The role of prezygotic events in the reproductive success of conifers

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Abstract

The purpose of this study was to determine if any prezygotic breeding barriers exist in conifers. Prezygotic events were analyzed for both in vivo and in vitro systems in conifers. The events that were monitored included archegonial development, pollen germination, pollen tube growth and penetration of female structures and gamete delivery.

Aspects of archegonial development in vivo were verified by serial sections of glycol methacrylate embedded sections. This study describes novel abnormal archegonial structures in Dunkeld larch. Normal archegonial development was also investigated in Dunkeld larch and Douglas-fir. This study demonstrates that the ratio between egg nuclear volume and egg cell volume in conifers is consistent with the linear relationship that is known to exist between cell and nuclear volume in all other land plants. In contrast to other plant cell nuclei, most of the DNA in the egg cell nuclei of conifers is localized at the periphery of these organelles. In addition, the relationship between DNA content and nuclear volume in conifer egg cells is not consistent with the linear relationship that is shown between nuclear DNA content and nuclear volume in angiosperms.

This study also examined the interactions between pollen and ovules as well as interaction of pollen and female gametophytes in vivo to determine if there were any prezygotic barriers to foreign pollen in conifers. Dissections of Dunkeld larch and Douglas-fir ovules that had been pollinated with either Dunkald larch, Douglas-fir, western white pine or Interior spruce pollens revealed that heterospecific pollen has a

reduced capacity to germinate in these ovules. Serial sections of glycol methacrylate embedded specimens showed that the nucellus also posed a barrier to some foreign pollen. Western white pine pollen was unable to penetrate the nucellus of either larch or Dougals fir. However, Douglas-fir pollen was able to penetrate not only the nucellus of larch ovules, but also megagametophytes and egg cells into which it delivered gametes. Larch pollen was also observed to penetrate Douglas-fir nucelluses and megagametophytes, though no gamete delivery was observed.

In vitro co-culture was also used to study the interaction of pollen and megagametophytes of different genera. Cells of megagametophytes provided no barrier to pollen tubes, and pollen tubes were able to penetrate any part of megagametophytes. Delivery of gametes was confirmed between spruce and larch. This study demonstrated that the megagametophyte plays no role in male selection.

To study the effects of culturing on megagametophytes of Douglas-fir and larch, cones were collected at the time of fertilization and the megagametophytes were removed and then placed on medium. A variety of cell types proliferated including prothallial, neck and jacket cells. Some of these multiplying cells showed a binucleate condition. This was the first report of neck cell multiplication and induction of a binucleate state for gymnosperm megagametophyte cells in vitro.

This study demonstrates that a number of prezygotic events can influence reproductive success in conifers.

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Chapter 1

General Introduction

The word conifer comes from the Latin roots: conus, meaning cone, and ferre, which means to bear. Most conifers produce seed in cones though there are some exceptions such as yew trees, which have red berry-like structures instead of cones. Junipers also have berry-like structures and these are used in making gin. Evergreen needle leaves also characterize these trees, though not all conifers are evergreen. Larch, cypress, *Metasequoia*, *Pseudolarix*, and *Taxodium* are all deciduous. Conifers have a worldwide distribution, and approximately 650 different species have been identified.

Conifers are the source of many commonly used products (Mabberley 1997). Wood from conifers is used to meet the majority of the world's lumber demands and is the raw material for most pulp and paper. It is even used in making cellophane and some plastics. In addition, conifers are used in the production of chemicals such as turpentine and tannins used in tanning leather. Some varnishes, printing inks, soaps, polishes and sealing waxes are also derived from conifers. Spruce wood is used in making violins and cellos. Oils from pines are used in men's colognes. Taxol from yew trees has been used as a treatment for ovarian cancer. North Americans and Europeans have had a long-standing tradition of bringing whole conifers into the home once a year for the purpose of Yuletide celebration, and this has spurred an industry that produces up to thirty-six million Christmas trees per year.

As a result of the many demands for conifer trees, a great deal of attention has been given to their reproductive cycles. Much of this research has been driven by the need to establish seed and seedling orchards to enable reforestation programs. The natural regeneration of a logged area is a slow process. Conifers take 5 to 30 years of growth before they reach sexual maturity (Kramer and Kozlowski 1979). In order to meet the needs of the timber industry, conifer reproductive cycles had to be studied so they could be manipulated in orchard settings, thereby ensuring abundant seed yields.

Conifers belong to a larger group of plants known as gymnosperms, which are characterized by naked seeds, or ovules. This in in contrast to angiosperm seeds, which are housed in ovaries. In conifers, ovules are found either on the scales of cones or directly on branches. At the tip of each ovule is an opening, the micropyle, which is the entrance for pollen grains. Many mechanisms that facilitate the movement of pollen through the micropyle and into ovules have been described (reviewed in Doyle 1945; Owens et al. 1998; Singh 1978; Takaso 1996; Tomlinson 1994; Tomlinson et al. 1997). When pollen grains are mature, they are released from their cones and are carried by wind to ovules in female cones. When a pollen grain germinates inside an ovule, it produces a pollen tube that grows through a layer of cells known as the nucellus, and then it enters the female gametophyte which houses the egg cells. The pollen tube then delivers a male gamete to an egg nucleus. This process of fertilization results in an embryo that develops within the ovule, which is then, called a seed. Under favorable conditions, the seed can germinate, forming a new conifer seedling.

Since pollen is borne on the wind, it is possible for the pollen of one conifer species to enter the ovule of another species. If fertilization is successful, a hybrid seed is produced. Many conifers hybridize naturally. In the interior region of British Columbia, white spruce and Engelmann spruce readily hybridize (Runion and Owens 1999). Hybrid swarms of red and black spruce are found in the forests of eastern Canada, while in the southeastern United States, *Pinus x sondereggei* (loblolly pine x long leaf pine) is a naturally occurring hybrid. Other conifers can hybridize if artificially pollinated by dusting female cones of one species with the pollen of another species. This has been repeatedly attempted with species of pines in the hope of producing hybrids with greater growth potential or a greater capacity for disease resistance (Critchfield 1986). Conifers that normally do not occupy the same habitat can be brought to the same location to facilitate hybridization. This can be deliberate or accidental. One of the more interesting accidents was found in a botanical garden where *Chamaecyparis nootkatensis* and *Cupressus macroca*rpa formed a hybrid known as Leyland's cypress (Zobel and Talbert 1984).

This is an extreme example of hybridization as it resulted from a wide cross between genera rather than a cross between species. Seed of Leyland's cypress is sterile, but the tree is easily cloned. As a result, it is used extensively in urban settings in Britain and North America.

Conifer hybridization is more often an exception rather than a rule. In the mixed stands of the Colorado Plateau Douglas-fir, white fir, limber pine, blue spruce, and ponderosa pine all grow together though they do not hybridize. However, where conifer distributions overlap, pollen of one species may enter the ovule of another species because of pollen pollution and this may result in seed loss.

Seed loss may be the result of either prezygotic or postzygotic breeding barriers. Traditionally, postzygotic breeding barriers have been the focus of studies that aimed to determine mechanisms of seed loss. It has been shown that self-pollinated conifers have reduced seed sets. This phenomenon, known as inbreeding depression, is the result of early embryo abortion due to increased homozygosity in recessive deleterious alleles (Fowler and Park 1983; Mergen et al. 1965; Orr-Ewing 1957). In addition, it has been shown that, sometimes, deliberate attempts to cross species of conifers can fail (Critchfield 1986). Seed loss in these situations were assumed to be due to mismatches in genetic complements of male and female gametes that resulted in embryo breakdown.

Recently, more attention has been given to understanding prezygotic factors that effect seed loss. Abnormal development in female gametophytes and cone abortion can have a significant influence on seed loss (Slobodník and Guttenberger 2000). Studies by Hagman (1975), McWilliam (1959), and Mikkola (1969) showed that foreign pollen has a reduced capacity to deliver gametes to egg cells, which also correlated with seed loss. However, none of these studies conclusively demonstrated the presence of prezygotic breeding barriers to hybridization.

The scope of this study is to examine the interactions between pollen and ovules as well as interactions between pollen and female gametophytes to determine if there are prezygotic breeding barriers in conifers. The three hypotheses that were tested in this study are as follows:

1) Prezygotic breeding barriers in conifers are maintained by the nucellus and/or secretions in the ovule.
2) Reproductive barriers that prevent hybridization in conifers may be overcome in vitro through the co-culture of pollen and megagametophytes.
3) Abnormal development in the female gametophytes of conifers is variable and can be observed both in vivo and in vitro.

Note on format

Chapter 6 of this dissertation has been published as Dumont-BéBoux et al. 1998. Chapter 7 has been published as Ma et al. 1998. These chapters, as they appear in this dissertation, are consistent with the publications with only minor changes to format. My contribution to the material presented in these chapters includes all works related to specimen fixation and embedding in glycol methacrylate, sectioning, light microscopy and interpretation of results. The writing of these chapters was a collaborative effort among all authors.

Chapter 2

Literature review

Introduction

Gymnosperms include all plants in which the seed bearing structures called ovules are not encapsulated by an ovary. There are four distinct groups within the gymnosperms including the cycads, ginkgos, conifers and gnetopsids. Gymnosperms have a reproductive strategy based on the separate development of male and female gametophytes. The male gametophyte develops in a pollen grain and the female gametophyte develops within an ovule that is maintained on the sporophyte. The two gametophytes are introduced to each other by pollination, where the principal method of pollen transfer is wind pollination (anemophily). Fossils of seed ferns, known also as pteridosperms, show that these plants were gymnospermous. In some pteridosperms, pollen grains germinated to release flagellated sperm that would swim in the ovule towards archegonia to fertilize egg cells (Taylor 1982). One fossil pteridosperm, *Callistophyton*, has a pollen grain that produced a pollen tube (Rothwell 1972). In extant gymnosperms, including conifers, pollen tubes deliver male gametes directly to archegonia. This process is known as siphonogamy.

Pollen development in the conifers is very diverse. Though all conifer pollens produce a pollen tube for the purpose of siphonogamy, the number of cells that develop in the pollen grain is variable, as is the surface structure of the pollen. Female gametophyte development is highly conserved within the conifers, as archegonial development is conserved not only in conifers but also among all archegoniate plants (Foster and Gifford 1974). However, ovule development is very diverse among the

conifers. This has been used as an important character for classifying conifers. Details of reproductive strategies in conifers have also been used to understand relationships among conifers as well as to establish protocols for conifer seed orchards.

In this chapter, I will outline the extant conifer families and then describe male and female gametophyte development, pollination mechanisms, and fertilization in conifers. I will then discuss what is known about conifer reproduction in the context of theories of reproductive isolation.

Conifer taxonomy

The nature of relationships between groups of conifers has been a topic of debate for the last century. As more information about morphology, gametophyte development, reproductive strategies, hybridization barriers and genetic sequence is gathered, a better understanding of the true phylogeny of conifers can be achieved. The most widely accepted classification of conifer species is based on Pilger's (1926) classification which recognizes seven families within the Coniferales including the Pinaceae, Cupressaceae, Taxodiaceae, Taxaceae, Podocarpaceae, Cephalotaxaceae and the Araucariaceae (Table 1). Several studies have examined whether this grouping accurately represents the phylogenetic relationships between species of conifers.

Systematic placement of the Taxaceae has been the most controversial. Florin (1948) argued that the megasporangiate structures characteristic of the Taxaceae were more likely derived from the sporangial structures found in *Rhynia* (Rhyniophyta), as the taxads lack female cones and instead bear their seeds on short lateral shoots. Both Florin (1948) and Sterling (1963) hypothesized that this characteristic was sufficient to place the taxads within their own order, the Taxales, in which the Taxaceae was the only family.

Table 1. The taxa of the Coniferales based on the classification scheme proposed by Pilger (1926).

Family	**Genera**
Pinaceae	*Pinus, Cedrus, Tsuga, Pseudotsuga, Abies, Picea, Larix, Pseudolarix, Cathaya, Keeteleeria*
Cupressaceae	*Cupressaceae, Juniperus, Thuja, Calocedrus, Libocedrus, Chamaecyparis, Biota, Thujopsis, Actinostrobus, Callitris, Fitzroya, Tetraclinis, Widdringtonia, Pilgerodendron*
Taxodiaceae	*Metasequoia, Sequoia, Taxodium, Sequioadendron, Cryptomeria, Athrotaxis, Cunninghamia, Glyptostrobus, Tiawania, Sciadopitys*
Taxaceae	*Taxus, Torreya, Amentotaxus, Austrotaxus, Nothotaxus*
Podocarpaceae	*Podocarpus, Dacrydium, Phyllocladus, Microcachrys, Saxegothaea, Pherosphaera, Acmopyle*
Cephalotaxaceae	*Cephalotaxus*
Araucariaceae	*Araucaria, Agathis*

The remaining coniferophytes were placed in the Coniferales. It is thought that the Coniferales, as well as all other seed plants, were derived from the Progymnospermophyta. This classification implies that all characteristics that Taxales share with the Coniferales, apart from megasporangiate structure, arose as the result of convergent evolution. This is not a parsimonious explanation for the evolution of conifers.

Another taxonomic description of the conifers, proposed by Buchholz (1934), recommended ten families for the Coniferales. This systematic scheme suggested that the conifers were not monophyletic and that they should be split into two clades, the Phanerostrobilares and the Aphanerostrobilares. The Phanerostrobilares were characterized by the presence of obvious ovulate cones, while the Aphanerostrobilares were characterized by the absence of strobili or cones. The latter group was most poorly defined, as Buchholz included genera in this grouping which have strobili (Chamberlain 1957). In addition, the suggestion that conifers are not monophyletic would necessitate the convergent evolution of morphological characters common to all conifers. Therefore, this proposed taxonomy was not satisfactory.

Other interpretations of the taxonomic structure of the conifers suggested either lumping or splitting of the seven families designated by Pilger (1926). Based on Eichler's (1889) account of conifer taxonomy, Engler (1926) proposed six families for the conifers. This classification, recognized by Chamberlain (1957), did not separate the Cephalotaxaceae from the Taxaceae. The existence of a close relationship or even the monophyly of the Cephalotaxaceae and the Taxaceae has been supported by one

phylogenetic analysis based on morphological data (Hart 1987) and another analysis that was based on molecular data (Stefanovic et al. 1998).

A recent phylogenetic analysis of the conifers suggested seven families for this assemblage; however, not all of these families were consistent with those proposed by Pilger (1926). This analysis, which was based on 28S rRNA sequence, proposed the combination of the Taxodiaceae and the Cupressaceae into one monophyletic clade (Stefanovic et al. 1998). Additionally, it was proposed that *Sciadopitys verticillata* be removed from the Taxodiaceae and placed in its own family, the Sciadopityaceae (Stefanovic et al. 1998). This classification of *Sciadopitys* was also supported by Hart's (1987) phylogenetic analysis that was based on morphological data. In both studies, the new family was designated the sister clade to the Taxodiaceae/Cupressaceae assemblage. Although it is likely that the classification scheme proposed by these studies will eventually be adopted into conifer nomenclature, most of the classical studies assumed the classification that was proposed by Pilger (1926). Therefore, for the sake of unity with the literature, families described in this review refer to those described by Pilger.

Development of the male gametophyte in conifers

All conifers have male pollen cones or microstrobili. During early development of these cones, groups of cells along a central axis elongate radially and then divide periclinally to form primordia similar to those of leaves. As the result of equal divisions on the abaxial and adaxial sides of the primordia, blunt appendages called microsporophylls are formed (Allen and Owens 1972). Microsporangia, also known as pollen sacs, are initiated from regions of meristematic cells that occur on the abaxial side of the microsporophylls (Singh 1978). The outermost cells of these regions divide anticlinally to form an

epidermis, while the inner cells divide in all directions to produce a mass of sporogenous cells. Sporogenous cells give rise to microspore mother cells that divide by meiosis to produce tetrads of microspores that, upon further development, become mature pollen grains.

Pollen grain maturation commences with the development of a thickened cell wall that is made up of an outer exine and an inner intine. In most members of the Pinaceae and the Podocarpaceae the exine may separate from the intine forming two or three air bladders known as sacci. In all other conifer families, air bladders are absent on pollen grains. When a pollen grain germinates, the intine grows to form a pollen tube that ultimately delivers sperm to an egg cell (Singh 1978). Pollen grains with sacci generally have a thicker exine than intine. In the Pinaceae, the area between the air bladders has a germinal pore through which the pollen tube may grow (Singh 1978). Pollen grains of the Podocarpaceae lack germinal pores (Tomlinson 1994). Pollen grains that do not have sacci generally have a thicker intine than exine and lack a germinal pore. In the absence of a germinal pore, the intine of a germinating pollen grain will swell causing the exine to split and then the pollen tube commences growth (Singh 1978).

In all groups of conifers excepting the Taxaceae and the Cupressaceae, the one-celled pollen grain undergoes one or more mitotic division before pollen is shed from pollen sacs. In the Pinaceae, mature pollen grains contain five cells (Singh 1978). The first and second cell divisions are both unequal and periclinal. These divisions give rise to two small prothallial cells that lie adjacent to one another and a large antheridial cell. These cells are often formed before the intine has completed development and as a result they usually become buried in this layer. The antheridial cell divides to form a tube cell

(which later forms the pollen tube) and a generative cell. The generative cell divides periclinally forming a stalk cell next to the prothallial cells and a body cell. At the time of fertilization, the body cell separates from the stalk cell and divides to form two sperm nuclei in the pollen tube.

Members of the Podocarpaceae have more than five cells in their mature pollen grains (Boyle and Doyle 1953; Doyle and O'Leary 1935a; Looby and Doyle 1944; Tomlinson 1994). These pollen grains also begin maturation with two unequal divisions that give rise to two adjacent prothallial cells and an antheridial cell. However, the prothallial cells then undergo several periclinal and anticlinal divisions resulting in a mass of prothallial cells in one hemisphere of the pollen grain. The antheridial cell divides to produce a generative cell and a tube cell. Pollen grains are often shed at this stage of development, though occasionally, body cells and stalk cells may also be found in pollen grains of podocarps during anthesis. Often, it is after the pollen grain has produced a pollen tube that the generative cell divides. This division is anticlinal and the stalk and body cell lie side by side. The body cell is larger than the stalk cell. At fertilization, the body cell divides unequally to form two gametes; the larger gamete is functional and participates in fertilization (Boyle and Doyle 1953; Looby and Doyle 1944; Wilson and Owens 1999).

Members of the Araucariaceae also have more than five cells at anthesis due to the divisions of first two prothallial cells that are formed (Haines et al. 1984; Owens et al. 1995). In addition to prothallial cells, a tube cell and a generative cell are present at the time of pollen shed. After the pollen tube is formed, the generative cell divides

anticlinally to produce a stalk cell and a body cell. An unequal division of the body cell gives rise to a small non-functional gamete and a large functional gamete.

In the Taxodiaceae, Cephalotaxaceae, Cupressaceae and Taxaceae, there are fewer than five cells in pollen grains at the time of pollen shed. In all of these taxa, prothallial cells are absent. In the Taxodiaceae (Brennan and Doyle 1956) and the Cephalotaxaceae (Singh 1961), pollen is shed at a two-celled stage. Within the pollen grain there is a tube nucleus and the antheridial initial. At the onset of pollen germination, the antheridial initial divides to give rise to a body cell and a stalk cell. The body cell divides at fertilization to yield the two male gametes that are the same size. In the Cupressaceae (Baird 1953; Mehra and Sircar 1948; Owens and Molder 1984a) and the Taxaceae (Pennell and Bell 1985; 1986a,b), pollen grains are shed in a uninucleate condition. Pollen grain germination in species of these taxa triggers an unequal division within the pollen grain that results in a large tube cell and a small generative cell. The generative cell divides to form a small stalk cell and a body cell, which at the time of fertilization, divides to yield two male gametes equal in size.

Development of ovules and the female gametophyte in conifers

Most conifers, with the exception of members of the Taxaceae and the Podocarpaceae, have prominent female cones in which megasporophylls, called ovuliferous scales, bear the ovules. Located beneath each ovuliferous scale is a bract. The ovuliferous scale is commonly regarded as originating from a fertile shoot (Foster and Gifford 1974). In fossils such as *Lebachia* (an early gymnosperm), the ovule is located at the tip of a fertile shoot and surrounded by megasporophylls. Beneath the ovule there are several sterile scales, and a single bract is positioned beneath the sterile scales. A reduction series in

which recurvation of fertile shoots coupled with the fusion of sterile scales (into a single sterile scale) is thought to have resulted in seed scale complexes like those found in fossils of *Voltzia*. This reduction series is believed to be the origin of ovuliferous scale morphology found in most extant conifers (Foster and Gifford 1974).

In conifers where ovuliferous scales and bracts are present they develop from primordia along the main axis of the cones. These are arranged in a spiral phyllotaxis. Primordia of megasporangial cells initiate the development of ovules on the adaxial surfaces of ovuliferous scales. As these cells proliferate, the inner cell layers differentiate to form the nucellus, and the outermost cells become the integument. The integument of an ovule is open at one end to allow for the entry of pollen grains at the time of pollination. This opening in the integument, or micropyle, is continuous with an internal chamber called the micropylar canal that terminates at the surface of the nucellus. The micropyle is variable in shape and size. Ovules are oriented on ovuliferous scales such that their micropyles face the cone axis. It has been shown that the morphology of conifer cones cause air eddies which facilitates the movement of pollen towards the micropylar tips of ovules (Niklas 1984).

In species within the Taxaceae, ovules are borne on apical meristems rather than on ovuliferous scales in strobili (Kemp 1959). Apical initials give rise to the nucellus and the integument develops from the flank meristem. Pith flank meristem and sub-apical initials form the chalazal end of the ovule. Bracts are absent in the Taxaceae. In the Podocarpaceae, the integument of the ovule is fused to a structure known as an epimatium (Gibbs 1912; Tomlinson et al. 1991). The epimatium (Gibbs 1912), otherwise referred to as an axillary ovuliferous structure (Boyle and Doyle 1953), is essentially an

ovuliferous scale that encapsulates the ovule (Gibbs 1912). A bract subtends this compound structure.

The integument of conifer ovules is also thought to have evolved from a reduction series (Foster and Gifford 1974; Singh 1978). The most primitive gymnosperm seed fossil is known as *Genomosperma kidstoni*. In ovules of the gymnosperm, lobes of integumental processes surround the nucellus. A more advanced ovule is seen in *G. latens* in which the integumentary lobes are partly fused along their length to form a rudimentary micropyle. The integumentary filaments are almost completely fused in *Eurystoma*. Complete fusion to form a single integument with a micropyle is seen in *Stamnostoma*. As mentioned previously, the micropyle is where pollen enters ovules at the time of pollination to reach the surface of the nucellus. Modifications of micropyles in extant conifers function in facilitating pollen capture (Doyle 1945). The role of micropylar morphology in pollination mechanisms will be discussed later.

Beneath the epidermis of the nucellus one or several cells function as archesporial cells. These cells divide to yield an upper primary parietal layer and a lower layer of primary sporogenous cells. The parietal layer undergoes cell division to add to the micropylar mass of the nucellus. The primary sporogenous cells will either divide to form one or two megaspore mother cells, or they simply differentiate into megaspore mother cells without further division (Singh 1978). Megaspore mother cells are enlarged as compared to other cells of the nucellus. Often, if more than one megaspore mother cell is formed only one is functional. The megaspore mother cell divides by meiosis, and the chalazal-most of the four resulting haploid megaspores give rise to a single megagametophyte. The other three haploid cells degenerate. Bell (1996) hypothesized

that the degeneration of the supernumerary megaspores is the consequence of selective apoptosis that is dependent on a bipartite locus. Such a system would require a directed chromosomal sorting throughout meiosis to ensure that the chalazal megaspore always survived.

The surviving megaspore enlarges and its nucleus divides, ultimately forming a large coenocytic megaspore with hexagonal arrays of nuclei arranged at the periphery of a central vacuole (Maheshwari and Singh 1967). Cellularization or alveolation is effected as anticlinal cell walls are laid down between the free nuclei. The cell walls extend from the megaspore cell wall towards the central vacuole. The periclinal divisions of alveoli result in the formation of prothallial cells that make up the bulk of the megagametophyte (Maheshwari and Singh 1967).

Archegonial development within a megagametophyte starts with the enlargement of prothallial cells at the micropylar end of the megagametophyte. These enlarged cells function as archegonial initials. The archegonial initials undergo unequal periclinal divisions forming neck cell initials and large central cells. The neck cell initials divide to form a neck that includes a minimum of one tier of cells, often with two to eight cells. In the Pinaceae (Maheshwari and Singh 1967) and some members of the Cupressaceae (Owens and Molder 1984a), the central cell divides unequally forming a small ventral canal cell, and a large egg cell. In the Taxaceae (Anderson and Owens 1999), Taxodiaceae (Brennan and Doyle 1956), some members of the Cupressaceae (Mehra and Sircar 1948), Araucariaceae (Owens et al. 1995), Cephalotaxaceae (Singh 1961), and Podocarpaceae (Boyle and Doyle 1953; Coker 1902; Looby and Doyle 1944; Sinnott 1913), the central cell nucleus divides, but no cell wall formation occurs between the

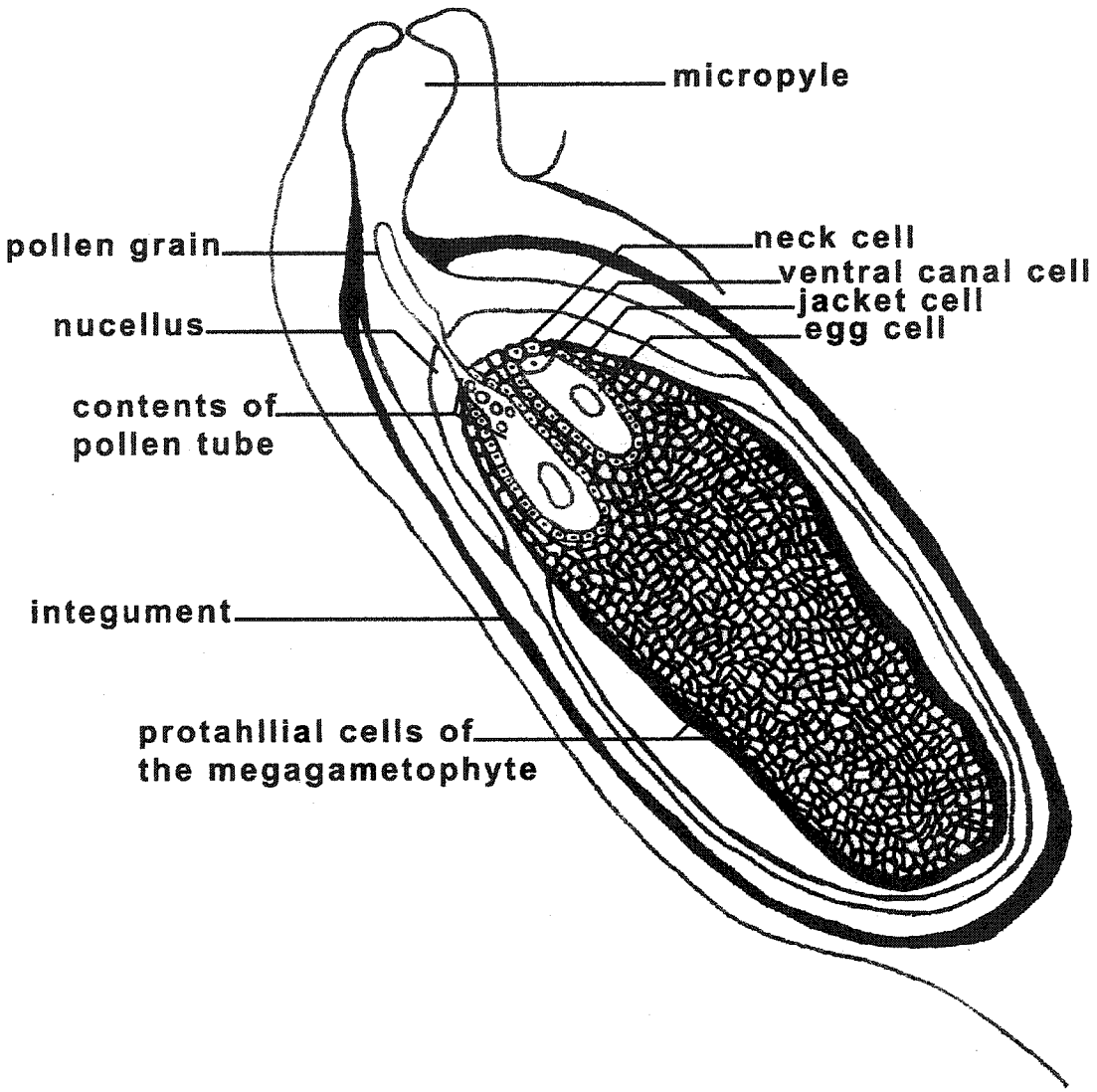
daughter nuclei. As the egg cell matures it enlarges. The egg nucleus also enlarges and descends in the cytoplasm from a micropylar position towards the center of the egg (Singh 1978). Mature egg cells of larch are about 600 μm in length and 350 μm in width and their nuclei are about 150 μm in diameter (Camefort 1967). Prothallial cells adjacent to archegonia differentiate to form jacket cells through the development of dense cytoplasm and prominent nuclei (Singh 1978). A mature ovule (Fig. 1) is achieved at the completion of archegonial development.

In Pinaceae, Podocarpaceae, Cephalotaxaceae and Taxaceae one to seven archegonia occur singly in the micropylar ends of megagametophytes with each individual archegonium bordered by jacket cells. In the Taxodiaceae (with the exception of *Sciadopitys*), Cupressaceae and Araucariaceae, archegonia develop in clusters, known as complexes, which include several archegonia bordering each other. The entire complex shares the same layer of jacket cells (Maheshwari and Singh 1967; Konar and Oberoi 1969). Archegonial complexes are initiated where pollen tubes contact the megagametophyte (Looby and Doyle 1944; Baird 1953; Brennan and Doyle 1956; Singh and Chatterjee 1963). As a result, these complexes are not only initiated at the apex of megagametophytes but also at lateral positions. Though archegonia are borne singly in the Podocarpaceae, their development is also initiated by pollen tube contact with the megagametophyte (Maheshwari and Singh 1967).

Pollination mechanisms in conifers

Pollination mechanisms in conifers refer to the means by which pollen is captured by an ovule and subsequently transferred into its micropylar canal. There is great diversity in pollination mechanisms among and within families of conifers (see reviews by Doyle

Fig. 1. Schematic of a mature ovule in Douglas-fir. Adapted from Allen and Owens 1972.


micropyle
pollen grain
neck cell
ventral canal cell
jacket cell
egg cell
nucellus
contents of
pollen tube
integument
protahlial cells of
the megagametophyte

1945; Owens et al. 1998; Singh 1978; Takaso 1996; Tomlinson 1994; Tomlinson et al. 1997). Though many pollination mechanisms have been described, there are likely many that remain undiscovered. A complex of evolved characteristics of male and female tissues allow for the capture and transfer of pollen into ovules. Features of the ovule that contribute to pollination mechanisms in conifers include modifications of the micropyle and ovular secretions. In addition, whether pollen grains have sacci also influences pollination mechanisms in conifers. In most conifers, these features work in concert to ensure pollen grains enter the micropylar canals of ovules where they germinate prior to fertilization.

Modifications of the micropyle in ovules that assist in pollen capture have been observed in many groups within the Pinaceae. In species of *Pinus* (Doyle and O'Leary 1935b) and *Picea* (Doyle and Kane 1943), two outgrowths that resemble pincers covered in small hairs capture pollen. Microdroplets are formed on the micropylar arms of *Picea* (Owens and Blake 1984) and *Cedrus* (Takaso and Owens 1995a) and assist in pollen capture. In *Pseudotsuga*, (Doyle and O'Leary 1935c; Allen and Owens 1972; Takaso and Owens 1995b) and *Larix* (Doyle and O'Leary 1935c; Villar et al. 1984), flaps with hair-like projections (stigmatic flaps), which are unequal in size, extend from the tip of the micropyle. After pollination, these flaps collapse and push pollen grains into the micropyle. In species of *Abies* (Doyle and Kane 1943) and *Cedrus* (Doyle and O'Leary 1935c; Chowdhury 1961; Takaso and Owens 1995a) the micropyle is funnel-shaped and stigmatic. In *Tsuga*, (Doyle and Kane 1943; Stanlake and Owens 1974) ovules have a large micropylar flare in which pollen can be collected. In the Araucariaceae (Haines et

al. 1984; Owens et al. 1995), another ovular adaptation is noted in which the nucellus grows beyond the tip of the micropyle to accommodate pollen in reaching it.

Ovular secretions in the form of pollination droplets also assist in capturing pollen. This is the case in the Cupressaceae, Taxodiaceae, Taxaceae, Cephalotaxaceae, Podocarpaceae (with the exception of *Saxegothaea*), and in some Pinaceae including *Pinus*, *Picea* and some species of *Tsuga* (Owens et al. 1998). In *Abies*, pollination droplets are absent (Arista and Talavera 1994) and pollen grains are thought to be washed into the micropyle by rainwater where they then come in contact with a pollination drop that is not exuded past the tip of the micropyle (Owens et al. 1998). In *Cedrus*, it was thought that pollen grains were washed into the micropyle by rainwater as well; however, pollination drops similar to those in *Pinus* and *Picea* were recently discovered (Takaso and Owens 1995a). In *Pseudotsuga* (Doyle 1945; Takaso and Owens 1994; Takaso et al. 1996; von Aderkas and Leary 1999a) and *Larix* (Doyle 1945; Takaso and Owens 1997; Villar et al. 1984; von Aderkas and Leary 1999b) the micropyle is flooded with a post-pollination pre-fertilization droplet that washes the pollen grains from stigmatic hairs. The only conifers in which pollen does not come in contact with an ovular secretion include species within the Araucariaceae (Haines et al. 1984; Owens et al. 1995), *Saxegothaea* (Doyle and O'Leary 1935a) and some species of *Tsuga* (Doyle and O'Leary 1935c; Doyle and Kane 1943). In these conifers, pollen grains germinate on ovuliferous scales and produce long pollen tubes that grow towards the nucellus (which exudes past the tip of the micropyle).

Pollination droplets were first described in conifers by Jean-Pierre Vaucher in 1841 (reviewed in Fujii 1903; Tison 1911). In 1903, Fujii stated that he felt that

pollination drops played an important role in the biology of pollen. Some studies have since addressed this postulation. In 1911, Tison observed that, in *Taxus baccata*, when pollen grains were wetted in pollination drops, the intine of the pollen grains swelled and split the exine, which is the first step of pollen germination. In addition, Takaso et al. (1996) demonstrated that ovular secretions collected from *Pseudotsuga* could induce pollen grains dissected from *Pseudotsuga* ovules to germinate in vitro.

Several studies have examined the role of ovular secretions in transporting pollen to the surface of the nucellus. In species within the Pinaceae and the Podocarpaceae that have saccate pollen, the female cones are upright at the time of pollination, and the ovules are positioned so their micropyles point downward. When saccate pollen lands on the pollination drop of an inverted ovule, it floats on the meniscus of the droplet (Owens et al. 1998). Pollen is drawn into the micropyle when the pollination drop is retracted (Tomlinson 1994). Retraction of these drops is the result of evaporation in some conifers but in others it is a metabolically active process (Tomlinson 1997). It has been shown that this mechanism is able to exclude non-saccate pollen from the micropyle of podocarps that are normally pollinated with saccate pollen (Tomlinson 1997).

In *Picea orientalis*, saccate pollen lands on upright rather than on inverted ovules. It has been demonstrated that the sacci on pollen grains of this species are porous and as a result, water enters the pollen grains and they sink (Runions et al. 1999). In taxa with non-saccate pollen that exude pollination drops, the ovules are upright and pollen sinks in the drop (Owens et al. 1998). *Pseudotsuga* and *Larix* are the exception to this rule as ovules are inverted in these genera. However, for *Larix*, Takaso and Owens (1997) interpreted pollen movement from stigmatic hairs in the micropyle to the surface

of the nucellus to be facilitated by the recession of post-pollination pre-fertilization ovular secretions. In *Pseudotsuga*, post-pollination pre-fertilization droplets trigger both pollen grain elongation through the micropyle and pollen tube formation once the pollen grain has reached the nucellus (Takaso and Owens 1994).

Fujii (1903) examined the pollination drop of *Taxus baccata* and showed that it contained sucrose, glucose, formic acid and amino acids. Tison (1911) added malic acid to the known composition of pollination drops in *Taxus baccata*. He also hypothesized that malic acid could have a role in chemotactic guidance of pollen tube growth. Pollination drops contain amino acids (Chesnoy 1993) and polypeptides (Ziegler 1959).

Ovular secretions are known to be involved in reproduction not only in most conifers, but also in other extant gymnosperms including *Ginkgo* and cycads (Singh 1978). In addition, evidence for pollination drops have been found in a fossil of *Callospermarion pusillum* – a Paleozoic Pteridosperm (Rothwell 1977). Therefore, it is clear that pollination drops are ancestral in seed plants and must be functionally significant in reproduction. In addition, when pollen enters the micropyle of an ovule, the first medium it must survive in is that of the ovular secretions. These secretions may have a role in pollen selection or as a defense barrier to foreign matter such as fungal spores or bacteria. However, no studies have been done to examine these possibilities.

Fertilization and zygote formation

After a pollen grain has germinated in the micropyle of an ovule and its pollen tube has penetrated the cells of the nucellus, the pollen tube then enters the megagametophyte. Generally, a pollen tube enters the megagametophyte via neck cells of an archegonium. The pollen tube then passes through a ventral canal cell (if it is present) and enters an egg

cell. The pollen tube ruptures at its distal end and two gametes are delivered into an egg cell. In conifer gametes, there is a significant inequality; male gametes are much smaller. A critical analysis of this male to female ratio has yet to be done (reviewed in Boyle and Doyle 1953). Only one male gamete will fuse with the female nucleus to form a zygote. The signals that cause one male gamete to be selected over the other for participating in fertilization are unknown. Furthermore, the mechanism by which a male gamete moves through the egg cytoplasm to the egg nucleus is unknown. It is possible that these processes are mediated by cytoskeletal elements. The role of actin in pollen tube growth has been studied (Lazzaro 1996); however, the role of actin and other components of the cytoskeleton in other aspects of conifer reproduction have not been studied. In addition, as a male gamete moves through the egg cell cytoplasm it enlarges. The mechanism by which this occurs has not been examined, nor has the functional significance of this phenomenon been explained.

When the male gamete approaches the female nucleus, the latter flattens at its micropylar end and invaginates so that it becomes cup shaped to receive the male nucleus (Camefort 1968; McWilliam and Mergen 1959). Zygote formation is initiated when the nuclear membranes of the two gametes fuse. Subsequently, pores form in the region of the membrane fusion, and these pores enlarge until the nucleoplasm of the gametes becomes continuous (Chesnoy and Thomas 1971). The first few mitotic divisions of the zygote do not involve the formation of cell walls. Therefore the early proembryo is coenocytic (Chesnoy and Thomas1971; Chowdhury 1962). The number of free nuclei that are formed in the proembryo is variable. In *Agathis*, as many as 64 free nuclei have been found in proembryos, while in *Sequoia*, the zygote divides only once before cell

wall formation commences (Chowdhury 1962). In the Pinaceae, four free nuclei are formed (Camefort 1968). Subsequently, the nuclei descend to the chalazal end of the egg cell and cellularization of the proembryo begins seed development (Camefort 1968).

Mechanisms of reproductive isolation in conifers

The biological species concept defines species as groups of interbreeding natural populations that are reproductively isolated from each other (Mayr 1942; Dobzhansky 1935; 1940; 1951; 1970). Since species and races often differ from one another in many genes, hybridization may result in the dilution of distinct characters that delimit species. Reproductive isolation serves as a mechanism to avoid dilution of the discrete genetic complements of individuals by inhibiting random interbreeding between members of different species (Dobzhansky 1951). Reproductive isolation can be subdivided into two categories including postzygotic isolation and premating or prezygotic isolation (Table 2).

Postzygotic reproductive isolation inhibits hybrid propagation by reducing the viability or fertility of hybrids (Dobzhansky 1970). If inviable hybrids are formed, inviability may be expressed at the level of zygote formation. Alternatively, adults may form and reproduce but the F2 generation of the hybrid may have reduced viability. In either case, it is correctly assumed that hybrid inviability is caused by between-locus incompatibilities that are the product of divergence between two genomes in isolation (Orr 1997).

Postzygotic reproductive isolation has the potential to be an expensive means of reproductive isolation in that gametes are wasted in non-viable mating efforts rather than used to increase numbers of viable progeny. In conifers, embryo formation stimulates the

Table 2. Reproductive isolation mechanisms. Reproduced from Dobzhansky 1970.

Mechanism	Description
a) Prezygotic isolation	Prevents the formation of hybrid zygotes.
1. Ecological isolation	Populations occur in different habitats in the same general region.
2. Seasonal or temporal isolation	Mating or flowering occurs during different seasons
3. Sexual or ethological isolation	Mutual attraction between sexes of different species is weak or absent.
4. Mechanical isolation	Physical incompatibility between genitalia of flower parts prevents copulation or pollen transfer.
5. Isolation by different pollinators	In flowering plants, related species attract specific insects as pollinators.
6. Gametic isolation	Female and male gametes may not be attracted to one another, or male gametophytes may be incompatible with female gametophyte or sporophyte tissues.
b) Postzygotic isolation	Reduced viability or fertility of hybrid zygotes.

accumulation and production of storage materials to nourish the embryo (Krasowski and Owens 1993; Owens et al. 1993). If the embryo is non-viable, then energy expended to accumulate nutrients is wasted. Therefore, one might expect selection to favor pre-zygotic over post-zygotic reproductive isolation mechanisms. However, it has been traditionally assumed that, in conifers, reproductive isolation is achieved postzygotically and is manifested as embryo abortion. Generally, the success of hybridization is measured by viable seed yield (Critchfield 1986). However, this method does not demonstrate whether any pre-zygotic breeding barriers exist (Hagman 1975).

Prezygotic isolation can be the result of one or more mechanisms, all of which are outlined in Table 2. Of these mechanisms, temporal isolation and gametic isolation are of particular relevance to conifer breeding systems. Sexual isolation does not influence reproduction in plants. Mechanical isolation does not affect conifer breeding systems, as any conifer pollen grain can fit into the micropyle of any other conifer. All conifers are wind pollinated and therefore no selection against breeding is effected by insect behaviour. Ecological isolation imposes reproductive barriers on organisms found in different habitats within the same general region. Ecological isolation may arise in three ways (Coyne and Orr 1998). Firstly, different species may confine mating behaviours to different populations. Secondly, species may be confined to subniches within a given area and therefore are effectively allopatric in distribution. Wind pollination reduces the likelihood of ecological isolation acting as a barrier to conifer interbreeding since pollen travels between populations in different locations. Thirdly, species may live allopatrically, though the distribution of the species may overlap allowing for hybrid formation. In the event that hybrids are maladapted, then reproductive isolation of the

two overlapping species is maintained. In conifers, this phenomenon is referred to as hybrid breakdown (Zobel and Talbert 1984); however, it has most often been observed in situations where species have been brought together artificially. For example, *Pinus sondereggeri*, a hybrid between *P. palustris* and *P. taeda* is frequently formed in seed orchards and in regions where there has been extreme ecological disturbance, but very few of these hybrids grow to maturity in naturally regenerated forests (Zobel and Talbert 1984).

Seasonal or temporal isolation is caused by the occurrence of distinct phenologies that serve to maintain reproductive isolation (Dobzhansky 1970). Different species of conifers often undergo pollination at different times of the year (Owens and Blake 1985), reducing the likelihood of hybridization. Temporal isolation may act to separate groups that, for reasons of genetic similarity, would otherwise easily hybridize. Two subspecies of *Juniperus phoenicea* (*Juniperus phoenicea* subsp. *phoenicea* and *Juniperus phoenicea* subsp. *turbinata*) are known to grow sympatrically; however they do not hybridize because they flower at different times of the year (Arista et al. 1997). Occasionally, extreme weather conditions, such as prolonged cold or wet periods in spring, or insect damage, can cause species that normally flower at different times to flower coincidentally (Zobel and Talbert 1984). Therefore, temporal isolation is not an entirely fail-safe means of enforcing reproductive isolation.

Gametic reproductive isolation refers to a mechanism of isolation enforced by incompatibility between gametes or gametophytes that enforces species specific mating (Dobzhansky 1970). Pollen grains must germinate in or near a micropylar canal, often in association with a pollination drop or a post-pollination pre-fertilization droplet. There

are some instances when pollen can be excluded from a given micropyle depending on the pollination mechanism that is employed to get pollen into the micropyle. In conifers where ovules are inverted and saccate pollen enters the micropylar canal by retraction of a pollination drop, non-saccate pollen will not enter the micropylar canal. However, heterospecific saccate pollen can still enter the micropylar canal (Tomlinson et al. 1997). Conifers with non-saccate pollen do not exclude any type of foreign pollen (Tomlinson 1997). Following germination, pollen tubes must successfully penetrate the sporophytic cells of the nucellus as well as gametophytic cells of the megagametophyte before it can deliver male gametes to the egg cell. Therefore, potential barriers to foreign pollen after it has entered a micropylar canal include ovular secretions, the nucellus, and cells of the megagametophyte.

The types and concentrations of sugars present in ovular secretions are variable among different conifers (Chesnoy 1993). As a result, different ovular secretions may have different osmotic potentials. This could influence the rate of hydration that pollen would experience in a given ovular secretion. In a dried state, the plasma membrane of pollen is in a gel state, porous and ineffective as an osmotic barrier. Membrane integrity is restored with rehydration (Heslop-Harrison 1992). In angiosperms, if hydration is too abrupt, preventing the plasma membrane from restoring integrity, the cell contents can leak out resulting in pollen death (Heslop-Harrison 1992). In Douglas-fir, slow rehydration of pollen grains prior to germination on media greatly improves pollen viability (Dumont-BéBoux et al. 1999); therefore, it is possible that pollen of this species would have a better germination rate in ovular secretions with a higher concentration of sugars.

Cells of the nucellus could provide a barrier to foreign pollen by either preventing or slowing pollen tube growth. Studies have shown that incompatible pollen will often be outperformed by compatible pollen in controlled crosses. McWilliam (1959), Mikkola (1969) and Hagman (1975) all observed that interspecific sterility in members of the Pinaceae is due to the reduced ability of pollen tubes to penetrate the nucellus of heterospecific ovules. These pioneering studies invoke the question: Do conifer ovules have pre-zygotic selection against foreign pollen? Further study with controlled experiments and statistical analyses need to be done to answer this question.

The role of cells of the megagametophyte in posing a barrier to foreign pollen has not been investigated. This has been due to the absence of a means to maintain megagametophytes outside of the ovule. Recently, a technique for conifer in vitro fertilization has been established (Fernando et al. 1998). This technique allows for the co-culture of pollen and megagametophytes in vitro. Therefore, it is now possible to examine pollen – megagametophyte interaction in the absence of other ovular tissues and secretions. Co-culture of heterospecific pollen and megagametophytes would reveal whether cells of the megagametophyte act as a barrier to foreign pollen.

Conclusions

Generally, reproductive success in conifers has only been assessed by determining seed yield. Little experimentation has been done to investigate how pre-zygotic events influence reproductive success. Some attention has been given to abnormal gametophyte development prior to fertilization and how this can have a negative impact on seed production potential (Slobodník and Guttenberger 2000). Tomlinson et al. (1997) has done some experimentation to assess the ability of foreign pollen to enter micropyles, and

demonstrated that pollination mechanisms do not pose impenetrable barrier to foreign pollen. McWilliam (1959), Mikkola (1969) and Hagman (1975) have made observations on the inability of heterospecific pollen to grow as well as like-pollen in nucellar cells. However, no studies have shown conclusively whether any part of conifer ovules or megagametophytes can act as a pre-zygotic breeding barrier in conifers. This situation is one that I set out to remedy.

Chapter 3

Archegonial abnormalities in Dunkeld larch

Introduction

Archegonia have been defined as structures with at least two cells including an egg cell that is separated from the outside of the female gametophyte by at least one cell forming the neck (Favre-Duchartre 1971). The development of megagametophytes and archegonia is highly conserved among conifers. This process involves the enlargement of a cell within the nucellus that functions as the megaspore mother cell. This cell divides by meiosis, and the chalazal-most of the four resulting haploid megaspores give rise to a single megagametophyte, while the other three cells degenerate (Bell 1996). The nucleus of the functional megaspore divides, ultimately forming a large coenocytic megaspore with hexagonal arrays of nuclei arranged at the periphery of a central vacuole (Maheshwari and Singh 1967). Cellularization or alveolation is effected as anticlinal cell walls are laid down extending from the megaspore cell wall towards the central vacuole. The periclinal divisions of alveoli result in the formation of prothallial cells that make up the bulk of the megagametophyte (Maheshwari and Singh 1967).

Prothallial cells at the micropylar end of the megagametophyte enlarge and differentiate to become archegonial initials. The archegonial initials undergo unequal periclinal divisions forming neck cell initials and large central cells. The neck cell initials divide to form a neck that includes a minimum of one tier of cells, often with two to eight cells. In the Pinaceae, including larch (Kosínski 1986) and some members of the Cupressaceae (Owens and Molder 1984a), the central cell divides unequally forming a small ventral canal cell, and a large egg cell. In the Taxaceae (Anderson and Owens

1999), Taxodiaceae (Brennan and Doyle 1956), some members of the Cupressaceae (Mehra and Sircar 1948), Araucariaceae (Owens et al. 1995), Cephalotaxaceae (Singh 1961), and Podocarpaceae (Boyle and Doyle 1953; Coker 1902; Looby and Doyle 1944; Sinnott 1913), the central cell nucleus divides, but no cell wall formation occurs between the daughter nuclei. As the egg cell matures, the egg nucleus descends in the cytoplasm from a micropylar position towards the center of the egg. As this happens, the egg nucleus expands until its diameter is about half the width of the egg cell. Prothallial cells adjacent to archegonia differentiate to form jacket cells through the development of dense cytoplasm and prominent nuclei (Singh 1978).

In *Ginkgo*, the cycads, Pinaceae, Podocarpaceae, Cephalotaxaceae and Taxaceae one to seven archegonia occur singly with each individual archegonium bordered by jacket cells. In the Taxodiaceae (with the exception of *Sciadopitys*), Cupressaceae and Araucariaceae, archegonia develop in clusters, known as complexes, which include several archegonia bordering each other. The entire complex shares the same layer of jacket cells (Maheshwari and Singh 1967; Konar and Oberoi 1969). Archegonial complexes are initiated where pollen tubes contact the megagametophyte, resulting in variable positions for these complexes (Looby and Doyle 1942; Baird 1953; Brennan and Doyle 1956; Singh and Chatterjee 1963). Though archegonia are borne singly in the Podocarpaceae, their development is also initiated by pollen tube contact with the megagametophyte (Maheshwari and Singh 1967).

Examples of abnormal megagametophyte and archegonial development have been observed. More than one megaspore may persist within the nucellus resulting in the development of two or more megagametophytes. These may develop beside one another

or stacked one above the other (Anderson and Owens 1999; Pennell and Bell 1987). Within the megagametophyte, lateral (Slobodník and Guttenberger 2000) and even chalazal (Chowdhury 1961) archegonia may initiate though this condition is also abnormal. Equally rare are conifer archegonia that develop in an inverted position (Dogra 1966). In cultures of *Ginkgo* megagametophytes, Favre-Duchartre (1956) noted swollen prothallial cells located between archegonia. He interpreted these cells as being archegonial initials. The occurrence of fused archegonia has also been reported (Anderson and Owens 1999; Chowdhury 1961; Dogra 1966; Slobodník and Guttenberger 2000; Wilson and Owens 1999). Dogra (1966) as well as Anderson and Owens (1999) have interpreted this abnormality as the result of the dissolution of prothallial cells between neighbouring archegonia (Dogra 1966; Anderson and Owens 1999).

This study describes novel archegonial structures in the European/Japanese hybrid larch, or Dunkeld larch (*Larix decidua* Miller x *Larix kaempferi* (Lamb.) Carrière = *Larix* x *marschlinsii* Coaz).

Materials and methods

Plant material

Between May 24, 2000 and June 9, 2000, female cones of European/Japanese hybrid, or Dunkeld larch were collected from 3 trees located on the campus of the University of Victoria. This collection period spanned the period of archegonial maturation that was immediately prior to fertilization through to the time of fertilization. Female cones and their ovules were dissected on the same day that they were collected. A total of 1, 984 ovules were dissected. Randomly selected megagametophytes were removed together with the micropylar portion of the nucellus.

Fixation and embedding

The megagametophytes together with their nucelluses were fixed in 2.5 % glutaraldehyde in 0.05 M phosphate buffer (pH = 7.2). Specimens were left in fixative at room temperature until further processing. Samples were washed overnight in 0.075 M phosphate buffer (pH = 7.2), followed by two more washes of 2 h each. The specimens were then dehydrated using a graded ethanol series up to 100 % ethanol. Subsequently, the specimens were placed overnight in a pre-infiltration solution made from equal parts of Technovit 7100 base and 100 % ethanol. The next day, specimens were infiltrated in a 1 % (w/v) solution of Technovit Hardener I in liquid base for 12 d. During this time, the specimens were maintained at 4 °C and the solution was changed three times. Specimens were then transferred to BEEM flat molds with 1:15 Hardener II and infiltration solution. A plastic sheet was placed over each mold to prevent air contact.

Sectioning, staining and microscopy

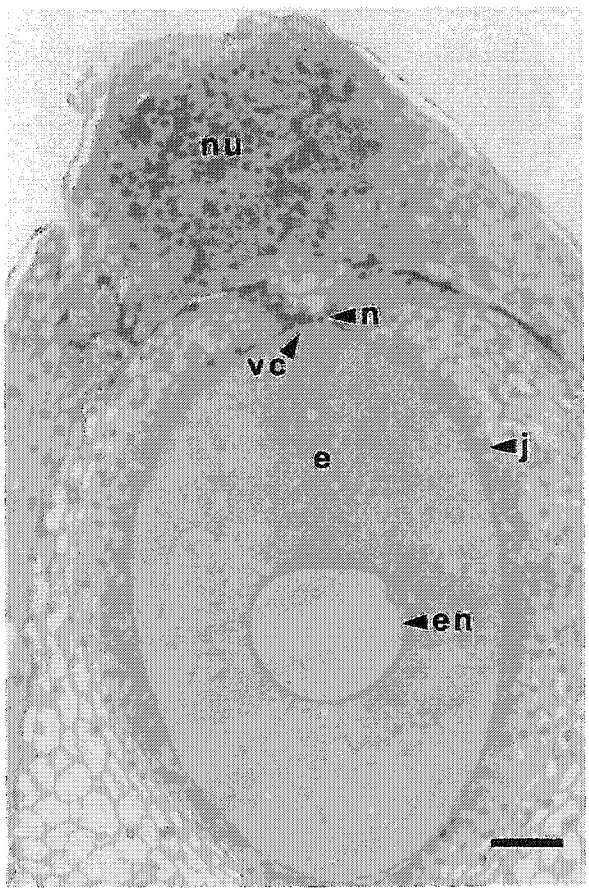
A total of 42 megagametophytes were sectioned longitudinally. Sections (8 μm thick) were cut using a Lieca SM2400 sledge microtome. Sections were stained using Toluidine blue O with NaOCl pretreatment according Gutmann (1995). Sections were viewed using either a Leitz Laborlux S or a Zeiss Axioplan microscope.

Results and discussion

Of the 42 megagametophytes of Dunkeld larch sectioned, three contained abnormal archegonial structures, while the other megagametophytes had normal archegonia. In *Larix decidua*, normal archegonia are made up of one tier of eight neck cells, a ventral canal cell and a large egg cell that is surrounded by a layer of jacket cells (Kosínski 1986). An example of a normal archegonium of Dunkeld larch is shown in Fig. 2. Normal archegonia in this hybrid larch were found to have a single tier of 4-6 neck cells, a ventral canal cell and a large egg cell that was surrounded by a layer of jacket cells. Also, the nucleus of mature egg cells had large nuclei approximately 150-190 μm in diameter; whereas, neighbouring sterile cells had nuclei about 10-15 μm in diameter.

Abnormal archegonia were observed either singly, or in groups bounded by a shared layer of jacket cells. Small egg cells that occurred in groups did not have ventral canal cells. In addition, they were either associated with a large egg cell with a ventral canal cell (Figs. 3-6), or they were observed to be separate from such egg cells (Figs. 7 and 8). Where a single abnormal archegonium was observed, it contained a central cell, and it appeared in an area of the megagametophyte that was morphologically distinct from the rest of the archegonium (Figs. 9 and 10). In all cases, the observed abnormal archegonial structures were not similar to abnormal archegonia that had been previously described for members of the Pinaceae. Furthermore, these abnormal structures meet the criteria for archegonia as they have an enlarged egg cell and are separated from the outside of the megagametophyte by sterile cells.

Fig. 2. Light micrograph of a longitudinal section of a megagametophyte showing a normal archegonium of Dunkeld larch. Egg (e); egg nucleus (en); jacket cells (j); neck cells (n); nucellus (nu); ventral canal cell (vc). Scale bar = 95 μm.


nu
n
vc
e
j
en

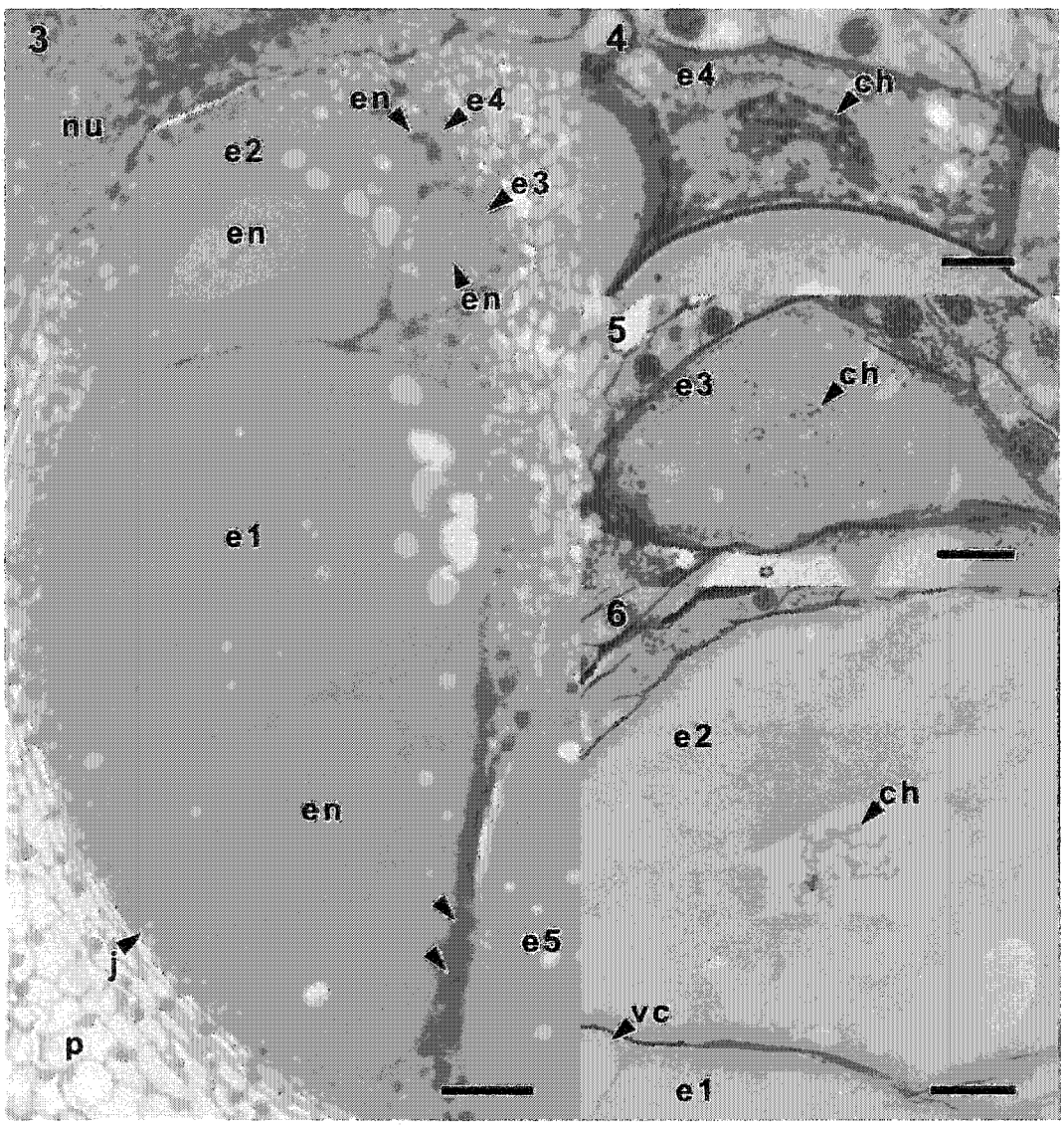
Figs. 3-6. Light micrographs of a longitudinal section of grouped archegonia. Sections are stained with toluidine blue O. Egg (e), egg nucleus (en), condensed chromatin (ch), jacket cells (j), nucellus (nu), prothallial cells (p), ventral canal cell (vc), region where jacket cells are absent between two neighbouring mature egg cells (arrow heads).

Fig. 3. One mature egg cell (e1) and four smaller egg cells (e2, e3, and e4) within a single layer of jacket cells (j). Also, a region where two mature egg cells are connected is shown (arrowheads). Scale bar = 80 μm.

Fig. 4. Small egg cell (e4) with condensed chromatin (ch) within the nucleus. Scale bar = 20 μm.

Fig. 5. Small egg cell (e3) with condensed chromatin (ch) within the nucleus. Scale bar = 25 μm.

Fig. 6. Small egg cell (e2) with condensed chromatin (ch) within the nucleus. Scale bar = 50 μm.


3
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6
e2
ch
vc
e1

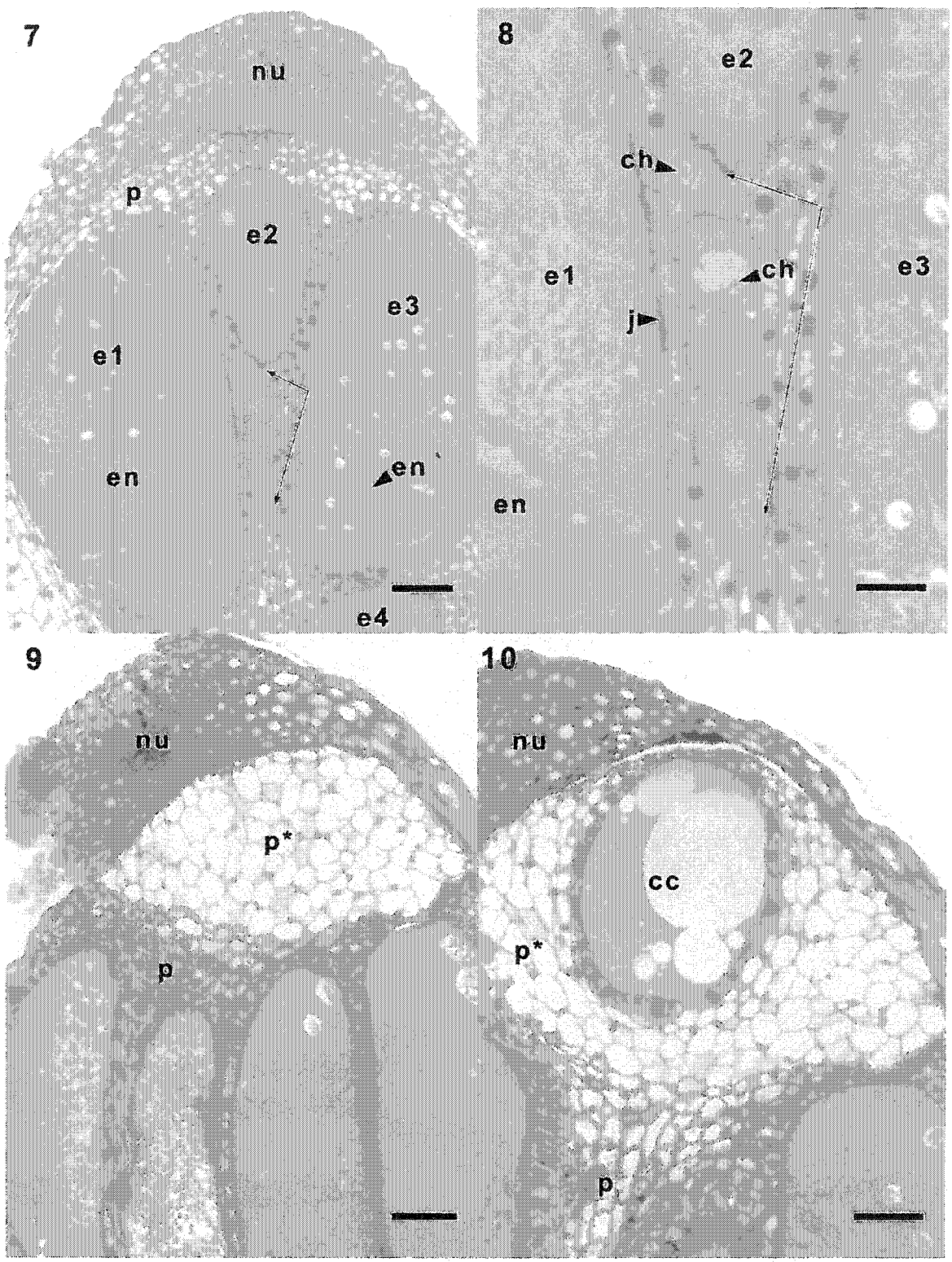
Figs. 7-10. Light micrographs of egg-like cells or a central cell-like cell within highly vacuolate prothallial cells of a megagametophyte. Sections are stained with toluidine blue O. Egg (e), egg nucleus (en), central cell-like cell (cc), condensed chromatin (ch), jacket cells (j), nucellus (nu), prothallial cells (p), highly vacuolate prothallial cells (p*) ventral canal cell (vc).

Fig. 7. An oblique section of four mature egg cells (e1, e2, e3, and e4). Located among the prothallial cells (p) between the mature eggs is a row of smaller cells (arrows). Scale bar = 100 μm.

Fig. 8. Close up of smaller egg cells shown in Fig. 6 (arrows). Condensed chromatin (ch) is apparent within the enlarged nuclei. Scale bar = 50 μm.

Fig. 9. Longitudinal section showing four archegonia with mature egg cells (e1, e2, e3, and e4). These mature egg cells are laterally displaced in relation to the apex of the nucellus (nu). Above the archegonia is a group of highly vacuolate prothallial cells (p*). Scale bar = 100 μm.

Fig. 10. Another section of the megagametophyte seen in Fig. 8. A central cell (cc) within the group of vacuolate prothallial cells (p*) is shown. Scale bar = 80 μm.


7
nu
p
e2
e3
e1
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e4
8
e2
ch
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ch
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9
nu
p*
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10
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cc
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p

Grouped abnormal archegonia

An example of an archegonium where one large, mature egg cell (e1) (Fig. 3) was the neighbour to three smaller egg cells (e2, e3, and e4) (Figs. 3, 4, and 5) was observed. All of these egg cells were observed to share one common jacket cell layer. A ventral canal cell (Fig. 6) was present in e1, and the egg nucleus was enlarged compared to the nuclei of all other types of haploid nuclei of the megagametophyte, including jacket cells, neck cells and prothallial cells. The ventral canal cell of the mature egg was laterally displaced in relation to the apex of the megagametophyte, and the smaller egg cells were located adjacent to the ventral canal cell and the apex of the mature egg. In e2, e3, and e4, ventral canal cells were not present; however, the nuclei of these cells were enlarged compared to those of sterile cells. The nuclei of these smaller egg cells ranged from 35 to 80 μm in diameter. It was also observed that chromatin was condensed in these smaller egg cells (Figs. 4, 5, and 6).

Alternatively, the cells e2, e3, and e4 may perhaps be interpreted as archegonial initials that arose from the differentiation of sterile cells that were adjacent to e1. This may account for the absence of ventral canal cells, since the division of an archegonial initial gives rise to a neck initial and a central cell, and a central cell in turn divides to give rise to a ventral canal cell and an egg cell. The presence of condensed chromatin in e2, e3, and e4 may be an indication that these cells are dividing, as chromosomes are known to condense during the M phase (prophase) of the mitotic cell cycle (Lodish et al. 2000). However, as previously mentioned, the nuclei of e2, e3, and e4 are enlarged, and large nuclei are usually associated with egg cells in conifer megagametophytes, not

archegonial initials (Singh 1978). Therefore, the original interpretation of e2, e3, and e4 as egg cells is likely correct.

There is a fifth mature egg (e5), seen in part, in Fig. 3. There are no jacket cells separating e1 and e5. The archegonia of e1 and e5 may simply have become connected because they had developed in close to one another. Connected archegonia have also been described for another species of larch: *Larix decidua* (Slobodník and Guttenberger 2000). This phenomenon has been described as the result of the dissolution of jacket cells in *Abies pindrow* (Dogra 1966), or as the result of a distortion caused by the expansion of a pollen tube at the apex of a megagametophyte in *Taxus brevifolia* (Anderson and Owens 1999). However, in both studies, no evidence was provided to back up these interpretations. In order to confirm the means by which archegonia became connected it would have been necessary for these researchers to examine them using electron microscopy to determine whether or not remnant jacket cell membranes exist in the region where the archegonia are joined.

A different type of grouped archegonia was found within one layer of jacket cells is shown in Fig. 7. These archegonia had small egg cells and were located between four surrounding normal archegonia. None of these small egg cells were in contact with a normal egg cell. They lacked ventral canal cells, their nuclei were enlarged, and they had condensed chromatin (Fig. 8). Favre-Duchartre (1956) described enlarged prothallial cells between mature archegonia in *Ginkgo* megagametophytes that had been kept in culture. He interpreted these enlarged prothallial cells as being archegonial initials capable of forming fully functional archegonia. The egg cells seen in Figs. 7 and 8 could be interpreted as archegonial initials since, due to the presence of condensed chromatin, it

is likely that they were in the process of dividing. However, the nucleus of these cells was enlarged relative to the nuclei of neighbouring sterile cells, as is characteristic of egg cells. Therefore, rather than dividing, the nuclei of these small eggs may simply be in the process of decondensing with egg cell maturation.

Single abnormal archegonium

In a megagametophyte, six mature archegonia were laterally displaced in relation to the apex of the nucellus (though only four of these archegonia are shown). In the region above these archegonia, there was a proliferation of vacuolate prothallial cells (Fig. 9). An enlarged cell with a morphology characteristic of the central cell stage (Camefort 1967) was observed within this modified region of prothallial cells (Fig. 10). The presence of an archegonium in a morphologically distinct region of cells within a megagametophyte has not been previously described.

Occasionally, more than one megaspore may develop within a nucellus resulting in stacked megagametophytes (Anderson and Owens 1999; Pennell and Bell 1987). However, there was no megaspore wall (which always surrounds a megagametophyte) that separated the area of vacuolate prothallial cells from the normal prothallial cells seen in Fig. 9 or 10. Therefore, these two regions belong to the same megagametophyte. In addition, the archegonium within the vacuolate prothallial cells was delayed in development in comparison to the archegonia located below it. Therefore, the development of this abnormal archegonium is not likely to have been coincident with the development of the archegonia located beneath it. It is more likely that this archegonium developed from the differentiation of a vacuolate prothallial cell after the development of the normal archegonia commenced.

Conclusions

Abnormal archegonial structures that have not been previously reported for any other conifer were described for Dunkeld larch. Irregularities in archegonial development are thought to have an adverse effect on the seed production potential of larch (Slobodník and Guttenberger 2000).

Chapter 4

Egg cell morphology in Dunkeld larch and Douglas-fir

Introduction

In conifers, archegonial development commences with the enlargement of prothallial cells at the micropylar margin of the megagametophyte. These enlarged cells are called archegonial initials (Maheshwari and Singh 1967). In genera of the Pinaceae including *Pseudotsuga* (Allen and Owens 1972), *Cedrus* (Chowdhury 1961), *Larix* (Kosínski 1986), *Pinus* (Owens and Molder 1977a), *Abies* (Owens and Molder 1977b), *Picea* (Runions and Owens 1999), *Tsuga* (Stanlake and Owens 1974), and some genera of the Cupressaceae (Owens and Molder 1984a), two to four archegonial initials are formed, each of which divide anticlinally to form a neck cell initial to the outside and a central cell to the inside. The neck cell initial gives rise in turn to a tier of neck cells. The central cell divides unequally, forming a ventral canal cell and an egg cell. However, in the genera of all other conifer families, the central cell nucleus divides and gives rise to a ventral canal nucleus and an egg cell nucleus that are not separated by a cell wall (reviewed in Konar and Oberoi 1969). Mature archegonia of conifers have one large egg cell surrounded by three types of cells including jacket cells, a ventral canal nucleus or cell, and neck cells.

As the egg cell matures, it enlarges. In *Larix decidua*, mature egg cells measure 600 μm in length and 350 μm in width (Camefort 1967). Jacket cells surrounding the egg cell divide to accommodate the enlargement of the egg cell (Singh 1978). During egg cell maturation, the nucleus descends through the cytoplasm from a position just beneath the ventral canal cell towards the middle of the egg cell (Camefort 1965). The

nucleus usually reaches the center of the egg cell at the time of fertilization in larch (Kosínski 1986) and Douglas-fir (Allen and Owens 1972). As the nucleus descends through the egg cytoplasm, it increases in diameter. In *Pinus laricio*, the mature egg nucleus is 100 μm in diameter (Camefort 1965), whereas in *Larix decidua*, the egg nucleus can be up to 150 μm in diameter (Camefort 1967). The nuclei of gymnosperm egg cells are thought to be the largest known in the plant kingdom (Singh 1978). Electron microscopy has revealed that, in conifer nuclei, the chromosomes are uncoiled and in 100 Å diameter fibrils (Camefort 1959; 1964). These fibrils were described as being sparsely distributed throughout the nucleoplasm. In general, DNA is distributed non-randomly in nuclei. In my study of Dunkeld larch (*Larix decidua* Miller x *Larix kaempferi* (Lamb.) Carrière = *Larix* x *marschlinsii* Coaz) and Douglas-fir (*Pseudotsuga menziesii* Dougl.), I focused on whether the distribution in egg nuclei was homogeneous.

In addition, this study examined the relationship between the volumes of conifer egg cells and their nuclei. In vascular plants, there is a linear relationship between nuclear volume and cell volume (Price et al. 1973). It was hypothesized that this linear relationship would be consistent for cells of angiosperms and the egg cells of Dunkeld larch.

Angiosperms also show a linear relationship between nuclear DNA content (as measured in pg) and nuclear volume (Baetcke et al. 1967; Bennett 1972). Furthermore, they exhibit a linear relationship between nuclear DNA content and chromosome volume (Bennett et al. 1983; Rees et al. 1966). In spite of the large size of nuclei in gymnosperms, the total amount of DNA in any gymnosperm cell only ranges from 12.6 to 38.8 pg (Dhillon 1987). In contrast, the nuclei of *Lilium longiflorum* shoot cells have

nuclei that are about eight times smaller in diameter than larch egg nuclei, yet they have a DNA content of 177 pg (Baetcke et al. 1967). Therefore, it was hypothesized that the relationship between nuclear DNA content and nuclear volume found in angiosperm cells would not be found in the egg cells of Dunkeld larch.

Materials and methods

Plant material

Seed cones of Dunkeld larch were collected between May 24 and June 1, 2000 on the University of Victoria campus. Some seed cones of Douglas-fir were collected between June 22 and June 23, 2000 from the Ministry of Forests Seed Orchard at Puckle Road, Victoria. Other seed cones of Douglas-fir were collected on the University of Victoria campus on July 23, 1999. For both species, seed cones were dissected the day they were collected. Megagametophytes with nucellus were removed from ovules and immediately fixed in 2.5 % glutaraldehyde in 0.05 M phosphate buffer (pH = 7.2) and left at room temperature until further processing.

Embedding and sectioning

Samples were washed overnight in 0.075 M phosphate buffer (pH = 7.2), followed by two buffer washes of 2 h each. The specimens were then dehydrated using a graded ethanol series up to 100 % ethanol. Subsequently, specimens were placed overnight in a pre-infiltration solution made from equal parts of Technovit 7100 base and 100 % ethanol. The next day, the specimens were infiltrated in a 1 % (w/v) solution of Technovit Hardener I in Technovit 7100 base for twelve days at 4 °C. The infiltration solution was changed three times. Specimens were then transferred to BEEM flat molds with 1:15 Technovit Hardener II and infiltration solution. A plastic sheet was placed over each mold to prevent air contact. Sections (8 μm thick) were cut using a Leica SM2400 sledge mictrotome.

Staining

A total of 12 larch and 12 Douglas-fir archegonia were sectioned and stained with

DNA-specific stains including DAPI and Acridine Orange. For DAPI staining, sections were treated with a 1 μg/ml solution of DAPI in distilled water for 30 min in the dark. Subsequently, slides were rinsed in distilled water and dried. Sections were then covered with Molecular Probes Slow fade component A, and a coverslip was applied. Sections were then viewed using a Zeiss – III RS fluorescence microscope. Images were captured using a DAGE – MTI CCD72 video camera and SCION Image software. Other sections were stained with an aqueous solution of 0.01 g Acridine Orange for 15 min. These sections were viewed using a Zeiss Axioplan epifluorescence microscope. In addition, sections of four larch archegonia were stained for protein using the Ninhydrin-Schiff reaction as described in Ruzin (1999).

Volume and nuclear DNA content estimations

Median sections were used to measure the diameter of 43 larch egg nuclei and 25 prothallial cell nuclei. Subsequently, the volumes of egg nuclei and prothallial cell nuclei, were estimated using the formula for the volume of a sphere. The volumes of 10 larch egg cells were approximated by assuming the volume of an egg to be equivalent to the volume of two stacked spheres. The diameter of the first sphere was equal to the diameter of the egg cell 1/3 of the distance from the egg apex (designated by the position of the neck cells), while the volume of the second sphere was equal to the diameter of the egg 2/3 of the distance from the egg apex. Egg cell and nuclear volumes for larch were compared. In order to compare total DNA content to egg nuclear volume in larch, the nuclear volume was calculated as indicated above. The DNA content of cells of the larch megagametophytes was assumed to be 12.3 pg of DNA per haploid nucleus as was reported by Pattanavibool et al. (1995).

Results and discussion

DNA and protein staining

Acridine Orange staining showed that DNA was not evenly distributed within the nuclei of Dunkeld larch and Douglas-fir (Figs. 11-14). As egg nuclear development in larch and Douglas-fir progressed, the nuclei descended in the egg cells. Concurrently, the diameter of the nuclei increased (Figs. 11-14). In young egg nuclei, DNA was found at the periphery of the egg nuclei and in the middle of the nuclei in condensed spots (Fig. 11). More mature egg cells had globules of DNA associated with the periphery of the egg nucleus (Figs. 12 and 13). Mature egg cells had an even band of DNA associated with the periphery of the egg nucleus (Fig. 14). This distribution of DNA has not been observed in any other organism.

DAPI staining gave further information about the morphology of the nucleus in larch and Douglas-fir. Young egg nuclei were lobed in their margins (Fig. 15). However, as egg nuclei matured their margin became entire (Fig 16). Similar developmental changes in nuclear shape have also been observed in *Pinus monticola* (Owens and Bruns 2000). Mature egg nuclei stained with DAPI also showed that DNA was localized at the periphery of egg nuclei, while the middle of the nuclei were devoid of DNA staining in these haploid cells. This was shown in both longitudinal (Figs. 15 and 16) and cross (Figs. 17 and 18) sections. Electron microscope studies by Camefort (1959; 1964), reported that DNA is found as fine fibrils of 100 Å in diameter throughout this organelle. Dense concentrations of DNA were not observed at the periphery of egg nuclei.
The amount of DNA in a cell directly affects its intensity of DAPI staining as it preferentially binds adenine-thymine base regions (Johnston et al. 1999). The cells of the

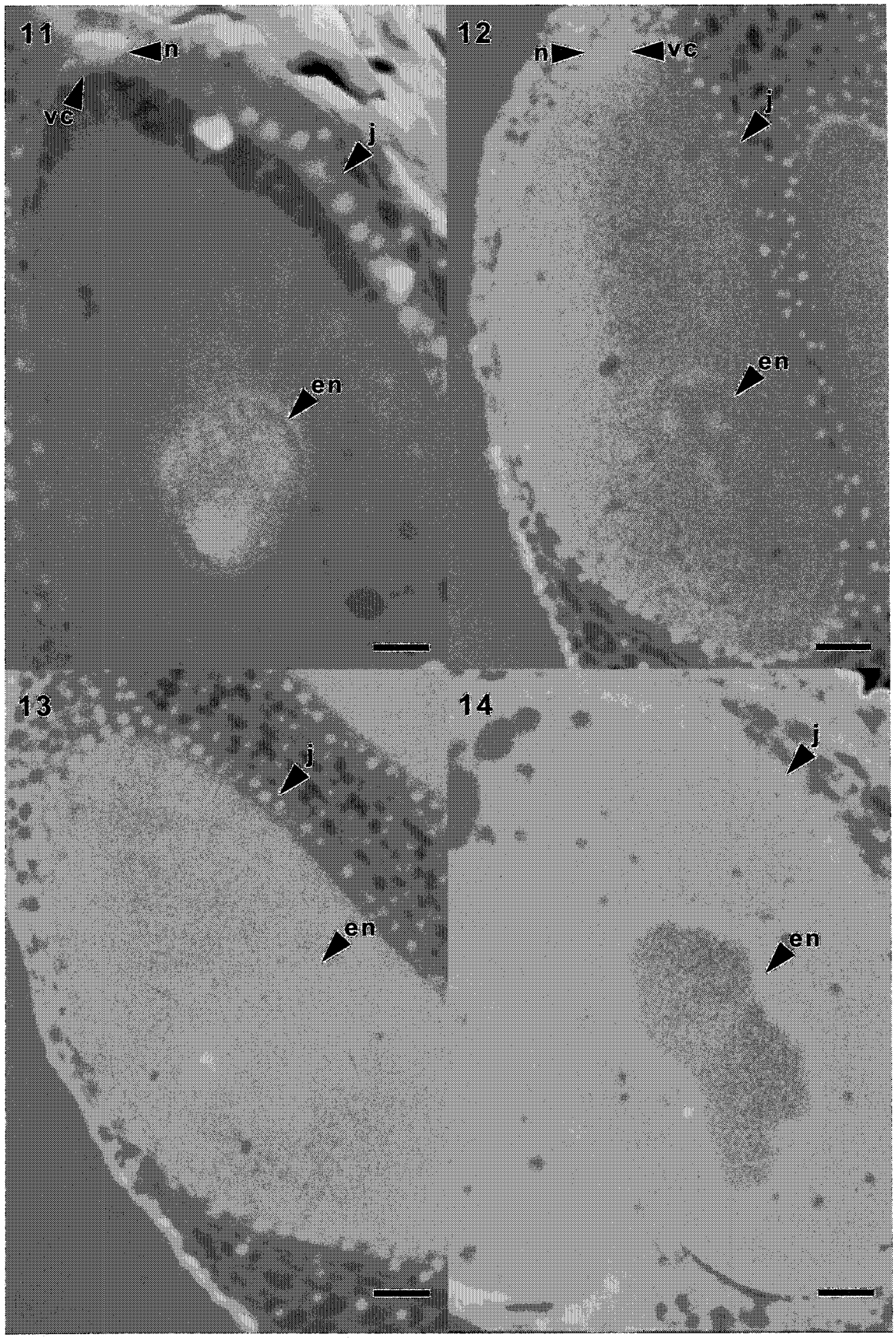
Figs. 11-14. Longitudinal sections of Acridine orange-stained Dunkeld larch and Douglas-fir megagametophytes. Egg nucleus (en); jacket cells (j); neck cells (n); ventral canal cell (vc).

Fig. 11. Median section of a Dunkeld larch archegonium with and early stage of egg cell development. The DNA is located at periphery of the nucleus and in several condensed areas within the egg nucleus (en). Scale bar = 40 μm.

Fig. 12. Section through the periphery of the egg nucleus (en) of Douglas-fir. Scale bar = 80 μm.

Fig. 13. Median section of the egg nucleus (en) also depicted in Fig. 8. Globules of DNA are located at the periphery of the egg nucleus (en). Scale bar = 65 μm.

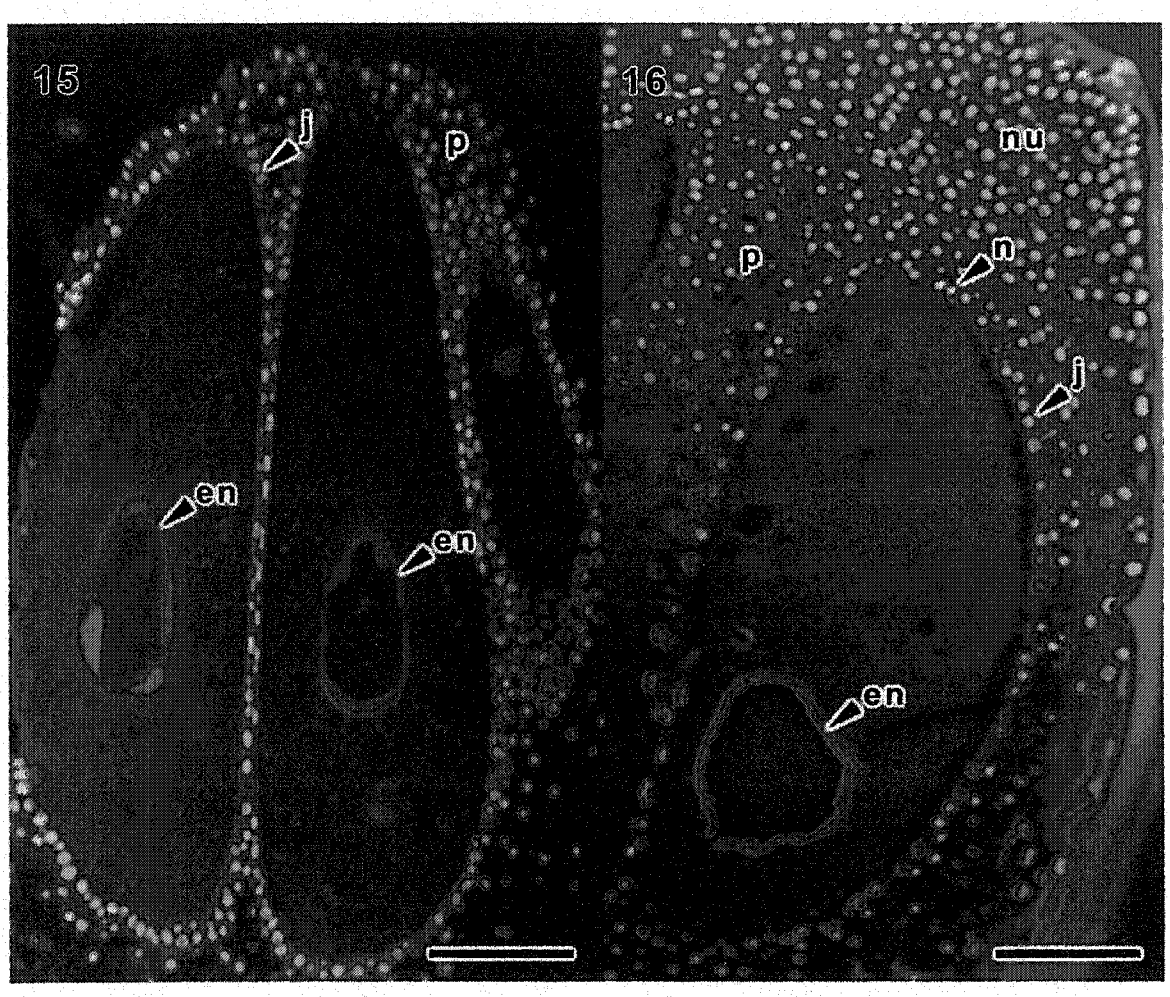
Fig. 14. Median section of a mature archegonium. The DNA forms a continuous band around the periphery of the egg nucleus (en). Scale bar = 60 μm.


11
n
vc
j
en
12
n
vc
j
en
13
j
en
14
j
en

Figs. 15-16. DAPI-stained longitudinal sections of Douglas-fir and Dunkeld larch megagametophytes. DNA stains bright blue. Egg nucleus (en); jacket cells (j); neck (n); nucellus (nu); prothallial cells (p).

Fig. 15. Median section of a Douglas-fir megagametophyte. DNA is localized around the periphery of the egg nuclei (en). The micropylar ends of the egg nuclei are lobed. Scale bar = 185 μm.

Fig. 16. Median section of a larch megagametophyte and nucellus (nu). The diploid nuclei of nucellar cells (nu) stain more brightly than the haploid nuclei of the prothallial cells (p) of the megagametophyte. Neck cells (n) above the egg cell also stain very brightly. Scale bar = 195 μm.


15
j
p
en
en
16
nu
p
n
j
en

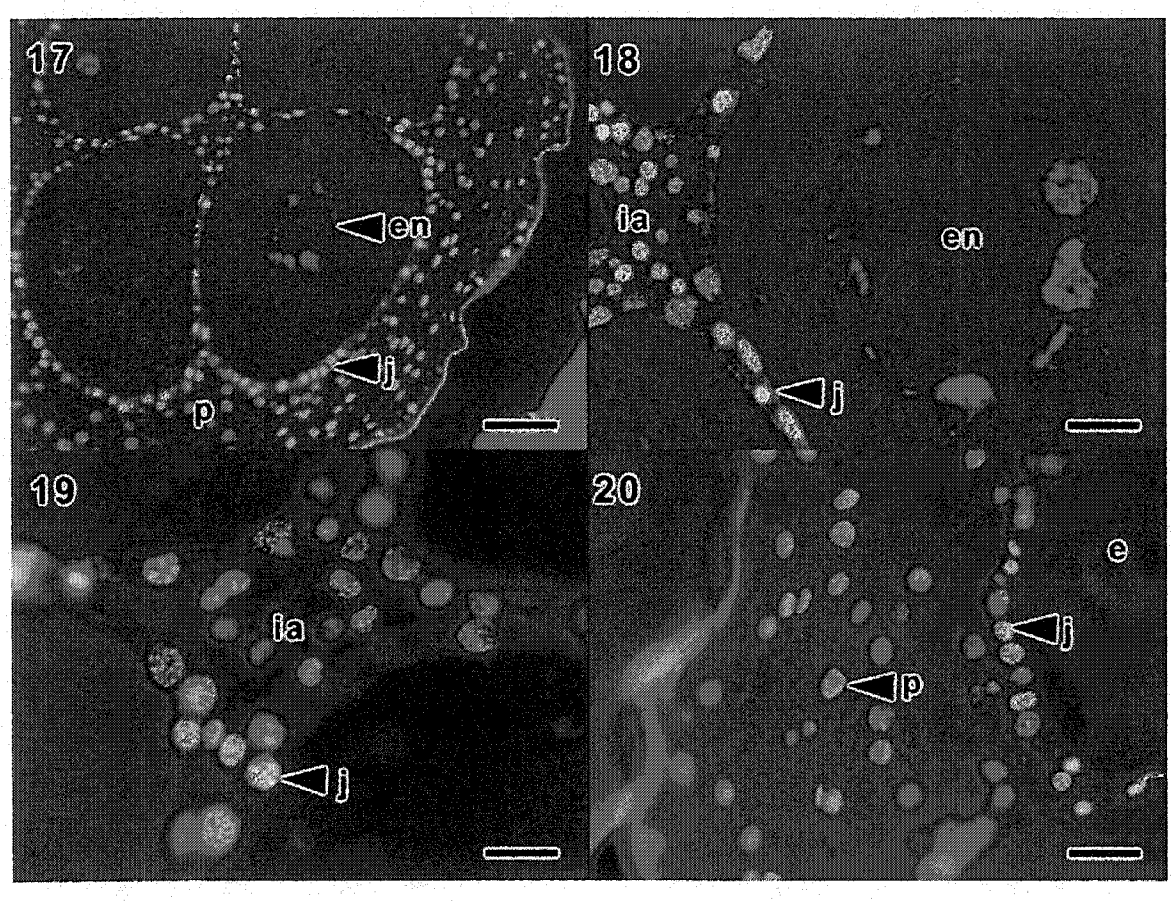
Figs. 17-20. DAPI stained cross sections of a Douglas-fir megagametophyte. Egg (e); egg nucleus (en); inter-archegonial prothallial cells (ia); jacket cells (j); prothallial cells (p).

Fig. 17. Section of a megagametophyte apex and four egg cells. A tip of an egg nucleus (en) is shown. DNA is restricted to the outer periphery of the nucleus. Scale bar = 130 μm.

Fig. 18. A median section of the egg nucleus (en) depicted in Fig.3. The DNA is restricted to the periphery of the egg nucleus (en). Jacket cells (j) and inter-archegonial prothallial cells (ia) adjacent to the egg cell have condensed regions of DNA in the periphery of the nuclei. Scale bar = 45 μm.

Fig. 19. Detail of jacket cells (j) and inter-archegonial prothallial cells (ia) showing condensed regions of DNA associated with the periphery of the nuclei. Scale bar = 35 μm.

Fig. 20. Detail of prothallial cells (p) adjacent to jacket cells (j). DNA is distributed evenly throughout the nuclei of prothallial cells (p). Scale bar = 45 μm.


17
en
j
p
18
ia
en
j
19
ia
j
20
e
j
p

nucellus stained more intensely with DAPI than did any cells of the megagametophyte (Fig. 16). Nucellar cells are sporophytic in origin and subsequently diploid in their chromosomal compliment, while all cells of the megagametophyte are derived from a single haploid megaspore (Singh 1978). The difference in intensity of staining between these cell types is due to relative DNA content. However, haploid neck cells in the megagametophyte also stained intensely (Fig. 16). Binucleate neck cells have been observed in Douglas-fir and larch megagametophytes kept in culture (Ma et al. 1998). It is possible that the intense staining seen in the neck cells of this megagametophyte is due to duplication of the neck cell chromosomes before a nuclear division. Alternatively, neck cells may simply be polyploid.

Jacket cell and inter-archegonial prothallial cell nuclei also showed an uneven distribution of DNA. Chromatin was condensed in discrete regions of these nuclei (Figs.18, 19 and 20). DNA in the jacket cells and inter-archegonial prothallial cells never appeared in an even strip of DNA around the periphery of the nucleus. Jacket cells are known to actively divide to accommodate the expansion of the maturing egg cell that they surround (Singh 1978). Therefore, the condensed chromatin in these cells may indicate that they are in an active cell cycle. In contrast, prothallial cells in the rest of the megagametophyte showed an even distribution of DNA in their nuclei (Fig. 20).

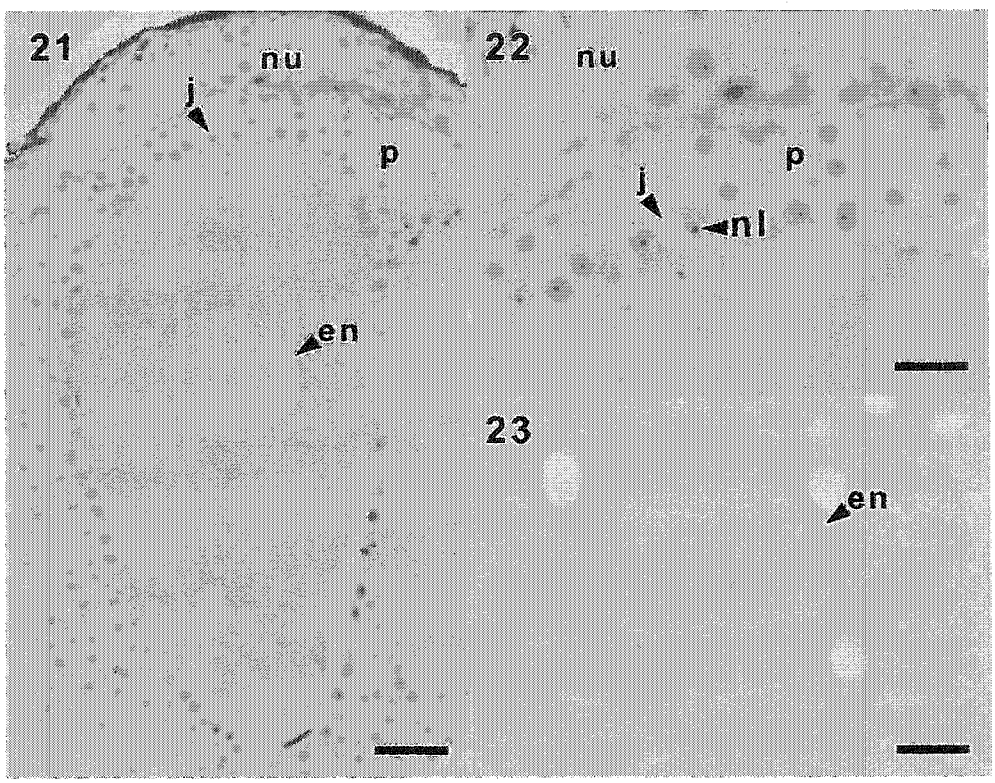
Sections stained by the Ninhydrin-Schiff reaction showed that protein is found in all cells of the megagametophyte and nucellus (Fig. 21). The cytoplasmically dense cells of the nucellus had more protein staining than did the more vacuolate cells of the megagametophyte. Concentrations of protein, likely due to the presence of ribosomes, were associated with the nucleoli of jacket and prothallial cells of the megagametophyte

Figs. 21-23. Longitudinal sections of a Dunkeld larch megagametophyte stained for protein using the Ninhydrin-Schiff reaction. Egg nucleus (en); jacket cells (j); nucleolus (nl); nucellus (nu); prothallial cells (p); transition between the cells of the nucellus and cells of the megagametophyte (t).

Fig. 21. Protein-staining the megagametophyte and nucellus. Scale bar = 90 μm.

Fig. 22. Detail of prothallial cells (p) showing staining for ribosomal protein the nucleolus (nl). Scale bar = 50 μm.

Fig. 23. Detail of egg nucleus (en) showing protein staining along the periphery of the nucleus. Scale bar = 50 μm.


21
nu
j
p
en
22
nu
p
j
nl
23
en

(Fig. 22). The periphery of the egg nucleus also stained intensely in these sections (Fig. 23). Since DNA is concentrated in this region of the egg nucleus, it is possible that this darkly staining region is due to the presence of histones. The use of histone immunolocalization would confirm this hypothesis. In addition, some darkly staining spots appeared within the egg nuclei (Fig. 23). These may be associated with the nucleoli that are known to occur within the egg nucleus (Camefort 1959; 1964).

Cell volume, nuclear volume and DNA content

The average volume of larch egg nuclei was calculated as $3.9 \times 10^6 \pm 1.9 \times 10^6 \mu m^3$. The estimated volume of larch egg cells was $3.5 \times 10^7 \pm 1.3 \times 10^7 \mu m^3$. These data for egg and egg nuclear volume were plotted with values of cell volume and nuclear volume previously published for 14 angiosperms (Price et al. 1973). The nuclear volume to cell volume ratio for larch egg cells is the same as that found for all higher plants investigated (Fig. 24). In addition, Price et al. (1973) reported that this ratio between cell volume and nuclear volume was also observed for pteridophytes and a gymnosperm, suggesting that the observed relationship holds for more than one group within the plant kingdom. Therefore, although the egg cells and egg nuclei of Dunkeld larch are unusually large, their relationship in terms of size is consistent with that found for other plants.

The DNA content of larch megagametophyte cells was reported to be 12.3 pg (Pattanavibool et al. 1995). Values for larch egg nuclear volume and DNA content were plotted with nuclear volume and DNA content values that were previously published for shoot cells of 28 angiosperms (Baetcke et al. 1967). This showed that the relationship between DNA content and nuclear volume found in angiosperms does not apply to larch egg cells (Fig. 25). The average volume of prothallial cell nuclei was

Fig. 24. Relationship between the nuclear volume and cell volume for apical meristem cells of 14 angiosperms and Dunkeld larch egg cells. Angiosperms cells, (▼); Dunkeld larch egg cells, (■). Adapted from Price et al. (1973).

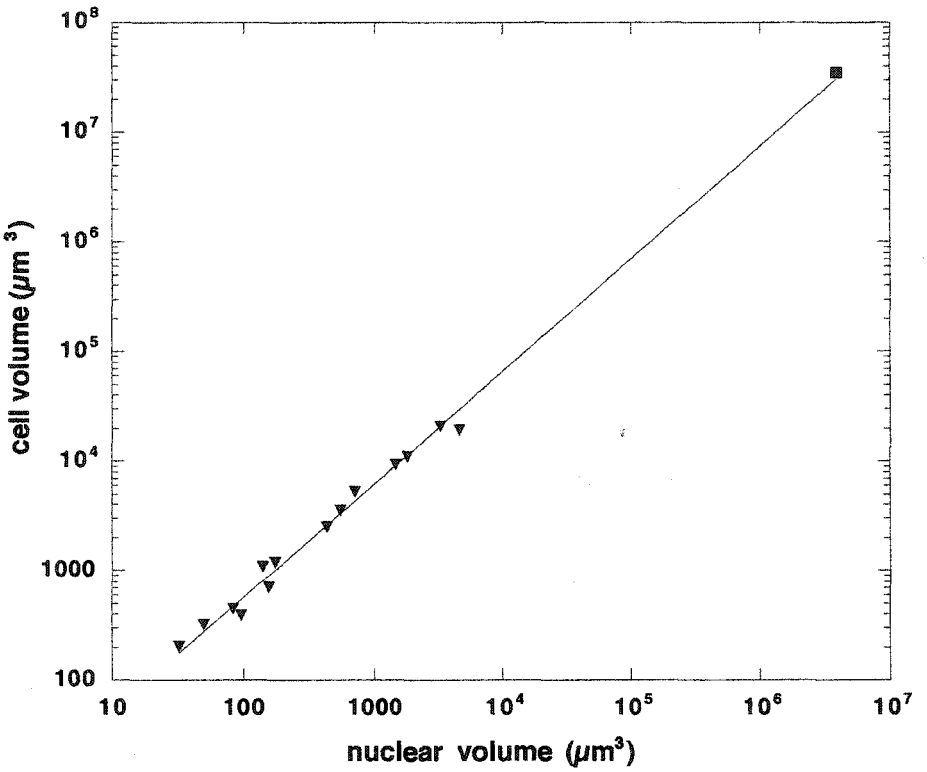
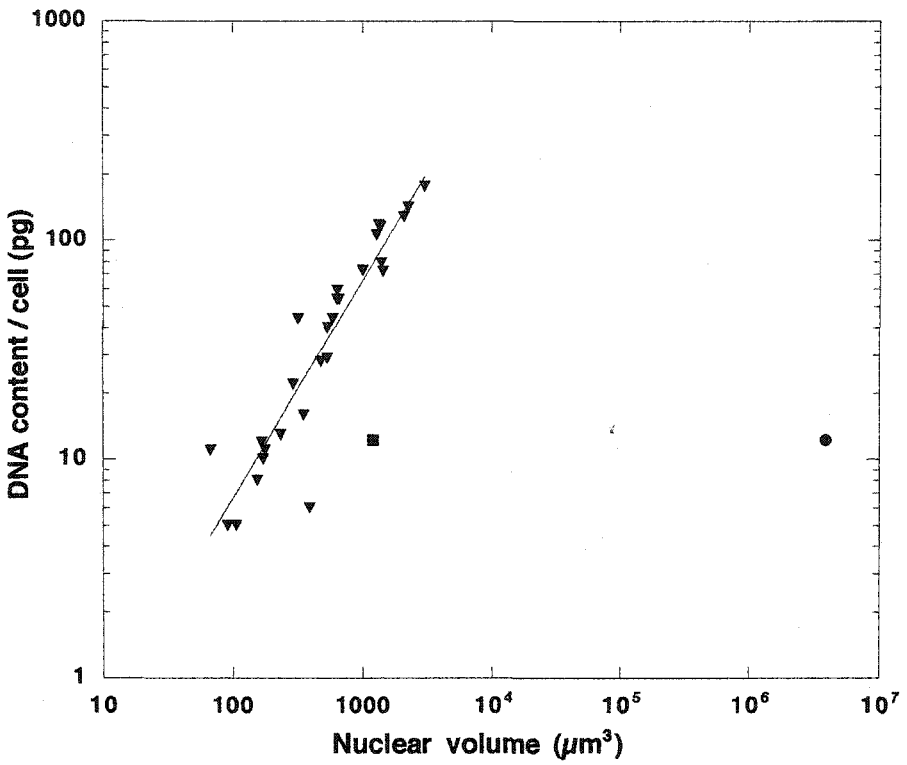

cell volume (μm 3)
nuclear volume (μm3)
10^8
10^7
10^6
10^5
10^4
1000
100
10
100
1000
10^4
10^5
10^6
10^7

Fig. 25. Relationship between the nuclear volume and DNA content for shoot cells of 28 angiosperms as well as Dunkeld larch prothallial and egg cells. Angiosperms cells, (▼); Dunkeld larch prothallial cells, (■); Dunkeld larch egg cells (●). Adapted from Baetcke et al. (1967).


1000
100
10
1
DNA content / cell (pg)
10
100
1000
10^4
10^5
10^6
10^7
Nuclear volume (μm^3)

$1200 \times 10^3 \pm 975\ \mu m^3$, which is 1000 times smaller than the volume determined for egg nuclei. When the average volume of prothallial cell nuclei was plotted in relation to its DNA content, the ratio of DNA content to nuclear volume was more consistent with that found for angiosperms (Fig. 25).

All gymnosperms are known to have egg cells and nuclei that are among the largest known (Singh 1978). It is likely that the ratio between egg cell volume and nuclear volume will be consistent with that which has been found for Dunkeld larch egg cells and angiosperms cells. It is also likely that all gymnosperms will have an unequal distribution of DNA in their egg nuclei. Dhillon (1987) reported that root cells from several genera of the Pinaceae have nuclei that range from 12.6 to 38.8 pg of DNA per nucleus. Therefore, haploid cells (including egg cells) in the Pinaceae would be expected to have between 6.3 and 19.4 pg of DNA per cell. As a result, the relationship between nuclear volume and DNA content that is found in angiosperms can never apply to gymnosperm eggs.

The large size of egg cells, egg nuclei and the distribution of DNA in these organelles may be of functional significance in gymnosperm reproduction. For example, large egg cells may increase the likelihood that pollen tubes, which penetrate the nucellus and megagametophyte to deliver sperm to eggs, actually find their targets. Large egg nuclei may provide a larger target for the sperm nuclei to optimize fertilization. Alternatively, large nuclei in conifer egg cells may simply be the result of the strong developmental relationship between cell volume and nuclear volume. This may also explain why a conifer sperm nucleus expands in volume as it descends in the egg cytoplasm until it fuses with the egg nucleus. However, other nuclei that do no take part

in fertilization are also released into egg cells by pollen tubes. These nuclei do not undergo a dramatic change in size. Therefore it is more likely that enlarged reproductive nuclei in gymnosperms are necessary to optimize efforts in reproduction.

Conclusions

During egg cell maturation in Douglas-fir and larch, the DNA becomes evenly confined to the periphery of the egg nucleus. This distribution of nuclear DNA has not been reported for any other eukaryotic cell. Conifer egg nuclei are approximately 1000 times larger in volume than the nuclei of neighbouring prothallial cells of the megagametophyte; however, the relationship between egg cell volume and egg nuclear volume is consistent with that found for other land plants. In contrast, the relationship between nuclear volume and DNA content in conifer egg nuclei is different from that which has been found for other land plants. Egg cell volume, egg nuclear volume and DNA distribution may be of functional significance in gymnosperm reproductive systems.

Chapter 5

Prezygotic breeding barriers in Dunkeld larch and Douglas-fir

Introduction

Conifer hybridization has been a useful breeding tool for producing improved trees with unique characteristics of wood quality or disease resistance, and therefore this practice has had great commercial value. Interspecific hybrids often display hybrid vigour, or heterosis, which may be defined as any quality of a hybrid tree (including growth rate, cold hardiness or pest resistance) that exceeds that of both of the parent species (Zobel and Talbert 1984). At times, situations arise when desired hybridizations fail. White pines in North America have been used in exploratory hybridization with the aim of acquiring resistance to white pine blister rust (*Cronartium ribicola*) (Critchfield 1986). This research has shown that sugar pine (*Pinus lambertiana*) will not successfully hybridize with any of the other North American white pines; however, it does hybridize with two pines native to eastern Asia (*P. armandii* and *P. koraiensis*) which are resistant to white pine blister rusts. No functional basis for the inability of *P. lambertiana* to hybridize with other North American white pines has been found.

Breeding barriers in conifers are generally assumed to be post-zygotic (Willson and Burley 1983). Consequently, the crossability of different conifer species is described in terms of viable seed yield (Critchfield 1986). This assumption does not take into account the many events that occur from the time pollen lands on a micropyle until it delivers a male gamete to fertilize an egg in a conifer ovule. Prior to fertilization, a pollen grain first must enter the micropyle of an ovule and then germinate to form a pollen tube. Subsequently, the pollen tube grows through cells of the nucellus. Finally, it

must penetrate cells of the megagametophyte until it reaches an egg cell into which it releases male gametes.

Among conifers, there are many different pollination mechanisms that deliver pollen into the micropyles of ovules. Pollen may enter micropyles by a pollination drop as is the case in the Cupressaceae, Taxodiaceae, Taxaceae, Cephalotaxaceae, Podocarpaceae, and in some Pinaceae including *Pinus*, *Picea*, *Cedrus* and some species of *Tsuga* (Owens et al. 1998). In *Abies*, pollen grains are thought to be washed into the micropyle by rainwater where they then come in contact with a pollination drop that is not exuded past the tip of the micropyle (Owens et al. 1998). In *Pseudotsuga* (Doyle 1945; Takaso et al. 1996; von Aderkas and Leary 1999a) and *Larix* (Doyle 1945; Takaso and Owens 1997; Villar et al. 1984; von Aderkas and Leary 1999b), pollen grains land on stigmatic hairs that bend into the micropyle. The micropyle is later flooded with an ovular secretion known as a post-pollination pre-fertilization droplet. In *Larix*, the droplet washes the pollen grains from the stigmatic hairs and draws them onto the nucellus (Takaso and Owens 1997). In *Pseudotsuga*, the droplets trigger the pollen grains to elongate through the micropyle until they reach the surface of the nucellus (Takaso and Owens 1994). The only conifers in which pollen does not come in contact with an ovular secretion include species within the Araucariaceae (Haines et al. 1984; Owens et al. 1995) and some species of *Tsuga* (Doyle and O'Leary 1935c; Doyle and Kane 1943). In these conifers, pollen grains germinate on ovuliferous scales and produce long pollen tubes that grow towards the nucellus (which exudes past the tip of the micropyle).

Ovular secretions are involved in reproduction in most conifers, as well as in other gymnosperms including ginkgo and cycads (Singh 1978). Evidence for pollination droplets has also been found in fossils of a Paleozoic pteridosperm, *Callospermarion pusillum* (Rothwell 1977). Therefore, ovular secretions must be functionally significant in gymnospermous plant reproduction; however, very few studies have been done to determine their role in reproduction apart from their physical role in moving pollen grains in ovules. Ovular secretions are known to contain sugars, malic acid, and amino acids (Chesnoy 1993; Fujii 1903; Tison 1911). Ziegler (1959) found that these droplets contained polypeptides. Tison (1911) hypothesized that malic acid in pollination drops may have a role in the chemotactic guidance of pollen tube, though no studies have demonstrated this. The chemical composition of ovular secretions is characteristic for each species (Chesnoy 1993). It is an uninvestigated possibility that the secretion may have a role in selecting against foreign pollen.

Some studies have investigated the role of the nucellus as a barrier to foreign pollen. Studies by McWilliam (1959), Mikkola (1969), and Hagman (1975) have all demonstrated that heterospecific pollen tubes can have a reduced capacity to grow through cells of the nucellus in conifer ovules. This suggests that some prezygotic barrier may exist in conifer reproduction, thought these studies did not conclusively demonstrate this with appropriate controls and statistical analysis.

The purpose of this study was to determine whether conifers have prezygotic breeding barriers to foreign pollen. Female cones of Douglas-fir (*Pseudotsuga menziesii* Dougl.) and Dunkeld larch (*Larix decidua* Miller x *Larix kaempferi* (Lamb.) Carrière = *Larix* x *marschlinsii* Coaz) were pollinated with either heterospecific or homospecific

pollens. We compared the germination rates of foreign and like pollen in situ. We also determined whether foreign pollen was able to penetrate cells of the nucellus. These experiments allowed us to test two hypotheses. The first null hypothesis of this study stated that pollen germination in a micropyle is independent of the type of pollen that is present in a given micropyle. The second null hypothesis stated that pollen germination in a micropyle is independent of whether the pollen that is in a given micropyle is homospecific or heterospecific in comparison to the surrounding female tissue.

Materials and Methods

Female cones of Dunkeld larch and Douglas-fir were pollinated with Dunkeld larch, Douglas-fir, western white pine (*Pinus monticola* Dougl.) or interior spruce (*Picea glauca* [Moench] Voss x *P. engelmannii* Parry) pollen. These female cones were collected at time of fertilization, ovule micropyles were dissected, and survivability as well as germination frequencies of pollens were assessed. In addition, the nucellus and megagametophyte together with any attached pollen were dissected from ovules, fixed and sectioned.

Pollen sources

The pollen used in these pollination experiments was collected during the 1999 conifer reproductive cycles. Pollen cones were monitored prior to anthesis. Microspores were removed and stained with 0.5 % aceto-carmine in 45 % glacial acetic acid and inspected by phase microscopy. Male cones were collected when they contained mature, five-celled pollen. Dunkeld larch pollen cones were collected from the University of Victoria campus between March 11 and 16, 1999. Douglas-fir pollen cones were collect from the University of Victoria campus as well as from the Ministry of Forests Research Station at Glyn Rd. between April 15 and 20, 1999. Interior spruce pollen cones were collected from the Glyn Rd. Research Station between April 27 and 30, 1999. Western white pine pollen cones were also collected from the Glyn Rd. Research Station on June 10, 1999.

After collection, pollen cones were surface-sterilized with one 30 s wash in 70 % ethanol followed by one 30 s wash in 1 % sodium hypochlorite. Subsequently, cones were rinsed in three 30 s washes in sterile de-ionized water. Pollen cones were then placed at the bottom of sterile Petri dishes lined with sterile 90 mm Whatman No. 1 filter paper. The top of the Petri dish was covered with a sterile 18.5 cm Whatman No. 1 filter

paper that was secured with a rubber band. The pollen cones were then allowed to dry at room temperature for 4 to 5 d until they had released their pollen. The dried pollen was then stored at 4 °C in sterile vials placed over silica beads until it was required. Just prior to use for pollination, each pollen type was sprinkled on 1/2 strength Litvay's medium to assess its viability. Western white pine and interior spruce pollen were considered viable if more than half of the pollen grains produced pollen tubes within three days of having been sown on the medium. Given that it is very difficult to get Dunkeld larch and Douglas-fir pollen to produce pollen tubes in vitro, these pollens were considered viable if more than half of the pollen grains elongated within three days of being sown on the medium. Only viable pollen was used for pollinating female cones of larch and Douglas-fir.

Female cones

Female buds on branches of Dunkeld larch and Douglas-fir were covered with bags to prevent pollination prior to bud break. Any male cones on the branches next to the female buds were removed. For larch trees located on the University of Victoria campus, female buds were bagged on March 7 and 8, 2000. On April 18, 2000, female buds of Douglas-fir were bagged on trees located in the Ministry of Forests Saanich Seed Orchard at Puckle Road.

Once the female buds of Dunkeld larch or Douglas-fir were receptive to pollen, the pollination bags were removed, and Dunkeld larch, Douglas-fir, interior spruce, or western white pine pollen was applied to the female cones. To avoid cross contamination of pollen types, only one bag was removed at a time to pollinate the female cones. Separate paintbrushes (No. 1 H.J. series 550 Sabeline) were used to apply each pollen

type. After pollination, the bags were replaced. When the female cones were no longer receptive to pollen, pollination bags were removed for the duration of the experiments. For Dunkeld larch, approximately 40 female cones were pollinated for each cross using 3 different trees on March 9, 2000. For Douglas-fir, about 20 female cones were pollinated for each cross using 8 trees on April 20 and 23, 2000. In addition, 25 female cones from 3 Douglas-fir trees on the University of Victoria campus were hand-pollinated with white pine pollen on April 19, 2000.

Female cone dissections

When ovular secretion in Dunkeld larch and Douglas-fir appeared female cone collection and ovule dissection was started. A total of 1,984 Dunkeld larch ovules were dissected between May 4 and June 1, 2000, and 870 Douglas-fir ovules were dissected between June 6 and June 23, 2000. The micropyles of these ovules were inspected for the presence of pollen using a Wild Heerbrugg dissecting microscope. The number of pollen grains as well as the number of those that were elongated or germinated was recorded. Pollen grains were then removed from the micropyles using insect pins, placed on glass slides in a drop of de-ionized water, and viewed with a Leitz Laborlux S light microscope to ascertain the germination status of the pollen grains. Pollen grains that had pollen tubes that were two times longer than the width of the pollen grain counted as germinated. In addition, any pollen grains that had pollen tubes growing into the surface of the nucellus were counted as germinated. The nucellus and megagametophyte of these specimens were removed from their ovule and fixed in 2.5 % glutaraldehyde in 0.05 M phosphate buffer (pH = 7.2), and left in fixative at room temperature until further processing. In order to observe later stages of pollen tube development, ovules of

Dunkeld larch were dissected on June 9, 2000 and ovules of Douglas-fir were dissected on July 14, 2000. When pollen tubes were found penetrating the nucellus, the nucellus and megagametophyte were dissected from the ovule and fixed. No pollen counts were recorded on these days.

Statistical analyses

Counts of germinated and ungerminated pollen were obtained from the dissection of ovules that had been pollinated with either homo- or heterospecific pollens. In order to test the null hypotheses, chi-squared tests for independence were performed on the data. Although 1,984 larch ovules and 870 Douglas-fir ovules were dissected, not all of these contained pollen. Furthermore, the number of pollen grains in a given micropyle was variable. Therefore, pollen counts that were used in the statistical analyses were different for each of the cross-pollinations. At least 57 pollen grains were counted for a given larch cross, and for Douglas-fir crosses, a minimum of 76 pollen grains were counted.

Embedding and sectioning

Fixed specimens were washed overnight in 0.075 M phosphate buffer (pH = 7.2) followed by two more washes of 2 h each. Specimens were then dehydrated in a graded ethanol series up to 100 % ethanol. Specimens were left in progressively more concentrated washes of ethanol for at least 1 h each. Subsequently, specimens were placed in a pre-infiltration solution of 1:1 Technovit 7100 base and 100 % ethanol overnight. The next day, specimens were placed in an infiltration solution of 1 % (w/v) Technovit Hardener I in liquid base for 12 d. During this time, the specimens were kept at 4 °C and the infiltration solution was replaced with freshly prepared solution at least three times. Specimens were then transferred to BEEM molds with a 1:15 ratio of

Hardener II to infiltration solution. A plastic sheet was laid over the BEEM molds to prevent contact with the air until curing was complete.

Sections (8 μm thick) were cut using a Leica SM2400 sledge microtome. Between 5 and 15 samples were sectioned for each cross with the exception of Douglas-fir crossed with interior spruce. In this cross, only one specimen was available to be fixed and sectioned. Sections were stained using Toluidine blue O with NaOCl pretreatment according Gutmann (1995). Sections were viewed using either a Leitz Laborlux S or a Zeiss Axioplan microscope.

Results and discussion

Ovule dissections and pollen development

When ovules of larch and Douglas-fir were dissected, pollen grains in the micropyles were found to be elongated, germinated or ungerminated (Figs. 26-29). Compared to dry pollen grains (Figs. 30-33), all pollen grains in micropyles were hydrated and swollen. Elongated Douglas-fir pollen grains (Fig. 26) are elliptical, and elongated larch pollen grains (Fig. 27) are either spherical or slightly elliptical. Germinated pollen grains had pollen tubes that were more than two times the diameter of the pollen grain in length. Only western white pine pollen grains were observed to germinate in micropyles (Fig. 28). When larch and Douglas-fir pollen germinated, the pollen tubes only grew into nucellar cells. Pollen tubes of these species did not grow in micropyles. Interior spruce pollen was not observed to germinate in micropyles, though occasionally short pollen tubes were observed (Fig. 29).

In the control crosses for Dunkeld larch and Douglas-fir the number of pollen grains that elongate and then germinate in micropyles increases with time (Figs. 34 and 35). Percent elongation and germination of heterospecific pollens are higher in the second half of the dissection period for both larch (Tables 3 and 4) and Douglas-fir (Tables 5 and 6). In larch (Barner and Christiansen 1960; Takaso and Owens 1997; Villar et al. 1984; von Aderkas and Leary 1999b) and Douglas-fir (Takaso and Owens 1994; Takaso et al. 1996; von Aderkas and Leary 1999a), post-pollination pre-fertilization droplet production does not begin until five or seven weeks after pollen enters the micropyle by the collapse of stigmatic flaps. This ovular secretion has been described as the trigger that ends pollen dormancy (Villar et al. 1984) and a trigger to

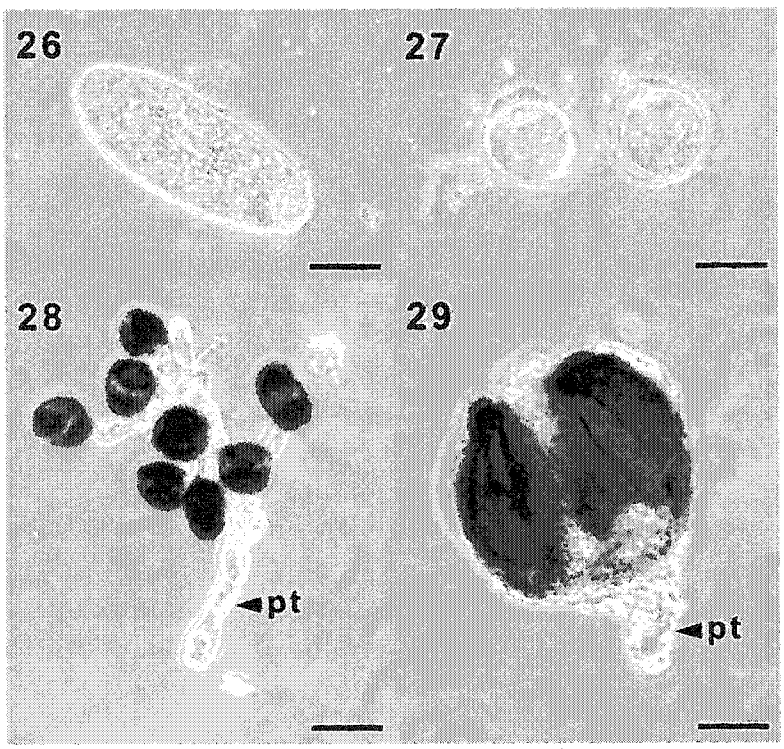
Figs. 26-29. Elongated and germinated pollen grains dissected from Douglas-fir micropyles. Pollen tubes (pt).

Fig. 26. An elongated pollen grain of Douglas-fir. Scale bar = 100 μm.

Fig. 27. Elongated pollen grains of Dunkeld larch. Scale bar = 100 μm.

Fig. 28. Germinated pollen grains of Western white pine. Scale bar = 65 μm.

Fig. 29. Interior spruce pollen grain with a short pollen tube (pt). Scale bar = 25 μm.


26
27
28
29
pt
pt

Figs. 30-33. Dried pollen. Sacci on pollen grains (s).

Fig. 30. Douglas-fir pollen. Scale bar = 165 μm.

Fig. 31. Dunkeld larch pollen. Scale bar = 165 μm.

Fig. 32. White pine pollen. Scale bar = 165 μm.

Fig. 33. Interior spruce pollen. Scale bar = 165 μm.

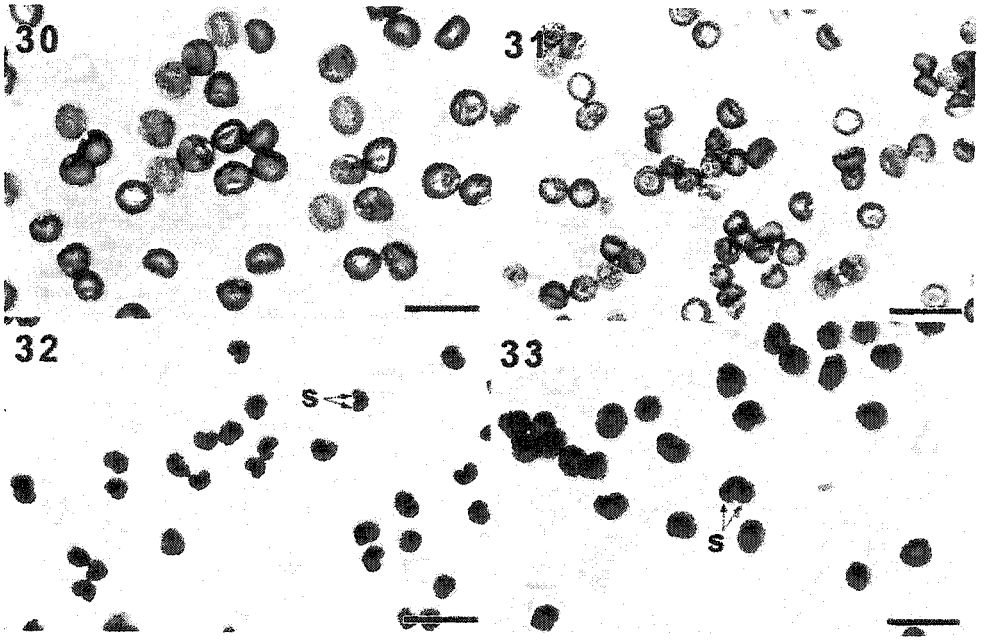

30
31
32
33
s
s

Fig. 34. Pollen elongation and germination over time for control crosses in Dunkeld larch. Plotted points represent average counts for each day that larch cones crossed with larch pollen were dissected. Number of micropyles dissected that contained pollen grains (■), number of pollen grains in micropyles (●), number of elongated pollen grains in micropyles (○), number of germinated pollen grains in micropyles (▼).

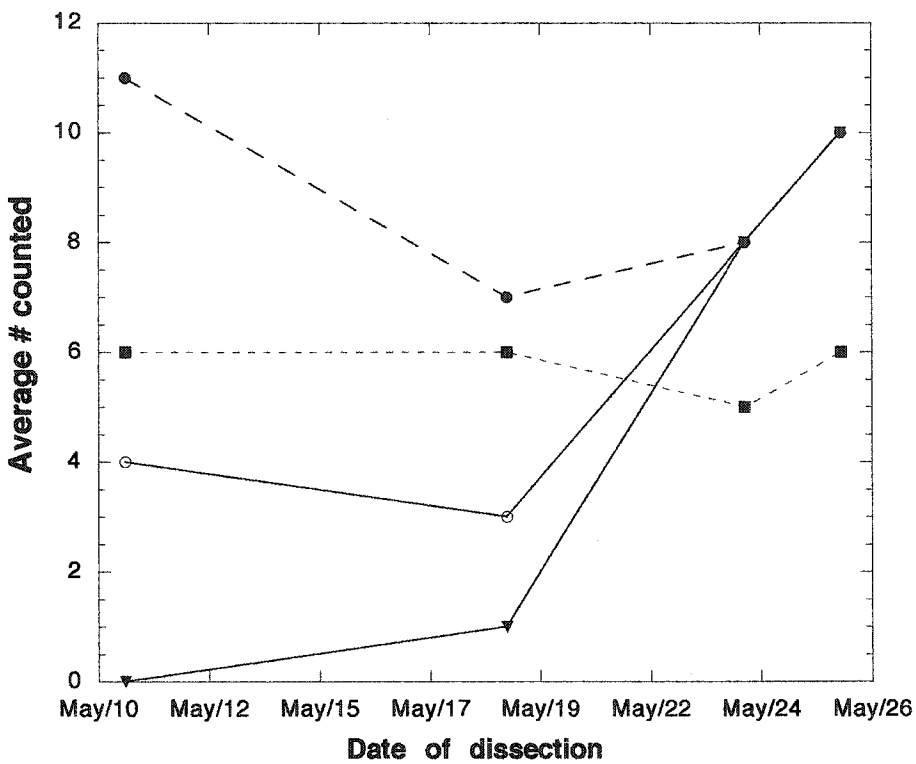

12
10
8
6
4
2
0
Average # counted
May/10
May/12
May/15
May/17
May/19
May/22
May/24
May/26
Date of dissection

Fig. 35. Pollen elongation and germination over time for control crosses in Douglas-fir. Plotted points represent average counts for each day that Douglas-fir cones crossed with Douglas-fir pollen were dissected. Number of micropyles dissected that contained pollen grains (■), number of pollen grains in micropyles (●), number of elongated pollen grains in micropyles (○), number of germinated pollen grains in micropyles (▼).

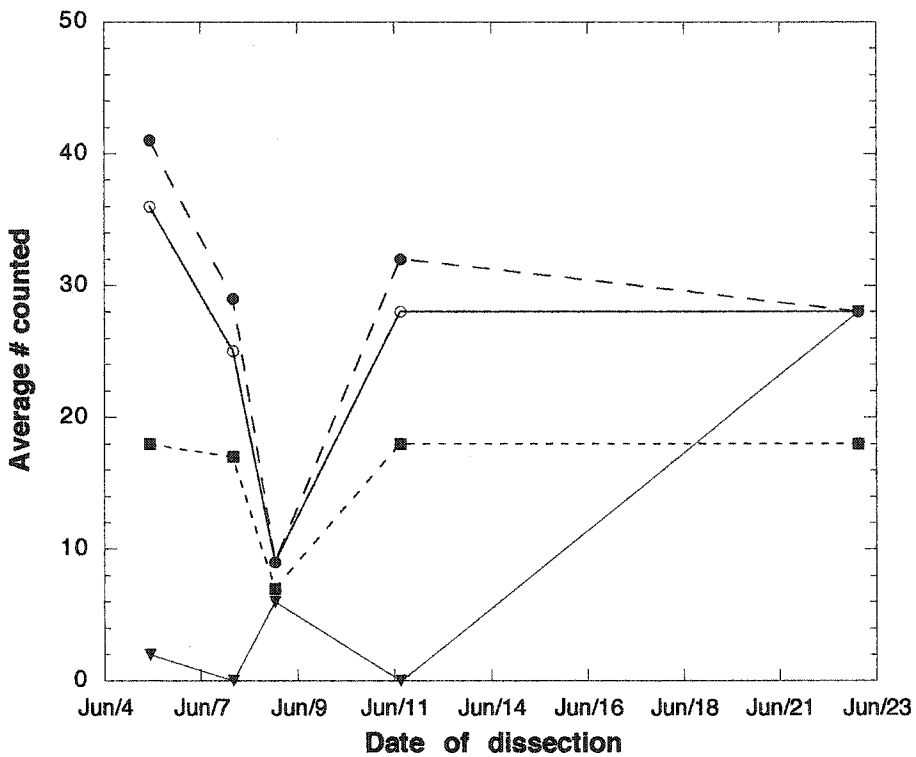

50
40
30
20
10
0
Average # counted
Jun/4
Jun/7
Jun/9
Jun/11
Jun/14
Jun/16
Jun/18
Jun/21
Jun/23
Date of dissection

Table 3. Ovule dissection and pollen count data for larch cones collected between May 4 and May 17, 2000 (which corresponded to the first half of the period of post-pollination prefertilization droplet production). Larch (Lx), Douglas-fir (Df), western white pine (Wp), interior spruce (Sp).

Cross	# ovules dissected	# micropyles containing pollen	# pollen grains counted	# pollen elongated	# pollen germinated	% elongation	% germination
Lx x Lx	242	18	32	13	0	41%	0%
Lx x Df	306	47	76	59	0	78%	0%
Lx x Wp	344	85	567	N/A	65	N/A	11%
Lx x Sp	186	17	42	N/A	0	N/A	0%

Table 4. Ovule dissection and pollen count data for larch cones collected between May 19 and June 1, 2000 (which corresponded to the second half of the period of post-pollination prefertilization droplet production). Larch (Lx), Douglas-fir (Df), western white pine (Wp), interior spruce (Sp).

Cross	# ovules dissected	# micropyles containing pollen	# pollen grains counted	# pollen elongated	# pollen germinated	% elongation	% germination
Lx x Lx	190	17	25	21	19	84%	76%
Lx x Df	232	40	48	42	25	88%	52%
Lx x Wp	324	99	482	N/A	263	N/A	55%
Lx x Sp	160	18	29	N/A	0	N/A	0%

Table 5. Ovule dissection and pollen count data for Douglas fir cones collected between June 6 and June 12, 2000 (which corresponded to the first half of the period of post-pollination prefertilization droplet production). Larch (Lx), Douglas-fir (Df), western white pine (Wp), interior spruce (Sp).

Cross	# ovules dissected	# micropyles containing pollen	# pollen grains counted	# pollen elongated	# pollen germinated	% elongation	% germination
Df x Df	190	60	111	98	8	88%	7%
Df x Lx	30	20	39	38	0	97%	0%
Df x Wp	122	60	226	N/A	102	N/A	45%
Df x Sp	92	30	76	N/A	0	N/A	0%

Table 6. Ovule dissection and pollen count data for Douglas fir cones collected between June 16 and June 23, 2000 (which corresponded to the second half of the period of post-pollination prefertilization droplet production). Larch (Lx), Douglas-fir (Df), western white pine (Wp), interior spruce (Sp).

Cross	# ovules dissected	# micropyles containing pollen	# pollen grains counted	# pollen elongated	# pollen germinated	% elongation	% germination
Df x Df	138	55	85	85	85	100	100
Df x Lx	86	23	37	29	12	78	32
Df x Wp	132	8	12	N/A	11	N/A	92
Df x Sp	80	29	80	N/A	0	N/A	0

pollen germination (Takaso and Owens 1994). This study confirms that pollen elongation and germination do coincide with the production of post-pollination pre-fertilization droplets; however, these processes of pollen development, in response to ovular secretions, appears to be gradual rather than immediate.

Western white pine pollen begins to germinate at the onset of post-pollination pre-fertilization droplet secretion in ovules of both larch and Douglas-fir (Tables 3 and 5). In addition, western white pollen had the highest percentage of germinated pollen during the first half of the dissection period in larch ovules, and the second highest percent germination in Douglas-fir ovulesin the second half of the dissection period. Interior spruce pollen, though it occasionally produced very short pollen tubes, did not successfully germinate in ovules of larch or Douglas-fir. Normally, pollen grains of western white pine (Owens and Molder 1977a) and interior spruce (Runions and Owens 1999) are hydrated in pollination drops and then germinate in the ovules of their own species within about two weeks after pollination. Female cones of larch were dissected 8 weeks after pollination, and Douglas-fir cones were dissected 7 weeks after pollination after ovular secretions had flooded the micropyle and pine and spruce pollen grains had been hydrated. Since dissections were continued over a period of four weeks, there had been sufficient time for pine and spruce pollen germination.

In the latter half of the dissection period, the percentage of homospecific pollen grains that germinated in larch ovules exceeded the percent germination of any heterospecific pollens (Table 4). The same was true for Douglas-fir ovules (Table 6). In larch and Douglas-fir ovules, it was also observed that western white pine pollen had the highest rate of germination in the first half of the dissection period. However, in the

latter half of the dissection periods for larch and Douglas-fir, the rate of homospecific pollen germination exceeded western white pine germination rates as well as the germination rates of any other heterospecific pollen type.

Statistical analysis

A chi-squared analysis was used to test the null hypothesis: pollen germination in a micropyle is independent of the type of pollen that is present in a given micropyle. For this analysis, the null hypothesis was rejected. Therefore, during the whole dissection period, the probability that a pollen grain would germinate in a given micropyle was dependent on the type of ovule in which it was located (Table 7). In the first half of the Douglas-fir dissection period, the percent germination of Douglas-fir pollen was significantly greater than pollen germination of any other type. In the second half of the dissection period, the percent germination of homospecific pollen was significantly greater than heterospecific pollen germination in ovules of both larch and Douglas-fir. However, this does not imply that homospecific pollen is always more likely to germinate that heterospecific pollen. Percent germination of western white pine pollen in the first half of the larch dissection period was significantly greater than percent germination for any other type of pollen.

A second chi-squared analysis was used to test the null hypothesis: pollen germination in a micropyle is independent of whether the pollen that is in a given micropyle is homo- or heterospecific with respect to the surrounding female tissues. The null hypothesis was not rejected for larch ovules that were collected between May 4 and May 17, 2000 (Table 8). Again, this shows that the germination of western white pine in larch ovules in the first half of the dissection period was significantly greater than

Table 7. X^2 values obtained in tests of the null hypothesis: Ho = Pollen germination in a micropyle is independent of the type of pollen that is present in a given micropyle. X^2 critical = 7.8 (df = 3, p = 0.05).

Ovule type dissected	Collection period	X^2
Dunkeld larch	May 4 – May 17, 2000	18.9
Dunkeld larch	May 19 – June 1, 2000	38.3
Douglas-fir	June 6 – June 12, 2000	107.9
Douglas-fir	June 16 – June 23, 2000	176.7

Table 8. X^2 values obtained in tests of the null hypothesis: Ho = pollen germination in a micropyle is independent of whether the pollen that is in a given micropyle is homo- or heterospecific with respect to the surrounding female tissues. X^2 critical = 3.8 (df = 1, p = 0.05).

Ovule type dissected	Collection period	X^2
Dunkeld larch	May 4 – May 17, 2000	3.3
Dunkeld larch	May 19 – June 1, 2000	5.9
Douglas-fir	June 6 – June 12, 2000	23.4
Douglas-fir	June 16 – June 23, 2000	138.3

germination of any other pollen type. The null hypothesis was rejected for all other dissection periods indicating that the percent germination of homospecific pollen was significantly greater than percent germination for heterospecific pollens.

Microscopic analyses

From sections of megagametophytes with nucellar tissues it can be seen that all pollen of different species show varying ability to penetrate nucellar cells and to fertilize larch egg cells. In control crosses, larch pollen was observed to penetrate nucellar cells, cells of the megagametophyte, and egg cells. Gametes were released into egg cells, zygotes were formed, and free –nuclear proembryos were observed near the chalazal end of egg cells (Fig. 36). Douglas-fir pollen was also able to penetrate nucellar cells of larch, as well as megagametophyte cells and egg cells (Fig. 37). In addition, gamete delivery by Douglas-fir pollen tubes into larch egg cells was also observed (Fig. 38). As expected, no embryos were found in larch egg cells that were penetrated by Douglas-fir pollen tubes. Western white pine pollen tubes were only found to penetrate the first layer of nucellar cells in larch ovules before coming to an abrupt stop (Fig. 39). Interior spruce pollen grains were arrested even earlier as they failed to germinate (Fig. 40).

In Douglas-fir ovules, Douglas-fir pollen grains were observed to penetrate through the nucellus and cells of like megagametophytes, and to deliver gametes to egg cells. Cellular pro-embryos (Fig. 41) were observed in the chalazal end of several eggs. Larch pollen also successfully penetrated the nucellus, megagametophyte and egg cells of Douglas-fir ovules (Fig. 42); however, gamete delivery by larch pollen tubes into Douglas-fir egg cells was not observed. Western white pine pollen was observed to

Figs. 36-44. Longitudinal sections of Dunkeld larch and Douglas-fir megagametophytes and nucelluses from ovules that contained homospecific and heterospecific pollens. Egg cell (e), egg nucleus (en), male gametes (mg), nucellus (nu), proembryo (pe), pollen grain (pg), pollen tube (pt), receptive vacuole (rv).

Fig. 36. Specimen of Dunkeld larch in which homospecific pollen has grown through the nucellus (nu) and into an egg cell (e) that has been fertilized. A free-nuclear proembryo (pe) is seen in the chalazal end of the egg cell (e) (inset). Scale bar = 145 μm.

Fig. 37. Specimen of Dunkeld larch where Douglas-fir pollen has grown through the nucellus (nu) and into an egg cell (e). The inset shows detail of the pollen tube (pt) in the tip of the egg cell (e). Scale bar = 145 μm.

Fig. 38. Specimen of Dunkeld larch where Douglas-fir pollen has grown through the nucellus (nu) and into an egg cell (e). Male nuclei (mn) are seen at the tip of the egg penetrated egg cell (e) (inset). Scale bar = 125 μm.

Fig. 39. Germinated pollen grain (pg) of western white pine on a Dunkeld larch nucellus (nu). Scale bar = 200 μm.

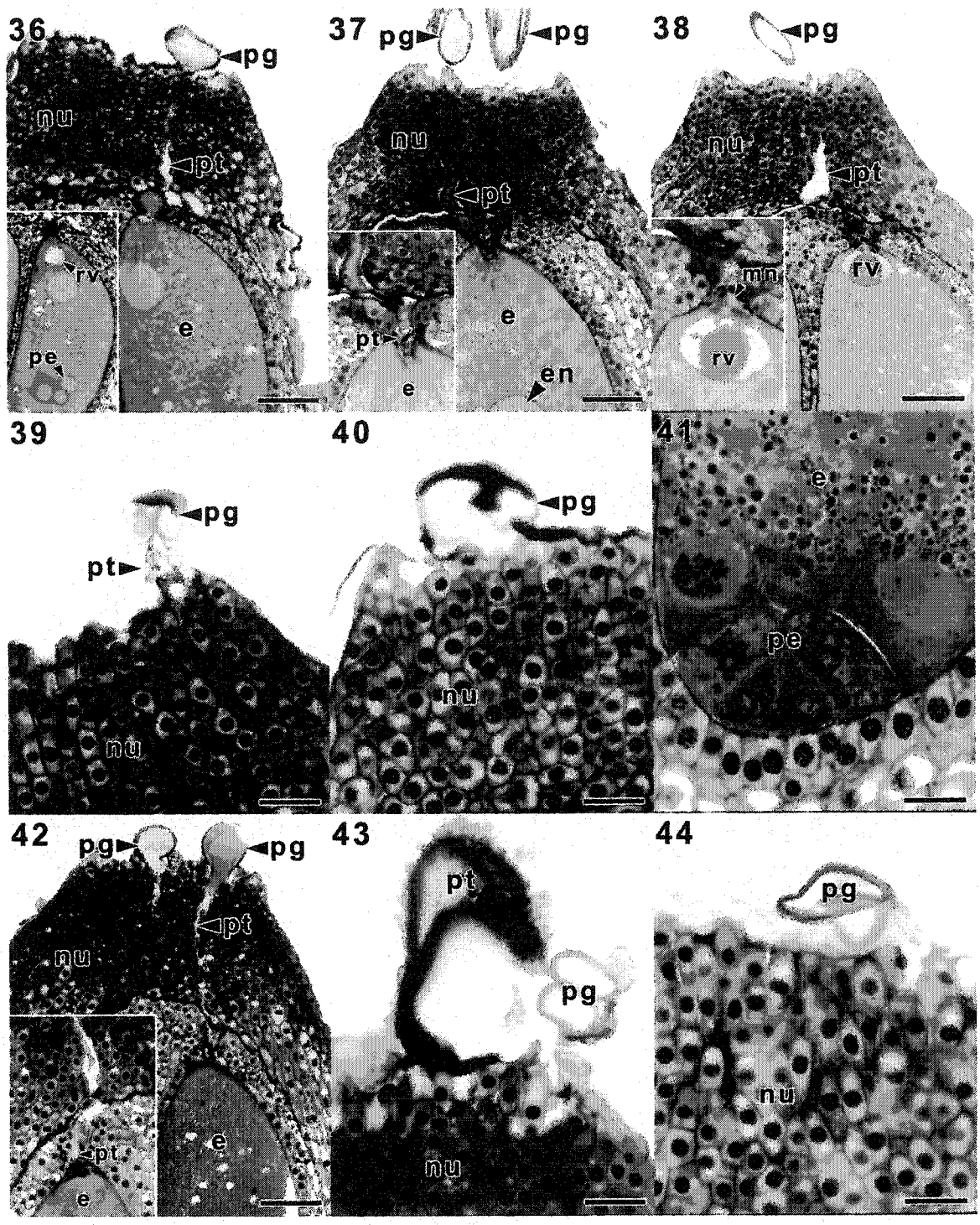
Fig. 40. Interior spruce pollen grain (pg) on the surface of a Dunkeld larch nucellus (nu). Scale bar = 285 μm.

Fig. 41. Cellular proembryo (pe) in the chalazal end of a Douglas-fir egg cell (e) that was fertilized by Douglas-fir pollen. Scale bar = 250 μm.

Fig. 42. Larch pollen tubes (pt) that have penetrated a nucellus (nu) and egg cell (e) (inset) from a Douglas-fir ovule. Scale bar = 145 μm.

Fig. 43. Germinated western white pine pollen on a Douglas-fir nucellus (nu). Scale bar = 250 μm.

Fig. 44. Interior spruce pollen grain (pg) on a Douglas-fir nucellus (nu). Scale bar = 200 μm.


36
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penetrate only the top-most layer of cells of the nucellus (Fig. 43). Spruce pollen did not produce pollen tubes in Douglas-fir ovules (Fig. 44).

Ovular secretions, through the inhibition of foreign pollen, may be the only prezygotic barriers between closely related conifers. Pollen from larch is able to penetrate the nucellus through to egg cells of Douglas-fir. Similarly, pollen from Douglas-fir is able to penetrate towards the egg cells of larch. Only ovular secretions, which reduce the germination rates of foreign pollen grains, provide prezygotic breeding barriers between these species. Species of larch and Douglas-fir belong to sister taxa (Gernandt and Liston 1999; Hart 1987). Since these groups are closely related it is possible that there has been insufficient divergence in aspects of pollen growth through the nucellus to result in a complete prezygotic barrier to hybridization. These conifers do not hybridize in nature owing to differences in chromosomal compliments. In addition, pollination in these species is separated by about six weeks.

The results also suggest that cells of the nucellus in ovules of larch and Douglas-fir may form a complete barrier to foreign pollen from species that are less related to these conifers. Pollen tubes of western white pine were unable to penetrate nucellar cells of either larch or Douglas-fir. This may be due a greater evolutionary separation, or it may be due to the pattern of development that is normally present in pine pollens. Western white pollen pine germinates within 2 weeks of pollination, but then it enters a dormant period that is not broken until the following growing season (Owens and Molder 1977a).

Conclusions

This study demonstrates that there are prezygotic breeding barriers in Dunkeld larch and Douglas-fir. Results from the ovule dissections and statistical analyses demonstrate that some condition of larch and Douglas-fir ovules reduces or inhibits germination of heterospecific pollen grains. It is possible that compounds in the ovular secretions, which flood the micropyles of these conifers, contribute to the observed selection against heterospecific pollen grains. Currently, the origin and exact content of these droplets is unknown. Future studies characterizing the chemical content may reveal the presence of inhibitors. Nucellar cells may impose a second prezygotic barrier to foreign pollen in larch and Douglas-fir ovules. This barrier is less effective against pollen grains that are from conifers that are more closely related.

Chapter 6

Intergeneric pollen – megagametophyte relationships of conifers in vitro

Introduction

In vitro fertilization (IVF) has recently been achieved in a gymnosperm, Douglas-fir (Fernando et al. 1998), consequently providing a tool for novel breeding strategies, the aims of which may be either to overcome prezygotic selection barriers against hybridization or to facilitate delivery of genetically engineered gametes. In this study we propose applying IVF methods to testing prezygotic reproductive barriers within the Pinaceae.

Prezygotic barriers show great variation within conifers. Examples of the relative weakness of prezygotic selection are the numerous hybrid swarms found in forests: in eastern Canada, *Picea rubens* x *mariana*; in western Canada, *Picea glauca* x *engelmannii*; and in southeastern United States, *Pinus* x *sondereggei*, to name but a few. Spontaneous hybridization, such as Dunkeld larch (*Larix decidua* x *leptolepis*) and Leyland's cypress (*Chamaecyparis nootkatensis* x *Cupressus macrocarpa*) are known to occur in arboreta (Mabberley 1990). Among the Pinaceae, a number of genera are more like species complexes or syngameons, in which species do not have well-defined reproductive boundaries (Otte and Endler 1989). However, examples of relative strength in prezygotic selection have been observed within *Picea* by Mikkola (1969) who found that alien pollen showed a pronounced inability to penetrate the nucellus. Whether prezygotic selection is strong or weak has never been the subject of experimental reproductive biology in conifers, and no mechanisms have been elucidated.

Pollen germination in most genera of the Pinaceae depends on liquid, either in the form of a pollination drop as in *Picea*, *Abies* and *Pinus* (Owens and Blake 1985) or as a post-pollination prefertilization drop as in *Larix* and *Pseudotsuga* (Said et al. 1991; Takaso et al. 1996). Beyond promoting the germination of pollen from the same species or even genus, the drop may well inhibit the germination of pollen from other genera (von Aderkas, unpublished).

Pollen penetration of the nucellus shows great variation among genera. In *Picea*, *Larix* and *Pseudotsuga*, the pollen pushes through this tissue rapidly (1 week), but in *Pinus* the pollen is arrested once within the nucellus and only resumes growth after nearly a year (Singh 1978).

When the pollen arrives at the megagametophyte, it moves into the archegonial chamber before penetrating the neck cells (Singh 1978). But other routes are also possible. Penetration through the wall of the archegonium had been reported for interior spruce (*Picea glauca* x *engelmannii*). This was attributed to abnormalities in ventral canal orientation, implying a role for this particular cell in directing pollen growth (Runions 1997). Some pollen may branch during penetration, either to provide anchorage (Willemse and Linskens 1969) or to optimize search strategies for archegonia.

Thus, barriers to foreign pollen may occur during pollen germination, pollen penetration of the nucellus and pollen penetration of the megagametophyte. These prefertilization processes have been well described in histological and ultrastructural studies, but no analysis of nucellar tissue or of any of the various interactions with the pollen has been carried out. This is largely due to the difficulties in working with intact cones, as well as the impossibility of mutation work with trees that have such long

breeding cycles. Likewise, selection of male gametes within the egg has received no attention in gymnosperms.

The purpose of the work presented here was to look at the interactions between pollen and megagametophyte and to determine whether any barriers to foreign pollen exist during the events from pollen presentation to male gamete delivery within the egg. We tried a variety of combinations of genera within the Pinaceae. To do this we used the in vitro fertilization system successfully applied to Douglas-fir (Fernando et al. 1998) in which the pollen was germinated in vitro and presented to megagametophytes isolated from ovules and free of any nucellar tissue. We hypothesized that pollen of one genus could penetrate and deliver its gametes into the archegonium of another genus. We wished to know if recognition of neck cells was necessary for penetration by pollen and whether any barriers to pollen penetration existed within megagametophytes. Furthermore, we were interested in any possible changes in megagametophytes during culture which may affect pollen behaviour.

Materials and Methods

Plant material

Male and female cones were collected from several sources. *Pinus monticola* male cones and *Pseudotsuga menzeisii* female cones came from British Columbia Ministry of Forests (BCMF) Glyn Road Station, Victoria. *Pinus monticola* female cones were collected at Timber West seed orchard (Saanich), *Picea sitchensis* male cones came from Nootka orchards (Pacific Forestry), *Larix occidentalis* male cones came from BCMF Kalamalka Research Station. Seed cones of *Larix* x *eurolepis* and *P. menzeisii* were collected on the campus of the University of Victoria. Male cones were collected in March and April of 1997, just prior to pollen shedding. Both bagged and unbagged female cones were collected from mid May to late June of the same year.

Pollen was collected from surface-sterilized cones prepared as described by Friedman (1987). The cones were washed for 15 s in 70 % ethyl alcohol, followed by 30 s in 1 % sodium hypochlorite. They were rinsed three times in a sterile distilled water and blot-dried on sterile filter paper. The sterile cones were kept at 23 °C in petri dishes covered with sterile filter paper until the shedding of pollen grains occurred. The dry pollen (≤ 9 % water content) was stored in airtight sterile glass vials over silica gel and kept at 4 °C until required. Larch pollen needed to be hydrated prior to culture. This was done in 100 % relative humidity according to Charpentier and Bonnet-Masimbert (1983). All manipulations were performed in a laminar flow hood.

The developmental stages of larch and Douglas-fir megagametophytes varied between central cell and late egg cell as determined by clearing in methyl salicylate

(Fernando and Cass 1996). White pine megagametophytes were either at or beyond fertilization. In late June, some open-pollinated Douglas-fir cones were also collected.

Seed cones were surface sterilized by flaming in 70 % ethyl alcohol. Megagametophytes were dissected and the nucellus removed. When open-pollinated Douglas-fir cones were dissected, care was taken to keep only those megagametophytes in which pollen attached to the nucellus was found in the micropyle.

Male and female gametes were co-cultured on Murashige and Skoog (1962) medium (M1) modified by Fernando et al. (1997) and supplemented with 150 mM sucrose and 10 % polyethylene glycol 4000 (Sigma). An exception to this was larch pollen, which was raised on a different medium (M2) containing Brewbaker and Kwack (1963) minerals and diluted 1:10, 7.5 % sucrose and 16 % polyethylene glycol 4000. All media were solidified with 0.4 % phytagel, and the pH was adjusted to 5.6 ± 0.1.

Pollen/megagametophyte interactions

Pollen was sown onto M1 and cultured at 23°C in the dark. Sitka spruce and white pine pollen were introduced to megagametophytes after 48 h on M1. Larch pollen was cultured on M2 for 5-7 d before being presented on a small block of M2 medium to megagametophytes cultured on M1.

Megagametophytes were placed in close proximity (approx. 100 μm) to growing pollen tubes. Megagametophytes in a variety of developmental states were used: unfertilized, fertilized, degenerating and dead. Megagametophytes were also bisected into the chalazal and micropylar halves. Both intact and cut ends were presented to pollen tubes. The male and female gametophytes were co-cultured for about 10 d or until penetration by pollen tubes had been observed. The intergeneric crosses attempted were

as follows (with male gametophyte indicated first in each cross): 1) *L. occidentalis* x *P. monticola*, 2) *P. monticola* x *P. menzeisii*, 3) *P. sitchensis* x *P. monticola*, 4) *P. sitchensis* x *L. x eurolepis*, *P. sitchensis* x *P. menzeisii*.

Histological analysis

Megagametophytes with attached pollen tubes were fixed in 2.5 % glutaraldehyde in phosphate buffer (pH 7.2). Five specimens of each kind of attempted cross were dehydrated using a graded series of ethyl alcohol and embedded in Technovit 7100. About 225 archegonia were sectioned. Serial sections (2-8 μm) were cut using glass knives mounted on a Sorvall JB4 ultramicrotome. Sections were stained for 1 m with 0.5 % Toluidine blue.

For electron microscopy, megagametophytes were embedded in Spurr's resin and cut into semi-thin (0.9-1 μm) and ultra-thin (50-90 nm) sections on a Leica ultramicrotome. The sections were examined and photographed with a Hitachi H-7000 electron microscope.

Results and discussion

Pollen and megagametophytes are not difficult to co-culture. Pollen reached the micropylar end of the female gametophyte (Fig. 45), penetrating the archegonium (Fig. 46, inset) and delivering male gametes to the egg (Fig. 46). No barriers to gamete delivery existed within the members of the Pinaceae tested in vitro. Penetration of isolated megagametophytes by pollen tubes was verified histologically in all of the crosses attempted (Table 9). Prezygotic events in vitro differ from those in situ in a number of ways: pollen behaviour, penetration sites, megagametophyte development, and male gamete development and release.

Pollen behaviour in vitro

In situ, pollen must penetrate a nucellus, which may direct or aid in penetration of the megagametophyte (Mikkola 1969). In our in vitro experiment, pollen developed normally, growing a tube and penetrating megagametophytes in the absence of nucellar tissue. Body cells migrated down the tubes and divided to generate two male gametes. Pine or spruce pollen germinated quickly, growing long, branching tubes. It was not uncommon to see several tubes anchored in megagametophyte apices. On some occasions, both branches of a pollen tube entered the megagametophyte (not shown). This branching phenomenon is common in vivo, and the extra branches are thought to anchor the tubes more firmly in the nucellus (Willemse and Linskens 1969) or else to correspond to haustoria-like structures (Dawkins and Owens 1993). Larch pollen, which is difficult to germinate in vitro, produced unbranched pollen tubes after 5-7 days in culture and successfully penetrated megagametophytes of *Pinus monticola* and *Pseudotsuga menzeisii*.

Figs. 45-56. Longitudinal sections of megagametophytes and pollen grains from in vitro co-cultures.

Fig. 45. Light micrograph of unpollinated Douglas-fir megagametophyte with spruce pollen grain (pg) anchored in its apex. One dead egg (e) appears as an opaque structure. Pollen tube (pt). Bar = 111 μm.

Fig. 46. Light micrograph of spruce pollen tube delivering gametes in an unpollinated larch megagametophyte (inset: serial section showing tube entering the archegonium). Female nucleus (fn), jacket (j), male nucleus (mn), neck cells (n). Bar = 33 μm.

Fig. 47. Light micrograph of spruce pollen tube penetrating into the neck of an unpollinated Douglas-fir megagametophyte and growing towards a dead archegonium. Bar = 33 μm.

Fig. 48. Light micrograph of spruce pollen tube penetrating beside the neck cells of an unpollinated Douglas-fir megagametophyte. The pollen tube grows towards a dead archegonium (e1) while a live archegonium (e2) lies adjacent to it. Bar: 33μm.

Fig. 49. Light micrograph of spruce pollen growing towards a dead archegonium (e2) after penetrating through the side of an unpollinated Douglas-fir megagametophyte cut into two halves. Prothallial cells (pc), megaspore membrane (mm). Bar = 40 μm.

Fig. 50. Light micrograph of spruce pollen invading a fertilized Douglas-fir megagametophyte (inset: developing embryo). Suspensor (s), proembryo (pe). Bar = 33 μm.

Fig. 51. Light micrograph of the chalazal end cut surface (c) of a Douglas-fir megagametophyte with spruce pollen penetrating and growing towards the chalazal tip. Bar = 40 μm.

Fig. 52. Light micrograph of a pollen tube penetrating through a side wound (w) of the chalazal half of a Douglas-fir megagametophyte. Bar = 40 μm.

Fig. 53. Electron micrograph of jacket cells of unpollinated Douglas-fir megagametophyte, showing nuclei at prophase and degenerating egg. Jacket nucleus (jn). Bar = 12.5 μm.

Fig. 54. Light micrograph of neck cells of an unpollinated Douglas-fir megagametophyte showing clusters of neck cells at the megagametophyte apex. Bar = 20 μm.

Fig. 55. Spruce pollen tube with two male gametes from body cell division within an unpollinated Douglas-fir megagametophyte. Bar = 40μm.

Fig. 56. White pine pollen tube with body cell (bc) dividing at a position outside of an open-pollinated Douglas-fir megagametophyte. Bar = 40 μm

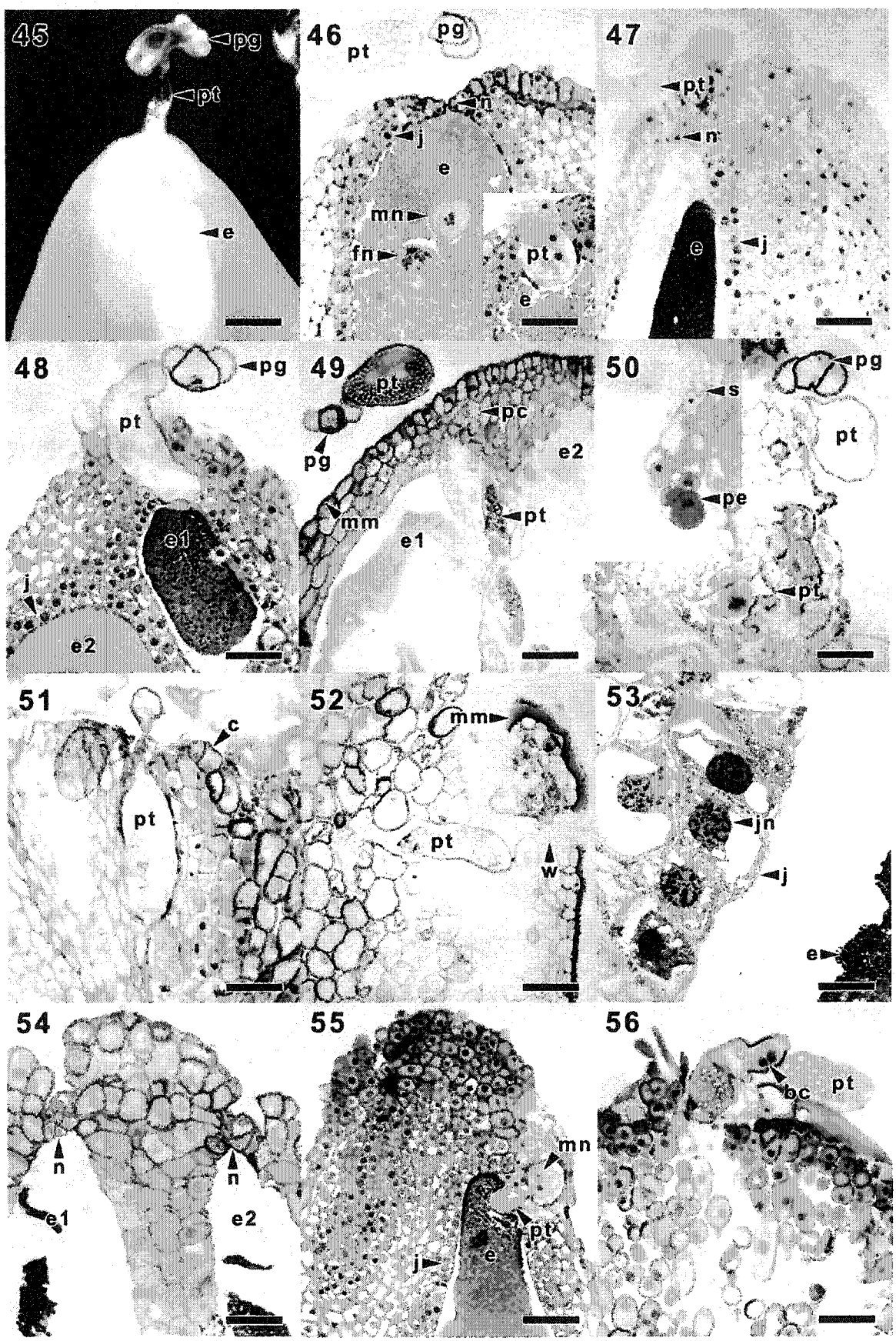
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Table 9. Intergeneric pollen megagametophyte co-cultures.

Male gametes	Female gametes	Penetration
L. occidentalis	*P. monticola*	+
P. monticola	*P. menzeisii*	+
P. sitchensis	*P. monticola*	+
P. sitchensis	*L.* x *eurolepis*	+
P. sitchensis	*P. menzeisii*	+

In vivo, pollen tube growth towards the archegonia may take a few days (*Larix*, *Pseudotsuga*), a few weeks (*Picea*) or a year (*Pinus*) (Dawkins and Owens 1993). In vivo, pollen tubes grow through the nucellus with which they are in close contact and which may possess mechanisms to control, regulate or prevent pollen-tube growth (Mikkola 1969). In contrast, cultured pollen tubes appeared within a few days of sowing, and no apparent mechanism regulating their growth was evident. Although they differ greatly amongst one another in their in vivo behaviour (Willemse and Linskens 1969; Allen and Owens 1972; Singh 1978; Owens and Molder 1979a; Owens and Molder 1984b), pine, Douglas-fir, larch and spruce pollen behaved similarly in vitro. In only a matter of days, all species were equally able to penetrate megagametophytes from different genera.

Pollen penetration

Although pollen tubes may enter the archegonia through the neck cells, this was seldom observed (Fig. 47). More commonly, pollen was seen growing into the megagametophyte through its apex. Pollen entrance was from cells beside the neck (Fig. 48) as well as through prothallial and jacket cells at the sides of the micropylar end of the megagametophytes (Fig. 49). A few pollen tubes missed the archegonium altogether, growing past it and through the prothallial cells which separated the archegonia from each other (not shown). The pollen point of entry had nothing to do with the internal organization of the archegonium, as many different points of entry were recorded for archegonia of normal structure and appearance.

It would appear unlikely that the ventral canal cell plays any role in pollen entry as has been suggested in the case of interior spruce (Runions 1997). Additionally, pollen

tubes were not strongly attracted to the neck cells, indicating that the neck was not involved in directing pollen-tube growth. Furthermore, the neck cells did not act as species-specific recognition sites as pollen from one genus was able to pass through the neck cells of megagametophytes from another genus (Fig. 47).

In vivo, it has been observed that Douglas-fir pollen would often reach the apex of a megagametophyte and then grow along the apical edge until an archegonial chamber was encountered, at which point the pollen would penetrate the megagametophyte (Owens and Morris 1990, 1991). Said et al. (1986) suggested that in *Larix leptolepis* the megaspore wall could provide some recognition mechanism at the apex of the megagametophyte. Our results clearly do not indicate the presence of any recognition mechanism, as the apices of all megagametophytes tested were unable to discriminate foreign pollen tubes. It may be possible that the apex is generally attracting the pollen, providing a signal of a very non-specific nature.

Megagametophyte development

The physiological state of the archegonia had no influence on pollen behaviour. Pollen penetrated living as well as dying eggs with equal facility (Table 10). Pollen were unable to distinguish living from dead eggs, as pollen were frequently seen entering dead archegonia immediately adjacent to lives ones (Fig. 48). Pollen was observed to penetrate non-functional archegonia which had been put into culture past the point of fertilization. In one example, pollen penetrated a megagametophyte in which an embryo was developing in the corrosion cavity (Fig. 50).

Such behaviour would suggest that no inductive signal exists in vitro promoting pollen tubes to invade a healthy archegonium, nor is there an inhibitory signal to prevent

Table 10. Co-culture of P. sitchensis pollen and various P. menzeisii megagametophytes

Megagametophytes	Penetration
Unpollinated	+
Pollinated/fertilized	+
Micropylar end (intact)	+
Chalazal end (intact)	−
Wounds, cut surfaces	+
Dead archegonia	+
Degenerating archegonia	+

pollen from entering a degenerating or fertilized one. In Douglas-fir, Owens and Morris (1991) reported that only one pollen tube could enter the archegonial chamber in situ and, consequently, the egg cell. In angiosperms, several studies have shown that ovules are penetrated by only one pollen tube and that pollen tubes are not attracted by fertilized ovules (Russell 1992). To our knowledge, no penetration of dead archegonia had been reported in vivo. In intraspecific IVF trials of Douglas-fir, a similar lack of pollen discrimination of archegonia has been noted (Dumont-BéBoux and von Aderkas, unpublished). The inability of the pollen to discriminate viable archegonia from the non-viable has implications for male selection during reproduction in conifers. Pollen compete during prezygotic stages, such that the fastest germinating, fastest growing pollen may be the first to successfully reach the archegonia (Willson and Burley 1983), but any initial advantage is lessened by the inability of the pollen to distinguish which archegonia are viable. Events in situ must be coordinated sufficiently well to prevent delays in pollen penetration which would lead to significant degeneration of archegonia. However, archegonial abortion has been recorded in *Picea* (Mikkola 1969), and in such cases pollen may also be unable to distinguish the living from the dead.

When megagametophytes were bisected into micropylar and chalazal halves prior to culture, pollen tubes penetrated both cut ends, growing either towards the archegonia (not shown) or towards the chalazal tip of the megagametophyte (Fig. 51). Pollen tubes also penetrated through the uncut portion of the chalazal tip of sides or the megagametophytes and grew towards the micropylar end; however, in such incidents pollen penetration was always associated with a wound in the megagametophyte

membrane (Fig. 52). At the chalazal end, pollen was never seen to penetrate through and intact megagametophyte membrane.

The megagametophyte is surrounded by a megaspore membrane similar in composition to the exine of pollen (Sedgley and Griffin 1989). Contrary to a report from Owens and Morris (1990), it was found that the megaspore wall was thinner at the micropylar end than at the chalazal end (compare Fig. 49 and 52). As in primitive gymnosperms (Pettitt 1977), conifer pollen is known to possess hydrolytic enzymes such as pectinase and cellulase in *Pinus sylvestris* (Willemse and Linskens 1969) and acid phosphatase and esterase in *Pinus*, *Picea*, *Abies* and *Cedrus* (Pettitt 1985). These enzymes are implicated in the penetration of the nucellus (Pettitt 1985) and possibly the megaspore membrane. In *Pinus contorta*, enzyme activity is higher in the presence of compatible pollination than in the presence of incompatible pollination (see Pettitt 1985). No penetration of the megaspore membrane, other than just above the archegonial chamber, has ever been reported in vivo. Our study did not reveal pollen penetration of the thick megaspore membrane of the megagametophyte chalazal end. The megaspore membrane presents a physical barrier, and possibly a chemical one, over the greater part of the megagametophyte that prevents the pollen tube from entering.

Megagametophytes continued to develop in vitro in unforeseen fashion. The cells of the apex continued to divide and expand, and so did various cell types of the archegonium. Even when the egg and ventral canal cell had died, jacket cells of the archegonium remained alive (Fig. 53), and neck cells continued to divide (Fig. 54). In spite of the increased number of actively secreting neck cells, pollen tubes were still not

strongly attracted to them (Fig. 54). It would appear that the secretion is not used in attracting pollen but may have some other, as yet undetermined, function.

Male gamete development

Division of the body cell to produce two male gametes occurred in a variety of locations and did not depend on pollen-tube entry of archegonia. Division was observed near the tip of pollen tubes that had penetrated either a megagametophyte (not shown) or an archegonium (Fig. 55) or away from the tip, in parts of the pollen tube that were still outside of the megagametophyte (Fig. 56). It took place under all circumstances, whether or not the archegonia were alive.

In white spruce pollen germinating in vivo, body cell division occurs early, when the tube is just entering the nucellus and the body cell is still at the proximal part of the tube (Dawkins and Owens 1993). In the same study, when pollen was germinated in culture, no division was observed and it was speculated that division was triggered by the nucellus (Dawkins and Owens 1993). This is in contrast to our results for spruce. Migration of the body cell and its division have already been reported for in vitro-raised Douglas-fir pollen (Dumont-BéBoux and von Aderkas 1997; Fernando et al. 1997). Gamete formation appears to be internally controlled as it occurs independently of the megagametophyte and in the absence of the nucellus.

Male gamete delivery

Male gamete delivery to the egg was confirmed between Sitka spruce pollen and a larch megagametophyte (Fig. 46). Serial sections revealed that the pollen tube bypassed the neck of the archegonium and entered through the cells of the apex of the megagametophyte. Upon penetration of the archegonium, the tip of the pollen tube ruptured and released its male gametes. The ventral canal/egg nucleus axis did not

appear to be necessary for successful delivery of gametes in vitro, although this is the only path portrayed in the histological literature (Singh 1978). The mechanism(s) which delivers male gametes to the egg nucleus appears to function irrespective of generic differences.

Conclusion

This is the first report of intergeneric crosses attempted in vitro for conifers. Male gametes can be delivered between different genera. We have shown that no recognition mechanism affects alien pollen growth in vitro. Furthermore, pollen can develop and grow towards and into megagametophytes belonging to different genera. The body cell is able to divide before and/or during penetration. Neck cells neither guide nor attract pollen. Penetration can occur through any undamaged part of the apex of the megagametophyte and through wounds. However, pollen will not penetrate unwounded chalazal ends and, therefore, cannot make its way through the thicker part of the megagametophyte membrane.

Chapter 7

Megagametophytes of Douglas-fir (*Pseudotsuga menziesii*) and larch (*Larix* x *eurolepis*) in culture: multiplication of neck cells and the formation of binucleate cells

Introduction

One of the goals of culturing tissues of plants is to provide genetically uniform material for novel breeding purposes. Androgenesis and gynogenesis are methods used to create haploid-cell lines, which when induced provide homozygous dihaploid breeding stock (Park et al. 1998). In conifers, only the female gamete producing tissue – the endosperm or megagametophyte – has been amenable to induction of embryos (von Aderkas et al. 1990) and subsequent production of trees (von Aderkas and Bonga 1993). If such material is to have value, then the question of genetic stability needs to be rigorously addressed. Each cell line developed in haploid tissue culture must be verified by chromosome squashes and/or DNA cytofluorometry. Studies of European larch haploid embryogenic culture have shown that polyploidization is frequent within a year or two of culture (von Aderkas and Anderson 1993). In short order, most of the lines are diploid (Pattinavibool et al. 1995). Aneuploidization, which also commonly occurs, may result in lines which become stable at odd chromosome numbers such as 30, compared to the initial number of 12 (Pattinavibool 1996). Although much of this instability is assumed to increase as the cultures age, the question arises whether some of this variation is due to initial genetic instability in the explant material, or whether it occurs early on in culture.

Increasingly, there is evidence that megagametophytes show mosaicism in situ. Nagl (1967) found 1C to 4C DNA levels among cells of the archegonial, or micropylar

end, of *Pinus nigra* megagametophytes. This mosaicism only occurred during a short period of megagametophyte development. Avanzi and Cionini (1971), looking at another gymnosperm, *Ginkgo biloba*, found 4C cells among the jacket cells of the archegonium. In a recent cytofluorometric study by Pichot and El Maatoui (1997), pinaceous conifer megagametophytes were found to be uniformly haploid, substantiating similar work on megagametophyte-derived protoplasts by Hakman et al. (1986). However, cupressaceous conifers showed unusual mosaicism, including all levels from 1C to 6C (Pichot and El Maatoui 1997).

There are different possible explanations for polyploidization. In the case of DNA doubling in *G. biloba* (Avanzi and Cionini 1971), endoreduplication can account for the increase. A more complex answer is required for cupressaceous conifers which have single-unit increases (Pichot and El Maatoui 1997). This can only come about if endomitoses are followed by nuclear fusions (Nagl 1995). For nuclear fusion to occur there must be multiple nuclei present.

Early this century, Carothers (1907) noted in *G. biloba* that even after megagametophytes had completed cellularization, there were persistent areas of coenocytic cells. A variant on this was noted by Owens and Molder (1974) in *Chamaecyparis nootkatensis* megagametophytes. As the tissue matured, cells often became multinucleate. To study the role of multinucleate cells as possible sources of nuclear fusion, it is first necessary to devise a system in which they can be produced.

Our study set out a single hypothesis, that megagametophytes of conifers can be induced to form multinucleate cells in vitro. We chose Douglas-fir and hybrid larch, as

these were organisms with which we had the greatest familiarity in megagametophyte culture (Takaso et al 1996; Fernando et al. 1997; von Aderkas et al. 1990, 1997).

Materials and methods

Plant material

Reproductive Douglas-fir trees in a breeding orchard were made available to us by the British Columbia Ministry of Forests, Glyn Road Research Station, Victoria, B.C. These were of genotype 3265 from a collection kept on site. European/Japanese hybrid larch (*Larix decidua* x *leptolepis* = *L.* x *eurolepis*) seed cones were removed from trees located on campus at the University of Victoria.

Culture conditions

Female cones were surface-sterilized and megagametophytes of different developmental stages were placed in petri dishes (9 x 150 mm) containing Murashige and Skoog (1962) medium modified according to Fernando et al. (1997) and supplemented with 150 mM sucrose and 10 % polyethylene glycol 4000 (Sigma). The medium was solidified with 0.4 % phytagel and the pH was adjusted to 5.6 ± 0.1. These were placed in a growth chamber at 23 °C for up to 10 d in complete darkness.

Sectioning

Megagametophytes of Douglas-fir, which had been dissected from cones over a 3-week period, were placed in vitro for up to 10 d. They were then collected and fixed in 2.5 % glutaraldehyde in phosphate buffer (pH 7.2). These were block-stained with 2 % aqueous uranyl acetate, dehydrated in an acetone series and then they were infiltrated in Spurr's resin (Spurr 1969). Both monitor and ultrathin serial sections were cut on a Reichert Ultracut E ultramicrotome and stained with saturated solutions of uranyl acetate in 70 % ethanol and lead citrate (Reynolds 1963). This material was examined with a Hitachi H-7000 electron microscope at 75 kV. A duplicate set of material was fixed in 2.5 % glutaraldehyde in phosphate buffer (pH 7.2), processed through an alcohol series,

infiltrated with glycol methacrylate, and polymerized. Serial sections (2 – 8 μm) were cut with glass knives on a Sorvall JB4 model ultramicrotome and stained with 0.5% toluidine blue O for 1 m. This material was examined with a Leitz Laborlux S light microscope. *Larix* x *eurolepis* megagametophytes were prepared in glycol methacrylate for light microscopy in a manner identical to that employed for Douglas-fir.

Results and discussion

Douglas-fir megagametophytes are composed of a variety of cell types (Fig. 57), some of which proliferated in culture conditions. The novel aspect of the study is that the neck cells of the archegonium not only proliferated (Figs. 60 and 61), but became binucleate (Figs. 58, 59, and 62). This was the only cell type of Douglas-fir to exhibit such free nuclear cells. The bulk of the megagametophyte is made up of prothallial cells, which eventually serve as storage cells. *Larix* x *eurolepis* megagametophytes, however, had prothallial and jacket cells which had two nuclei each (Fig. 63).

The apical prothallial cells also multiplied. Other archegonial cells, such as the ventral-canal cells and the unfertilized egg, generally died within a number of days being placed in culture (Figs. 59-62), in agreement with previous findings (Fernando et al. 1997). The remaining cells of the archegonium – the jacket cells – were still alive and beginning to expand (Figs. 59-62 and 69).

Neck cells are not unique to conifers, but are found in all archegoniate plants. There is no record of these cells continuing to multiply after egg degeneration, whether in fern, moss, or gymnosperm. Gymnosperms may vary widely in the number of neck cells. *Ephedra* and podocarps have approximately 40, whereas most cycads possess four, and conifers have from two to four, depending on genus (Singh 1978). Reports of more than one tier of neck cells have been made for *Picea engelmannii* (Owens and Molder 1984b) *and P. glauca* (Owens and Molder 1979b). In the case of podocarps, the neck cells may even form a multicellular column (Quinn 1964), rather than the more common tier of cells. In our sections, it was found that Douglas for neck cells of gametophytes in situ are generally four in number. Once in culture, however, the Douglas-fir neck cells

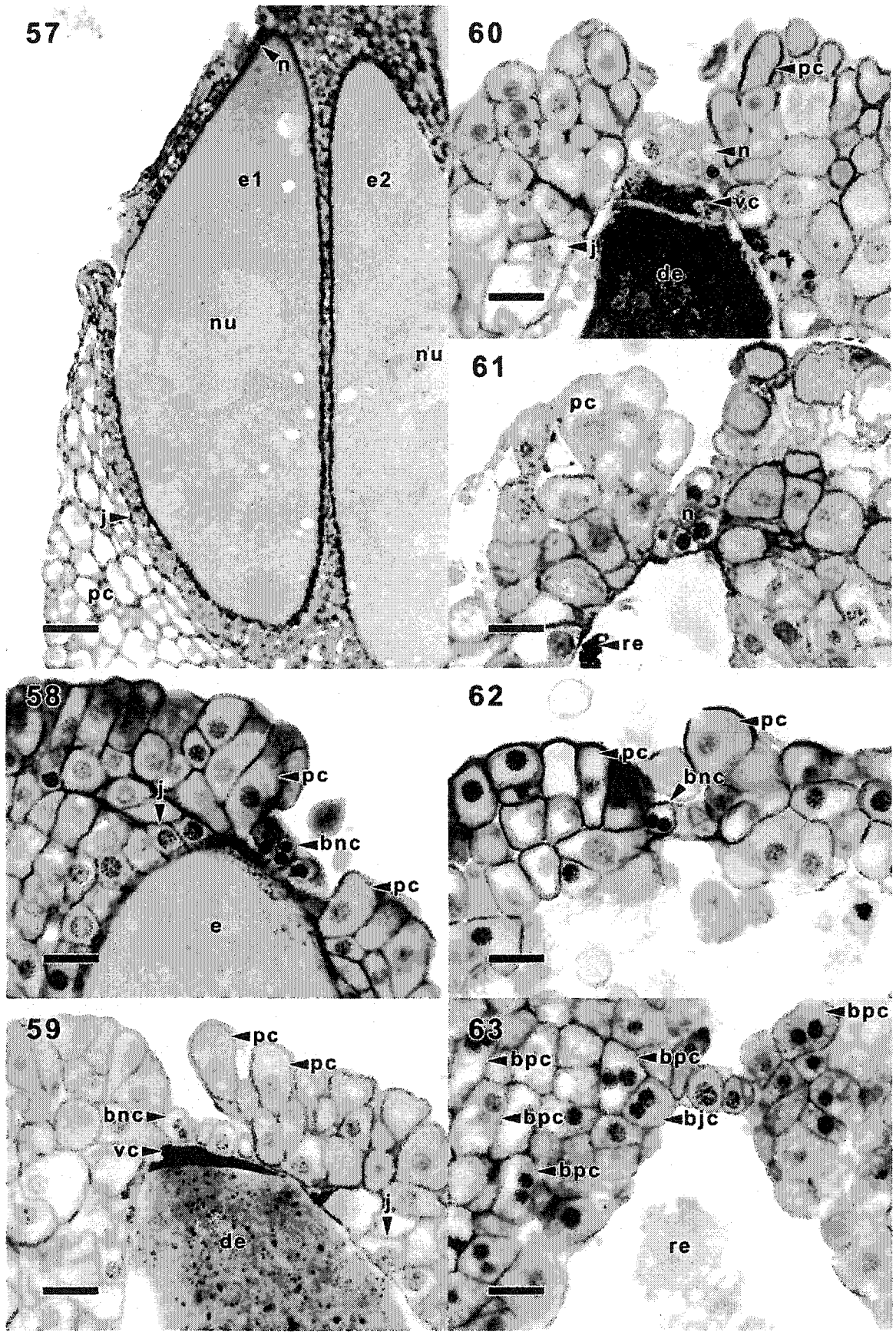
Figs. 57-63. Light micrographs of glycol methacrylate-embedded megagametophytes stained with toluidine blue O. Binucleate jacket cell (bjc), binucleate neck cell (bnc), binucleate prothallial cell (bpc), degenerating or dead egg (de), egg (e), jacket (j), neck (n), nucleus (nu), prothallial cell (pc), remnants of egg (re), ventral-canal cell (vc).

Fig. 57. Douglas-fir, complete archegonial axis with surrounding neck (n), jacket (j), and prothallial cells (pc). Bar = 30 μm.

Fig. 58. Douglas-fir, apex of a healthy archegonium. A binucleate neck cell (bnc) is shown). Bar = 17 μm.

Fig. 59-62. Douglas-fir, progressive stages of archegonial and ventral-canal cell (vc) degeneration. The megagametophyte in Fig. 6 was collected and put in culture at a stage of development well beyond fertilization. Notice the presence of binucleate neck cells and that jacket cells and prothallial cells are expanding. Bars in Figs. 3, 4, and 6 = 17 μm. Bar in Fig. 5 = 7.5 μm.

Fig. 63. Megagametophyte of larch. Here binucleate cells are found among jacket and prothallial cells. Bar = 17 μm.


57
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Figs. 64-69. Electron micrographs of Spurr-resin-embedded Douglas-fir megagametophytes stained with uranyl acetate and lead citrate. Coated pits (cp), coated vesicle (cv), egg (e), endoplasmic reticulum (er), Golgi complex (gc), mitochondrion (m), microtubule (mt), multivesicular complex (mvc), nucleus (nu), plasma membrane (pm), starch grain (sg), vesicle (v), vacuole (va), cell wall (w).

Fig. 64. Douglas-fir, binucleate neck cell with thick cell wall (w) material. Bar = 2.2 μm.

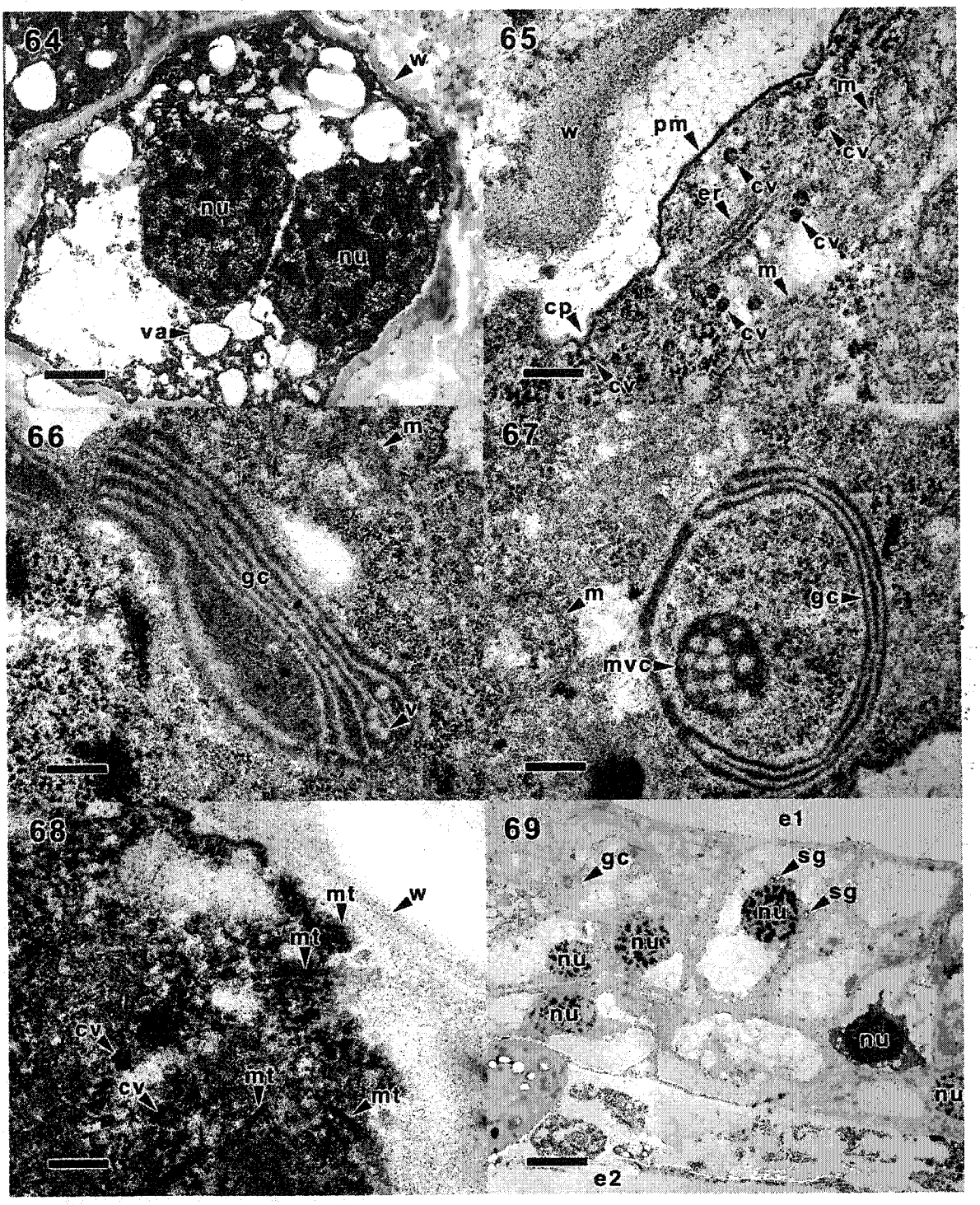
Fig. 65. Coated vesicles (cv), coated pits (cp), endoplasmic reticulum (er) and mitochondria (m) at the periphery of a neck cell indicating metabolic activity prior to fixation. Bar = 0.2 μm.

Fig. 66. Golgi complex (gc) with associated vesicles (v) from a jacket cell. Bar = 0.4 μm.

Fig. 67. Golgi complex (gc) with associated multivesicular complex of a neck cell. Bar = 0.4 μm.

Fig. 68. Microtubules (mt) and coated vesicles (cv) at the periphery of a neck cell. Bar = 0.2 μm.

Fig. 69. Rows of jacket cells separating two archegonia (e1 and e2). Bar = 11 μm.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proliferated to form a plug, which continued to divide. The number of cells ranged form four to more than eight in our study. These continued to grow after the egg and ventral-canal cell had long since degenerated (Figs. 59-62). In cycads, it has been noted by Norstog (1972), that the two-celled binucleate nature of the neck cells were due to poor observation, as closer inspection showed that these neck cells possessed another thinner wall in each cell which was difficult to detect. We did not observe such walls in our serially sectioned material.

Electron microscopy demonstrated that the cell contents of these multiplying neck cells (Figs. 65-68) are similar to previously published micrographs of in situ neck cells in western white pine, *Pinus monticola* (Bruns and Owens 1989). These cells have thick cell walls (Figs. 64, 65, and 68) and, similar to results presented for *Cunninghamia lanceolata* (Singh et al. 1976), also possess large and small vacuoles. Additionally, the neck cells were observed to possess coated vesicles (Figs. 65 and 68), partially coated endoplasmic reticulum (not shown), Golgi complexes (Fig. 66), coated pits (Fig. 65), and multivesicular complexes (Fig. 67). These organelles are known to be involved in endocytotic pathways (Fowke et al. 1991). Endocytotic pathways are proposed as mechanisms for recycling plasma membrane to Golgi complexes via internalization, and the return route is mediated via coated pits and coated vesicles; this pathway is thought to compensate for secretory activities (Fowke et al. 1991). Furthermore, the presence of coated pits and coated vesicles is positively correlated with cell wall deposition, and they are often associated with the cell plate region of dividing cells (Fowke et al. 1991, Franke and Herth 1974, Nakamura and Miki-Hirosige 1982, Robertson and Lyttleton 1982,

Ryser 1979). Thus, the neck cells observed in this study have ultrastructural characteristics common to actively dividing and secreting cells.

In spite of the presence of thick cell walls and evidence for cell wall deposition mediated by coated vesicles in neck cells, there is no evidence that neck cells produce chemotactic signals to attract pollen. In vitro fertilization experiments have shown that in intraspecific (Fernando et al. 1998) and interspecific and intergeneric crosses among pinaceous species (Dumont-BéBoux et al. 1998), pollen were able to penetrate any archegonia presented to them by a process that rarely involved entry by the neck. The neck may represent a weak point in the apex of the megagametophyte, the secretions aiding in the movement of these cells past one another as the pollen enters.

Unlike neck cells of conifers previously illustrated, neck cells of megagametophytes placed in vitro are frequently seen to possess two nuclei. Serial sections showed these to be separate and not U-shaped or bowed single nuclei. No nuclear fusion was seen. Nagl's (1978) suggestion that endopolyploid events typify storage cells of angiosperm endosperm cannot be extrapolated to gymnosperm storage tissue, as none of the prothallial storage cells exhibit this phenomenon.

Experimentally, it has been possible to create multinucleate cells from protoplasts of somatic embryos (Gupta and Durzan 1987, Fowke et al. 1990) and haploid embryos (von Aderkas 1992). Polyethylene glycol-mediated fusion leads to creation of coenocytes composed of varying nuclear number. These are able to divide synchronously and spontaneously cellularize, eventually forming embryos (Fowke et al. 1990). Because of the even distribution of nuclei around the margins, the coenocytes more closely resemble an early-stage free-nucleate proembryo. Morphological

appearances are deceptive, as similar-looking coenocytes derived from haploid protoplasts do not become megagametophytes, but normal embryos (von Aderkas 1992).

In future studies it would be interesting to assess the DNA level. There were not enough neck cells available for processing for DNA cytofluorometric study. Other studies which will be carried out will focus in trying to culture these neck cells to see if they have any potential for further differentiation.

Chapter 8

General discussion

Successful individuals must be able to reproduce. They must also be able to persevere through the avoidance of interbreeding with other species, which may result in the dilution of distinct characters that delimit a species.

Archegonial morphology and reproduction

Conifer gametophytes have adaptations that are thought to promote the reproductive success of members of this assemblage. Male gametophytes, or pollen, in conifers are small and are able to be distributed by wind to increase the chances of random mating. Female gametophytes are retained and protected by the sporophyte in cones that direct pollen towards ovules (Niklas 1984), and ovules that are modified to capture pollen (Doyle 1945; Owens et al. 1998; Singh 1978; Takaso 1996; Tomlinson 1994). When pollen is captured by an ovule it produces a pollen tube that grows directly into egg cells to release its gametes, which increases the likelihood of successful fertilization. Conifer egg cells are very large. They can be up to 1000 times larger than neighbouring prothallial cells in the megagametophyte. This may be of functional significance in that it may increase the chances of a pollen tube finding an egg in a megagametophyte.

In this study, Dunkeld larch and Douglas-fir egg cell morphology were analyzed. It was observed that there was a linear relationship between egg cell volume and nuclear volume. This linear relationship was consistent with the linear relationship between nuclear volume and cell volume that is found in other land plants (Price et al. 1973). A linear relationship also exists between nuclear content and nuclear volume in

angiosperms (Baetcke et al 1967; Bennett 1972); however, this relationship is not found in conifer egg cells. The nuclei of conifer egg cells are much larger than would be expected given their total DNA content. DAPI staining also showed that most of the DNA in conifer egg cells is found at the periphery of the egg nuclei, while the middle of the nuclei are devoid of DAPI staining. This distribution of DNA has not been found in any other eukaryote. Egg cell volume and egg nuclear volume in conifers may be modified to provide a larger target for pollen tubes to increase reproductive success.

Also investigated in Dunkeld larch were abnormalities in archegonial development. This study described patterns of abnormal development that were previously unknown. Documented abnormal development in conifers included archegonial fusion and megagametophyte twinning. This study showed that in the Dunkeld larch, small archegonia could be found in clusters. Additionally, abnormal development in megagametophytes may give rise to regions of cells that house late developing archegonia. Whether such abnormal archegonia are viable is unknown, though it is likely that they would have a reduced capacity to take part in normal reproduction, and therefore diminish the number of valid targets for pollen. Abnormal archgonial development may result in reduced seed sets in larch (Slobodník and Guttenberger 2000). This should be further investigated in other conifer.

Prezygotic breeding barriers in conifers

Aspects of conifer reproduction promote gametic reproductive isolation. A reduced capacity of foreign pollen to germinate in ovules and a reduced ability of foreign pollen tubes to penetrate cells of the nucellus were observed for Dunkeld larch and Douglas-fir. Therefore, there are prezygotic breeding barriers to foreign pollen in these

species. Ovular secretions may have a role in reducing germination of heterospecific pollen. The nucellus appears to be a barrier to foreign pollen. The effectiveness of the nucellus as a barrier depends upon the relatedness between the heterospecific pollen and the ovule. Larch and Douglas-fir are sister taxa (Gernandt and Liston 1999). The nucellus of larch does not bar pollen tube growth of Douglas-fir pollen, and Douglas-fir nucellus does not bar larch pollen.

Future studies should use a bioassay to determine the effects of directly applying ovular secretions to pollen. It would be interesting to see if germination and pollen tube growth are influenced. Further studies should investigate properties of ovular secretions that would enable it to inhibit foreign pollen germination. Chesnoy (1993) demonstrated that the chemical content of ovular secretions in different conifers is unique. Ziegler (1959) found polypeptides in ovular secretions. It is possible then that there may be proteins in these droplets that could function in inhibiting foreign pollen.

Properties of the nucellus should also be investigated to determine if proteins in the extra-cellular matrix of these cells have the ability to influence pollen tube growth. Similar research has been done in angiosperms. Whether proteins influence pollen tube growth though the transmitting tissues of angiosperms styles has been investigated. Substrate adhesion molecules have been immunolocalized in angiosperm styles (reviewed in Mascarenhas 1993). This suggests that a specific interaction between pollen tubes and transmitting tissues may exist. In support of this, Klips (1999) as well as Carney and Arnold (1969) showed that pollen tube growth rates in angiosperms are slower in interspecific hybrid crosses than in intraspecific crosses. This study, as well as studies by Mikkola (1969), McWilliam (1959) and Hagman (1975), demonstrates that

heterospecific pollen has a reduced ability to penetrate cells of the nucellus in conifers. It is possible that similar mechanisms regulate pollen tube growth through nucellar cells and transfusion tissues.

This study also demonstrated that breeding barriers present in the ovule could be overcome in vitro. In vitro co-culture of megagametophytes, where cells of the nucellus and ovular secretions are removed, allowed pollen from any genus to penetrate megagametophytes from any other genus. Gamete delivery was observed between pollen and egg cells of different genera. Embryo formation was not observed. This was partly due to the fact that egg degeneration occurs rapidly when megagametophytes are placed in culture. Techniques to improve culture conditions should be developed. Future advancements in this new technology could enable hybrid formation between species that are incompatible by traditional methods for crossing.

Further analysis of this in vitro system showed that changes occur in megagametophytes as a result of the culture condition. Neck cells of Douglas-fir, and neck cells, prothallial cells and jacket cells of larch were observed to become binucleate as a result of the culture conditions. Cells of the apex of the megagametophytes also proliferated. Further study of these changes need to be made to determine if such changes could compromise the genetic stability of cultured tissues.

Conclusions

The studies presented in this dissertation supported the hypotheses that were tested. Prezygotic breeding barriers to foreign pollen are maintained by the nucellus and secretions in the ovule. These reproductive barriers can be overcome in vitro through pollen – megagametophyte co-culture. Novel abnormal archegonia morphologies were

found in vivo and in vitro. This suggests that abnormalities in archegonial development are more variable than previously shown.

Literature Cited

Allen, G.S., and Owens, J.N. 1972. The life history of Douglas fir. Forestry Service, Environment Canada, Ottawa.

Anderson, E.D., and Owens, J.N. 1999. Megagametophyte development, fertilization, and cytoplasmic inheritance in *Taxus brevifolia*. International Journal of Plant Science, **160:** 459-469.

Arista, M., Ortiz, P.L., and Talavera, S. 1997. Reproductive isolation of two sympatric subspecies of *Juniperus phoenicea* (Cupressaceae) in southern Spain. Plant Systematics and Evolution, **208:** 225-237.

Arista, M., and Talavera, S. 1994. Phenology and anatomy of the reproductive phase of *Abies pinsapo* Bioss. (Pinaceae). Botanical Journal of the Linnean Society, **116:** 223-234.

Avanzi, S., and Cionini, P.G. 1971. A DNA cytophotometric investigation on the development of the female gametophyte of *Ginkgo biloba*. Caryologia, **24:** 105-116.

Baetcke, K.P, Sparrow, A.H., Nauman, C.H., and Schwemmer, S.S. 1967. The relationship of DNA content to nuclear and chromosome volumes and to ratio sensitivity (LD_{50}), **58:** 533-540.

Baird, A.M. 1953. The life history of *Callitris*. Phytomorphology, **3:** 258-284.

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

Bell, P.R. 1996. Megaspore abortion: a consequence of apoptosis? International Journal of Plant Science, **157:** 1-7.

Bennett, M.D. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. Proceedings of the Royal Society of London. Series B, **181:** 109-135.

Bennett, M.D., Heslop-Harrison, J.S., Smith, J.B., and Ward, J.P. 1983. DNA density in mitotic and meiotic metaphase chromosomes of plants and animals. Journal of Cell Science, **63:** 173-179.

Boyle, P., and Doyle, J. 1953. Development in *Podocarpus nivalis* in relation to other podocarps. I. Gametophytes and fertilization. Scientific Proceedings of the Royal Society of Dublin, **26:** 179-205.

Brennan, S.J., and Doyle, J. 1956. The gametophytes and embryogeny of *Athrotaxis*. Scientific Proceedings of the Royal Dublin Society, **27:** 193-257.

Brewbaker, J.L., and Kwack, B.H. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. American Journal of Botany, **50:** 859-865.

Bruns, D., and Owens, J.N. 1989. Mechanisms of cytoplasmic inheritance in western white pine. *In* 47th Annual Meeting of the Electron Microscopy Society of America. *Edited by* Bailey, G.W. San Francisco Press, San Francisco, pp. 766-767.

Buchholz, J.T. 1934. The classification of the Coniferales. Transcripts of the Illinois State Academy of Sciences. **25:** 112-113.

Camefort, H. 1959. Cytologie végétale. -Structures des filaments de chromatine de noyau de l'oosphere chez le *Pinus laricio* Poir. (var. *austriaca*): étude en microscopie électronique. Comptes Rendus Hebdomadaires des Sceances. Académie des Sciences (Paris), **249:** 1790-1792.

Camefort, H. 1964. Cytologie végétale. -Observations sur la structure des chromosomes et des nucléoles de l'oosphere des Pins. Comptes Rendus Hebdomadaires des Sceances. Académie des Sciences (Paris), **259:** 4335-4338.

Camefort, H. 1965. Une interpretation nouvelle de l'organisation du protoplasme de l'oosphere des Pins. In: travaux dedies a Lucien Plantefol, Masoon (Ed.), Paris, pp. 407-436.

Camefort, H. 1967. Observations sur les mitochondries et les plastes de la cellule centrale et de l'oosphere du *Larix decidua* Mill. (*Larix europea* D.C.). Comptes Rendus Hebdomadaires des Sceances de l'Académie des Sciences (Paris) Série D, **265:** 1293-1296.

Camefort, H. 1968. Cytologie de la fécondation et de la proembryogénèse chez quelque gymnospermes. Bulletin de la Société Botanique de France, Actualités botanique, **115:** 137-160.

Carney, S.E., and Arnold, M.L. 1997. Differences in pollen-tube growth rate and reproductive isolation between Louisiana irises. The Journal of Heredity, **88:** 545-549.

Carothers, J.E. 1907. Development of ovule and female gametophyte in *Ginkgo biloba*. Botanical Gazette, **43:** 116-130.

Chamberlain, C.J. 1957. Gymnosperms: structure and evolution. The University of Chicago Press. Chicago, Illinois.

Charpentier, J.P., and Bonnet-Massimbert, M. 1983. Influence d'une réhydration préalable sur la germination in vitro du pollen de douglas (*Pseudotsuga menziesii*) après conservation. Annales des Sciences Forestieres, **40:** 309-317.

Chesnoy, L. 1993. Les sécrétions dans la pollinisation des Gymnospermes. Acta Botanica Gallica, **140:** 145-156.

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

Chowdhury, C.R. 1961. The morphology and embryology of *Cedrus deodara* (Roxb.) Loud. Phytomorphology, **11:** 283-304.

Chowdhury, C.R. 1962. The embryogeny of conifers: a review. Phytomorphology, **12:** 313-338.

Coker,W.C. 1902. Notes on the gametophytes and embryo of *Podocarpus*. Botanical Gazette, **33:** 89-107.

Coyne, J.A., and Orr, H.A. 1998. The evolutionary genetics of speciation. Philosophical Transactions of the Royal Society of London B, **353:** 287-305.

Critchfield, W.B. 1986. Hybridization and classification of the white pines (*Pinus* section *Strobus*). Taxon, **35:** 647-656.

Dawkins, M.D., and Owens, J.N. 1993. In vitro and in vivo pollen hydration, germination, and pollen tube growth in white spruce, *Picea glauca* (Moench) Voss. International Journal of Plant Science, **154:** 506-521.

Dhillon, S.S. 1987. DNA in tree species. *In* Cell and tissue culture in forestry. Volume 1. General principles and biotechnology. *Edited by* Bonga, J.M., and Durzan, D.J. Martinus Nijhoff Publishers, Dordrecht, Netherlands, pp. 298-313.

Dobzhansky, T. 1935. A critique of the species concept in biology. Philosophical Science, **2:** 344-345.

Dobzhansky, T. 1940. Speciation as a stage in evolutionary divergence. American Naturalist, **74:** 312-321.

Dobzhansky, T. 1951. Genetics and the origin of species. Third Edition. Columbia University Press, New York.

Dobzhansky, T. 1970. Genetics of the evolutionary process. Columbia University Press, New York.

Dogra, P.D. 1966. Observations in *Abies pindrow* with a discussion on the question of occurrence of apomixis in gymnosperms. Silvae Genetica, **15:** 11-20.

Doyle, J. 1945. Developmental lines in pollination mechanisms in the Coniferales. Scientific Proceedings of the Royal Dublin Society, **24:** 43-62.

Doyle, J., and Kane, A. 1943. Pollination in *Tsuga pattoniana* and in species of *Abies* and *Picea*. Scientific Proceedings of the Royal Dublin Society, **23:** 57-72.

Doyle, J., and O'Leary, M. 1935a. Pollination in *Saxegothaea*. Scientific Proceedings of the Royal Dublin Society. **21:** 175-179.

Doyle, J., and O'Leary, M. 1935b. Pollination in *Pinus*. Scientific Proceedings of the Royal Dublin Society, **21:** 181-190.

Doyle, J., and O'Leary, M. 1935c. Pollination in *Tsuga*, *Cedrus*, *Pseudotsuga*, and *Larix*. Scientific Proceedings of the Royal Dublin Society, **21:** 191-206.

Dumont-BéBoux, N., and von Aderkas, P. 1997. In vitro pollen tube growth in Douglas fir. Canadian Journal of Forest Research, **27:** 674-678.

Dumont-BéBoux, N., Anholt, B., and von Aderkas, P. 1999. In vitro Douglas fir pollen germination: influence of hydration, sucrose and polyethylene glycol. Annals of Forest Science, **56:** 11-18.

Dumont-BéBoux, N., Weber, M., Ma, Y., and von Aderkas, P. 1998. Intergeneric pollen-megagametophyte relationships of conifers in vitro. Theoretical and Applied Genetics, **97:** 881-887.

Eichler, A.W. 1889. Coniferae. *In* Die Natürlichen Pflanzenfamilien, *Edited by*, Engler, A., and Prantl, K. **2:** 108.

Engler, A. 1926. Gymnospermae. *In* Die Natürlichen Pflanzenfamilien, *Edited by*, Engler, A., and Prantl. Second Edition.

Favre-Duchartre, M. 1956. Essaya de cultiver le gamétophyte femelle de *Ginkgo*. Revue de Cytologie et de Biologie Vegetales - Le Botaniste, **17:** 214.

Favre-Duchartre, M. 1971. A propos de la notion d'archégone. Bulletin de la Sociétié Botanique de France, **118:** 525-536.

Fernando, D.D., and Cass, D.D. 1996. Development and structure of ovule, embryo sac, embryo, and endosperm in *Butomus umbellatus* (Butomaceae). International Journal of Plant Science, **157:** 269-279.

Fernando, D.D., Owens, J.N., and von Aderkas, P. 1998. In vitro fertilization from co-cultured pollen tubes and female gametophytes of Douglas fir (*Pseudotsuga menziesii*). Theoretical and Applied Genetics, **96:** 1057-1063.

Fernando, D.D., Owens, J.N., von Aderkas, P., and Takaso, T. 1997. In vitro pollen tube growth and penetration of female gametophyte in Douglas fir (*Pseudotsuga menziesii*). Sexual Plant Reproduction, **10:** 209-216.

Florin, R. 1948. On the morphology and relationships of the Taxaceae. Botanical Gazette, **110**: 31-39.

Foster, A.S., and Gifford, E.M. 1974. Comparative morphology of vascular plants. Second edition. W.H. Freeman and Company, San Francisco.

Fowke, L.C., Attree, S.M., Wang, H., and Dunstan, D.I. 1990. Microtubule organization and cell division in embryogenic protoplast cultures of white spruce (*Picea glauca*). Protoplasma, **158:** 86-94.

Fowke, L.C., Tanchak, M.A., and Galway, M.E. 1991. Ultrastructural cytology of endocytotic pathways in plants. *In* Endocytosis, exocytosis and vesicle traffic in plants. *Edited by* Hawes, C.R., Coleman, J.O.D, and Evans, D.E. Cambridge University Press, New York, pp. 15-40.

Fowler, D.P., and Park, Y.S. 1983. Population studies of white spruce. I. Effects of self-pollination. Canadian Journal of Forestry Research, **13:** 1133-1138.

Franke, W.W., and Herth, W. 1974. Morphological evidence for de novo formation of plasma membrane from coated vesicles in exponentially growing cultured plant cells. Experimental Cell Research, **89:** 447-451.

Friedman, W.E. 1987. Morphogenesis and experimental aspects of growth and development of the male gametophyte of *Ginkgo biloba* in vitro. American Journal of Botany, **74:** 1816-1830.

Fujii, K. 1903. Über die Bestäubungstropfen der Gymnospermen. Sitzung, **24:** 211-217.

Gernandt, D.S., and Liston, A. 1999. Internal transcribed spacer region evolution in *Larix* and *Pseudotsuga* (Pinaceae). American Journal of Botany, **86:** 711-723.

Gibbs, L.S. 1912. On the development of the female strobilus in *Podocarpus*. Annals of Botany. **26:** 516-572.

Gupta, P.K., and Durzan, D. 1987. Somatic embryos from protoplasts of loblolly pine proembryonal cells. BioTechnology, **5:** 710-712.

Gutmann, M. 1995. Improved procedures for photographic documentation of phenolic deposits in semithin sections of plant tissue. Journal of Microscopy, **179:** 277-281.

Hagman, M. 1975. Incompatibility in forest trees. Proceedings of the Royal Society of London, **188:** 313-326.

Haines, R.J., Prakash, N., and Nikles, D.G. 1984. Pollination in *Araucaria* Juss. Australian Journal of Botany, **32:** 583-594.

Hakman, I., von Arnold, S., and Fellner-Feldegg, H. 1986. Isolation and DNA analysis of protoplasts from developing female gametophytes of *Picea abies* (Norway spruce). Canadian Journal of Botany, **64:** 108-112.

Hart, J.A. 1987. A cladistic analysis of conifers: preliminary results. Journal of the Arnold Arboretum, **68:** 269-307.

Heslop-Harrison, J.S. 1992. Pollen capture, adhesion and hydration. *In* Sexual plant reproduction. *Edited by* Cresti, M, and Tiezzi, A. Springer-Verlag, New York, pp. 81-88.

Johnston, J.S., Bennett, M.D., Rayburn, A.L., Galbraith, D.W., and Price, H.J. 1999. Reference standards for determination of DNA content of plant nuclei. American Journal of Botany, **86:** 609-613.

Kemp, M. 1959. Morphological and ontogenetic studies on *Torreya californica* II. Development of the megasporangiate shoot prior to pollination. American Journal of Botany, **46**: 249-261.

Klips, R.A. 1999. Pollen competition as a reproductive isolating mechanism between two sympatric *Hibiscus* species (Malvaceae). American Journal of Botany, **86:** 269-272.

Konar, R.N., and Oberoi, Y.P. 1969. Recent work on reproductive structures of living conifers and taxads – a review. The Botanical Review, **35:** 89-116.

Kosínski, G. 1986. Megagametogenesis, fertilization, and embryo development in *Larix decidua*. Canadian Journal of Forest Research, **16:** 1301-1309.

Kramer, P.J., and Kozlowski, T.T. 1979. Physiology of woody plants. Academic Press Inc., Orlando.

Krasowski, M., and Owens, J.N. 1993. Ultrastructural and histochemical postfertilization megagametophyte and zygotic embryo development of white spruce (*Picea glauca*) emphasizing the deposition of seed storage products, Canadian Journal of Botany, **71:** 98-112.

Lazzaro, M.D. 1996. The actin microfilament network within elongating pollen tubes of the gymnosperm *Picea abies* (Norway spruce). Protoplasma, **194:** 186-194.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., and Darnell, J. 2000. Molecular cell Biology. Fourth Edition. W.H. Freeman and Company, New York.

Looby, W.J., and Doyle, J. 1942. Formation of gynospore, female gametophyte, and archegonia in *Sequoia*. Scientific Proceedings of the Royal Dublin Society, **23:** 35-54.

Looby, W.J., and Doyle, J. 1944. The gametophytes of *Podocarpus andinus*. Scientific Proceedings of the Royal Dublin Society, **23:** 222-237.

Ma, Y., Weber, M., Dumont-BéBoux, N., Webber, J., and von Aderkas, P. 1998. Megagametophytes of Douglas fir (*Pseudotsuga menziesii*) and larch (*Larix* x *eurolepis*) in culture: multiplication of neck cells and the formation of binucleate cells. Protoplasma, **204:** 219-225.

Mabberley, D.J. 1990. The plant book. A portable dictionary of the higher plants. Cambridge University Press, Cambridge, UK.

Mabberley, D.J. 1997. The plant-book. A portable dictionary of the vascular plants. Second Edition. Cambridge University Press. Great Britain.

Maheshwari, P., and Singh, H. 1967. The female gametophyte of gymnosperms. Biological Review, **42:** 88-130.

Mascarenhas, J.P. 1993. Molecular mechanisms of pollen tube growth and differentiation. The Plant cell, **5:** 1303-1314.

Mayer, E. 1942. Systematics and the origin of species. Columbia University Press, New York.

McWilliam, J.R. 1959. Interspecific incompatibility in *Pinus*. American Journal of Botany, **46:** 425-433.

McWilliam, J.R., and Mergen, F. 1959. Cytology of fertilization in *Pinus*. Botanical Gazette, **119:** 246-249.

Mehra, P.N., and Sircar, M.K. 1948. The structure and development of the female and male gametophytes in *Cupressus funebris*. Proceedings of the National Institute of Sciences of India, **15:** 15-23.

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

Mikkola, L. 1969. Observations on interspecific sterility in *Picea*. Annales Botanici Fennici, **6:** 285-339.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum, **15:** 473-497.

Nagl, W. 1967. Mikrophotometrishe DNS-Messungen an Interphase- und Ruhekernen sowie Mitosen in der Samenanlage von *Pinus sylvestris*. Z Pflanzenphysiol, **56:** 40-56.

Nagl, W. 1978. Endoployploidy and polyteny in differentiation and evolution: towards an understanding of quantitative and qualitative variation of nuclear DNA in ontogeny and phylogeny. Elsevier North-Holland, Amsterdam.

Nagl, W. 1995. Cdc2-kinases, cyclins and the switch from proliferation to polyploidization. Protoplasma, **188:** 143-150.

Nakamura, S., and Miki-Hirosige, H. 1982. Coated vesicles and cell plate formation in the microspore mother cell. Journal of Ultrastructure Research, **80:** 302-311.

Niklas, K.J. 1984. The motion of windborne pollen grains around conifer ovulate cones: implications on wind pollination. American Journal of Botany, **71:** 356-374.

Norstog, K. 1972. Role of archegonial neck cells in *Zamia* and other cycads. Phytomorphology, **22:** 125-130.

Orr, H.A. 1997. Haldane's rule. Annual Review of Ecology and Systematics, **28:** 195-218.

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

Otte, D., and Endler, J.A. 1989. Speciation and its consequences. Sinauer Associates, Sunderland, Mass.

Owens, J.N., and Blake, M.D. 1984. The pollination mechanism of Sitka spruce (*Picea sitchensis*). Canadian Journal of Botany. **62:** 1136-1148.

Owens, J.N., and Blake, M.D. 1985. Forest tree seed production: a review of the literature and recommendations for future research. PNFI, Canadian Forestry Service. Information Report PI-X-53.

Owens, J.N., and Bruns, D.M. 2000. Western white pine (*Pinus monticola* Dougl.) reproduction: I. Gametophyte development. Sexual Plant Reproduction, **13:** 61-74.

Owens, J.N., Catalano, G.L., Morris, S.J., and Aitken-Christie, J. 1995. The reproductive biology of kauri (*Agathis australis*). I. Pollination and prefertilization development. International Journal of Plant Science, **156:** 257-269.

Owens, J.N., and Molder, M. 1974. Pollination, female gametophyte and seed development of yellow cedar (*Chamaecyparis nootkatensis*). Canadian Journal of Botany, **53:** 186-189.

Owens, J.N., and Molder, M. 1977a. Seed-cone differentiation and sexual reproduction in western white pine (*Pinus monticola*). Canadian Journal of Botany, **55:** 2574-2590.

Owens, J.N., and Molder, M. 1977b. Sexual reproduction of *Abies amabilis*. Canadian Journal of Botany, **55:** 2653-2667.

Owens, J.N., and Molder, M. 1979a. Sexual reproduction of *Larix occidentalis*. Canadian Journal of Botany, **57:** 2673-2690.

Owens, J.N., and Molder, M. 1979b. Sexual reproduction of white spruce (*Picea glauca*). Canadian Journal of Botany, **57:** 152-169.

Owens, J.N., and Molder, M. 1984a. The reproductive cycles of Western redcedar and Yellow-cedar. Ministry of Forests, British Columbia.

Owens, J.N., and Molder, M. 1984b. The reproductive cycle of Interior spruce. British Columbia Ministry of Forests, Forestry Division, Research Branch, Victoria.

Owens, J.N., and Morris, S.J. 1990. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. I. Pollen tube and archegonial development. American Journal of Botany, **77:** 433-445.

Owens, J.N., and Morris, S.J. 1991. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. II. Fertilization and proembryo development. American Journal of Botany, **78:** 1515-1527.

Owens, J.N., Morris, S.J., and Misra, S. 1993. The ultrastructural, histochemical, and biochemical development of the post-fertilization megagametophyte and the zygotic embryo of *Pseudotsuga menziesii*. Canadian Journal of Forest Research, **23:** 816-827.

Owens, J.N., Takaso, T., and Runions, C.J. 1998. Pollination in conifers. Trends in Plant Science, **3:** 479-485.

Park, Y.S., Bonga, J.M., and Mullin, T.J. 1998. Clonal forestry. *In* Forest genetics and tree breeding. *Edited by* Mandel, A.K., and Gibson, G.L. CBS Publishers, Daryanganj, New Delhi, pp. 143-167.

Pattinavibool, R. 1996. Somatic cell genetics in larch (*Larix* spp.). Ph.D. thesis. University of Victoria, Victoria, British Columbia, Canada.

Pattinavibool, R., von Aderkas, P., Hahnhijärvi, A., Simola, L.K., and Bonga, J.M. 1995. Diploidization in megagametophyte-derived cultures of the gymnosperm *Larix decidua*. Theoretical and Applied Genetics, **90:** 671-674.

Pennell, R.I., and Bell, P.R. 1985. Microsporogenesis in *Taxus baccata* L.: the development of the archaesporium. Annals of Botany, **56:** 415-427.

Pennell, R.I., and Bell, P.R. 1986a. The development of the male gametophyte and spermatogenesis in *Taxus baccata* L. Proceedings of the Royal Society of London, **228:** 85-96.

Pennell, R.I., and Bell, P.R. 1986b. Microsporogenesis in *Taxus baccata* L.: the formation of the tetrad and development of the microspores. Annals of Botany, **57:** 545-555.

Pennell, R.I., and Bell, P.R. 1987. Megasporogenesis and the subsequent cell lineage within the ovule of *Taxus baccata* L. Annals of Botany, **59:** 693-704.

Pettitt, J.M. 1977. Detection in primitive gymnosperms of proteins and glycoproteins of possible significance in reproduction. Nature, **266:** 530-532.

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

Pichot, C., and Maatoui, M. 1997. Flow cytometric evidence for multiple ploidy levels in the endosperm of some gymnosperm species. Theoretical and Applied Genetics, **94:** 865-870.

Pilger, R. 1926. Gymnospermae. *In* Die Natürlichen Pflanzenfamilien, *Edited by* Engler, A., and Prantl, K. Second Edition.

Price, H.J., Sparrow, A.H., and Nauman, A.F. 1973. Correlations between nuclear volume, cell volume and DNA content in meristematic cells of herbaceous angiosperms. Experientia, **29:** 1028-1029.

Quinn, C.J. 1964. Gametophyte development and embryogeny in the Podocarpaceae II: *Dacrydium laxifolium*. Phytomorphology, **15:** 37-43.

Rees, H., Cameron, F.M., Hazarika, M.H., and Jones, G.H. 1966. Nuclear variation between diploid angiosperms. Nature, **211:** 828-830.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology, **7:** 208-212.

Robertson, J.G., and Lyttleton, P. 1982. Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clover. Journal of Cell Science, **58:** 63-78.

Rothwell, G.W. 1972. Evidence of pollen tubes in paleozoic pteridosperms. Science, **175:** 772-774.

Rothwell, G.W. 1977. Evidence for a pollination-drop mechanism in Paleozoic Pteridosperms. Science, **198:** 1251-1252.

Runions, C.J. 1997. An investigation of sexual reproduction in *Picea* (Pinaceae) including prezygotic stages, fertilization, and proembryo formation. Ph.D. thesis, University of Victoria, Victoria, BC, Canada.

Runions, C.J., and Owens, J.N. 1999. Sexual reproduction of interior spruce (Pinaceae). I. Pollen germination to archegonial maturation. International Journal of Plant Science, **160:** 631-640.

Runions, C.J., Rensing, K.H., Takaso, T., and Owens, J.N. 1999. Pollination of *Picea orientalis* (Pinaceae): saccus morphology governs pollen buoyancy. American Journal of Botany, **86:** 190-197.

Russell, S.D. 1992. Double fertilization. International Review of Cytology, **140:** 357-388.

Ruzin, S.E. 1999. Plant microtechnique and microscopy. Oxford University press, Inc. New York.

Ryser, U. 1979. Cotton fiber differentiation: occurrence and distribution of coated vesicles and smooth vesicles during primary and secondary cell wall formation. Protoplasma, **98:** 223-239.

Said, C., Villar, M., and Zandonella, P. 1991. Ovule receptivity and pollen viability in Japanese larch (*Larix leptolepis* Gord.). Silvae Genetica, **40:** 1-6.

Said, C., Zandonella, P., and Villar, M. 1986. Pollen-ovule interactions in *Larix leptolepis*. New data and hypothesis. *In* Biotechnology and ecology of pollen. *Edited by* Mulcahy, D. Mulcahy, G.B. and Ottaviano, E. Springer, Berlin Heidelberg New York, pp. 507-510.

Sedgley, M., and Griffin, A.R. 1989. Sexual reproduction of tree crops. Academic Press, London.

Singh, H. 1961. The life history and systematic position of *Cephalotaxus drupacea* Sieb. Et Zucc. Phytomorphology, **11:** 153-197.

Singh, H. 1978. Embryology of the gymnosperms. Gebrüder Borntraeger, Berlin.

Singh, H., and Chatterjee, J. 1963. A contribution to the life history of *Cryptomeria japonica* D. Don. Phytomorphology, **13:** 449-445.

Singh, H., Kaur, G., and Lal, M. 1976. Some aspects of oogenesis in *Cunninghamia lanceolata*. Journal of Cytology and Genetics, **11:** 112-115.

Sinnott, E.W. 1913. The morphology of the reproductive structures in the Podocarpineae. Annals of Botany, **27:** 39-82

Slobodník, B., and Guttenberger, H. 2000. Ovule, megaspores, and female gametophyte formation in *Larix decidua* Mill. (Pinaceae). Acta Biologica Cracoviensia Series Botanica, **42:**93-100

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research, **26:** 31-43.

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

Stefanovic, S., Jager, M., Deutsch, J., Broutin, J., and Masselot, M. 1998. Phylogenetic relationships of conifers inferred from partial 28S rRNA gene sequences. American Journal of Botany, **85:** 688-697.

Sterling, C. 1963. Structure of the male gametophyte in gymnosperms. Biological Reviews, **38:** 167-203.

Takaso, T. 1996. Pollination mechanisms in conifers. Acta Phytotaxonomica Et Gebotanica, **47:** 253-269.

Takaso, T., and Owens, J.N. 1994. Effects of ovular secretions on pollen in *Pseudotsuga menziesii* (Pinaceae). American Journal of Botany, **81:** 504-513.

Takaso, T., and Owens J.N. 1995a. Pollination drop and microdrop secretions in *Cedrus*. International Journal of Plant Science, **156:** 640-649.

Takaso, T., and Owens, J.N. 1995b. Ovulate cone morphology and pollination in *Pseudotsuga* and *Cedrus*. International Journal of Plant Research, **156:** 630-639.

Takaso, T., and Owens, J.N. 1997. Pollen movement in the micropyles canal of *Larix* and its simulation. Journal of Plant Research, **110:** 259-264.

Takaso, T., von Aderkas, P., and Owens, J.N. 1996. Prefertilization events in ovules of *Pseudotsuga*: ovular secretions and its influence on pollen tubes. Canadian Journal of Botany, **74:** 1214-1219.

Taylor, T.N. 1982. Reproductive biology in early seed plants. Bioscience, **32:** 23-28.

Tison, A. 1911. Remarques sur les gouttelettes des ovules de conifères. Memoires de la Societe Linneenne de Normandie. **24:** 51-61.

Tomlinson, P.B. 1994. Functional morphology of saccate pollen in conifers with special reference to Podocarpaceae. International Journal of Plant Science, **155:** 699-715.

Tomlinson, P.B., Braggins, J.E., and Rattenbury, J.A. 1991. Pollination drop in relation to cone morphology in Podocarpaceae: a novel reproductive mechanism. American Journal of Botany, **78:** 1289-1303.

Tomlinson, P.B., Braggins, J.E., and Rattenbury, J.A. 1997. Contrasted capture mechanisms in Phyllocladaceae and certain Podocarpaceae (Coniferales). American Journal of Botany, **84:** 214-223.

Villar, M., Knox, R.B., and Dumas, C. 1984. Effective pollination period and nature of pollen-collecting apparatus in the gymnosperm, *Larix leptolepis*. Annals of Botany, **53:** 270-284.

von Aderkas, P. 1992. Embryogenesis from protoplasts of haploid European larch. Canadian Journal of Forest Research, **22:** 397-402.

von Aderkas, P., and Anderson, P. 1993. Aneuploidy and polyploidization in haploid tissue cultures of *Larix decidua*. Physiologia Plantarum, **88:** 73-77.

von Aderkas, P., and Bonga, J.M. 1993. Plants from haploid tissue culture of *Larix decidua*. Theoretical and Applied Genetics, **87**: 225-228.

von Aderkas, P., and Leary, C. 1999a. Micropylar exudates in Douglas fir – timing and volume production. Sexual Plant Reproduction, **11:** 354-356.

von Aderkas, P., and Leary, C. 1999b. Ovular secretions in the micropylar canal of larches (*Larix kaempferi* and *L. eurolepis*). Canadian Journal of Botany, **77:** 531-536.

von Aderkas, P., Chiwocha, S., Takaso, T., Dumont-BéBoux, N., Fernando, D., and Owens, J.N. 1997. Towards in vitro fertilization in conifers: gametophyte culture and interaction. In 1997 Biological Sciences Symposium Proceedings, TAPPI, Atlanta, pp. 63-74.

von Aderkas, P., Klimaszewska, K, and Bonga, J.M. 1990. Diploid and haploid embryogenesis in *Larix leptolepis*, *L. decidua* and their reciprocal hybrids. Canadian Journal of Forest Research, **20:** 9-14.

Willemse, M.T.M., and Linskens, H.F. 1969. Dévelopment du microgamétophyte chez le *Pinus sylvestris* entre la méiose et la fécondation. Revue de Cytologie et de Biologie Vegetales - Le Botaniste, **32:** 121-128.

Willson, M.F., and Burley, N. 1983. Mate choice in plants. Tactics, mechanisms, and consequences. Princeton University Press, Princeton, N.J.

Wilson, V.R., and Owens, J.N. 1999. The reproductive biology of Totara (*Podocarpus totara*) (Podocarpaceae). Annals of Botany, **83:** 401-411.

Ziegler, H. 1959. Über die Zusammensetzung des Bestäubungstropfens und den Mechanismus seiner Skretion. Planta, **52:** 587-559.

Zobel, B., and Talbert, J. 1984. Applied Forest Tree Improvement. John Wiley and Sons, Inc. New York.