. LM. Bar=100 μ m.
- Fig. 113 In clone 1944, pollen tubes sometimes penetrated to the megagametophyte before egg cell maturation. Male gametes (σ^7) aborted if pollen tubes arrived at the megagametophyte during the central cell (cc) stage. nc - first tier of neck cells. LM. Bar=50 μ m.

Fig. 114 In clone 1944, the nucellus (nu) sometimes did not completely enclose the megagametophyte (mg) which, therefore, lay appressed to the seed coat (sc) along one side. In this situation, central cells (cc) aborted. LM. Bar=200 μ m.

Fig. 115 As in fig. 114. The side of the megagametophyte (mg) that was in contact with the tapetal layer of the nucellus (t) was covered by a megaspore wall (mw) that had a thick and elaborate ectexine (fig. 115a). Where the megagametophyte was not enclosed by the nucellus it lay adjacent to the seed coat (sc) and the megaspore wall was thin. LM. Bar=5 μ m.



Figures 116-121

Figs. 116-117 Abnormal ventral canal cell. LM. Bar=50 μ m.

Fig. 116 Often the ventral canal cell (vc) formed to one side of the apex of the egg cell (ec) rather than subtending the neck (nc). In this case, the ventral canal cell has begun to deteriorate and a thick polysaccharide deposit coats its cell wall.

Fig. 117 Sometimes the ventral canal cell nucleus (n_1) was not enclosed by the cell wall (cw) but remained free in the micropylar half of the egg cell space. A pollen tube (pt) grew through the megagametophyte to contact the egg cell wall and then turned towards the apex before abortion of the male gametes occurred.

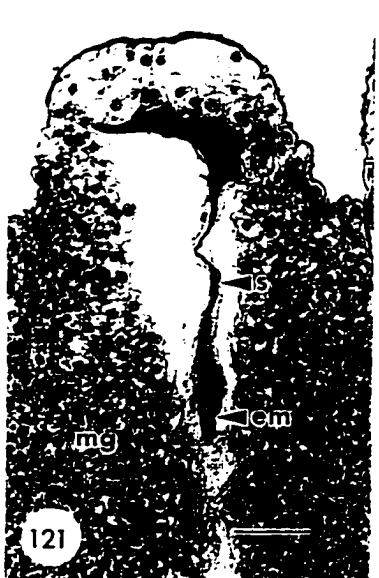
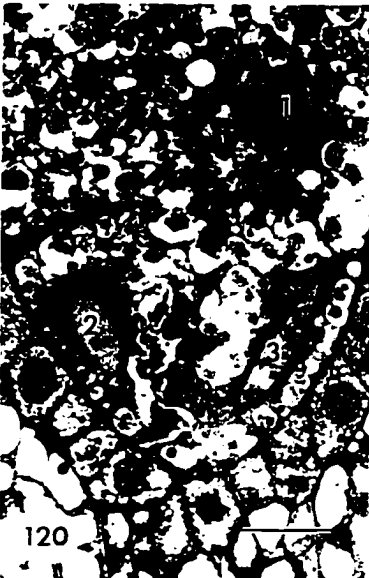
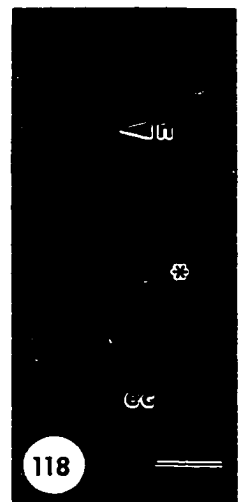
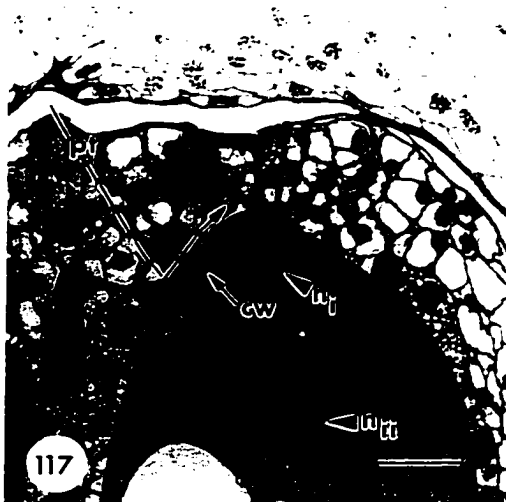
Figs. 118-121 Postzygotic failures

Fig. 118 On one occasion, two aborting nuclei (n) were observed within the egg cell cytoplasm. The two nuclei may have been the gametes, in which case fertilization failed, or they may have been the products of zygotic mitosis, in which case failure was postzygotic. Perinuclear zone mitochondria remained clustered near one nucleus (*). LM. Bar=25 μ m.

Fig. 119 The ventral canal cell nucleus (by this stage deteriorated) had been free in the egg cell cytoplasm as evidenced by the cell wall which formed across the centre part of the egg cell. This type of cell wall formation, between the egg nucleus and the free ventral canal cell nucleus, was common and never fully divided the archegonium. This was the only case in which fertilization was successful through the neck of an egg cell with an abnormal ventral canal cell. LM. Bar=50 μ m.

Fig. 120 As in fig. 119. Even though fertilization was successful, the free nuclei of the proembryo (1-3) became distorted and had apparently aborted. LM. Bar=25 μ m.

Fig. 121 Abortions of early embryos (em) were observed in several samples. The suspensor cells (s) elongated, forcing the embryo into the prothallial tissue (mg) but the embryonal cells deteriorated. LM. Bar=500 μ m.



Discussion

Very early abortion of the megagametophyte or gross deformity within the pre-fertilization ovule of interior spruce resulted in most of the prezygotic failures observed in this study. Quantification of losses by category of prezygotic failure is difficult in a study which utilizes resin embedded tissue because of the time consuming nature of the work and the uneven sample size sectioned at each stage. Preliminary estimates made using paraffin embedded tissue collected in 1992 indicated that lack of pollination, and ovule abortion at approximately the time of pollination could account for as much as a 50 percent reduction in seed set. Lack of pollen, however, was a problem in 1992 because pollen cone production was low. Pollination success was much higher in 1993 (unpublished results) when pollen cones were abundant. Promotion of pollination success was discussed by Runions and Owens (1996).

Megagametophyte abortion

Various spruce species have been reported to abort unpollinated ovules immediately after the pollination period (Dogra, 1967). In this study, unpollinated ovules, or ovules pollinated with dead pollen, developed to archegonial maturity and persisted throughout the fertilization period which contradicts Dogra's observation. The contradiction is seemingly in cause and effect. Many ovules were aborted before pollination, and ovules aborted at the free nuclear stage of the megagametophyte may not produce a pollination drop. If not, pollination should not be possible. In other words, lack of pollen might not have caused the reported early abortion, the early abortion may have prevented pollination. This matter requires further investigation as the origin of the pollination drop remains uncertain. It may originate in the nucellus (Owens, 1993), in which case pollination could occur after megagametophyte abortion, or it may be homologous with lesser secretions which originate

in the megagametophyte of Douglas fir (Takaso et al., 1996), in which case pollination should not be possible after megagametophyte abortion. Rainwater can float pollen into ovules (Runions and Owens, 1996) which could explain pollen in the pollen chamber of ovules with early aborted megagametophytes.

Bell (1996) has proposed a genetic mechanism which might govern selective apoptosis of megaspores at the tetrad stage. Potentially, this mechanism can be used to explain failure of megagametophytes to form or mature. His bipartite locus model predicts that megaspores that contain a pair of recessive alleles are fated for immediate cell death, and that megaspores with one dominant and one recessive allele may be apoptotic but rescued by a 'survival signal' originating in the chalazal pole of the tetrad. These predictions are well supported by experimental observations of *Mirabilis* by Correns (cited in Bell, 1996). In this model, as many as 25 percent of megaspores would abort at formation or shortly thereafter. Cellular structure was often absent in the remains of aborted megagametophytes of interior spruce and this could be indicative of very early abortion for genetic reasons.

Other aborted megagametophytes had been clearly cellular prior to abortion. External factors which might account for ovule abortion were not deemed significant in this study. Insect predation was minimized because developing seed cones were enclosed in bags that contained an insecticidal chemical, and weather conditions were not extreme during the stages observed. Ovule abortion may result from intrinsic regulation by the maternal sporophyte. Wiens et al. (1987) describe selective ovule abortion as a means of maximizing nutrient use efficiency during ovule partitioning. Seed cones of *Pinus* abort if a threshold number of ovules are not pollinated (Sweet, 1973). Mass abortion of ovules or seed cones was never observed in interior spruce although seed cones from entirely unpollinated trees were not studied. Early ovule abortion was apparently random within

seed cones and occurred, on occasion, even when adjacent ovules contained plentiful pollen.

Pollen germinated and pollen tubes grew through the nucellus in the general direction of the aborted megagametophyte. Pollen tubes of *Picea* develop readily in water (Webber, 1991) so pollen does not seem to require a chemical signal for pollen tube initiation from the megagametophyte as does the pollen of *Pseudotsuga* (Takaso et al., 1996).

Archegonial abortion

Archegonia rarely aborted in cellular megagametophytes prior to the fertilization period. In only two ovules examined had all archegonia aborted at this stage. Archegonial initials had the same genetic complement as surrounding megagametophyte tissue which appeared healthy. The developmental stage within failed archegonia was not determinable. Only on one occasion was a deteriorating archegonium observed within an ovule which contained other healthy appearing archegonia. This abortion occurred prior to egg cell maturation and could have been discounted as a case of poor fixation had not neighboring archegonia appeared healthy.

Unfertilized egg cells began to deteriorate approximately 20 days after the pollination period when other egg cells in the same, or adjacent ovules had been fertilized. Abortion of unfertilized egg cells occurred by discrete stages. The cell walls between egg cell and ventral canal cell deteriorated, then the egg nuclear membrane and modified plastids deteriorated, and finally, the small inclusion-bound cytoplasmic fraction (which was the majority of egg cell cytoplasm at this stage) became vacuolate.

Miscellaneous prezygotic abnormalities

Other than early abortion of megagametophytes or archegonia, prezygotic failure resulted from, i) pollen tube arrival at necks of immature archegonia, ii) deformed nucellus, iii) misplaced ventral canal cell, and perhaps iv) failure of gametes to fuse within the egg cell cytoplasm. The first three of these points resulted in the failure or the impossibility of fertilization in 25.5 % (14 of 55) of ovules sectioned in this part of the study. In cases where deformity occurred only in one archegonium within an ovule, fertilization of adjacent archegonia was still possible. A conservative estimate is that these abnormalities prevented fertilization in 14.5 % (eight of 55) ovules sectioned. This figure tallies well with the estimate of seed losses due to 'other prezygotic factors' in *Pseudotsuga* (Owens et al., 1991). Failure of gamete fusion after male gametes had entered the egg cell cytoplasm was not observed with certainty.

In four of 18 ovules from clone 1944, egg cells were not mature when pollen tubes reached the neck. Central cell mitosis had not occurred, or had only just occurred and the egg nucleus had not migrated to a central position within the cytoplasm. Pollen tubes did not push between the neck cells and the male gametes deteriorated. Clonal variation in the timing of developmental stages is common in *Picea* (Runions et al., 1995), but poor timing resulting in loss of pollen viability was surprising. Despite clonal variation in the timing of receptivity for pollination, it would seem that pollination drops, which move pollen into the ovule and initiate germination, should only be produced once the ovule is sufficiently advanced so that the archegonia will mature by pollen tube arrival. For some reason, in clone 1944, pollination drop production and pollen transport into the ovule occurred too early. Seed cones were protected within paper isolation bags so rain could not have been responsible for premature movement of pollen into the ovules.

A deformed nucellus resulted in failure of fertilization in three of 18 ovules from clone 1944. The megaspore mother cell is usually surrounded by several layers of nucellar

cells at formation (Owens and Molder, 1979a). For a portion of the megagametophyte not to be enclosed by the nucellus requires that either the megaspore mother cell arose in a peripheral layer of the sporogenous tissue, rather than from deeper cells, or that the nucellus ruptured at some point during early megagametophyte growth. In the evolution of heterospory, megaspore retention within the sporophyte occurred prior to ovule enclosure by an integument (Stewart, 1983). Ovules of clone 1944 were all enclosed by a normal looking integument so this condition would seem to be a gross abnormality rather than reflection of a plesiomorphic condition. Failure of archegonia to develop beyond the central cell stage indicates that the abnormality was detrimental yet it seems to have persisted in this clone. The exposed megagametophyte remained surrounded by the megaspore wall; a definitive condition in heterosporous seed plants. However, along the unenclosed flank of the megagametophyte, where it was next to the inner layer of the integument, the megaspore wall was very thin. This demonstrates that the origin of, at least, the exine layer of the megaspore wall is from the inner, tapetal layer of the nucellus, and that diffusion of wall components from this layer occurs. Pollen were never observed within an ovule with an incomplete nucellus. Perhaps deformation of the nucellus resulted in a loss of pollination drop formation. The implication here is that the pollination drop was not secreted, which suggests that its origin is in the nucellus. This sort of speculation is anecdotal, however, because many normal appearing ovules remained unpollinated as well.

Ventral canal cell abnormalities and a proposed function

In seven archegonia studied, the ventral canal cell was abnormal. This represents approximately 4 % of the egg cells studied and provides for insight to the function of the ventral canal cell. All but one case of ventral canal cell abnormality resulted in failure to produce embryos. Fertilization through the side of the egg cell near a displaced ventral

canal cell, which resulted in a normal appearing proembryo, was reported as a special case of fertilization in chapter seven. In five of seven abnormal archegonia, ventral canal cell displacement resulted in prezygotic failure and, in one case, fertilization occurred but abnormal appearing proembryo free nuclei aborted during migration to the chalazal pole.

Displacement of the ventral canal cell occurred in one of two ways. In the first way, at central cell nuclear division, the ventral canal cell nucleus was not held at the micropylar pole of the egg cell during formation of the intervening cell walls, but moved into the egg cytoplasm (which therefore contained egg cell, and ventral canal cell cytoplasm and can not be considered an egg cell proper). Only by its position was the micropylar-most nucleus considered the ventral canal cell nucleus. In all respects, including formation of a perinuclear zone, it resembled the egg nucleus. Cell wall formation between the two nuclei was initiated across the central region of the egg cell space but never completely traversed the space that was $>200\ \mu\text{m}$ across. Cell walls resembled the walls formed between nuclei in the open tier of the proembryo (Chapter 7) which projected into the egg cell space but ended blindly. It appears as if cell walls can only be produced across a finite distance between nuclei. In the second type of displacement observed, central cell division was not aligned across the long axis of the archegonium parallel with the plane of archegonial initial division. The result was that the ventral canal cell appeared normal but did not subtend the neck of the archegonium. Instead, it was located to one side of the egg cell, in the micropylar region, adjacent to archegonial jacket cells.

The ventral canal cell is genetically equivalent to the egg cell, but its function is unknown. Maheshwari and Singh (1967) reviewed morphology of the megagametophyte in gymnosperms and described the ventral canal cell as 'ephemeral,' and they did not ascribe it any function. Ventral canal cells occur in all taxa of gymnosperms, either with cell walls as in Ginkgoales and Pinaceae, or as free nuclei in the micropylar pole of the egg cell as in Cycadales, Araucariaceae, Cupressaceae, and Podocarpaceae. Central cell

division was thought not to occur in Taxaceae (Maheshwari and Singh, 1967), but Pennell and Bell (1987) report that it does. In Ephedrales, a ventral canal cell nucleus forms (Friedman, 1990) and, after fertilization of the egg cell, the ventral canal cell nucleus fuses with the second male gamete nucleus. Friedman (1990) describes this double fertilization as evolutionarily homologous with double fertilization in angiosperms, and suggests that the resultant supernumerary embryo may be the evolutionary progenitor of endosperm (Friedman, 1994). Gnetales also have double fertilization but no egg cell or ventral canal cell differentiation is apparent within the free nuclear megagametophyte prior to fertilization. Rather, all of the free nuclei seem to be potential gametes and proximity to the discharged male gametes seems to be the criterion that governs which nuclei participate in fusion (Carmichael and Friedman, 1995).

Persistence of the ventral canal cell or nucleus in all taxa of gymnosperms probably means that it is evolutionarily conserved. Observations of its normal development and alteration in pollen tube behaviour when the ventral canal cell was displaced in interior spruce suggest that the ventral canal cell has a function. When in normal position subtending the neck cells, the ventral canal cell began to deteriorate as pollen tubes grew through the nucellus. Deterioration of the ventral canal cell nucleus at this stage is common to most conifers (Bell, 1994), and cycads (Steyn et al., 1996). Pollen tubes grew between neck cells into the deteriorating ventral canal cell. If the ventral canal cell nucleus was free, and occupied the micropylar part of the egg cell, pollen tubes still penetrated between the neck cells. When the ventral canal cell was displaced and enclosed by a cell wall so that it lay adjacent to archegonial jacket cells, pollen tubes did not grow towards the neck but grew into the megagametophyte, and attempted (with one success) to penetrate the egg cell wall adjacent to the ventral canal cell. My interpretation of these observations is that the neck cells act as a passive conduit through which the pollen tube is attracted by secretions which originate in the apoptotic ventral canal cell. In support of this idea it is worth noting

that when central cell division had not occurred in clone 1944, pollen tubes ceased growth at the neck cells and male gametes deteriorated. Perhaps the signal of archegonium maturation is nothing more than formation and deterioration of the ventral canal cell or nucleus with liberation of an, as yet undetermined, chemical signal which attracts the pollen tube.

Summary of seed losses

Maximum seed efficiency in this study was in clone 1926 which produced seeds from $50.5 \pm 20.0\%$ of ovules. Historically, this 50 percent reduction from potential seed set would have been attributed to lack of pollen and postzygotic failure. A conservative estimate in any of the clones studied is that 15% of seeds were lost due to lack of pollen or early ovule abortion. In clone 1926, 7 to 10% (2 or 3 of 30 ovules sectioned) were lost for other reasons during prezygotic stages. About 25% of seed losses therefore would have occurred postzygotically for genetic reasons. Clone 1944 fared much worse, it had the lowest seed production efficiency ($35.0 \pm 20.4\%$), and the greatest number of prezygotic abnormalities which may have prevented fertilization (28 to 32%, 7 or 8 of 25 ovules sectioned) which means that approximately 20% of seed losses were for postzygotic reasons.

Abortion of proembryos and early embryos was observed occasionally in this study but postzygotic seed losses were not quantified. Losses from the early embryo stage of development are considered one of the most significant reasons for reduced seed production. Postzygotic failure is due to accumulation of recessive lethal, or sub-lethal alleles at fertilization (Fowler and Park, 1983). Quantification of postzygotic seed losses would be possible from the specimens of interior spruce collected for this research but this survey has not been undertaken.

Seed losses after self-pollination are much higher than after cross-pollination.

Chapter 9 is a report of abnormalities in prezygotic development after self-pollination in interior spruce.

Chapter 9

Evidence of self-incompatibility in a gymnosperm

Introduction

Conifers, in general, produce fewer sound seeds from self-pollination than from cross-pollination (Orr-Ewing, 1957; Sarvas, 1962; Mergen et al., 1965; Koski, 1973). Reduction in seed set is said to be due to abortion during postzygotic development (Mergen et al., 1965; Fowler and Park, 1983). Prezygotic mechanisms which prevent self-fertilization have not been observed and conifers have therefore been considered self-compatible (Seavey and Bawa, 1986; Zavada and Taylor, 1986; Sage et al., 1994).

We have recently conducted an ultrastructural study of reproductive development in interior spruce (white spruce (*Picea glauca* (Moench) Voss), or Engelmann spruce (*P. engelmannii* Parry), or their hybrid in British Columbia), which included cross-, and self-pollinations. The rationale for including self-pollinations was that direct observation had very rarely been used to evaluate the timing of seed losses in a conifer. Seed loss during postzygotic stages occurs because of inviability, a manifestation of inbreeding depression. Conifers have high numbers of recessive embryonic lethal alleles (Bishir and Namkoong, 1987) which when expressed in homozygous condition can result in embryo abortion. An assumption that gamete fusion has occurred is required before seed losses after self-pollination can be categorized as postzygotic. This assumption might not be valid.

Two types of prezygotic developmental abnormalities which were unique after self-pollination, and which prevented gamete fusion were observed in this study. Prezygotic mechanisms which prevent fertilization after self-pollination in white spruce constitute a mechanism of self-incompatibility acting in a gymnosperm.

Materials and Methods

Tree selection and pollination

Trees used in this study had been established as grafts in a clonal seed orchard at the Kalamalka Seed Centre near Vernon, British Columbia in 1982. Individual ramets of three clones (QL orchard clones 1926, 1944, and 4752) were chosen in April 1993. Each clone had an abundant supply of developing pollen cones, and seed cone buds. Pollen cone buds were removed and isolation bags placed over branches bearing seed cones prior to the pollination period. Cross-pollen was collected from 12 clones which were not otherwise included in the study, and mixed. Self-pollen was collected from branches on which pollen cones had been left to mature until pollen shedding. Cross-, and self-pollen was tested to ensure that it was between 5 to 10% moisture content prior to artificial pollinations. Cross-pollinations were done using the freshly collected pollen as well as pollen which had been stored for one year. On May 6 and 7, pollination was accomplished by injecting 0.5 ml aliquots of pollen into each bag daily during the period when seed cones appeared maximally receptive. Once seed cones had become postreceptive, the paper bags were replaced with mesh bags which excluded insects.

Anatomical study of fertilization period

Beginning one week after the final pollination, ovules were collected and prepared for the developmental study. Every two days for 18 days, two seed cones were collected per treatment per tree. Median longitudinal sections less than 1 mm in thickness were cut from ten ovules from the centre region of each cone, fixed in glutaraldehyde, post-fixed in OsO₄, dehydrated and embedded in Spurr's resin. A detailed study of development following cross-pollination was completed prior to the study of self-pollinated ovules.

Fifty-five cross-pollinated ovules and 15 self-pollinated ovules were examined. In each study, ovules were serially sectioned at 0.8 μm with a Riechert Ultracut E microtome, and stained with toluidine blue (CI 52040) at pH 11.0 for light microscopy (O'Brien and McCully, 1981). For transmission electron microscopy, sections were cut at 0.06 μm , picked up on 300-mesh or 75-mesh copper grids, stained with uranyl acetate and lead citrate and examined with an Hitachi TEM. All of the developmental stages described herein occurred during the eight day period from 13 to 20 days after the final pollination.

Seed production efficiency

Ten seed cones were left within mesh bags on each control pollinated branch until seed maturation in August 1993. Cross-, and self-pollinated cones were collected prior to seed shed. Seeds were extracted and X-rayed to determine whether they were viable or empty. Viable seeds are X-ray opaque. Seed efficiency percent (SEF%) was calculated as the seed to ovule ratio ($\text{SEF}\% = (\# \text{ seeds} / \# \text{ ovules}) \times 100$).

Observations and results

Seed production after cross-, and self-pollination

Seed production was significantly lower after self-pollination than after cross-pollination in all three clones ($p < 0.001$, table 7). Large variance in SEF% after self-pollination in clones 1926 and 4752 result from one cone in each case which had unusually high seed production.

Table 7: Seed production after cross-, and self-pollination in white spruce

Pollen type	SEF% {(# filled seeds / # ovules) x 100}		
	clone 1926	clone 1944	clone 4752
cross	50.5±20.0	35.0±20.4	39.0±26.7
self*	13.3±18.8	3.1±2.1	13.7±18.6

* for all clones, self-pollination resulted in significantly lower seed production than cross pollination ($p < 0.001$)

Reproductive development after cross-pollination

Development within the ovule was followed between the time of pollen germination and emergence of the early embryo into the megagametophyte. Details of this part of the study were published separately (see. Chapters 6-8) but, in general, normal development was as described in Owens and Molder (1979a). Figure 122 summarizes the normal development of *Picea* during the period of zygote formation.

Pollen germinated without delay and pollen tubes grew vigorously through the nucellus. Body cell cytoplasm was dense and contained two male gametes which had formed from mitosis without subsequent cell wall formation (fig. 123). Male gamete nuclei were large (ca. 20 to 30 μm dia.) and surrounded by plastids and mitochondria. Neck cells of each archegonium deteriorated to some extent prior to contact with a pollen tube. The pollen tube grew between them to contact the ventral canal cell wall which lysed and the pollen tube contents were released into the egg cytoplasm (fig. 124). Prezygotic development continued until one male gamete nucleus and the egg nucleus fused. This occurred very quickly (< 1 day) after release of the male gametes from the pollen tube.

Many abnormalities of development were disruptive enough to preclude fertilization of ovules. Table 8 summarizes the types of abnormalities observed in cross-pollinated ovules. In all cases, these abnormalities were independent of the type of pollen applied. Gross deformities of the ovule included, megagametophytes or egg cells which had aborted prior to pollination, megagametophytes incompletely surrounded by nucellar tissue, and atypical mitosis leading to deformed or misplaced neck and ventral canal cells. Pollen germinated in these situations but fertilization occurred on only one occasion.

Reproductive development after self-pollination

Many of the abnormalities observed after cross-pollination were also observed after self-pollination but, as described, these were independent of pollen type. Two categories of developmental defect, however, were unique to the self-pollinated situation (table 8). Both types of defect prevented fertilization of the egg cell.

In three of four ovules of clone 1944, pollen tubes aborted within the nucellus as the tube tip came in proximity to the megagametophyte. In two of these cases, both pollen tubes had aborted, in the third case, one of two pollen tubes had aborted and in the final case, one egg cell had been fertilized. Figure 125 shows a pollen tube aborted within the nucellus. Male gamete nuclei had become small, round and darkly staining and cytoplasm of the body and tube cells appeared deteriorated. No plastids or mitochondria were visible in the body cell cytoplasm.

The second type of developmental anomaly unique to self-pollinated ovules occurred in clone 1926. In two of four ovules studied, pollen tubes grew into the neck of an archegonium but did not then rupture to release male gametes into the egg cell cytoplasm. Pollen tubes continued growing through the ventral canal cell into the egg cell cytoplasm (fig. 126). Subsequent growth and branching of the pollen tube throughout the egg cell cytoplasm resulted in deterioration of the egg cell. Figure 127 shows a long branch of a pollen tube within the egg cell cytoplasm, almost in contact with the egg nucleus. A shorter branch of the same pollen tube (marked by an asterisk in fig. 126) contains the male gametes (fig. 128). Male gamete nuclei in this case appear healthy (compare figs. 123 and 128) and are surrounded by plastids and mitochondria.

When abnormalities unique to the self-pollinated situation occurred, egg cells (those adjacent to disrupted ones) appeared mature and competent to be fertilized, i.e. the egg nucleus was large and centrally located within the egg and the neck and ventral canal cells were normally developed.

Table 8: Types of abnormalities observed within pollinated ovules of interior spruce.

Type of pollen	Type of abnormality
Abnormality independent of the type of pollen applied	i) early abortion of megagametophyte and / or egg cell
	ii) megagametophyte not completely enclosed by nucellus
	iii) misplaced ventral canal cell
	iv) immature egg cell when pollen tube arrives
Abnormality unique after self-pollination	i) pollen tubes arrested within the nucellus and male gametes aborted (clone 1944)
	ii) pollen tubes penetrate the egg cell without releasing male gametes (clone 1926)

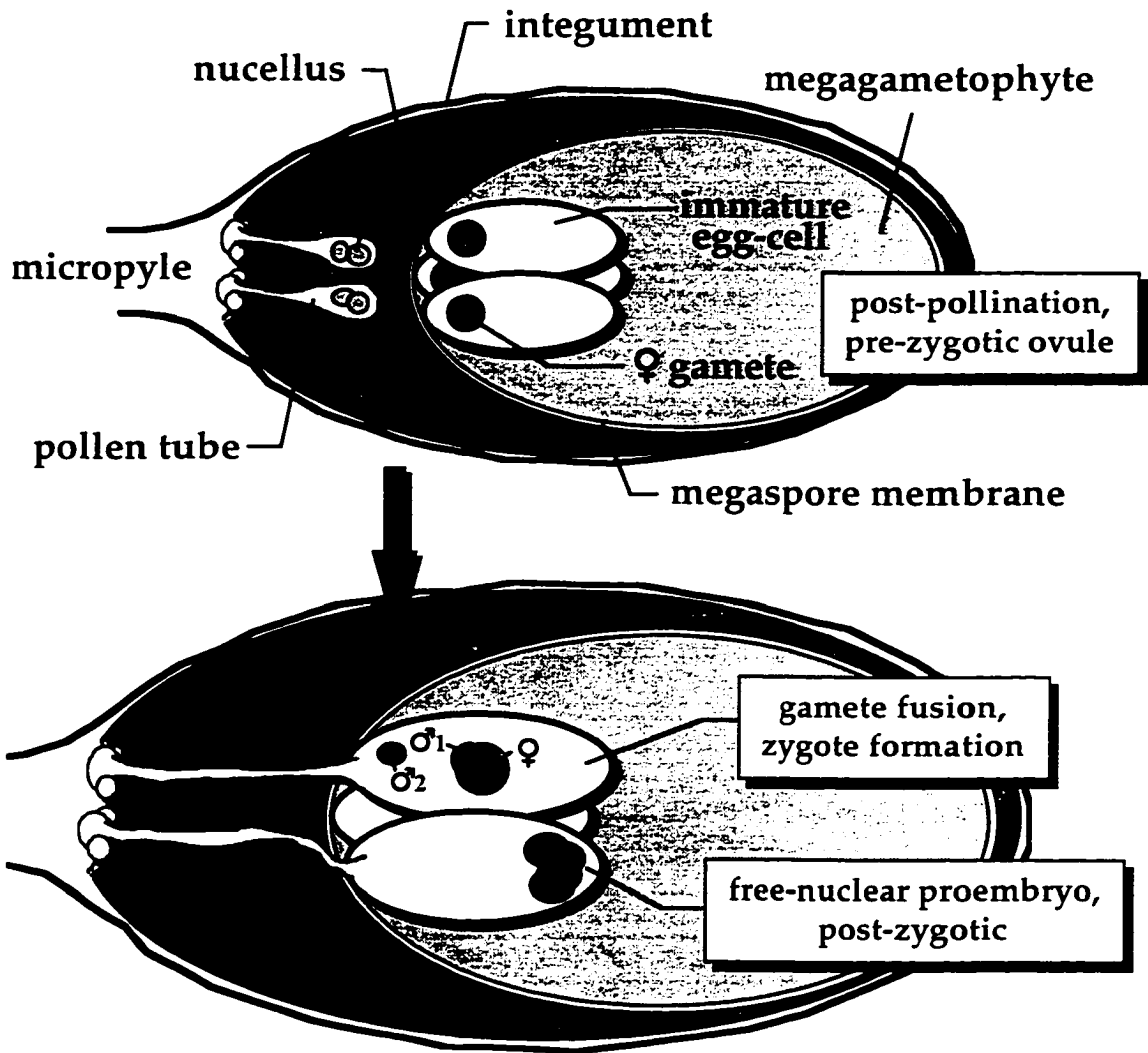


Figure 122. Normal development within the ovule of interior spruce during fertilization.

Figures 123-128

Figs. 123-124 Transmission electron micrographs showing normal development during the prezygotic stage.

Fig. 123 Longitudinal section of the pollen tube within the nucellus. Tube cell (TC) cytoplasm is peripheral and vacuolate. The male gamete nuclei (M_1 and M_2) are contained within the body cell which, when healthy appearing is full of mitochondria (Mt) and plastids (Pl). The stalk cell (SC) is surrounded by a very thin cell wall. Bar=5 μ m

Fig. 124 When a pollen tube (PT) encounters the neck cells (NC) of an archegonium it forces between them and into the ventral canal cell (VCC). The tip of the pollen tube ruptures and its contents are released into the egg cell (EC) cytoplasm through a small opening (arrow). Bar=5 μ m

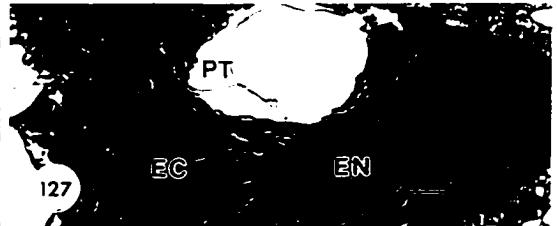
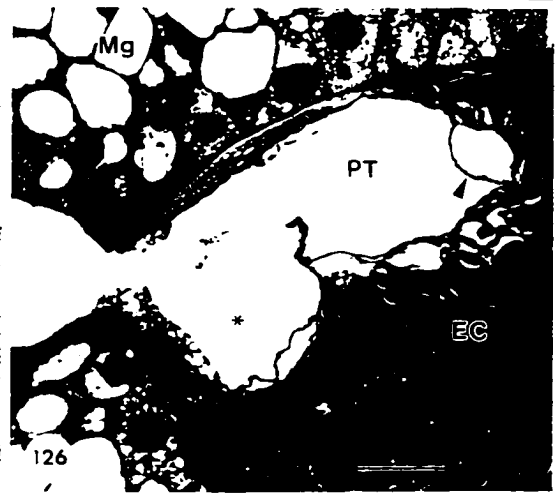
Figs. 125-128 Light micrographs which illustrate the types of development unique to the self-pollinated case. PT - self-pollen tube, EC - egg-cell, Mg - megagametophyte.

Fig. 125 Self-pollen tube aborted within the nucellus (N). Male gamete nuclei are small, round and dark staining and no organelles can be seen in the pollen tube cytoplasm. Bar=20 μ m

Fig. 126 A self-pollen tube has penetrated into the egg cell cytoplasm without rupturing to release the male gametes. The cell wall of the pollen tube (arrowhead) is visible within the egg cell cytoplasm. * - marks the location of male gametes in adjacent sections (see fig. 128). Bar=100 μ m

Fig. 127 Pollen tube penetration of the egg cell cytoplasm reaches the egg nucleus (EN). The egg cell cytoplasm is deteriorating. Bar=100 μ m

Fig. 128 A branch of the pollen tube (* in Fig. 126) within the egg cell cytoplasm contains the male gamete nuclei which appear healthy and are surrounded by cellular organelles. Bar=50 μ m



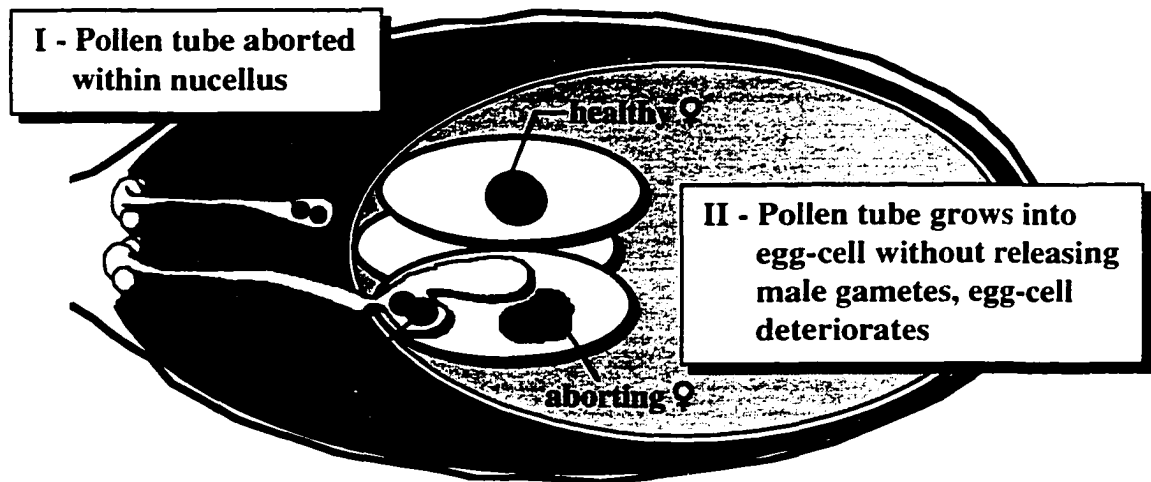


Figure 129. Self-incompatibility types observed in interior spruce

Discussion

Very few studies of conifer seed development have examined the period of gamete fusion in detail sufficient to observe prezygotic failure. Orr-Ewing (1957) found no developmental anomalies within the ovule of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco. Plym-Forshell (1974) conducted similar studies on Scots pine, *Pinus sylvestris* L., with the same result and Mergen et al. (1965) found no barriers to self-fertilization in white spruce. In all of these cases, early embryo abortion was common after self-fertilization and our observations bear this out (unpublished). A preliminary survey of paraffin embedded ovules, however, indicated to us that developmental abnormalities such as early deterioration of the megagametophyte without fertilization could be mistaken for abortion of the early embryo. Deterioration of tissues occurs quickly and remnants of a proembryo or early embryo are difficult, if not impossible, to distinguish. In most cases of apparent embryo abortion we could not say with confidence that gamete fusion had occurred. In this study, tissue preparation for ultrastructural examination preserved structures well enough to allow detailed analysis of prezygotic events, with surprising results.

Self-Incompatibility

Two types of prezygotic departure from normal development were observed. In each case, these abnormalities prevented fertilization and can be considered the result of self-incompatibility (SI) reactions.

Three categories of SI have been described for angiosperms; gametophytic SI (GSI, Newbigin et al., 1993), sporophytic SI (SSI, Nasrallah and Nasrallah, 1993), and late-acting or ovular SI (Seavey and Bawa, 1986; Sage et al., 1994). The first two types, GSI and SSI are relatively well understood systems (Haring et al., 1990) which are under

genetic control and mediated by interactions of S-locus gene products. In families with GSI, eg. Solanaceae, expression of S-locus genes in the haploid genome of the pollen determines the SI response (Newbigin et al., 1993). Pollen germinates on the stigma and pollen tubes begin to grow through the style but abort before reaching the ovule. In families with SSI, eg. Brassicaceae, S-locus products of the pollen producing sporophyte determine the SI response (Nasrallah and Nasrallah, 1993). Pollen generally are prevented from germinating on the stigma.

Late-acting or ovular systems of SI are widely reported but not as clearly defined as GSI or SSI systems (Sage et al., 1994). Pollen tubes grow at least to the micropyle prior to arrest, which occurs within the ovary (Seavey and Bawa, 1986). Kenrick et al. (1986) found that self-pollen tubes of *Acacia retinodes* arrested within the nucellus. In extreme cases, male gametes of SI pollen may even be released into the embryo sac (Gibbs and Bianchi, 1993). Timing of late-acting SI in the various reports suggests that more than one type of mechanism exists although the way in which these work is not understood.

Timing of the SI reactions observed in this study is illustrated in Figure 129. Pollen tube arrest within the nucellus appears analogous with the GSI mechanism or with later acting SI mechanisms like that described by Kenrick et al. (1986) for *A. retinodes* in which pollen tube arrest is within the nucellus. Pollen tube growth that extends into the egg cell without release of the male gametes can be considered late-acting SI but no description of a similar SI mechanism acting in an angiosperm exists. It is not possible, from these initial observations of SI in white spruce to define a mechanism. The timing of disruption is prezygotic in each case but otherwise the two systems are quite different. In the first case, male gametes die in the pollen tube while it is within sporophytic tissue (nucellus). This occurred occasionally after cross-pollination as well but in the cross-pollinated case, archegonia were immature when pollen tubes arrived. Here, after self-pollination, pollen tube abortion occurred even though the archegonia were mature. In the

second case, male gametes appear healthy but they have not been released into the egg cell. We can only speculate about chemical interactions that might result in pollen tube arrest or failure of the pollen tube to lyse in these cases. Pollen tube arrest occurs within the nucellus after some interspecific crosses of *Pinus* (Hagman, 1975). Pollen of the uncrossable species was found to have different polysaccharide composition. Pettitt (1985) was able to demonstrate an array of proteins emitted from growing pollen tubes of a number of conifer species. Interaction of sugars, proteins or other substances secreted from growing pollen tubes with substances that are secreted within the ovule (Takaso and Owens, 1996) must be the basis of the types of SI response we have observed in white spruce.

Low level seed production after self-pollination means that SI is not a complete barrier to self-fertilization in interior spruce. Self-incompatibility systems need not be absolute in their action, some angiosperm species exhibit partial SI in which low amounts of seed are produced after self-fertilization (eg. *Medicago sativa*; Brink and Cooper, 1938; Sayers and Murphy, 1966). Sedgley and Griffin (1989) report that selection against SI in certain commercial fruit trees can weaken the mechanism so that low levels of seed set are possible. Sage et al. (1994) suggest that late-acting SI systems may represent nothing more than 'weak' GSI systems in which pollen tube inhibition is not complete within the style. Weak SI mechanisms may be sufficient to prevent fertilization in most but not all cases. A few SI systems are mediated by the products of more than a single locus (Hayman and Richter, 1992). If the SI reactions depend on interactions of gene products from more than one locus, with differential expression between pollen and ovule, or if dominance interactions exist between alleles from a single S-locus (Thompson and Taylor, 1966) then partial SI can result.

Zavada and Taylor (1986) see the origin of SI mechanisms as one of the significant developments during early angiosperm evolution. Reduced inbreeding depression (Mau et

al., 1990) and the possibility of mate selection (Zavada and Taylor, 1986) would have increased the fitness of those species with SI systems and this might explain the success of early angiosperms during the Cretaceous. Primitive SI mechanisms need not have first appeared within basal angiosperms, however. Kenrick et al. (1986) downplay the role of stylar tissue in the origin of SI systems by suggesting that nucellar tissue or any other structure on which pollen germinated could have provided a site for SI interactions. Our observations of late-acting, partial SI within the nucellus of white spruce give evidence of an SI system which may predate the origin of angiosperms. Whether the various SI systems evolved from a common ancestral system or arose independently is unclear and it is difficult, therefore, to ascribe evolutionary significance to our results.

Postzygotic seed losses in conifers

Reduced seed production after self-pollination in conifers is well documented (Koski, 1973; Johnsson, 1976; Fowler and Park, 1983; Owens et al., 1990, 1991). Self-pollination in our study resulted in seed production at only 10-30 % of that after cross-pollination. This reduced seed set was quantified by X-ray of seed extracted from cones at the end of the seed maturation period. Timing of seed losses after self-pollination would have been described as postzygotic had development within the ovule not been carefully observed during the prezygotic period.

Self-fertilization in conifers can result in embryos with high genetic load because of accumulation of sub-lethal recessive alleles in homozygous condition (Johnsson, 1976). When the number of embryonic lethal equivalents is high, empty seeds result (Fowler and Park, 1983). Sub-lethal genetic load is reflected in various levels of inbreeding depression in subsequent development (Williams and Savolainen, 1996). Inbreeding and, in the extreme case, selfing can be used as tools in plant breeding to purge lethal recessive alleles from a breeding population to produce elite, homozygous lines. Williams and Savolainen

(1996) suggest that selfing may not be the best strategy in conifer breeding because of the unusually high number of embryonic lethal equivalents in most trees. Too many desirable gene combinations would be lost in the purge of lethal recessives. If a significant level of seed loss was attributed to prezygotic failure, models which predict the number of embryonic lethal equivalents based on postzygotic seed losses (Savolainen et al., 1992; Namkoong and Bishir, 1987) would need revision. In this scenario, the estimated average number of embryonic lethal equivalents per tree would be reduced. Advocates of selfing in production of elite lines may perceive a benefit from this reduction. Any benefit, however, would likely be outweighed by the risk of losing valuable genes to self-incompatibility.

Conclusion

This report is of preliminary observations that give the first evidence of self-incompatibility acting in a conifer. Two types of barrier to fertilization, that were unique to the self-pollinated case, occurred in interior spruce. Sample sizes were too small to allow quantification of seed losses due to self-incompatibility in this case. Once self-pollination experiments have been conducted on a larger scale, we will know how common prezygotic barriers to fertilization are in this species. A recent report of egg cell rejection of some spermatozoids in *Encephalartos villosus* Lem. (Zamiaceae; Steyn et al., 1996) is the first report of a system of prezygotic discrimination between intraspecific male gametes in a gymnosperm. Incompatible male gametes were not necessarily from self-pollen in that study but the result supports our contention that cell-to-cell signaling and recognition play a role in gymnosperm sexual reproduction.

Chapter 10

General discussion and directions for future research

Sexual reproduction in interior spruce can fail for various reasons during prezygotic stages. The time scale of development from pollination to embryo formation is presented in fig. 130. Reasons for failure to produce seed, and the stage during which they occurred, are indicated. Three categories of prezygotic failure have been identified: i) lack of viable pollen or early ovule abortion, ii) physical defects in the ovule that prevent fertilization, and iii) incompatibility reactions that prevent fertilization. My conservative estimate is that 15% of ovules were not pollinated or had aborted at a very early stage, and that about 15% of ovules were not fertilized due to physical abnormality or incompatibility. Owens et al. (1991) reported that 11% of seed losses in *Pseudotsuga menziesii* were due to prezygotic factors other than lack of pollen or early ovule abortion.

Pollination

The pollination mechanism of seed orchard interior spruce was found to function even in the hot, dry environment of the southern Interior of British Columbia. Pollination drops were produced as seed cones closed at the end of the receptivity period. This alleviated our fears that pollination drops might be reduced in volume or not secreted, therefore making pollination inefficient or impossible in this environment. Within pollination control bags, humidity was high and pollination drops were more voluminous and persistent than in unenclosed seed cones. This high humidity might more closely mimic the moist natural range of this species.

Pollen was found to float into inverted ovules in the pollination drop. The role of sacci as floatation devices was demonstrated by observation of interior spruce pollen

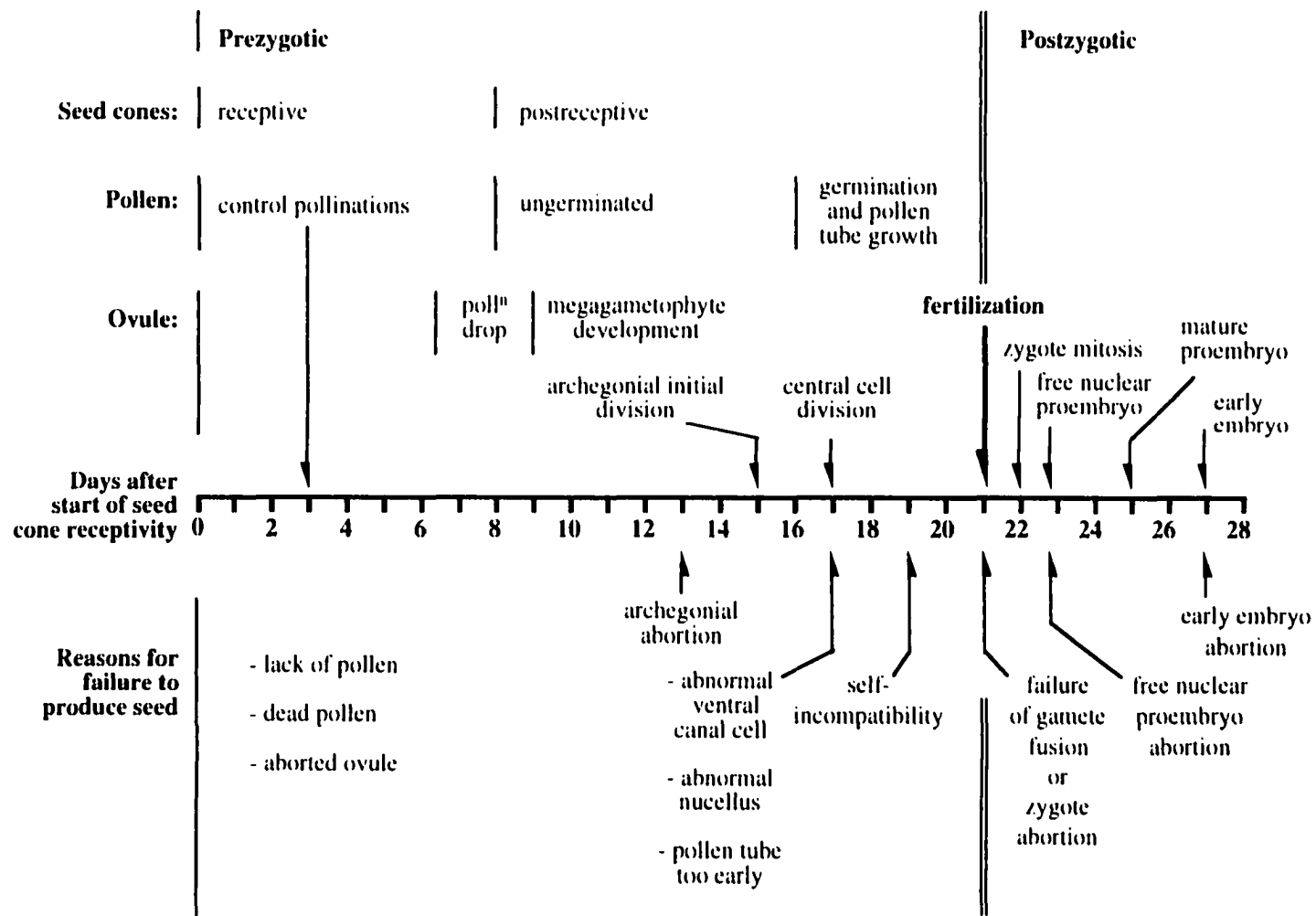


Figure 130. Developmental stages and abnormalities observed during sexual reproduction of interior spruce.

behaviour in vivo, and in model systems. In interior spruce, as in some Podocarpaceae (Tomlinson, 1994), the large pollination drop can scavenge pollen from seed cone structures near the micropylar arms and thereby increase the efficiency of pollination.

Saccus morphology has evolved in oriental spruce (*Picea orientalis*) so that they provide only a brief (30 to 60 sec.) period of floatation before pollen sink into the upright ovules. Pollination by this exceptional mechanism in *Picea* allowed study of the correlated evolution of characters, in this case, ovule orientation at pollination and saccus functional morphology. Ultimately, the primary role of sacci is in pollen floatation and the character that makes pollen buoyant has changed in the species in which more than brief floatation would be inefficient in the pollination mechanism.

Ovule and seed development

From pollen germination until early embryo formation, development was as reported in earlier literature (see Owens and Molder, 1984a). Examination of resin embedded specimens allowed detailed description of fertilization and proembryo development. The normal course of megagametophyte maturation was described and enabled description of prezygotic failure that resulted from abnormal development or incompatibility. Abnormal developments observed within ovules included, archegonial abortion, misplaced ventral canal cells, and abnormal nucelli. In each of these types, fertilization did not occur or was not successful. The only type of incompatibility observed after cross-pollination was temporal; male gametes aborted if pollen tubes reached the megagametophyte prior to egg, and ventral canal cell maturation.

Misplaced ventral canal cells were common and pollen tube behaviour near archegonia with this condition suggested that deterioration of the mature ventral canal cell attracts the pollen tube and signals egg cell maturity. When the ventral canal cell was misplaced to one side of the archegonium, pollen tubes were observed to ignore the neck

cells and grow through the prothallial tissue of the megagametophyte towards the side of the archegonium where the ventral canal cell was located. On one occasion, a pollen tube was able to affect fertilization through the side of the egg cell when the ventral canal cell nucleus was free in the micropylar half of the archegonium, but the free nuclear proembryo aborted shortly after formation.

Two types of incompatibility were observed after self-pollination. In the first type, male gametes aborted while the pollen tubes were within the nucellus even though the egg cell was mature. The second type was later acting. Pollen tubes grew into egg cells but instead of rupturing to release the male gametes, they continued to grow and destroyed the egg. Self-incompatibility in a gymnosperm had not previously been reported but, increasingly, lines of evidence suggest that interactions between gymnosperm pollen or male gametes and ovules occur (Takaso and Owens, 1996; Steyn et al., 1996).

Future research: seed orchard management

Failure of sexual reproduction during prezygotic stages is a factor that reduces seed set in commercial seed orchards. Three recommendations are made based on this study.

- 1) Supplemental mass pollination must be used, early and repeatedly, even during years when pollen cones are produced in abundance. The goal of supplemental mass pollination is to provide a large quantity of high quality pollen but a situation of diminishing returns exists if pollen is applied too late during seed cone receptivity. Self-, and contaminating pollen occupy the micropylar arms and physically compete with later applied pollen.
- 2) Control pollinations were generally inefficient because pollen adhered to the moist inner surface of control pollination bags when the air inside was too humid and condensation occurred. The best solution is probably to use more pollen per application and to

perform pollinations later in the day when humidity in the bags is lowest. Early in the day the bags tended to be very wet because of dew and during the hottest part of the day transpiration tended to elevate humidity within the bags.

- 3) Fashler and El-Kassaby (1987) have used an overhead misting system to retard the onset of pollen shedding and seed cone receptivity, and to prolong the receptive period once it begins in a seed orchard of *Pseudotsuga menziesii*. Alteration of reproductive bud phenology in this manner meant that seed orchard trees were pollinated after the majority of pollen from contaminating natural sources had been shed. The same sort of overhead misting could be beneficial in interior spruce seed orchards. Particularly in hot, dry settings, misting briefly once or twice a day might humidify the microenvironment around each seed cone and prolong receptivity as observed within the humid environment of the control pollination bags. A second benefit which might derive from overhead misting is in increased pollination efficiency due to pollen floatation. Pollen was observed clustered in the micropyle after a brief rain and in subsequent model experiments to simulate rain. Rainwater or mist might even collect pollen in the air and deposit it within seed cones in which the only hydrophilic surfaces are the micropylar arms and micropyles. This idea has yet to be tested.

Future research: plant reproductive biology

Many of the observations of sexual reproduction suggested new ideas or areas that could usefully be studied by different techniques.

- 1) The ventral canal cell may play a role in fertilization. The mechanism might best be elucidated by molecular techniques but, at this point, we have no knowledge of what the signaling mechanism or molecules might be.

- 2) The mechanism of cytoplasmic inheritance remains incompletely worked out in interior spruce. Paternal plastids and mitochondria entered the egg cell but paternal mitochondria are reportedly not a component of the progeny cytoplasm (David and Keathley, 1996) and their exclusion from the proembryo was not observed. They clustered at a distance from the zygote and by the free nuclear proembryo stage, all mitochondria of the neocytoplasm were uniform in appearance. Are the neocytoplasmic mitochondria all maternal or do paternal and maternal mitochondria conform in appearance and mix in the neocytoplasm? Perhaps the fate of cytoplasmic organelles with genomes would be better followed by labelling DNA with fluorochromes and using confocal microscopy, or by reconstruction of serial ultrathin sections using the software applications now available (see, Mogensen, 1996).
- 3) Self-incompatibility should be studied again in a similar but larger scale study to characterize prezygotic effects of self-pollination.
- 4) Small inclusions and cytoskeleton in the conifer egg cell would be better studied after cryofixation, using confocal microscopy, or followed by freeze-substitution and electron microscopy, than after chemical fixation. The mechanism of nuclear and organelle movement within the egg cell remains unclear.

Future research: population genetics

Seed losses resulting from other than lack of pollen and extrinsic factors like freezing or predation have always been considered postzygotic losses in conifers (Kärkkäinen and Savolainen, 1993)

- 1) Observed prezygotic losses are difficult to quantify but they reduce the estimated number of recessive lethal alleles which is reportedly high in conifers (Namkoong and Bishir, 1987). Existence of a self-incompatibility mechanism in interior spruce would reduce

the estimated number of lethal recessive alleles even further. Perhaps conifers do not carry as heavy a genetic load as has been thought.

- 2) Occurrence of prezygotic abnormalities varied on a clonal basis. The possibility of observing a self-incompatibility mechanism might increase if clones that are poor seed producers in general (eg. clone 1944 in this study) are chosen for further study.

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