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Reproductive Biology of Pacific yew (*Taxus brevifolia*)

by

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B.Sc., University of Victoria, 1997

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

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

Taxus brevifolia Nutt., commonly known as Pacific or Western yew, is a conifer native to the Pacific Northwest of North America. Contrary to other *Taxus* species, *T. brevifolia* staminate strobili are usually located on two-year old foliage though they may occur on foliage from one to five years old. This delayed staminate strobilus development may be an adaptation to the low light environment where *T. brevifolia* grows. Microsporogenesis was found to occur in the fall preceding pollination. Isobilateral tetrads were visible as early as mid-October. Over-wintering staminate strobili usually contained separate microspores. In 1996 through 1999, pollination occurred in March and April in two natural forest sites on southern Vancouver Island, British Columbia. Low amounts of airborne pollen and a prolonged pollination period indicated low pollination success within *T. brevifolia.* Female receptivity was measured by the presence of a pollination drop and protandry up to 18 days was observed. *In vitro* pollen germination was moderate to good, ranging from 65% to 88%. DAPI fluorescence staining showed successful male gametophyte development *in vitro*.

The phenology of megasporogenesis and free nuclear mitosis within the megagametophyte was variable and this development occurred anytime between early February until the end of June. One megaspore mother cell developed from the sporogenous tissue and underwent meiosis forming a linear tetrad of megaspores. Though up to three of the megaspores may be functional, the chalazal megaspore developed faster than the others and became the dominant megaspore. Cellularization of the

megagametophyte began in mid-April and continues until early June. The presence of an ephemeral ventral canal nucleus was confirmed. Fertilization was observed in June in 1996. The mature egg cell cytoplasm and sperm structure was used to infer paternal inheritance of plastids and biparental inheritance of mitochondria.

To examine this further, DNA was extracted from hybrid embryos of *T. brevifolia* and *T. x media* Rehd.. Paternal contribution of mitochondria was confirmed using the probe *rp*S14-*cob*. The *T. x media* parents produced two bands of 526 and 970 bp in length, whereas the *T. brevifolia* parents produced only one band (526 bp). The chloroplast probes were not effective at amplifying *Taxus* DNA although appropriate sized bands were produced in *Pinus contorta*.

Proembryos occurred from mid-May to mid-June. Sixteen nuclei were present before cellularization. Early embryos were present from mid-May to mid-August. Simple polyembryony was observed up to the massive embryo stage and differential growth of the embryonal cells was interpreted as incomplete cleavage polyembryony. Mid-embryos were present from mid-June to late August and had a distinct protoderm and focal zone. Late embryos were visible from mid-July onwards. Starch began accumulating at the early embryo stage, whereas, proteins and lipids accumulated in the late embryo stage. The presence of a red aril corresponded to increased amounts of lipid in the megagametophyte cells. Individual seeds matured from July until November. The seed efficiency ranged from 0% to 16% and averaged 5%. Pre-zygotic loss was the most common fate of ovules, followed by post-zygotic loss. Possible causes of this poor seed efficiency are poor pollination success, insect damage or light limitation.

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DEDICATION

Excerpt from Ann Landers newspaper column in the Times Colonist:

"Love is friendship that has caught fire. It is quiet understanding, mutual confidence, sharing and forgiving. It is loyalty through good times and bad times. It settles for less than perfection and makes allowances for human weaknesses.

Love is content with the present, it hopes for the future, and it doesn't brood over the past. It's the day-in and day-put chronicles of irritation, problems, compromises, small disappointments, big victories and working towards common goals."

Thank you D.

Chapter 1

General Introduction

Taxaceae is a conifer family with a circumpolar distribution in the Northern Hemisphere (Dallimore and Jackson 1966). This family is considered an evolutionary mystery as the absence of a compound woody cone has puzzled scientists for decades. Instead, a single seed is borne in the axil of a leaf with a fleshy aril surrounding the seed at maturity. No fossils have been found which explain the development of this deceptively simple structure. Therefore, scientists have had an ongoing debate regarding the taxonomy of Taxaceae. The primary disagreement is whether Taxaceae should be a family within Coniferales or whether it should be elevated to the level of order and renamed Taxales (Pant 2000).

The most widely distributed genus in this family is *Taxus* or the yews. *Taxus* is distinguished from the other Taxaceae by having radially arranged microsporophylls, red arils with no intercalary growth and no foliar resin canals (Price 1990). The red arils and the variable foliage make this genus extremely important to the horticultural industry. In 1966, there were over 71 cultivars of *Taxus* recognized (Dallimore and Jackson 1966).

Recent events have triggered enormous pharmaceutical interest in *Taxus*. In the 1960s, a program screening natural products for anti-cancer activity included *Taxus brevifolia* bark for testing. The National Cancer Institute of the United States measured promising anti-leukemic and anti-tumor activities (Wani *et al.* 1971). The active ingredient, Taxol® (paclitaxel), has a unique mode of action that stabilizes microtubules thereby preventing cell division and causing cell death (Schiff *et al.* 1979). Since then,

taxanes have been found in virtually every *Taxus* species (Parmar *et al.* 1999) and several fungi (*Taxomyces andreanae, Pestalotiopsis microspora, Seimatoantlerium tepuiense, Seimatoantlerium nepalense*) (Stierle *et al.* 1993; Strobel *et al.* 1996, 1999 Bashyal *et al.* 1999).

The study of reproduction is fundamental to the understanding of a species and is useful both to the academic community and to industry. One important industrial application of reproductive knowledge is species regeneration after harvest. Semi-synthesis of taxanes has largely replaced the need to collect bark from wild trees (Nicolaou *et al.* 1996). Nevertheless, as long as the primary method of harvest in British Columbia is clear-cutting, *T. brevifolia* will be removed during harvesting (Campbell and Nicholson 1995). This necessitates research on the reproductive biology of *T. brevifolia* to ensure its regeneration.

Three scientists have made major structural and development contributions to our knowledge of *Taxus* reproduction. Dupler (1917, 1919, 1920) meticulously examined ovulate structures, staminate strobili and gametophytes in *Taxus canadensis*. Pennell and Bell (1985, 1986a, 1986b, 1987, 1988) described microsporogenesis, male gametophyte development, spermatogenesis, megasporogenesis, archegonial development and fertilization in *T. baccata*. Sterling (1948a, 1948b, 1949, 1963) covered the gametophyte development, proembryo development, early embryogeny and embryonic differentiation in *Taxus cuspidata*. Similar research has never been published regarding *Taxus brevifolia*.

Additional biological information on *Taxus* is available from ecological studies by Allison (1987, 1990a, 1990b, 1990c, 1991, 1992, 1993) and DiFazio (DiFazio 1995;

DiFazio *et al.* 1996, 1997, 1998). Allison (1987, 1990a, 1990b, 1990c, 1991, 1992, 1993) described the effects of herbivory and pollen limitation on sex expression and seed production in *T. canadensis*. DiFazio (DiFazio 1995; DiFazio *et al.* 1996, 1997, 1998) examined the strobilus production, seed production, sex expression and growth of *T. brevifolia* under a range of overstory conditions.

This research covers the sexual reproductive cycle of *T. brevifolia* using predominantly structural and developmental techniques. Developmental stages covered include microsporogenesis, pollination, male gametophyte development, megasporogenesis, megagametophyte development, archegonial development, fertilization, cytoplasmic inheritance, embryo development, storage product accumulation, and seed efficiency. Additional *in vitro* research and field monitoring are included to address specific questions regarding the nature of the sperm, the taxonomic placement of *Taxus*, and the limiting factors affecting seed efficiency. Molecular techniques were also used to explore cytoplasmic inheritance within *Taxus*. Background information concerning all of these areas is found in the individual chapter introductions.

Chapter 2

Literature Review

Evolution and Taxonomy

The oldest fossils having uniovulate structures similar to *Taxus* include *Paleotaxus* from the upper Triassic and *Taxus jurassica* from the Jurassic (Florin 1948). More recently, *T. jurassica* was renamed *Marskea*, an extinct genus within Taxaceae (Miller 1977). *Lebachia*, a member of the extinct order Voltziales which is thought to have given rise to the modern female compound strobilus in conifers, has fossils present from the Carboniferous and Permian. It has been proposed that the family Taxaceae has a different ancestor from the other conifers because evolution may occur too slowly for *Paleotaxus* to have formed from *Lebachia* (Florin 1948). The fossil record has no evidence showing the evolution of the uniovulate structure whether it arose from *Lebachia* or another ancestral species (Florin 1948; Keng 1969). Nevertheless, it has been suggested that the uniovulate structure in Taxaceae arose by reduction of the megasporangiate strobilus of Podocarpaceae (Takhtajan 1953). Alternately, a plausible theory showing the development of the uniovulate structure of *Taxus* from *Lebachia* has been proposed (Harris 1976).

The phylogenetic position of Taxaceae remains controversial today. A few scientists advocate the exclusion of Taxaceae from Coniferales (Sahni 1920; Florin 1948, 1954). However, the majority of literature supports the inclusion of Taxaceae within the conifers (Table 1). Developmental, biochemical and molecular studies support the close

Table 1. Chronological perspective on the controversy over the inclusion of Taxaceae within Coniferales.

Reference	Significance
Sahni 1920	Excluded Taxales from Coniferales based on ovule vasculature in *Taxus*, *Torreya* and *Cephalotaxus*.
Florin 1948	Excluded Taxales from Coniferales due to ovulate structure, pollen cone morphology, non-saccate pollen and early appearance in the fossil history.
Takhtajan 1953	Included Taxaceae in Coniferales. Proposed evolution from Podocarpaceae based on reproductive structures.
Harris 1976	Included Taxaceae in Coniferales. Proposed theory explaining the evolution of a *Taxus* ovule from Voltziales.
Hart 1987	Included Taxaceae in Coniferales after analyzing 123 morphological, anatomical, chemical and chromosomal characters.
Raubeson and Jansen 1992	Included Taxaceae in Coniferales using a rare chloroplast DNA structural mutation shared by all conifer families.
Chase et al. 1993	Included Taxaceae in Coniferales after sequencing a plastid gene in 499 seed plants.
Chaw et al. 1993	Included Taxaceae in Coniferales based on comparisons of 18S rRNA sequences of *Taxus, Pinus, Podocarpus* and *Gingko.*
Stefanovic et al. 1998	Included Taxaceae in Coniferales after sequencing a partial 28S rRNA gene in 47 plant species.
Cheng et al. 2000	Included Taxaceae as a sister group to Taxodiaceae/Cupressaceae after sequencing the chloroplast matK gene and nuclear rDNA ITS region in 19 conifer species.
Pant 2000	Included Taxaceae in Coniferales after reviewing morphological and developmental evidence.

relationship of Cephalotaxaceae to Taxaceae (Keng 1969; Hu *et al.* 1986; Hart 1987; Chaw *et al.* 1993, 1995; Stefanovic *et al.* 1998; Wolff *et al.* 1999; Cheng *et al.* 2000; Pant 2000). Cupressaceae and Taxodiaceae are the next closest relatives to Cephalotaxaceae and Taxaceae, according to comparison of nuclear DNA, chloroplast DNA, ribosomal RNA, pollen grain structure and pollination mechanism (Doyle 1945; Sterling 1963; Chase *et al.* 1993; Stefanovic *et al.* 1998; Cheng *et al.* 2000). Large-scale molecular and structural investigations on the phylogeny of conifers confirm that Coniferales is a monophyletic group, including Taxaceae (Hart 1987; Raubeson and Jansen 1992; Chase *et al.* 1993; Stefanovic *et al.* 1998).

Currently, five genera are included in Taxaceae and they are separated into two tribes. Tribe Torreyeae includes *Amenotaxus* and *Torreya.* Tribe Taxeae includes *Austrotaxus, Pseudotaxus* (previously named *Nothotaxus*) and *Taxus* (Miller 1988; Price 1990; Cheng *et al.* 2000). Distinguishing features of Taxeae from Torreyeae include the absence of foliar resin canals, radially arranged microsporangia and an aril that exhibits no intercalary growth in the former (Price 1990; Cope 1998).

Taxus includes seven to twelve species distinguished primarily by geographical distributions (Dallimore and Jackson 1966; Wilde 1975; Miller 1977; Price 1990; Cope 1998). Dallimore and Jackson (Dallimore and Jackson 1966) list eight species: *T. baccata, T. brevifolia, T. canadensis, T. celebica, T. cuspidata, T. floridana, T. globosa* and *T. wallichiana.* Because of their similar morphology and ability to hybridize, *Taxus* species are occasionally considered several subspecies of one large species of *T. baccata* (Bialobok 1978; Cope 1998). Numerous cultivars and hybrids have been created from *T. baccata, T. canadensis, T. cuspidata* and *T. wallichiana.* Only three cultivars are

recognized in *T. brevifolia* : cv *erecta* (a columnar form), cv *nana* (a dwarf form) and cv *nutallii* (a drooping form) (Taylor and Taylor 1981; Bolsinger and Jaramillo 1990).

Distribution and Ecology

Taxus brevifolia is found scattered in the understory of forests from southern Alaska through British Columbia, Washington and Oregon to northern California. This species grows inland to the Rocky Mountains in British Columbia and to the Lewis Range in Montana. Generally, it grows at low to moderate elevations (Taylor and Taylor 1981; Bolsinger and Jaramillo 1990). *Taxus brevifolia* only forms a dominant forest cover in north central Idaho (Crawford and Johnson 1985; Bolsinger and Jaramillo 1990).

Taxus brevifolia grows in a wide range of climate, moisture and soil types. Sites with *T. brevifolia* usually have long growing seasons with high precipitation and humidity (Taylor and Taylor 1981). Nevertheless, it can be found in warmer and drier climates on streamside areas and north-facing slopes. It grows best on deep, moist or rich, rocky or gravelly soils though it is found on many other soil types (Bolsinger and Jaramillo 1990; Daoust 1992; Campbell and Nicholson 1995).

Numerous plant species are found in association with *T. brevifolia*. In British Columbia, *T. brevifolia* is common in coastal Douglas-fir, coastal western hemlock, and interior cedar-hemlock biogeoclimatic zones. More rarely, it is found in interior Douglas-fir, montane spruce, mountain hemlock and Engelmann spruce-subalpine fir biogeoclimatic zones (Campbell and Nicholson 1995). *Tsuga heterophylla* is the most common species associated with *T. brevifolia* in western Oregon and Washington (Busing *et al.* 1995). *Taxus brevifolia* occurs in the highest abundance and coverage in

old-growth forests where there has been no harvesting or wildfire for several hundred years (Spies 1991).

Taxus brevifolia has a limited ability to acclimate or adapt to natural or human disturbances. Though *T. brevifolia* is shade tolerant, it can acclimate to increased exposure to sun by modifying leaf morphology and decreasing light capture and use (Mitchell 1998). In some cases, *T. brevifolia* will increase basal growth in response to partial canopy removal (Bailey 1997). However, it is susceptible to natural wildfire or broadcast burning after harvest. After a fire, regeneration is slow and depends primarily on seed germination (Busing *et al.* 1995).

Economic Botany

Taxus brevifolia has been used by the native peoples of the Pacific Northwest for thousands of years. The strong and resilient wood was used for many different types of tools (Taylor and Taylor 1981; Bolsinger and Jaramillo 1990; Hartzell 1991; Turner 1998). For example, the Nitinaht people of British Columbia formed yew wood into digging sticks, wedges, needles, bows and even whaling harpoons (Turner *et al.* 1983). Yew wood was used for ceremonial purposes such as scrubber branches for bereaved persons or red paint made from ground yew wood and fish oil (Turner *et al.* 1990; Turner 1998). Many tribes also used needles or bark of *T. brevifolia* in medicines (Taylor and Taylor 1981; Turner *et al.* 1990; Hartzell 1991). The Makah and Nuu-Chah-Nulth tribes crushed the yew needles in hot water producing an astringent bath for elderly people or young children (Taylor and Taylor 1981; Hartzell 1991). The Coast Salish people of British Columbia used a steeped solution of four barks including *T. brevifolia* to treat

ailments of the stomach, digestive tracts, liver, kidney and even tuberculosis (Turner and Hebda 1990).

In the 1960s, a potent anticancer compound was isolated from the bark of *T. brevifolia* (Wani *et al.* 1971). This compound was originally named Taxol®. This name has been registered by the Bristol-Myers Squibb Company so paclitaxel is now the generic name (Seki and Furusaki 1996). Paclitaxel and other related taxanes have a unique mechanism that selectively kills cells such as tumor cells. Taxanes promote microtubule assembly preventing segregation of the chromosomes thereby killing rapidly dividing cells (Schiff *et al.* 1979). In clinical trials, paclitaxel and related taxanes have been shown effective against ovarian, breast, lung, neck, head, and gastrointestinal tract cancers and to a lesser extent malignant melanoma (Borman 1991; Foa *et al.* 1994; Seki and Furusaki 1996). The low supply of taxanes limited the development of cancer treatments for several years. Now taxanes can be commercially produced by semi-synthesis using derivatives from the needles of cultivated *Taxus* species (Denis *et al.* 1988; Nicolaou *et al.* 1996). Handling methods, such as water stressing the cultivated *Taxus* plants, increase the taxane production (Hoffman *et al.* 1999). Other methods including tissue or fungal cultures are also being developed (Jaziri *et al.* 1996).

Taxus brevifolia is still harvested in British Columbia. This limited resource needs careful consideration as bark harvest girdles and kills the tree. Approximately ten large trees are needed to produce one cancer treatment (Hartzell 1991). Furthermore, it is estimated if all trees in a stand greater than 5 cm diameter at breast height are removed, the stand would take at least 200 years to recover (Busing and Spies 1995). Therefore, the Ministry of Forests has created harvest guidelines to maintain *T. brevifolia* populations.

Currently, a permit is required and only areas designated for logging will have bark collection, though limited collections have been recommended in other areas. Stump sprouting increases with stump height and the percentage intact bark (Minore and Weatherly 1996). Therefore, the stump must be at least 15 cm high and the bark surrounding it left intact to promote sprouting (Robson 1991; Campbell and Nicholson 1995). The Forest Service in the United States instated similar regulations where only areas slated to be logged allowed to be harvested (Lowe 1993).

Despite concerns over the harvest of *T. brevifolia*, this species is not considered a threatened or endangered species in British Columbia (Campbell and Nicholson 1995). An inventory on northern Vancouver Island averaged 1.5 *T. brevifolia* trees per hectare (de Jong and Bonnor 1995). Nevertheless, it is considered rare in the United States (Scher 1996). A recent checklist of threatened conifers listed *T. brevifolia* as having a low risk but "near threatened" as it may potentially become vulnerable to extinction in the wild in the medium-term future (Farjon and Page 1999).

Sex Expression

Almost all *Taxus* species, including *T. brevifolia*, are dioecious. Generally, the male to female tree ratio is approximately one (Melzack and Watts 1982; Daoust 1992; DiFazio 1995). Cosexual individuals occasionally occur in *T. brevifolia*, *T. cuspidata*, *T. baccata*, and *T. globosa* (Keen and Chadwick 1954; Chadwick and Keen 1976; Hogg *et al.* 1996). Functionally male trees have the ability to produce ovules but female trees very rarely produce pollen cones (Keen and Chadwick 1954; DiFazio 1995; DiFazio *et al.*

1996). The percentage of cosexual individuals in a *T. brevifolia* population averages 15% and may increase with elevation (DiFazio 1995; DiFazio *et al.* 1996).

Taxus canadensis is the only species within the *Taxus* genus that is monoecious. Sex expression within *T. canadensis* is under partial genetic control and partial environmental control. Deer browsing also affects the proportion of male to female trees and male to female strobili produced on monoecious trees (Allison 1987, 1992). Young trees become male first and have greater flexibility in male to female bud production from year to year than older trees. Often single sex trees are present in a population and monoecious trees tend to be primarily male or female (Allison 1987, 1991; Wilson *et al.* 1996). There is no spatial separation of male and female strobili on the trees (Allison 1987, 1993).

Staminate Development

Pollen cones are initiated in the leaf axils in the year preceding pollination. Greater than 99% of the pollen cones of *T. canadensis* are located on the foliage of the current year (Allison 1987, 1993). The pollen cone apex differentiates in July and microsporophylls are initiated in late August (Dupler 1919). In *T. baccata*, each pollen cone has from 10-12 or up to 15 microsporophylls and the number of microsporangia per microsporophyll is extremely variable ranging from three to eight or four to 11 (Wilde 1975; Pennell and Bell 1985). Microsporangia are peltate and borne in a circle around each microsporophyll axis. This arrangement has been considered very unusual in comparison with the abaxial, dorsiventrally symmetric microsporangia of Pinaceae (Florin 1948). The pollen cone structure in Taxaceae may be quite derived and have

arisen by reduction and fusion from a pollen cone structure similar to Cephalotaxaceae (Takhtajan 1953; Wilde 1975).

Archesporial initiation occurs within the developing pollen cone in the early fall preceding pollination. The tapetal layer is recognizable in *T. baccata* and *T. canadensis* from October onwards. At the same time, the sporogenous cells differentiate into pollen mother cells (Dupler 1919; Pennell and Bell 1985). Dyads can be observed within *T. baccata* by the end of November and tetrads appear one week later. The tetrads soon separate, forming rounded microspores by mid-December (Pennell and Bell 1986a). The microsporangia within a strobilus are not necessarily synchronized during this development (Dupler 1917). Nevertheless, before dormancy all microsporangia contain separate microspores in *T. baccata* and *T. canadensis* (Dupler 1919; Pennell and Bell 1986a). The microspores acquire a thick cell wall and increase in size before anthesis (Dupler 1917; Pennell and Bell 1986a). Concurrently, the tapetum deposits sporopollenin on the outermost layer of the exine (Rohr 1977). The mature pollen in *T. brevifolia* is non-saccate, approximately 21 μm in diameter, and has numerous orbicules of variable sizes (Owens and Simpson 1986).

Once inside the ovule, pollen grains shed their exines and begin to elongate. The first division forming the tube cell and the generative cell occurs 10-12 days after pollination (Dupler 1917). A cell wall keeps the generative nucleus at one end of the pollen tube while the tube nucleus moves down through the elongating pollen tube. This cell wall is ephemeral and degenerates approximately three weeks after germination. The generative nucleus then divides forming the sterile nucleus and the nucleus of the spermatogenous cell. The spermatogenous nucleus acquires a cytoplasm and cell wall de

novo (Pennell and Bell 1986b). The tube nucleus, sterile nucleus and spermatogenous cell remain in close association travelling down in the pollen tube (Dupler 1917; Sterling 1948a; Pennell and Bell 1986b).

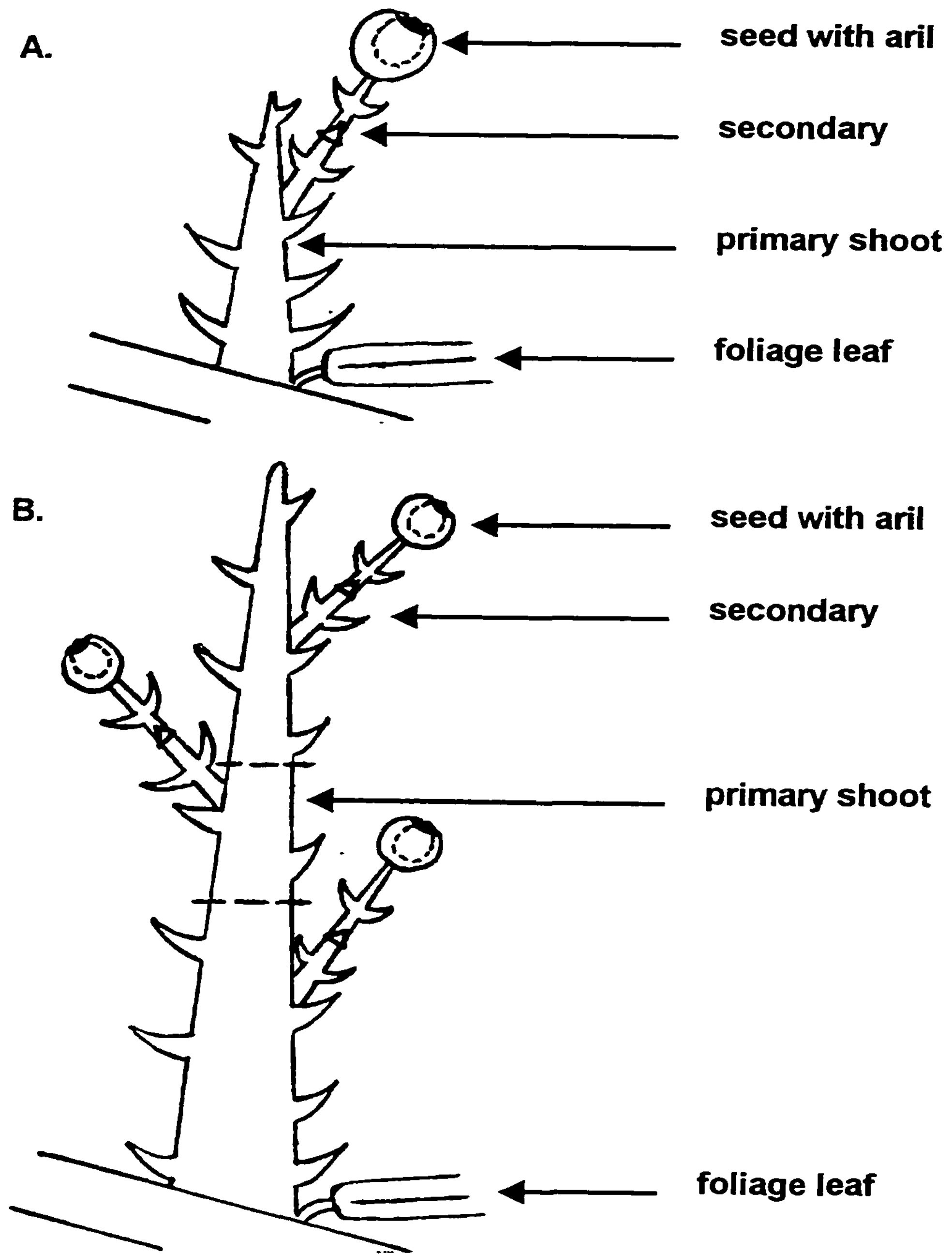
There have been several published accounts of unequal sperm in *Taxus* (Robertson 1907; Dupler 1917; Sterling 1948a; Gianordoli 1974). This misinterpretation is due to the spermatogenous nucleus dividing in one hemisphere of the cell. Equal nuclei with different amounts of cytoplasm are formed before entering the archegonium. A complete cell plate never forms between the sperm except in vitro and the spermatogenous cell wall containing them soon degenerates (Favre-Duchartre 1958, 1960; Rohr 1973; Pennell and Bell 1986b). Thus sperm in *Taxus* are released as equal nuclei (Pennell and Bell 1986b).

Ovulate Development

Ovulate structures occur in the axil of leaves on current and older shoots. In *T. canadensis*, greater than 33% of ovulate structures are found on shoots two years old or older. They are not concentrated in the upper or lower branches in the tree (Allison 1987, 1993). Furthermore, increased amounts of ovulate structures are produced on vigorous shoots and under a more open canopy in *T. brevifolia* (DiFazio 1995; DiFazio *et al.* 1997).

The ovulate structure in *Taxus* is extremely reduced compared with the compound female strobilus found in the majority of conifers. A primary axis is produced in the leaf axils (Fig. 1). This primary axis produces two opposite scales, then several spiral scales.

Figure 1. Diagrammatic representation of the ovulate structure in *Taxus*. Adapted from Andre (1956). A. Mature ovulate structure after one year of growth. B. Mature ovulate structure after three years of growth. Dotted lines delineate each year's growth. Ovules and seeds do not usually remain attached over consecutive years.


A.
seed with aril
secondary
primary shoot
foliage leaf
B.
seed with aril
secondary
primary shoot
foliage leaf

In the axil of one of the uppermost spiral scales, a secondary shoot is initiated. This shoot produces three decussate pairs of scales then a terminal ovule. The following year, the primary shoot may initiate additional scales and a new secondary shoot (Aase 1915; Dupler 1920; Andre 1956; Loze 1965). Over consecutive seasons, this ovulate structure resembles a compound strobilus. One interpretation is that the primary shoot represents the cone axis and all the fertile secondary shoots have been lost except on the uppermost bract (Miller 1988). This ovulate structure was likely first described by Van Tieghem (Van Tieghem 1869), though he believed the ovule was axillary to the last scale initiated on the secondary shoot. Despite some disagreement (Andre 1956), developmental studies have confirmed the terminal nature of the ovule on the secondary shoot (Aase 1915; Dupler 1920; Loze 1965; Shi and Wang 1989). Modifications to the normal ovulate structure involve the primary shoot either producing two or more secondary shoots thus two or more ovules or resuming normal vegetative growth the year following ovule production (Aase 1915; Dupler 1920; Andre 1956; Loze 1965).

Megagametophyte development begins with the differentiation of sporogenous tissue within the center of the nucellus. The sporogenous cells have denser cytoplasm and larger nuclei than the surrounding cells (Sterling 1948a). One and occasionally two megaspore mother cells differentiate. Over-wintering ovules tend to contain megaspore mother cells though other stages are also present (Dupler 1917). The megaspore mother cell undergoes meiosis forming a linear tetrad of megaspores or rarely a T-shaped tetrad (Dupler 1917; Sterling 1948a; Pennell and Bell 1987; Brukhin and Bozhkov 1996). More than one tetrad of megaspores does occasionally occur in *T. canadensis* (Dupler 1917). Generally, the chalazal megaspore of the tetrad of megaspores inherits the greater

proportion of organelles and becomes the functional megaspore. However, the maturation of two, three or even four megaspores does occur rarely in *T. baccata* and *T. cuspidata* and more commonly in *T. canadensis* (Cecchi Fiordi *et al.* 1991). This can result in more than one megagametophyte developing within an ovule (Dupler 1917; Sterling 1948a).

The free nuclear megagametophyte divides seven to eight times before cell walls are laid down (Dupler 1917; Sterling 1948a; Pennell and Bell 1987; Brukhin and Bozhkov 1996). The cell wall formation occurs in a centripetal fashion with no cell divisions until cellurization is complete. This produces long, thin cells, historically called alveoli, within the early cellular megagametophyte (Sterling 1948a). Periclinal cell divisions then occur filling the megagametophyte with cells containing relatively small nuclei. Archegonial initials, recognizable by their larger size, usually occur at the micropylar end of the megagametophyte (Dupler 1917; Sterling 1948a; Pennell and Bell 1987; Brukhin and Bozhkov 1996).

Archegonial initials divide unequally forming a primary neck cell and a central cell. The primary neck cell immediately divides again forming a single tier of neck cells. The central cell becomes vacuolate and the nucleus increases in size (Dupler 1917; Sterling 1948a; Pennell and Bell 1987; Brukhin and Bozhkov 1996). There is some dispute to whether the central cell divides forming an ephemeral ventral canal cell and an egg cell or whether it functions directly as the egg cell. The majority of literature supports the latter view (Robertson 1907; Dupler 1917; Favre-Duchartre 1958; Brukhin and Bozhkov 1996). Nevertheless, this cell division was observed in *T. baccata* and *T. cuspidata*. Unfortunately, it was called a neck canal cell in *T. baccata* so its significance

went unnoticed and it was only observed three times in *T. cuspidata* so it was considered abnormal (Sterling 1948a; Pennell and Bell 1987).

The number of archegonia per megagametophyte varies. *Taxus canadensis* averages four to eight archegonia per megagametophyte (Dupler 1917), whereas three to 17 archegonia have been observed in *Taxus baccata* (Robertson 1907; Favre-Duchartre 1958; Brukhin and Bozhkov 1996). *Taxus cuspidata* usually contains eight to 14 though the range was from six to 25 archegonia (Sterling 1948a).

Jacket cells differentiate around each archegonium. The degree of specialization depends on the number of archegonia present. Jacket cells tend to be smaller cells with larger nuclei compared to the surrounding megagametophyte cells (Sterling 1948a). Often archegonia are in direct contact with each other without intervening jacket cells (Robertson 1907; Dupler 1917; Sterling 1948a; Brukhin and Bozhkov 1996).

Pollination

An eleven-year study showed that *T. baccata* pollen cones are relatively insensitive to the accumulation of temperature sums in the spring (Richard 1985). Nevertheless, a sharp drop in temperature will delay dehiscence. Anthesis generally occurs 100 days ± 5 after the daily mean temperature drops below 10°C in the fall preceding pollination. In Montpellier, France, anthesis of *T. baccata* occurs between late February and late March (Richard 1985). In *T. canadensis* growing in Pennsylvania, anthesis occurs in late April (Dupler 1917). Pollen cones of *T. brevifolia* shed pollen in June in Washington or Oregon or in April or May in British Columbia (Rudolf 1974; Campbell and Nicholson 1995).

The pollination mechanism in *Taxus* involves a pollination drop. This aqueous drop contains of fructose, other sugars and several types of free amino acids (Seridi-Benkaddour and Chesnoy 1988). The phenology of pollination drop production varies among individual trees. Pollination drops have been observed during and after pollen cone anthesis in *T. brevifolia* (DiFazio 1995). The pollination drop increases pollen capture at the micropyle (Niklas 1985). The non-saccate pollen sinks in the pollination drop so the variable orientation of the ovule is puzzling (Doyle 1945). The pollination drop may be retracted soon after pollination as occurs in *Chamaecyparis nootkatensis* (Owens *et al.* 1998). Dew or rain may also help by moving pollen down the sides of the ovules to the micropyle (Niklas 1985).

Fertilization

Fertilization occurs in *T. baccata*, *T. canadensis* and *T. cuspidata* in May or June (Dupler 1917; Sterling 1948b; Favre-Duchartre 1958). The mature archegonium contains a central egg nucleus surrounded by a perinuclear zone of mitochondria and lipid droplets. Plastids are conspicuously absent from the egg cytoplasm as the plastids are modified into large inclusions (Pennell and Bell 1987). The pollen tube digests through the megaspore and egg cell walls and releases the sperm and associated cytoplasm into the egg cell. The functional sperm accompanied by male cytoplasm moves towards the egg nucleus (Pennell and Bell 1988). The other sperm, sterile nucleus and tube nucleus remain at the micropylar end of the archegonium and eventually degenerate (Dupler 1917; Favre-Duchartre 1958). Fusion occurs along many points of contact between the functional sperm and egg (Pennell and Bell 1988).

Cytoplasmic inheritance refers to the relative contributions of the male and female parents of cytoplasmic organelles such as chloroplasts and mitochondria. In *T. baccata*, the maternal plastids are modified into large inclusions so all the chloroplasts in the proembryo are inherited from the male parent. The majority of mitochondria are probably inherited from the female cytoplasm as the perinuclear zone concentrates the maternal mitochondria for participation in the neocytoplasm (Chesnoy 1987b; Pennell and Bell 1988; Mogensen 1996). However, paternal mitochondria are also included in the neocytoplasm so mitochondrial inheritance is biparental (Chesnoy 1987a; Pennell and Bell 1988). This type of cytoplasmic inheritance is similar to that found in Pinaceae (Chesnoy 1987a; Mogensen 1996).

Seed Development

The proembryo undergoes four or rarely five free nuclear divisions. This produces 16 or 32 nuclei that are primarily located in the chalazal one-third of the archegonium. Cell walls then form between these nuclei. The arrangement of the cellular proembryo is not in well-defined tiers. At first, there are two clusters of cells, the embryonal cells and the cells open to the micropylar end of the archegonium (Sugihara 1946; Sterling 1948b; Brukhin and Bozhkov 1996). These cells divide asynchronously producing the suspensor cells (Sugihara 1946). The number of cells in each tier is variable and the distinction between tiers subjective. For example, it has been reported that the dysfunctional suspensor tier is absent, sometimes present or always present (Buchholz 1929; Sterling 1948b; Brukhin and Bozhkov 1996).

Simple polyembryony occurs in *Taxus* (Buchholz 1929). In *T. cuspidata* and *T. baccata*, one type of cleavage polyembryony has also been reported. Primary suspensor cells split off the suspensor system and continue development with a variable number of embryonal cells at the elongating tip (Sterling 1948b; Brukhin and Bozhkov 1996). Another type of cleavage polyembryony has been reported in *T. baccata* where the embryonal cells cleave off forming additional embryos (Brukhin and Bozhkov 1996). In addition, there are reports of embryos forming from the proliferation of dysfunctional suspensor cells. Historically, these are called rosette embryos. There are doubts to whether these rosette embryos should be considered embryos (Sterling 1948b).

The early embryo is pushed into the megagametophyte by the elongation of the primary suspensors. The embryonal cells then divide periclinally producing embryonal tubes that function as secondary suspensors (Sterling 1948b). Concurrently, the embryonal cells divide and increase in number. Cotyledons and a radicle are initiated and the procambial cylinder becomes visible. The embryo will continue to increase in size and mature for three to four months (Sterling 1949; Brukhin and Bozhkov 1996).

Seeds of *T. brevifolia* mature asynchronously from August until October (Rudolf 1974; Bolsinger and Jaramillo 1990; Walters-Vertucci *et al.* 1996). Similarly, seeds of *T. canadensis* mature asynchronously from July until September (Wilson *et al.* 1996). Mature embryos are up to 1.5 mm long with two or rarely three cotyledons (Sterling 1949; Brukhin and Bozhkov 1996). They contain 30% lipids and 3.9% sugars measured by dry weight while megagametophytes contain 71% lipids, 19% proteins and 2.2% sugars measured by dry weight (Walters-Vertucci *et al.* 1996). Mature seed coats are quite hard as the sclerotesta is composed of extremely thick-walled cells (Dupler 1920).

An unusual feature of *Taxus* seeds is the formation of a bright red aril. The aril is a fleshy growth from the base of the developing seed. It forms a cup-like extension surrounding the seed at maturity. The developmental origins of the aril are unknown, though it has been suggested that it is a late-appearing endotesta of the seed coat (Dupler 1920). The red color and fleshy nature of the aril increase seed dispersal and predation by birds and rodents. The birds and rodents cache the seed for food reserves or digest the aril and excrete the undigested seed (Bialobok 1978; Bolsinger and Jaramillo 1990; Wilson *et al.* 1996).

Taxus seeds have a compound dormancy and usually remain in the forest litter for at least two years before germinating (Rudolf 1974; Minore *et al.* 1996). Suggested stratification conditions are warm temperatures for three to seven months then cold temperatures for two to six months (Rudolf 1974; Daoust 1992). Warm temperatures caused the embryo to double in size and the abscisic acid levels to drop. The cold treatment may function by increasing the levels of gibberellins or the sensitivity of the seed to gibberellins (Chien *et al.* 1998). The timing of germination and the germination rates vary among provenances of *T. baccata*. Even with an extensive stratification treatment, the maximum seed germination after 14 months was only 32% (Melzack and Watts 1982). Many of the seeds that do not germinate during the first year may germinate in successive years (Rudolf 1974). Abscisic acid is likely responsible for causing the dormancy in mature seeds as exogenously applied abscisic acid imposed dormancy on excised embryos (Le Page-Degivry 1973). However, seed dormancy can be rapidly broken using excised embryos without applying any abscisic acid. In *T. baccata*, 100%

germination was achieved after leaching the seeds for seven days in water, removing the seed coat, and culturing the excised embryos for seven more days (Zhiri *et al.* 1994).

Reproductive Constraints

Reproductive bud production is affected by a variety of abiotic and biotic factors. Strobilus production in *T. brevifolia* increases with more light availability through the canopy. To a lesser extent branch vigor is also correlated with strobilus production (DiFazio 1995; DiFazio *et al.* 1997). Browsing by white-tailed deer (*Odocoileus virginianus*) significantly reduces strobilus production in *T. canadensis.* Intermediate to high levels of browsing decrease the number of male strobili produced and high levels of browsing decrease the number of female strobili produced (Allison 1987, 1990a). Ungulates are known to browse *T. brevifolia* but the effects on reproduction have not been studied (Campbell and Nicholson 1995). Several species of mites are known to affect young vegetative and reproductive buds in *T. baccata* and *T. brevifolia.* Many of the severely effected buds never develop (Bialobok 1978; Mitchell *et al.* 1997).

Pollination success is extremely variable in *T. canadensis.* Individual trees in a population had pollination successes ranging from 5% to 100% (Wilson *et al.* 1996). Pollination success depends primarily on male strobilus production and nearest neighbor distance (Allison 1987, 1990c). There is speculation that monoecy may have evolved in *T. canadensis* because it was chronically pollen limited and self-pollination may enhance seed set (Allison 1987, 1993). In addition, ungulate browsing on *T. canadensis* has been shown to indirectly limit pollination success thus reducing seed production (Allison 1987, 1990b). In *T. brevifolia*, supplemental pollination doubled seed efficiency. However, this

only increased seed efficiency up to 15%. Overstory openness and vertebrate predation accounted for some of the remaining seed losses (DiFazio 1995; DiFazio *et al.* 1998).

Predation by rodents or birds is a significant factor affecting seed production in *T. baccata*, *T. brevifolia* and *T. canadensis*. Rodents may remove the developing seeds from the branch or eat the seed after it falls to the ground (DiFazio 1995; Hulme 1996; Wilson *et al.* 1996; DiFazio *et al.* 1998). Fallen seeds located under shrubs are particularly prone to rodent predation (Hulme 1996). In addition, birds such as nuthatches (*Sitta*) break open *Taxus* seeds and eat the contents (Bialobok 1978).

Even if a seed escapes predation, microsite availability may limit germination in mature sites (Hulme 1996). The absence of woody, fleshy-fruited shrubs such as *Juniperus* and *Berberis*, shown to act as effective nurse plants, increased seedling mortality from summer drought stress and herbivore damage in southern Spain (Garcia *et al.* 2000). Similarly, disturbances to Himalayan forests have decreased the crown cover of the dominate tree species and the soil nutrient status while increasing the soil pH, thus, making the sites unsuitable for natural regeneration of *T. baccata*. The population structure in the heavily disturbed sites reflects this trend with no *T. baccata* seedlings or saplings present (Rikhari *et al.* 2000).

Chapter 3

Microsporogenesis, pollination, pollen germination and male gametophyte development

Introduction

Taxus brevifolia Nutt., commonly known as Pacific or western yew, grows along the Pacific northwest of North America. This understory conifer was largely ignored until the discovery of a novel cancer drug within the bark called Taxol® (paclitaxel). In addition, the taxonomy of *T. brevifolia* remains controversial as some scientist exclude it from Coniferales. Members of Taxaceae produce an unusual ovulate structure that matures into a single seed with a fleshy aril instead of a compound woody strobilus (Price 1990). All *Taxus* species including *T. brevifolia* are functionally dioecious except for *Taxus canadensis* (Marshall), which is monoecious (Allison 1993).

The phenology and development of microsporogenesis have been described in *Taxus baccata* L. and *T. canadensis* (Dupler 1917; Pennell and Bell 1985, 1986a; Krizo and Korínеková 1989). Microsporogenesis in *T. brevifolia* has never been described and meiosis was assumed to occur in the spring (Owens and Simpson 1986).

Several comprehensive studies cover the pollination ecology of *T. canadensis* and the reproductive ecology of *T. brevifolia* (Allison 1990c, 1993; Wilson *et al.* 1996; DiFazio *et al.* 1998). The focus of these studies was primarily ecological. Wind pollination in *Taxus cuspidata* (Siebold & Zucc.) has been discussed in relation to air disturbance patterns (Niklas 1985). Anthesis of *T. baccata* was related to weather data in Europe (Richard 1985).

The morphology of staminate strobili has been described in *T. baccata* and *T. canadensis* (Dupler 1919; Wilde 1975). Male gametophyte structure and development has been described in *T. baccata*, *T. canadensis* and *T. cuspidata* (Robertson 1907; Dupler 1917; Sterling 1963; Pennell and Bell 1986b). The terminology surrounding male gametophyte development in gymnosperms varies in the literature. This paper follows the terminology proposed by Sterling (1963) except that sperm is used instead of male gamete. This change reduces unnecessary terminology differentiating angiosperms and gymnosperms. The structure of sperm in *T. baccata* has been debated due to controversy over whether they are equal or unequal and whether they are cells or nuclei (Favre-Duchartre 1960; Rohr 1973; Gianordoli 1974; Pennell and Bell 1986b).

There are no known structural or developmental studies published on microsporogenesis, staminate strobili morphology, pollen germination or male gametophyte development in *T. brevifolia*. This information will be useful to maximize seed collections as *T. brevifolia* is currently harvested in British Columbia. The objective of this study is to describe microsporogenesis, pollination, pollen germination and male gametophyte development within this species. This information is compared to other *Taxus* species.

Methods

Two natural forest sites on southern Vancouver Island were selected based on the presence of relatively large *T. brevifolia* trees. Both sites were approximately 1ha in area, at an elevation of 60m and within the coastal Douglas-fir biogeoclimatic zone. All reproductive *T. brevifolia* trees within a portion of the University of Victoria (UVic)

campus and Goldstream Provincial Park (Gold) were surveyed. There were 32 female trees, 31 male trees and one cosexual tree at UVic, whereas Goldstream had 19 female trees, 17 male trees and two cosexual trees. Additional collections to study microsporogenesis were made from another undeveloped forest site near UVic.

Microsporogenesis was examined using pollen squashes and paraffin embedded samples. Two large male trees at each site were selected based on the presence of numerous developing staminate strobili. At least six developing staminate strobili from each of these four trees were collected bimonthly or weekly from September 23, 1998 to December 7, 1998. Branches were wrapped in moist paper towel and placed in a cooler for transportation to the laboratory. Using a dissecting microscope, the budscales were removed and the median 5mm sections were fixed in formalin-acetic acid-alcohol (FAA). Specimens were embedded in paraffin and stained with safranin-hematoxylin following the procedure detailed in Anderson and Owens (1999). At least two more developing staminate strobili from each of these four trees were collected weekly or biweekly from September until December in 1998 and 1999. These cones were squashed, stained with aceto-carmine and viewed using a compound microscope. As aceto-carmine stains the chromatin red, it allowed rapid determination of the phenology within the male strobili.

Pollination was monitored from 1996-1999 at UVic and 1997-1999 at Goldstream. Microscope slides were coated with petroleum jelly and mounted on one of two wind vanes at each site. Each slide was mounted vertically with a horizontal cover protecting it from precipitation and a triangular tail that directed the slide into the wind. The wind vanes were mounted on 3 m poles and positioned at either end of the two approximately rectangular shaped sites. The slides were changed biweekly from February

until May. The aerial pollen collected within the 1cm^2 marked area was identified and counted using a compound microscope. All temperature data was obtained from an automated weather station on the University of Victoria campus. In the degree day calculations, the threshold temperature used was 5°C and the start date was January 1 in all four years. The number of days with an average temperature >5°C was also calculated as another measure of phenology.

Mature *T. brevifolia* pollen was dusted on aluminum SEM stubs and gold coated before viewing with a JEOL JS M-35 SEM at 15 kV. The budscales of *T. brevifolia* staminate strobili were removed and strobili were mounted without further preparations onto carbon tabs. They were viewed using a Hitachi S-3500N SEM at 10 kV.

Female receptivity was noted by the presence of pollination drops on two large female *T. brevifolia* trees at each site. The presence of pollination drops was recorded biweekly from February until May from 1996-1999 at UVic and 1997-1999 at Goldstream.

Pollen from four male trees was germinated *in vitro* in 1998 and 1999. Thirty to sixty unshed staminate strobili were collected per tree, surface sterilized and allowed to shed under sterile conditions following the procedure described by Fernando et al. (1997). Pollen was plated on 8% sucrose Brewbaker and Kwack (1963) media, incubated at 24°C in continuous darkness. In both years, there were three petri dishes plated per tree and 109-206 pollen grains were counted per petri dish. In 1998, germination was counted every two days and germination plateaued between eight to 12 days of culture. Therefore, germination was assessed only at day 10 and day 12 in 1999. For both years, the germination percentage data and the standard errors were calculated for day 12. An

analysis of variance (ANOVA) using SPSS software was done to find the significant factors influencing pollen germination. The assumptions underlying ANOVA are random sampling, independence of errors, homogeneity of variance and normality of errors (Health 1995). It was necessary to transform the germination percentage data using the arcsine of the square root of the proportion to conform to the normality assumption. The following linear model was used with all factors considered fixed, except the error term.

$$Y_{ijkl} = \mu + D_i + S_j + DS_{ij} + T/S_{(j)k} + DT/S_{i(j)k} + e_{(ijk)l}$$

where Y_{ijkl} = transformed germination percentage

μ = mean transformed germination percentage

D_i = effect of the ith year of collection (i=1,2)

S_j = effect of the jth site (j=1,2)

DS_{ij} = effect of the year by site interaction

$T/S_{(j)k}$ = effect of the tree nested within site (k=1,2)

$DT/S_{i(j)k}$ = effect of the year by tree within site interaction

$e_{(ijk)l}$ = experimental error (l=1,2,3)

In vitro male gametophyte development was examined using germinating pollen grown under the same conditions as describe above. Every two to seven days, germinating pollen was fixed in 4% paraformaldehyde for 20 min, rinsed three times in phosphate buffered saline for 15 min and stained with 1 x 10^{-4}% DAPI (4',6-diamidino-2-phenylindole) fluorescent stain for 5 min. Male gametophytes were examined and photographed using a compound fluorescence microscope.

Observations

Developing staminate strobili were recognizable in the axils of the leaves in the fall preceding pollination. Developing staminate strobili were usually present on two-year old foliage while one-year old foliage contained potentially staminate meristems (Figs. 2, 3). Latent meristems were also observed on foliage older than one-year old. These are assumed to develop in consecutive years because mature staminate strobili were observed on up to five-year old foliage. In addition, developing staminate strobili were occasionally observed on one-year old foliage.

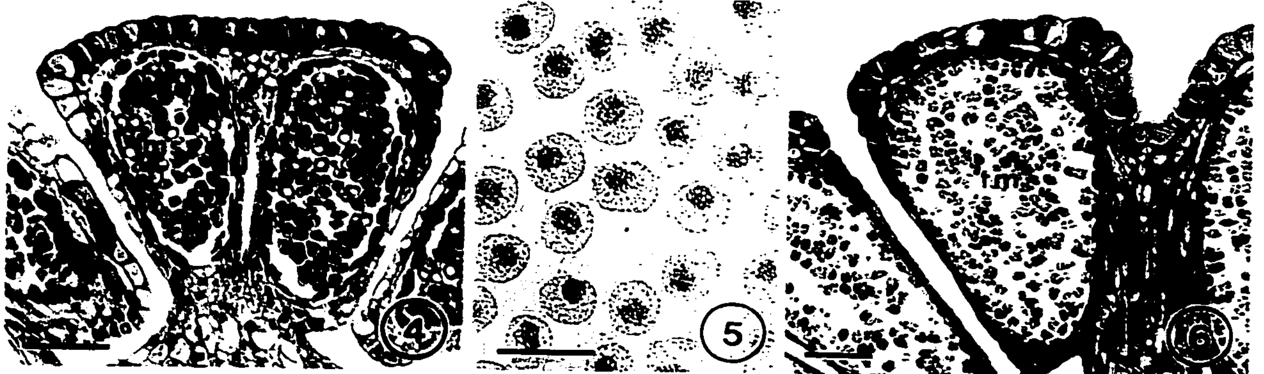
Microsporocytes were recognizable when their cell walls rounded up and their chromosomes condensed (Figs. 4, 5). Meiotic divisions were synchronous within individual microsporangia but not within the entire strobilus. First dyads formed, followed by tetrads of microspores (Figs. 6, 7). The cell walls of the microspores thickened and the tetrads of microspores eventually separated (Figs. 8, 9). Binucleate tapetal cells were visible as early as the microsporocyte stage onwards (Fig. 10).

Microsporogenesis occurs in the fall preceding pollination (Fig. 11). In 1998, sporogenous tissue was recognizable in late September. Meiosis occurred in late October and early November. By early December the majority of staminate strobili contained separated microspores. The aceto-carmine squashes confirmed similar phenology in 1999 except meiosis at Goldstream began in mid-November rather than mid-October.

Mature staminate strobili contained nine to eleven microsporophylls each with four to six peltate microsporangia arranged around the tip of each microsporophyll (Fig.12). Immediately prior to anthesis, the axis of the staminate strobilus extended, pushing the microsporophylls out of the decussate budscales (Fig. 13). The epidermis

Figures 2-10. Staminate strobilus morphology and microsporogenesis in *T. brevifolia*. (bars for all paraffin embedded sections = 100µm and bars for all aceto-carmine squashes = 50µm) Fig. 2. Branch with developing staminate strobili (ds) on two-year old foliage and potentially staminate buds (pb) on one-year old foliage. Notice the budscale scar (bs) separating the two years growth (bar = 1cm). Fig. 3. Median longitudinal section showing the meristem of a potentially staminate bud. Figs. 4-5. Microsporocytes (ms) had condensed chromosomes as early as mid-October. Figs. 6-7. The formation of tetrads of microspores (tm) was synchronous within each microsporangium. Figs. 8-9. Staminate strobili typically contain separate microspores (sm) before dormancy. Separate microspores artificially clump together after dehydration and paraffin embedment. Fig. 10. A tangential section through the tapetum showing binucleate cells (bc).

pb
bc
ds
2
pb
3



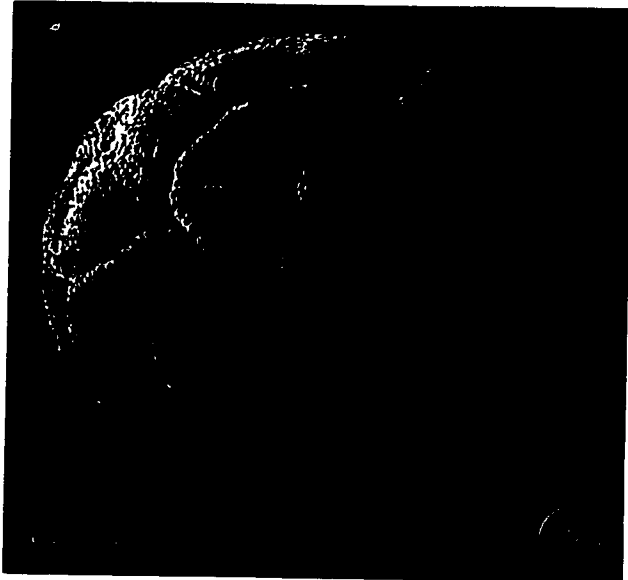
4
5
6

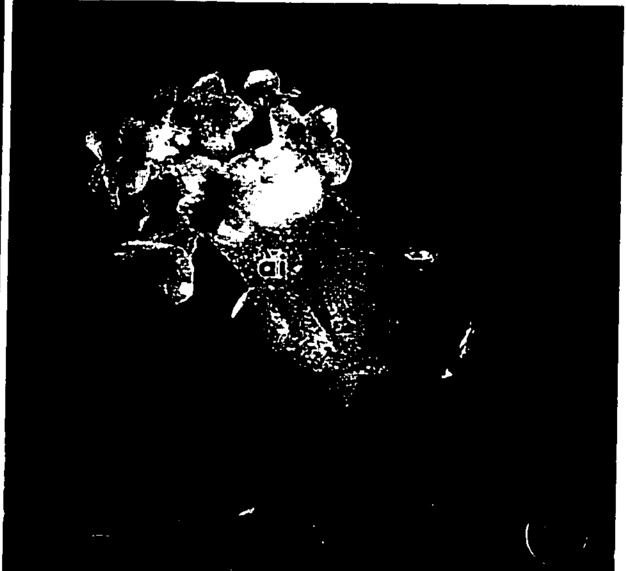
7
8
9
bc
10

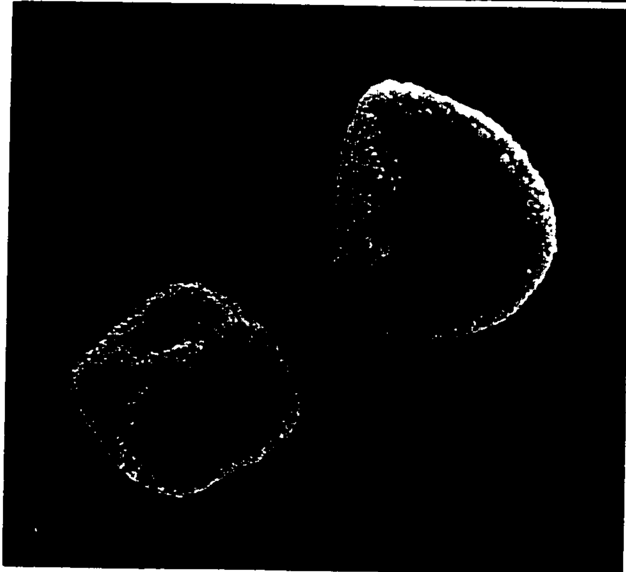
Figure 11. Phenology of 193 *Taxus brevifolia* staminate strobili collected bimonthly or weekly from September 23 to December 7, 1998, at UVic and October 15 to December 7, 1998, at Goldstream. When more than one stage was present within a staminate strobilus, only the most advanced stage was included in this figure.

SITE	STAGES	SEP	OCT	NOV	DEC
UVIC	SPOROGENOUS CELLS				
	MICROSPORE MOTHER CELLS				
	MEIOSIS				
	MICROSPORES				
GOLD	SPOROGENOUS CELLS				
	MICROSPORE MOTHER CELLS				
	MEIOSIS				
	MICROSPORES				

Figures 12-15. Mature *T. brevifolia* reproductive structures. Fig. 12. SEM of a staminate strobilus one month prior to anthesis, showing the surface of microsporophylls. One typical microsporophyll has four microsporangia arranged around the tip of a microsporophyll (bar = 2mm). Fig. 13. Staminate strobilus during anthesis. The central axis (ai) of the male strobilus elongated and pushed the microsporophylls out of the budscales (b). (bar = 2mm). Fig. 14. SEM of the non-saccate pollen covered with orbicules (bar = 20μm). Fig. 15. The underside of a branch with an ovulate structure. A single ovule (o), protected by budscales (b), produces a prominent pollination drop (d) (bar = 2mm).







connecting the underside of the microsporangia to the microsporophyll axis ruptured allowing pollen release. Mature pollen was approximately 25 μm in diameter, non-saccate with numerous orbicules (Fig. 14). The ovules exuded prominent pollination drops (Fig. 15). The orientation of the ovules appeared upright, oblique or inverted with no consistent orientation.

Airborne pollen was captured during March and April in 1996-1999 at UVic and in 1997-1999 at Goldstream (Fig. 16). Anthesis lasted between 20 to 44 days. The density of pollen collected on the slides was low, with a maximum value of 55 grains/cm^2 collected from March 21-25, 1996. The range of degree days and number of days >5°C to pollination and the peak of pollination was large with the minimum figures occurring in 1996 and the maximum figures occurring in 1999 (Table 2).

Other *Taxus* pollen is virtually indistinguishable from *T. brevifolia* pollen. Therefore, ornamental *T. x media* near UVic was monitored for anthesis. Anthesis *T. x media* occurred before *T. brevifolia* anthesis in all the years studied. No sources of *Taxus* pollen were known at Goldstream. It is assumed that there are extremely low amounts of regionally dispersed *Taxus* pollen as the majority of ornamentally planted individuals are female.

Female receptivity was observed in March and April (Fig. 17). Pollination drops were observed starting the same day as airborne pollen or up to 18 days later indicating a slight protandry. The range of degree days and number of days >5°C to the beginning of pollination drop production was also large with the minimum figures occurring in 1996 and the maximum figures occurring in 1999 (Table 2). Individual ovules produced pollination drops for up to two weeks with the maximum volume observed in the early

Figures 16-17. Pollination and female receptivity. Fig. 16. Periods when aerial pollen was captured are marked. Pollination occurred in March and April, lasting between 20 and 44 days. Fig. 17. Pollination drop production. Female receptivity showed protandry of up to 18 days.

Fig. 16.

YEAR	SITE	MARCH	APRIL
1996	UVIC		
1997	UVIC		
	GOLD		
1998	UVIC		
	GOLD		
1999	UVIC		
	GOLD		

Fig. 17.

YEAR	SITE	MARCH	APRIL
1996	UVIC		
1997	UVIC		
	GOLD		
1998	UVIC		
	GOLD		
1999	UVIC		
	GOLD		

Table 2. Degree days and the number of days >5°C until the beginning of pollen capture, the period of maximum pollen capture and pollination drop production. For both calculations, the start date was January 1 and the threshold temperature was 5°C.

Year	Values	Beginning Of Pollen Capture	Maximum Pollen Capture	Pollination Drop Production
1996	Degree Days	33.7	105.8	76.3
	Days >5°C	35	52	45
1997	Degree Days	99.1	141.0	131.5
	Days >5°C	55	68	65
1998	Degree Days	141.0	141.0	141.0
	Days >5°C	54	54	54
1999	Degree Days	179.0	180.3+[a]	179.0
	Days >5°C	75	76+[a]	75

[a] The temperature datum for April 1999 was not available so both of these values are underestimates.

morning. However, when pollination drops were withdrawn within 30 min of hand pollination, pollinated ovules produced no further drops in consecutive days.

Success of *in vitro* pollen germination ranged from 65.1% to 84.0% in 1998 and 72.0% to 85.9 % in 1999 (Table 3). Tree #1 from Goldstream had the highest pollen germination rates in both years, whereas, tree #7 from Goldstream had the lowest pollen germination rate in 1998 and tree #8 from UVic had the lowest pollen germination rate in 1999. The ANOVA showed four factors contributing to mean pollen germination percentage (Table 4). The tree nested within site was an extremely important source of variance, followed by the year, the interaction between site and year, and the interaction between year and tree nested within site.

Soon after hydration, mature pollen shed their exines (Fig. 18). Within three days of *in vitro* culture, many microspores had divided formed a generative cell and tube cell (Fig.19). The generative cell remained within one end of the elongating pollen tube until at least day 17. Then the cell wall separating the generative cell from the tube cell dissolved and the generative nucleus divided to form the sterile nucleus and spermatogenous nucleus (Fig. 20). This division was relatively equal though the spermatogenous nucleus increased in size following mitosis while the sterile nucleus remained the same size. By day 24, the spermatogenous nucleus was surrounded by a cell wall while the sterile nucleus remained unchanged. The spermatogenous cell had dense cytoplasm compared to the tube cytoplasm (Fig. 21). In other pollen tubes at day 24, the spermatogenous cell had already divided forming two sperm of equal size (Fig. 22). The sperm shared the remains of the spermatogenous cell cytoplasm. A cell wall isolating them from each other was never observed. An abnormal situation was once observed,

Table 3. Percentage pollen germination after 12 days of culture (mean ± standard error).

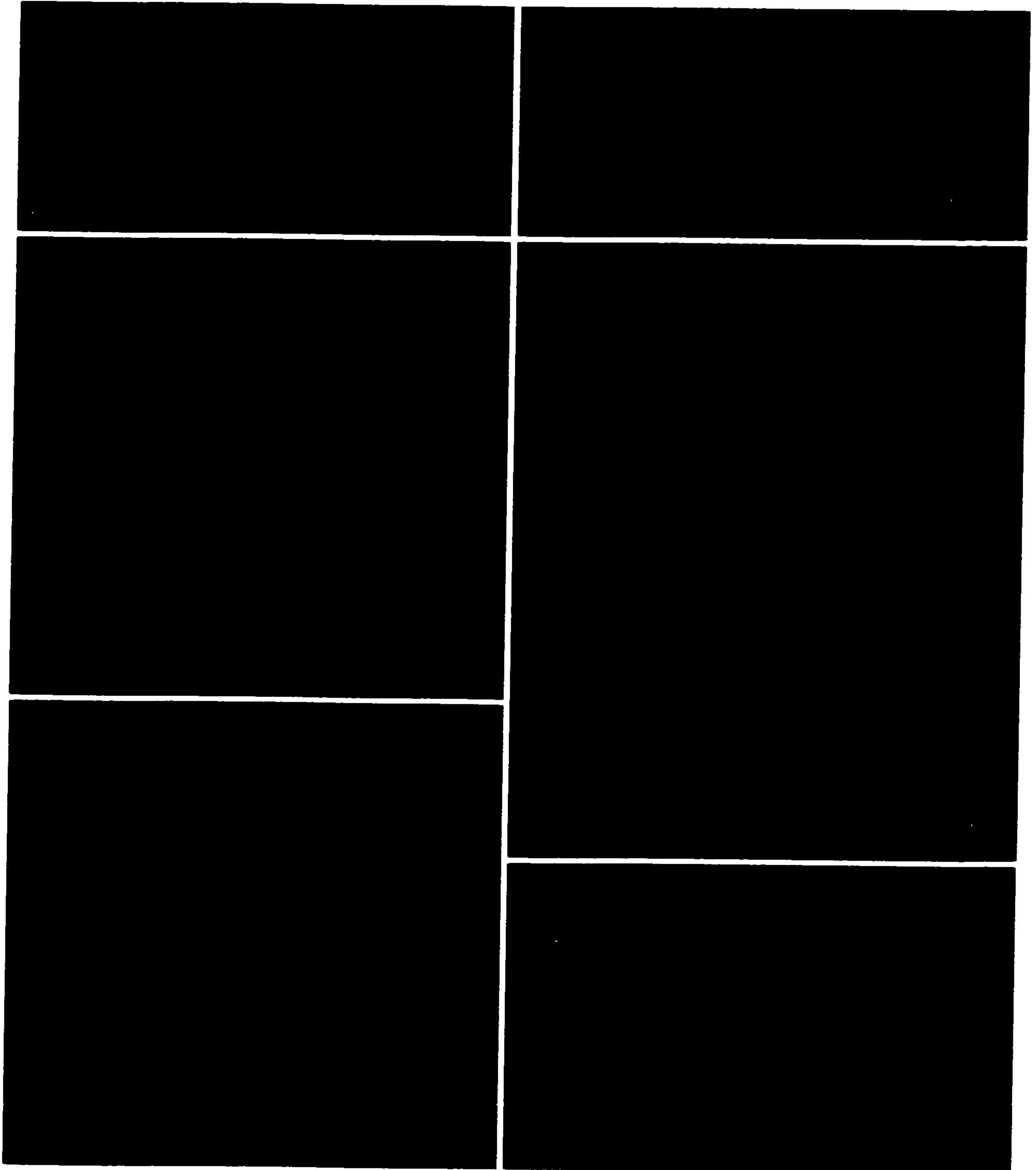
Site & Tree	1998	1999
UVic 8	76.9 ± 3.6	72.0 ± 1.5
UVic 27	80.8 ± 1.4	85.9 ± 2.1
Gold 1	84.0 ± 1.1	88.1 ± 1.2
Gold 7	65.1 ± 4.0	79.3 ± 2.9

Table 4. Results of the nested analysis of variance for the pollen germination rates of four *T. brevifolia* trees growing at UVic and Goldstream in 1998 and 1999.

Source Of Variation	Degrees Of Freedom	Mean Sum Of Squares	F-Ratio	P-Value
Year	1	2.0×10^{-2}	7.70	0.02 [a]
Site	1	3.0×10^{-4}	0.12	0.74
Year By Site Interaction	1	1.6×10^{-2}	6.18	0.03 [a]
Tree Within Site	2	6.2×19^{-2}	23.41	0.00 [a]
Year By Tree Within Site Interaction	2	1.0×10^{-3}	3.79	0.05 [a]
Error	16	2.6×10^{-3}		
Total	23			

[a] Significant at 0.05.

Figures 18-23. DAPI fluorescence of developing male gametophytes. (bar = 50μm for Figs. 18-23). Fig. 18. Microspores quickly expanded and shed their exines (ei). Fig. 19. The tube nucleus (t) and generative cell (g) formed from division of the microspore. Fig. 20. The generative cell divided forming the sterile nucleus (st) and spermatogenous nucleus (sn). Fig. 21. The spermatogenous nucleus acquired cytoplasm and a cell wall, while the sterile nucleus remained associated with it. Fig. 22. The spermatogenous cell divided forming two sperm (sp) of equal size. Fig. 23. Abnormal occurrence of five nuclei within one pollen tube. The extra nucleus (Nu) may have resulted from division of the sterile nucleus or one of the sperm.



presumably due to the prolonged *in vitro* culture. After 24 days in culture, a pollen tube had five nuclei (Fig. 23).

Discussion

Staminate strobili in *T. brevifolia* are located on two-year old foliage. This is contrary to the situation in *T. canadensis* where staminate strobili are almost exclusively on one-year old foliage (Dupler 1919; Allison 1993). However, the description of the broad apices identified as differentiating staminate meristems in *T. canadensis* resembles the potentially staminate apices observed on one-year old foliage in *T. brevifolia* (Dupler 1919). This suggests that *T. brevifolia* initiates the staminate strobili on the current year foliage but these meristems remain dormant until the second year. This could be an adaptation to the low light environment where *T. brevifolia* grows, so the developing staminate strobili have an entire summer to enlarge and develop rather than just the fall. Delayed staminate strobilus development has not been reported before in conifers; however, there is evidence that conifers have modified reproductive cycles in response to their environment. For example, *Chamaecyparis nootkatensis* (D. Don) Spach delays seed maturation for a second year presumably because it grows at higher elevations with shorter growing seasons (Owens and Molder 1975).

Microsporogenesis in *T. brevifolia* has successive divisions forming dyads then tetrads. This type of meiosis produces isobilateral tetrads where all four microspores are in one plane. This description is consistent with observations in *T. baccata* (Pennell and Bell 1986a). During microsporogenesis, tapetal cells in *T. canadensis* were usually uninucleate but not infrequently binucleate (Dupler 1919). *T. baccata* had many

binucleate tapetal cells and some tapetal cells were trinucleate just prior to anthesis (Pennell and Bell 1986a). *T. brevifolia* is similar to *T. baccata* in that it has more binucleate tapetal cells than uninucleate cells though trinucleate tapetal cells were not observed before dormancy.

In Pennsylvania, Michigan and Illinois, *T. canadensis* underwent meiosis in mid-October and over-wintered at the microspore stage (Dupler 1917). In England, meiosis in *T. baccata* was observed in mid- to late November (Pennell and Bell 1985). Only a few cultivated *T. baccata* individuals in central Slovakia underwent meiosis after winter dormancy (Krizo and Korínecková 1989). Therefore, the phenology of microsporogenesis in *Taxus* species in natural habitats occurs in the fall preceding pollination. This is consistent with our results in *T. brevifolia.*

The morphology of the mature staminate strobilus in *T. brevifolia* is similar to that of other *Taxus* species. The number of microsporangia per microsporophyll ranged from four to eight in *T. canadensis*, four to six in *T. brevifolia* and three to 11 in *T. baccata* (Dupler 1919; Wilde 1975; Pennell and Bell 1985). In addition, *T. brevifolia* had nine to 11 microsporophylls per strobilus while *T. baccata* had 10-15 microsporophylls per strobilus (Wilde 1975; Pennell and Bell 1985).

The staminate strobilus in *Taxus* has been interpreted as both primitive and derived. In fact, the radially arranged microsporangia and non-saccate pollen have been used as reasons to exclude Taxaceae from Coniferales (Florin 1948). The staminate strobilus in *Taxus* may be an intermediate form between the radially symmetric microsporophylls in Cordaitales and Ginkgoales and the dorsiventrally flattened microsporophylls in the majority of conifers (Takhtajan 1953). Another hypothesis, based

on a study of vasculature of Cephalotaxaceae and Taxaceae, showed that *Taxus* staminate strobili may be derived by reduction and fusion of a compound staminate strobilus as represented in *Cephalotaxus* (Wilde 1975).

Pollen in *T. baccata* and *T. canadensis* are shed as single cell microspores (Dupler 1917; Krizo and Korínekovà 1989). This was confirmed in *T. brevifolia* pollen. The tapetal cells in *T. baccata* contributed the orbicules of sporopollenin that are obvious on the pollen exine (Pennell and Bell 1986a). It is likely that orbicules have the same origin in *T. brevifolia*.

The pollination mechanism combining a pollination drop with non-saccate pollen occurs in Cupressaceae *sensu lato*, Cephalotaxaceae and Taxaceae (Owens *et al.* 1998). Although *Taxus*, *Cryptomeria*, and *Cupressus* have non-saccate pollen, their ovules are not consistently upright as predicted (Doyle 1945). Instead, rapid withdrawal of the drop after pollination was observed in *C. nootkatensis* (Owens *et al.* 1980) and now in *T. brevifolia*. A study of the mode of pollen deposition on *T. cuspidata* ovules showed that pollen deposition occurred directly on the windward side and by sedimentation on the leeward side. In addition, water may help to move this deposited pollen down to the micropyle or pollen may land directly in the pollination drop (Niklas 1985). Given the large surface area of the drop observed in *T. brevifolia*, it is likely that some pollen lands directly in the drop in *T. brevifolia* also. The pollination drop in *T. baccata* contained sugars and free amino acids (Chesnoy 1993).

Anthesis has been recorded for *T. baccata*, *T. brevifolia*, *T. canadensis* and *T. cuspidata* (Sterling 1948a; Rudolf 1974; Bialobok 1978; Pennell and Bell 1986a; DiFazio *et al.* 1998). It ranged from late February to early March for *T. baccata* in England to June

for *T. brevifolia* in Washington (Pennell and Bell 1986a). Our results are most similar to those found in western Oregon for *T. brevifolia* where it pollinated in March and April (DiFazio *et al.* 1998).

Our study confirmed that *Taxus* pollination can not be accurately predicted using heat units such as degree days or the number of days >5°C. An eleven year study found that *T. baccata* is relatively insensitive to temperature sums (Richard 1985). In Richard's (1985) study, anthesis began 100 days ± 5 after the last day <10°C in the fall preceding pollination (Richard 1985). At UVic, the number of days since the last day <10°C in the fall ranged from 70 days in 1997/1998 to 121 days in 1996/1997. Clearly, pollination of *T. brevifolia* growing on southern Vancouver Island cannot be accurately predicted using Richard's (1985) hypothesis either. This poor correlation between temperature and phenology may be due to the omission of other factors influencing development, such as precipitation and microsite, from the degree day measurements (Wang 1960).

T. canadensis had a rapid anthesis of only 24 hours in some cases (Allison 1993). This is contrary to our results where the duration of anthesis was 20-44 days in *T. brevifolia*. Perhaps this difference is due to more distinct environmental cues at the *T. canadensis* study site. Their study site was a small island in Minnesota with a continental climate. The coastal climate on Vancouver Island is much milder than Minnesota.

A study near Vancouver, British Columbia recorded abundance of wind borne pollen up to 100 grains/cm^2/day produced by *Thuja plicata* (Donn ex D. Don in Lambert) or by *Alnus rubra* (Bong.) (McLennan and Mathews 1984). In the United Kingdom, *T. baccata* produced between 9 grains/cm^2/day to 138 grains/cm^2/day (Proctor *et al.* 1996).

Compared to these studies, *T. brevifolia* produced very low amounts of pollen (maximum 14 grains/cm^2/day).

Pollination success refers to the successful arrival of pollen to the micropyle of an ovule. *T. canadensis* had abundant pollen and pollination success in one study (Dupler 1917), whereas another study measured pollination success ranging from 27% to 94% depending on the site (Wilson *et al.* 1996). The majority (86%) of variation in pollination success has been explained by nearest neighbor distance and pollen production (Allison 1990c). As *T. canadensis* is monoecious, pollination success was also correlated with pollen production (Wilson *et al.* 1996). *T. brevifolia* is dioecious so it cannot self-pollinate like *T. canadensis*. *T. brevifolia* had low amounts of pollen and low densities of individual trees at both UVic and Goldstream. For these reasons, pollination success may be quite low in *T. brevifolia* growing in their natural habitat.

Low pollination success may be a recent problem for *T. brevifolia*. A study of plant macrofossils at Marion Lake, British Columbia suggested that *T. brevifolia* used to be more common before the arrival of *Thuja plicata* approximately 6000 years before present (bp). From approximately 6500 to 5400 years bp *T. brevifolia* needles were as abundant as *Abies amabilis*. However, *T. brevifolia* needles were rarely found since 5400 years bp (Waiman and Mathewes 1987). Perhaps *T. plicata* out-competed *T. brevifolia* as they have similar microsite requirements. In the process, the density of *T. brevifolia* may have reached critically low levels for efficient wind pollination.

Overall, *T. brevifolia* fails to meet several criteria for efficient wind pollination. These criteria include: the production of large amounts of pollen, organization of inflorescence structures designed to trap pollen out of moving air, close spacing of

compatible plants, a low probability of rainfall during anthesis, and unambiguous environmental cues to coordinate flowering (Whitehead 1983). In *T. brevifolia,* low amounts of pollen were observed. No compound ovulate strobilus exists to generate wind eddies to capture pollen. However, the ovulate structure itself does aid in pollen movement towards the micropyle (Niklas 1985). *T. brevifolia* are scattered in the understory so individuals are not closely spaced. Pollination occurs in March and April, months that often have abundant precipitation. Finally, the prolonged pollination window may indicate poor coordination between individual trees. Pollen must be a limiting factor in *T. brevifolia.* Indirect evidence to support this is that supplemental pollination doubled seed set. However, other factors such as light availability and vertebrate predation of seeds must also be important as factors limiting seed set because seed set of hand pollinated ovules is still <15% (DiFazio *et al.* 1998).

In *Picea*, seed set was moderate to good when pollen germination values are between 50% to 80% (Webber 1991). By these standards, pollen germination found in *T. brevifolia* was moderately good (Table 2). The only previous reference to pollen germination in *T. brevifolia* was a supplemental pollination experiment that indicated that only pollen with >90%viability was included (DiFazio *et al.* 1998). The highest pollen germination measured in this study was 88.1% and further comparisons cannot be made without more information about the other pollen viability tests. Pollen germination in this study was strongly affected by both microsite and climate (Table 4).

In *T. baccata*, the exine ruptures quickly due to hydration of the intine (Pennell and Bell 1986b; Chesnoy 1987a). The first mitosis forming the tube and generative cells occurred three to four days in culture in *T. baccata* (Pennell and Bell 1986b). In *T.*

brevifolia, this mitosis was also observed after three days. However, *in vivo* development was longer in *T. canadensis*, as the generative and tube cells were formed after 10-12 days (Dupler 1917). In *T. baccata,* the division of the generative cell was unequal and occurred after 21 days (Pennell and Bell 1986b). The division of the generative cell in *T. brevifolia* produced relatively equal nuclei after 17 days. However, the spermatogenous nucleus increased in size while the sterile nucleus remained the same size. This may explain the difference in interpretation between *T. baccata* and *T. brevifolia.*

In vivo structure of the spermatogenous cell, sterile nucleus and tube nucleus, in *T. brevifolia* and *T. cuspidata,* was identical to that observed in culture. The spermatogenous cell has a cell wall, dense cytoplasm and remains closely associated with the tube nucleus and sterile nucleus (Sterling 1948a; Anderson and Owens 1999). The dense cytoplasm of the spermatogenous cell contained numerous mitochondria and plastids (Favre-Duchartre 1960; Gianordoli 1974; Anderson and Owens 1999). Throughout development *in vivo* the pollen tube continued to grow longer and the tip increased in diameter in *T. canadensis* and *T. cuspidata* (Dupler 1917; Sterling 1948a). The pollen tubes grown *in vitro* continued to elongate but the tip did not swell as dramatically as had been observed *in vivo.* Prior to sperm formation, the spermatogenous cell increased dramatically in size and the number of organelles increased in *T. baccata* (Chesnoy 1987a).

Sperm formation in *Taxus* has been a controversial topic in the literature. The division of the spermatogenous cell is not centered within the spermatogenous cell wall so many authors have interpreted the sperm as unequal cells because as they appear to receive unequal amounts of cytoplasm (Robertson 1907; Dupler 1917; Sterling 1948a).

Though the beginning of a cell plate has been observed in *T. baccata*, there was never a continuous cell wall between the sperm (Favre-Duchartre 1960; Gianordoli 1974; Pennell and Bell 1986b). Recently, *in vivo* studies have shown that the sperm are equal in size and are nuclei in both *T. baccata* and *T. brevifolia*. Both of these sperm remain associated with the spermatogenous cell cytoplasm (Pennell and Bell 1986b; Anderson and Owens 1999). Previous research culturing male gametophytes of *T. baccata* either showed no development after the three nucleate stage or produced abnormal sperm after 25-60 days in culture (Rohr 1973; Pennell and Bell 1986b). There is only one report of successful development *in vitro* in *T. baccata* (Bransheidt (1939) in (Singh 1978)). Our work has confirmed that normal appearing sperm can be produced *in vitro*.

Occasionally, excessive mitoses occur in cultured male gametophytes (Singh 1978). In addition, an extra division of one of the sperm has been observed *in vivo* in *Pseudotaxus chienii* (W.C. Chen) (Chen and Wang 1979). The presence of five nuclei within a pollen tube in *T. brevifolia* is considered abnormal compared to the normal situation of four nuclei within the mature male gametophyte.

Conclusions

This chapter has provided structural and developmental information concerning microsporogenesis, pollination and male gametophyte development in *T. brevifolia*. The discovery of delayed staminate strobilus development warrants further study as a possible adaptation to a low light environment. Investigations into the seed development and production are currently underway as the low pollination success indicates an extremely

low seed set. Finally, the successful production of normal sperm *in vitro* may become important because pollen is a useful vector for gene transfer (Fernando *et al.* 2000).

Chapter 4

Megagametophyte development, fertilization, and cytoplasmic inheritance

Introduction

Taxus brevifolia is commonly known as Pacific or western yew. Its range extends from the southern tip of Alaska through British Columbia to northern California and east to the Rocky mountains. *Taxus brevifolia* is scattered in the understory of many forest types and only forms a dominant forest cover in northern Idaho (Bolsinger and Jaramillo 1990).

The wood is dense with cinnamon-brown heartwood surrounded by pale yellow sapwood. Presently, the use of Pacific yew wood is limited to specialty markets, such as musical instruments and long bows (Hartzell 1991). However, this tree has been used by the Pacific Northwest indigenous peoples for tools and for medicinal purposes for ailments ranging from liver disease to tuberculosis (Turner and Hebda 1990). An effective family of anticancer compounds called taxanes has recently made this species famous. In 1970, a molecule called Taxol® (paclitaxel) was discovered which is effective against several types of cancers, including ovarian, breast, head, and neck tumors (Wani *et al.* 1971). This drug has a unique mechanism which functions by stabilizing microtubules rather than inhibiting their formation like other cancer drugs (Schiff *et al.* 1979; Horwitz *et al.* 1986). Taxanes are currently harvested from natural *T. brevifolia* in British Columbia.

Taxus brevifolia is dioecious and has a single ovule in the axil of a leaf instead of a compound ovulate strobilus normally found in conifers. Occasionally, two ovules

develop in one leaf axil. The mature seed is surrounded by a bright red, fleshy aril (Bolsinger and Jaramillo 1990). These features have caused the taxonomy of this genus to be debated.

Some researchers advocate elevating the Taxaceae family to an order separate from the Coniferales. It has been hypothesized that Taxaceae descended directly from Cordaitales. This theory is based on the position of vascular bundles within the seeds of *T. baccata* (Sahni 1920). Unfortunately, no one has confirmed the presence of a vascular bundle supplying the aril on which this hypothesis was based (Florin 1948). Florin (1948, 1954) separated Taxaceae from the conifers because the earliest Taxaceae fossils occurred in the Triassic and Jurassic. This may have been too early to have been derived from *Lebachia*, the hypothesized ancestor of the compound ovulate strobilus in conifers. *Lebachia* occurred in the Carboniferous and Permian. However, if evolution occurred more quickly during the Paleozoic and Mesozoic, Taxaceae could have evolved from *Lebachia* (Keng 1969). Three major differences of Taxaceae from the conifers were recognized: (1) the terminal, erect uniovulate strobilus, (2) the radially symmetric microsporophylls and (3) the non-saccate pollen (Florin 1948). The male strobilus of Taxaceae could have arisen by reduction and fusion of the male strobilus of Cephalotaxaceae (Wilde 1975), which Florin (1948) considered a conifer. Many conifers including Araucariaceae, Cupressaceae and Taxodiaceae have non-saccate pollen (Doyle 1945). Most recently, Taxaceae was separated from the conifers based on ovulate features which were assigned primitive or derived states and analyzed (Miller 1988), although Miller (1977) himself expressed dissatisfaction with separating Taxaceae outside the conifers.

Other research supports the inclusion of Taxaceae within the conifers. Cephalotaxaceae is often closely linked with Taxaceae. Vegetative characteristics in common include spiral tertiary thickenings on the tracheid walls, whorled juvenile phyllotaxy, spiral mature phyllotaxy, (Hart 1987) and needle peroxidase composition (Hu *et al.* 1986). Reproductive similarities include many aspects of embryogeny (Buchholz 1929; Keng 1969; Wang *et al.* 1979), annular thickenings on the pollen wall, erect ovules (Hart 1987), and seed peptide compositions (Hu *et al.* 1986). Comparisons of the chloroplast DNA (Chaw *et al.* 1993) and 18S rRNA (Chaw *et al.* 1995) yielded remarkable similarities as well. More general investigations confirm the monophyly of conifers. A survey of chloroplast DNA found that all conifers, including Taxaceae, have only one copy of a specific inverted repeat while pteridophytes and non-coniferous seed plants have two copies (except for the angiosperm subfamily Fabaceae) (Raubeson and Jansen 1992). Phylogenetic relationships based on research of partial 28S rRNA support the inclusion of Taxaceae within the conifers (Stefanovic *et al.* 1998). Furthermore, comparisons of 123 morphological, anatomical, chemical and chromosomal characteristics between 63 conifer genera strongly support including Taxaceae in Coniferales (Hart 1987).

The majority of research on the reproductive biology of *Taxus* occurred early in this century; therefore, the majority of observations are limited to light microscopy. Anatomical descriptions of ovules exist for *T. canadensis* (Dupler 1917) and *T. cuspidata* (Sterling 1948a). However, the majority of literature covers the ovulate development in *T. baccata* (Favre-Duchartre 1958; Brukhin and Bozhkov 1996; Pennell and Bell 1987, 1988). Ultrastructural studies on *T. baccata* were done by Pennell and Bell (1987, 1988)

and Cecchi Fiordi et al. (1991). There is no description of megagametophyte development and fertilization in *T. brevifolia*.

The origins of mitochondria and plastids are variable within the conifer families. Cytoplasmic inheritance is not exclusively maternal as in the majority of angiosperms (Mogensen 1996). Cupressaceae and Taxodiaceae have exclusively paternal inheritance of organelles, whereas, Pinaceae has paternal inheritance of plastids and primarily maternal inheritance of mitochondria. Ultrastructural work in Taxaceae has indicated paternal inheritance of plastids and biparental inheritance of mitochondria (Chesnoy 1987b). The absence of plastids in the mature egg cell is associated with the presence of large inclusions, which are modified plastids that have enlarged and engulfed egg cell cytoplasm. If modified plastids are present there is no maternal inheritance of plastids (Camefort 1963, 1965, 1968). Conifer families that have sperm cells typically have exclusively paternal inheritance of all organelles, whereas, families with sperm as nuclei have biparental inheritance of organelles (Chesnoy 1987b). The presence of a perinuclear zone of mitochondria is also associated with maternal or biparental inheritance of mitochondria. Thus the ultrastructure of the egg cell cytoplasm, sperm and cytoplasm accompanying the sperm can be used to predict maternal or paternal inheritance of organelles (Singh 1978; Chesnoy 1987b; Owens and Morris 1991).

Taxus brevifolia merits study because it is a commercially harvested species of which little is known concerning its reproductive biology. In addition, the unusual ovulate structure has taxonomic and evolutionary implications within the conifers. The main objectives of this study are to determine the phenology, megagametophyte development and the structural mechanisms of cytoplasmic inheritance in *T. brevifolia*.

These observations will be discussed in relation to the inclusion of Taxaceae within the Coniferales.

Methods

Six female trees, large enough for repeated sampling and having accessible branches, were selected from a natural forest in Victoria, British Columbia. Overall, 64 *T. brevifolia* trees were clumped within a one hectare area. Of these, 32 were female, 31 were male and one was cosexual. This forest, dominated by *Pseudotsuga menziesii* and *Abies grandis*, is at an elevation of 60 meters and is located south of the University of Victoria campus. Branches bearing ovules were collected weekly from February 8 to June 24, 1996. Weekly collections included at least eight ovules per tree, from at least two trees. The phenology was confirmed in a second year of collections from February 4 to June 3, 1997. Sampled branches were placed in a cool, moist plastic bags or upright in beakers of water then transported to the laboratory for dissection.

Approximately one-fourth of the collected ovules were fixed with minimal dissection in formalin-acetic acid alcohol (FAA) (Johansen 1940) for observations using SEM. For remaining ovules, the budscales were removed and the ovules sliced longitudinally on opposite sides. Five mm thick median sections were fixed in Navashin's fixative (Berlyn and Miksche 1976) for embedment into paraffin and 1-2 mm thick median sections were fixed in glutaraldehyde (Bozzola and Russell 1992) for embedment in Spurr's resin. All specimens were aspirated several times until they sank into the fixative.

FAA-fixed specimens remained in fixative for several weeks at 21°C. They were then rinsed, dehydrated in a graded ethanol series, critical point-dried, mounted on aluminum stubs with silver paste or nail polish, and sputter-coated with gold. These specimens were photographed in a JOEL JS M-35 SEM at 15 kV.

Navashin-fixed specimens also remained in fixative for several weeks at 21°C. They were rinsed, dehydrated in a graded ethanol-tertiary-butyl alcohol series of solutions and embedded in Tissue Prep 2 (Fisher Scientific). The paraffin blocks were softened in a modified Gifford's solution (Gifford 1950) for two or three weeks at 37 °C. Specimens were serially sectioned at 6 µm and mounted on glass slides with a 1% gelatin and 15% glycerol adhesive. The slides were stained with 1% aqueous safranin and 0.5% aqueous hematoxylin, then observed and photographed using light microscopy.

Micrographs of serial sections of early cellular megagametophytes were used to count the number of mitoses within the free nuclear megagametophytes. No mitosis occurs within the cellular megagametophyte until all the cell walls are laid down. Therefore, the early cellular megagametophyte contains the same number of nuclei as the mature free nuclear megagametophyte. Individual nuclei are recognizable by their shape, size, position and cell walls. Individual nuclei were only counted once, though many of the nuclei were present in two micrographs.

Serial sections were also used to estimate pollination success. Germinated *T. brevifolia* pollen grains were identified by the presence of a spermatogenous cell, tube nucleus, and sterile nucleus within the pollen tube. The number of ovules with at least one pollen grain, divided by the overall number of ovules, estimated the pollination success.

Glutaraldehyde-fixed specimens remained at 2°C for a variable period of time until they were post-fixed in 1% osmium. They were then dehydrated in an ethanol series and propylene oxide was gradually substituted for ethanol, embedded in Spurr's resin and hardened for 24 hours at 60°C. These ovules were sectioned using a Lecia Reichert ultracut E ultramicrotome at 0.6-0.8 μm and 60 nm thickness. The thick sections were mounted on glass slides, stained with Richardson's stain (Richardson *et al.* 1960) and observed and photographed using light microscopy. The thin sections were collected on 200 mesh copper grids, stained in 2% aqueous uranyl acetate and 0.2% aqueous lead citrate and photographed using a Hitachi H-7000 TEM.

Observations

At pollination, ovules were found in the axils of a leaves (Fig. 24). Three sets of decussate budscales surrounded and protected the ovule. The integument completely enclosed the ovule forming a small micropyle (Fig. 25). A pollination drop was secreted through this micropyle. Ovules with pollination drops were observed from March 14 to April 28, 1996 and March 25 to April 22, 1997.

Stages from undifferentiated sporogenous tissue, megaspore mother cells (MMC) and free nuclear megagametophytes were visible in the first collections on February 8, 1998 (Fig. 33). Often two or more sporogenous cells resembled early MMCs. However, only one clearly differentiated MMC was observed in each ovule. Differentiated MMCs were identifiable by their central location in the sporogenous tissue and their larger size, up to 50 μm in diameter (Fig. 26). The MMC then underwent meiosis forming a dyad separated by a distinct cell wall, then a tetrad of megaspores.

Figures 24-32. *T. brevifolia* ovules. Fig. 24. Branch with an ovule (o) with the budscales removed, March 2,1996. (Bar = 1 mm). Fig. 25. SEM of an ovule showing the external details of the integument, April 21, 1996. (Bar = 500 μm). Figs. 26-32. LM of *T. brevifolia* ovules embedded in resin or paraffin. Fig. 26. Megaspore mother cell (mm), May 5, 1996. (Bar = 50 μm). Fig. 27. Tetrad of megaspores, May 26, 1996. The chalazal megaspore (c) is the largest. (Bar = 100 μm for Figs. 27-31). Fig. 28. Free nuclear megagametophyte (fn), May 26, 1996. Fig. 29. Cellularization of a megagametophyte. Note the long, thin cells called alveoli (al), April 14, 1996. Fig. 30. Typical cellular megagametophyte (cm) with a pollen tube (pt) containing a spermatogenous cell. A central cell (cc) is visible at the micropylar end of the megagametophyte. Fig. 31. Ovule containing one free nuclear megagametophyte, one cellular megagametophyte, and a pollen tube, June 3, 1996. The pollen tube bypassed the free nuclear megagametophyte in favor of the more developed chalazal megagametophyte. Fig. 32. Ovule containing two free nuclear megagametophytes, one cellular megagametophyte, and a pollen tube, June 4, 1997. The two free nuclear megagametophytes were confirmed by serial sections. The pollen tube has undulations in the wall due to the pressure from the surrounding nucellar cells. Again the pollen tube grew past the micropylar megagametophytes in favor of the chalazal one.

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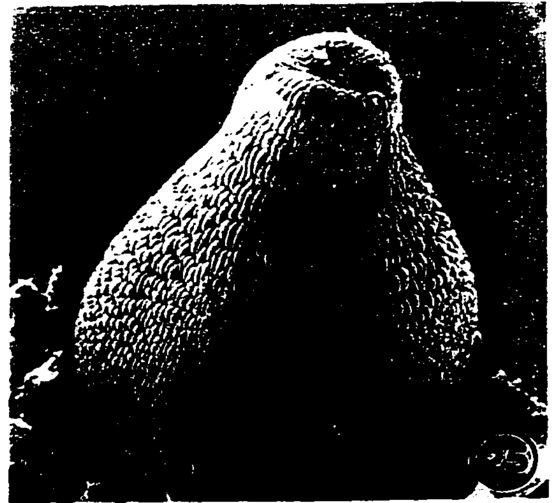
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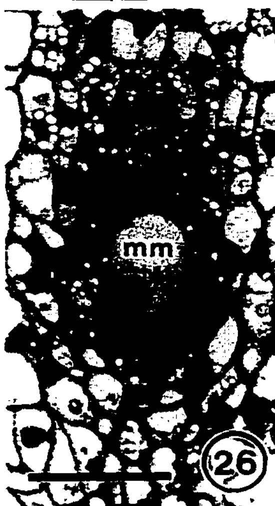
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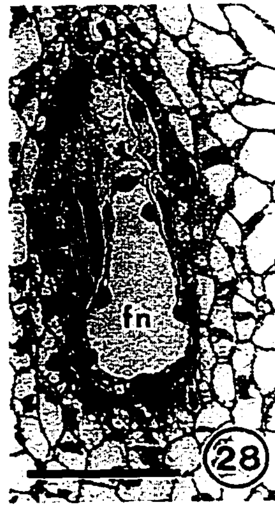
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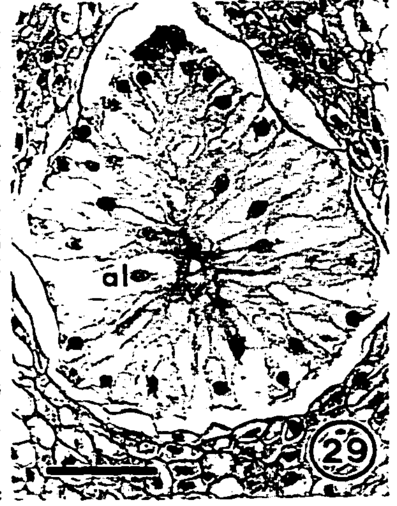
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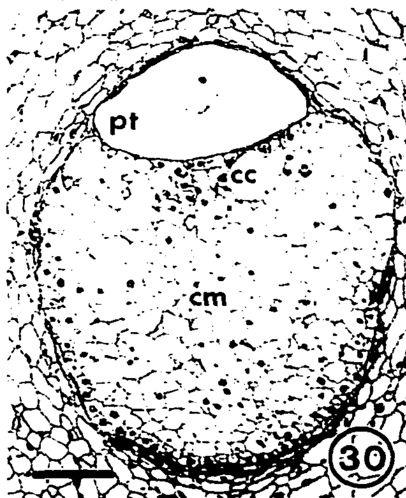
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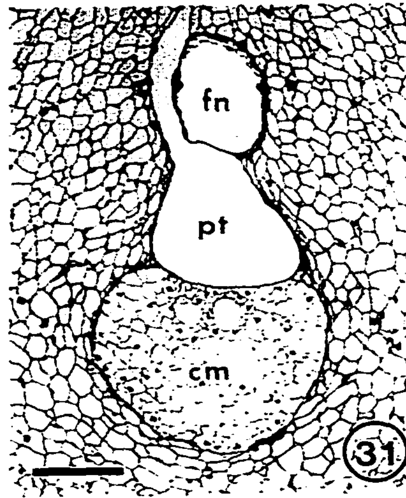
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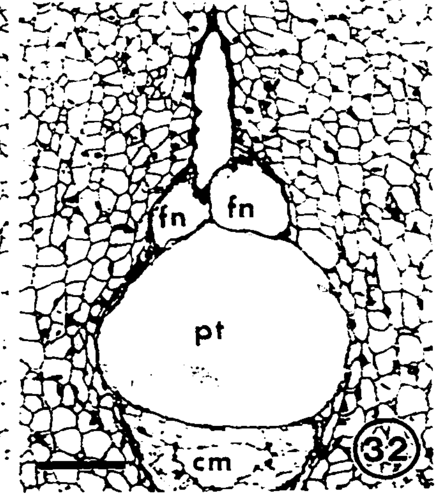
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Figure 33. Phenology of 236 *T. brevifolia* ovules collected weekly from February 8 until June 24, 1996. Megasporogenesis includes stages from differentiation of sporogenesis tissue to tetrads of megaspores. Only pre-zygotic cellular megagametophytes are included in the stage by that name. The dotted lines indicate that the stage was found in the first or last collection. A dotted line begins fertilization because proembryos were observed May 26 even though fertilization itself was not observed until June 3, 1996. When more than one megagametophyte is present, only the chalazal megagametophyte is included.

Months / Stages	FEB	MAR	APR	MAY	JUN
Megasporogenesis					
Free Nuclear Megagametophyte					
Cellular Megagametophyte					
Fertilization					

Tetrads of megaspores were observed on the first collection date, February 8, until June 19, 1996. However, they must occur earlier as free nuclear megagametophytes were also found in early February (Fig. 33). Linear-shaped tetrads were commonly found, though a single T-shaped tetrad was observed. Up to three enlarged megaspores within an ovule were observed. However, the chalazal megaspore was always the largest and differentiated into the functional megagametophyte (Fig. 27).

The most common stage observed from February 8 to June 24, 1996, was the free nuclear megagametophyte. Nuclei were located in the thin peripheral layer of cytoplasm. A large, central vacuole occupied most of the cell (Fig. 28). Cellularization occurred after at least nine simultaneous free nuclear divisions. Cell walls formed in a centripetal direction, creating long, thin cells called alveoli (Fig. 29). Once cellularization was complete, periclinal divisions occurred. Cells located at the chalazal end of the megagametophyte were usually smaller and denser than those elsewhere (Fig. 30).

A single megagametophyte usually formed in each ovule but two megagametophytes were found in 5% of the ovules and three megagametophytes in 1% of the ovules. More than one megagametophyte was due to the enlargement of more than one megaspore from a single tetrad of megaspores because more than one tetrad of megaspores was never seen within an ovule. In cases where there was more than one megagametophyte, the chalazal megagametophyte was always more developed than the micropylar ones. If a pollen tube was present it preferentially grew to the chalazal megagametophyte (Fig. 31, 32).

Archegonia form at the micropylar end of the megagametophyte from archegonial initials recognizable by their larger size compared to the surrounding cells. Archegonial

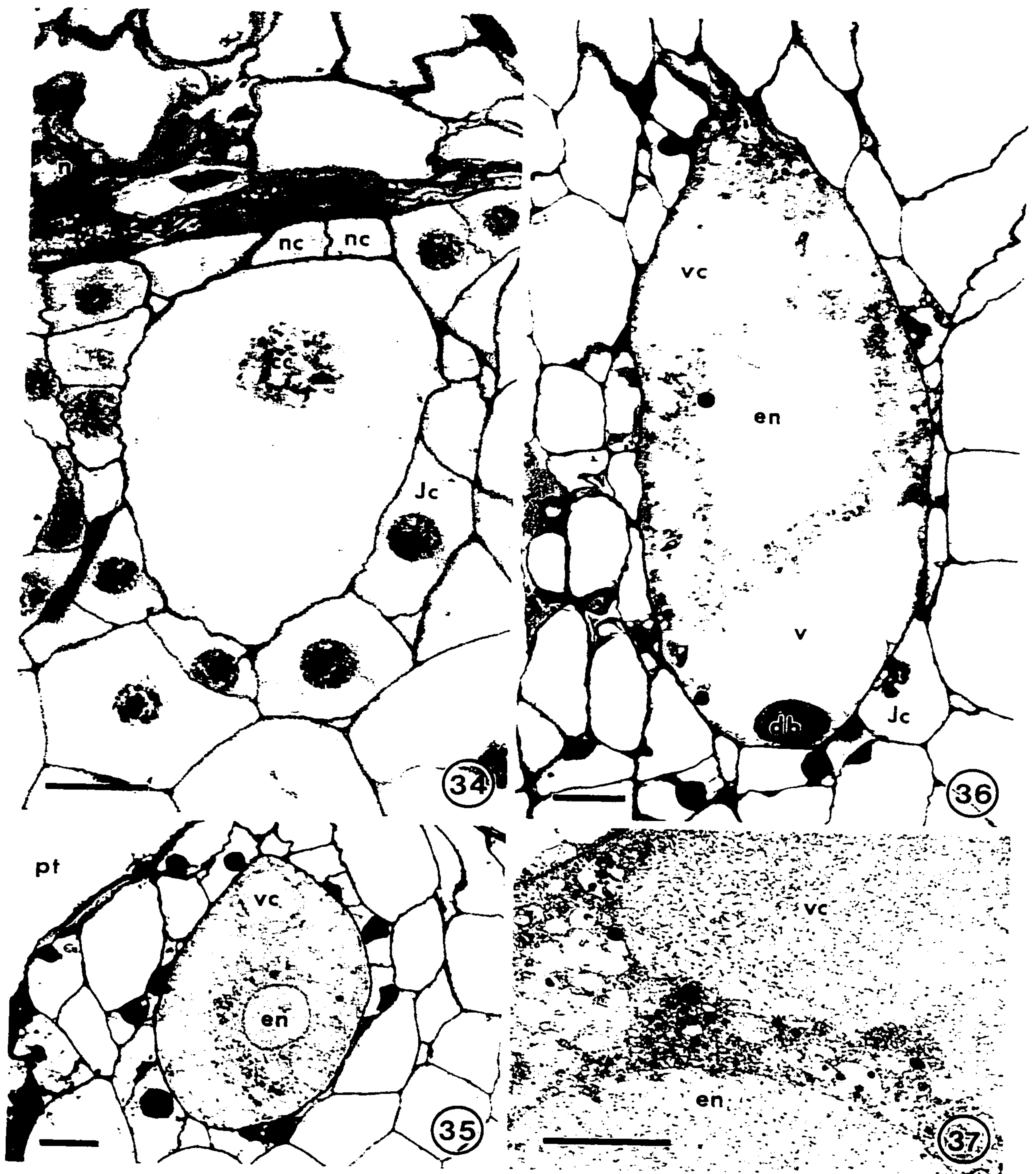
initials divide periclinally forming a small primary neck cell and a large central cell. The primary neck cell divides anticlinally immediately forming a single tier of neck cells just inside the megaspore cell wall. The central cell and its nucleus both enlarge and the cytoplasm becomes quite vacuolate (Fig. 34).

The central cell divides forming a ventral canal nucleus and an egg nucleus (Fig. 35). The egg nucleus moves to a central location within the egg and the numerous vacuoles present at the central cell stage coalesce into a large vacuole (Fig. 36). The ventral canal nuclear contents were comparable in density and composition to the egg nuclear contents (Fig. 37). The ventral canal nuclear membrane is assumed to degenerate quickly as it was never observed surrounding the ventral canal nucleus.

Jacket cells differentiate from megagametophyte cells surrounding the developing central cell. They form a single cell layer of small, dense cells. Jacket cells are not uniform in size or shape and as a result the jacket layer is not always continuous (Fig. 35, 37). Rarely, archegonia shared a single layer of jacket cells or no jacket cells between them. In these cases, a pollen tube had expanded above the megagametophyte distorting the shape of the megagametophyte and possibly forcing the archegonia together.

Mature archegonia were found from May 12 until June 24, 1996. The average number of archegonia within an ovule was four (range of one to eight). Mature egg cells had a 5-6 μm thick perinuclear zone containing mitochondria and lipid droplets around the nucleus (Fig. 38). The perinuclear zone bordered most of the egg nucleus, but was not continuous. Along the periphery of the egg cell, large whorls of membrane were present (Fig. 39). These whorls were recognizable as rough ER by presence of ribosomes and characteristic shape associated with autophagous function. Modified plastids were visible

Figures 34-37. Archegonia of *T. brevifolia*. Fig. 34. Light micrograph (LM) of a central cell, May 26, 1996. Notice the central cell nucleus (cc) is surrounded by numerous vacuoles. Jacket cells (Jc) surround the archegonium and a single tier of neck cells (nc) surmount it. Several nucellar cells are collapsing (n) presumably due to the pressure of the pollen tube which is just out of the plane of section. (Bar = 20 µm for Figs. 34-36). Fig. 35. LM of an archegonium showing a ventral canal nucleus (vc) and egg nucleus (en), June 4, 1996. A pollen tube (pt) is just above the archegonium. Fig. 36. LM of a mature archegonium containing an egg nucleus, ventral canal nucleus, large vacuole (v), and a darkly staining body (db), June 4, 1996. The neck cells are out of the plane of section. Notice the jacket layer is less distinct than in Fig. 34. Fig. 37. Transmission electron micrograph (TEM) of a portion of archegonium in Fig. 35 showing the similarities between the contents of the egg nucleus and the ventral canal nucleus. (Bar = 10 µm).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Figures 38-43. Figs. 38-41. TEMs of the cytoplasmic contents of *T. brevifolia* archegonia collected June 4,1996. Fig. 38. The perinuclear zone bordering the egg nucleus (en) contains mainly mitochondria (m) and lipid bodies (l). (Bar = 1 μm for Figs. 38-40, 43). Fig. 39. Whorls of ER (w) are conspicuous within the egg cytoplasm. These whorls are associated with numerous ribosomes. Fig. 40. A modified plastid which has engulfed egg cytoplasm (ec) and a whorl of ER. Fig. 41. Large, darkly staining bodies (db) are quite conspicuous within the mature egg cell. (Bar = 2 μm). Fig. 42. TEM of a spermatogenous cell (sc) preparing to divide to form the sperm, June 4, 1996. (Bar = 10 μm). The spermatogenous cell nucleus (sn) is in a peripheral position and the chromatin (ch) is condensed. Fig. 43. Close-up of the nuclear membrane (nm) of the spermatogenous cell. Numerous mitochondria are located within the cytoplasm.

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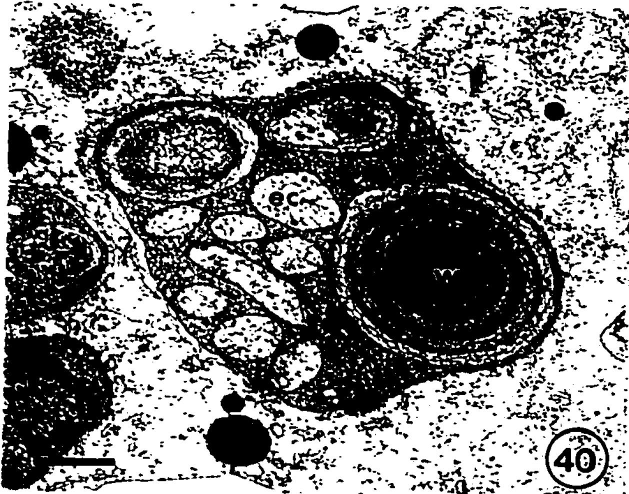
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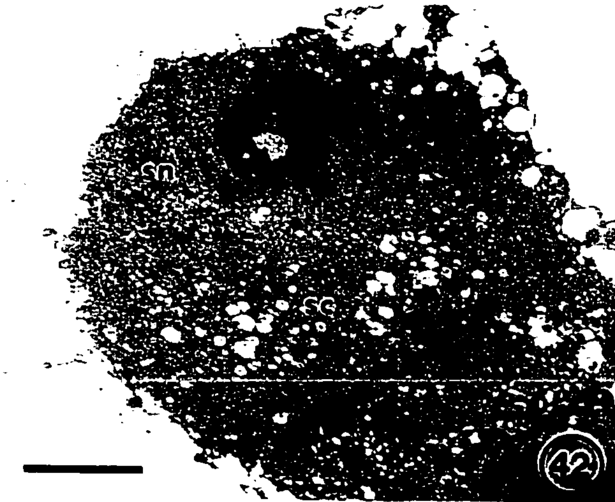
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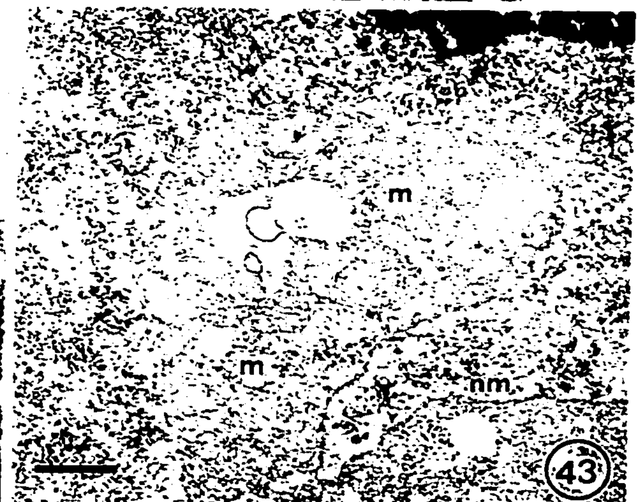
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after the central cell stage (Fig. 40). They were concentrated at the micropylar end and had engulfed large amounts of egg cytoplasm. In the mature egg, large darkly staining, granular bodies were found (Fig. 36, 41). These darkly staining bodies are too granular to be lipids and have no membranes surrounding them (Fig. 41). Their composition is unknown. No plastids were seen within the mature egg cell.

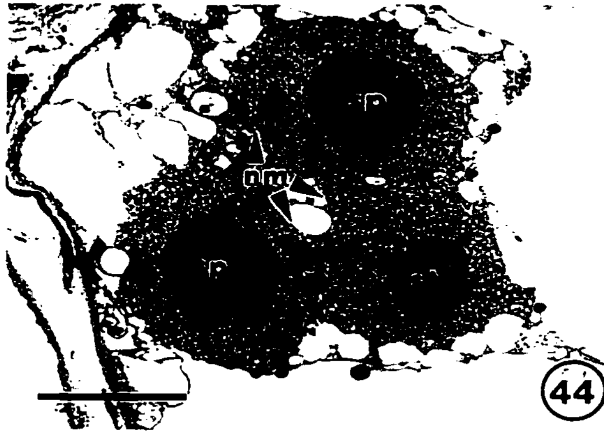
Elongating pollen tubes were visible from April 28, 1996 onwards. Only 35% of the ovules collected were pollinated with *T. brevifolia* pollen. The pollen tube nears the center of the ovule at stages varying from sporogenous tissue to cellular megagametophyte. Once in contact with the megagametophyte, the pollen tube enlarges, greatly distorting the micropylar end of the megagametophyte (Figs. 30-32).

The tube nucleus, sterile nucleus and spermatogenous cell are the first recognizable contents within the pollen tube. They move down the pollen tube in close association. The tube and sterile nucleus precede the large spermatogenous cell. The spermatogenous nucleus (Fig. 42) is located centrally while the spermatogenous cell travels down the pollen tube. The nucleus moves to a peripheral position as the spermatogenous cell nears the megagametophyte. The spermatogenous cell cytoplasm contains numerous organelles including mitochondria (Fig. 43).

Two equal sized sperm formed before the pollen tube entered the archegonium. The sperm were nuclei (Fig. 44) that remained in close association with the sterile nucleus. The tube nucleus degenerated soon after sperm formation. Numerous plastids and mitochondria remained adjacent to the sperm (Fig. 45).

The pollen tube does not penetrate through the neck cells of the archegonium as the neck cells degenerate before fertilization. Instead, the pollen tube enters the

Figures 44-49. Fig. 44. TEM of sperm (sp) and a sterile nucleus (st), June 4, 1997. Each set of condensed chromatin is surrounded by a nuclear membrane (nm). The degenerating tube nucleus is out of the plane of section. (Bar = 10 μm). Fig. 43. Numerous plastids (pl) and mitochondria (m) are located between the sperm just before entry into the archegonium. (Bar = 1 μm for Figs. 45, 48, 49). Fig. 46. LM of the fusing egg nucleus (en) and sperm, June 10, 1996. The pollen tube (pt) entry point is just out of the plane of section, the location is marked by an arrowhead. Within the archegonium, the existing vacuole remains intact and a vacuolate area (*) is created by the pollen tube discharging its contents. (Bar = 20 μm). Fig. 47. TEM showing the fusing nuclear membranes (arrows) and numerous cytoplasmic inclusions within the sperm. (Bar = 2 μm). Fig. 48. TEM of the neocytoplasm bordering the sperm. It contains plastids, mitochondria and abundant ribosomes. Fig. 49. TEM of the neocytoplasm bordering the egg nucleus. It contains plastids, mitochondria and abundant ribosomes also.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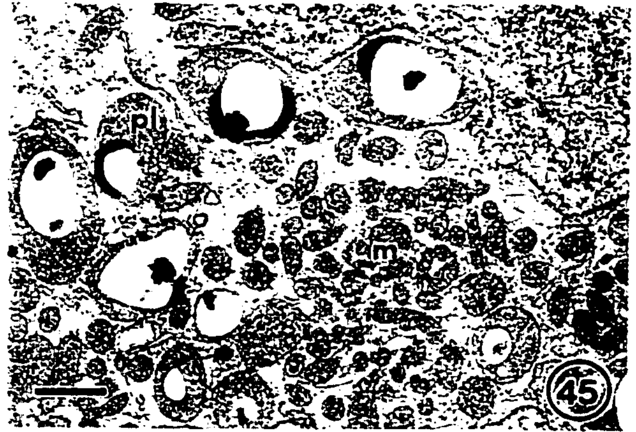
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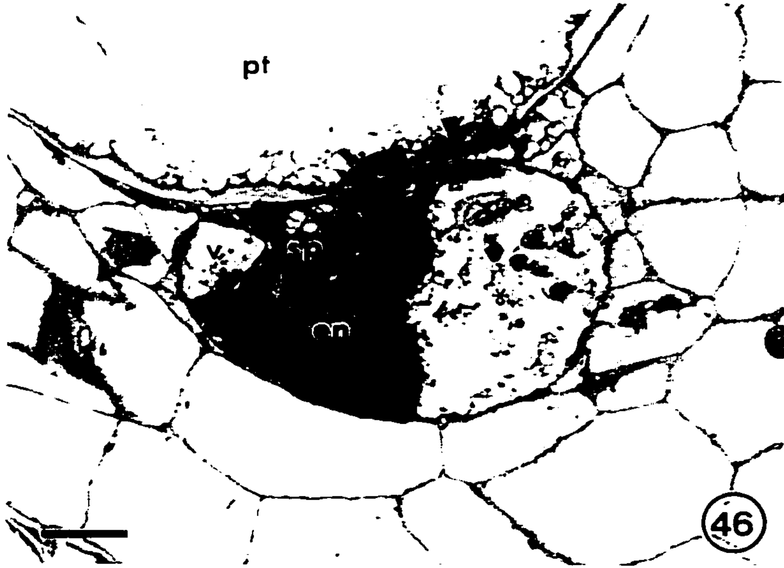
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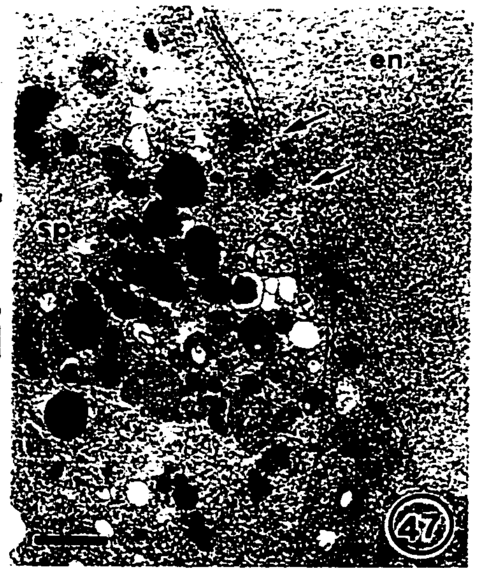
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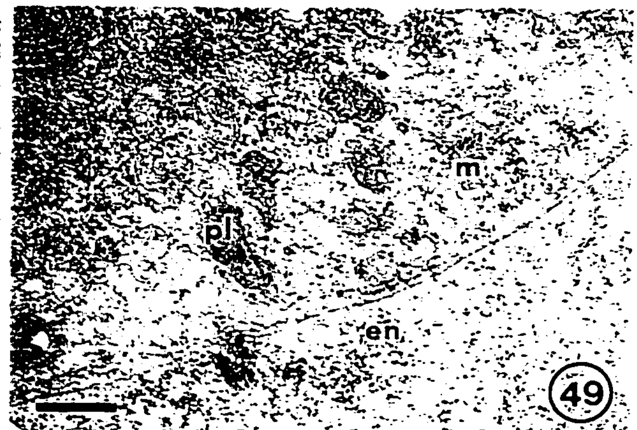
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archegonium through the thinnest area between the egg cell and pollen tube. The entry of pollen tube contents into the archegonium is quite disruptive, leaving a large vacuolate area in the egg cell (Fig. 46). The second sperm and the tube nucleus enter the vacuolate area within the archegonium and degenerate. The egg nuclear membrane and the sperm nuclear membrane fuse along their many points of contact. The sperm contains cytoplasmic inclusions while the egg nucleus has none (Fig. 47). However, the sperm inclusions are darkly staining and appear to be degenerating so their function may be limited. The cytoplasm associated with the sperm is released into the egg along with the sperm. The majority of this cytoplasm is from the spermatogenous cell cytoplasm that remained in close association with the sperm after division. It contains numerous plastids and mitochondria. The paternal mitochondria and plastids intermingle with the maternal perinuclear mitochondria. This forms the neocytoplasm bordering the fusing sperm and egg nucleus (Fig. 48, 49).

Discussion

The phenology of *Taxus brevifolia* is remarkable in the large range of ovule stages found at any one time. Though the development of individual ovules can occur relatively quickly, the development of ovules from even one tree is extremely variable. Similar variation was described in *T. cuspidata* ovules (Sterling 1948a) and over-wintering *T. canadensis* ovules (Dupler 1917). No mention of this variation has been made for *T. baccata*. In fact, Pennell and Bell (1987) observed synchrony of ovules within individual *T. baccata* trees. The variability of the ovules during development reported within *T. brevifolia*, *T. canadensis* and *T. cuspidata* could reflect insensitivity to

environmental conditions (such as temperature or humidity) or indistinct environmental conditions. Poor pollination success could be another result of poor coordination with environmental conditions. This study confirmed a low pollination success of 35% in *T. brevifolia*. Favre-Duchartre (1967) claimed pollination success in *T. baccata* was 100%. He justified this complete pollination success by the unpollinated ovules aborting and falling off the tree before collections were made making this measure of pollination success inaccurate. The variability observed within *T. brevifolia*, *T. canadensis* and *T. cuspidata*, could reflect an environmental insensitivity or no distinct environmental threshold during development and may contribute to low pollination success.

Reproductive features can often cautiously indicate relationships within a genus. However, no trend is discernable in *Taxus* in the species researched thus far. Perhaps this reflects that *Taxus* species are based on geographic separations and are actually very closely related (Hartzell 1991). All studied *Taxus* species have linear tetrads of megaspores (Dupler 1917; Sterling 1948a; Favre-Duchartre 1958; Brukhin and Bozhkov 1996). The T-shaped tetrads have been only rarely seen in *T. brevifolia* and *T. baccata* (Pennell and Bell 1987) and could have been overlooked in the other species. At least nine free nuclear divisions within the megagametophyte occurred in *T. brevifolia*, as reported in *T. cuspidata* (Sterling 1948a); whereas, *T. baccata* and *T. canadensis* have only seven or eight free nuclear divisions (Dupler 1917; Favre-Duchartre 1958; Pennell and Bell 1987; Brukhin and Bozhkov 1996). Two megagametophytes were seen in 5-6% of the ovules of *T. brevifolia* and *T. cuspidata* (Sterling 1948a) but more rarely in *T. baccata* (Cecchi Fiordi *et al.* 1991). Any of the megaspores may be functional in *Taxus*. More than one megagametophyte is formed from the development of more than one

megaspore. Only *T. canadensis* produced up to five megagametophytes and two megagametophytes were quite common (Dupler 1917). The occurrence of up to five megagametophytes was explained by the development of more than one of the megaspores within a tetrad as well as the occurrence of more than one tetrad of megaspores within a *T. canadensis* ovule (Dupler 1917). More than one tetrad of megaspores was not observed in *T. brevifolia*. There were no clear archegonial complexes in *T. brevifolia*. Two archegonia pushed together by the apparent pressure from the expanded pollen tube were observed; however, they never had a common jacket layer. Archegonial complexes have been reported in *T. baccata*, *T. canadensis*, and *T. cuspidata* (Dupler 1917; Sterling 1948a; Brukhin and Bozhkov 1996). The average number of archegonia within an ovule of *T. brevifolia* was four, ranging from one to eight. This is at the lower end of the range reported for *T. baccata* (Favre-Duchartre 1958; Brukhin and Bozhkov 1996) and *T. canadensis* (Dupler 1917). *Taxus cuspidata* had the most numerous archegonia with commonly eight to 14 archegonia and up to 25 archegonia within one ovule (Sterling 1948a). Sterling (1948a) reported that the degree of differentiation of the jacket layer was inversely proportional to the number of archegonia in *T. cuspidata*. Ovules in *T. brevifolia* appeared to follow this trend with the isolated archegonia having more distinct jacket layers.

The lack of a ventral canal nucleus has been reported several times in *Taxus* (Dupler 1917; Favre-Duchartre 1958; Brukhin and Bozhkov 1996). Sterling (1948a) reported three examples of what could have been a ventral canal nucleus. More recently, a distinct ventral canal cell was reported in *T. baccata* but it was given a different name as fern terminology was used to describe archegonial development (Pennell and Bell

1987). Comparison of this terminology with the standard terminology used in conifers shows that a ventral canal cell is equivalent to a neck canal cell (Pennell and Bell 1987). A ventral canal nucleus was seen within 15 archegonia in six resin-embedded *T. brevifolia* ovules. The rare observation of the ventral canal nucleus indicates that it degenerates quite quickly. *Torreya* and *Pseudotaxus* also have an ephemeral ventral canal nucleus (Favre-Duchartre 1964, 1965; Chen and Wang 1979). Nevertheless, the last division forming the egg nucleus and ventral canal nucleus is conserved in *Taxus*.

Large darkly staining bodies in the mature archegonia were also seen in *T. baccata*. They were identified as modified plastids (large inclusions) filled with ribosomes (Pennell and Bell 1987). There were no membranes associated with these structures in *T. brevifolia*; therefore, they could not be modified plastids. They are too granular in texture to be lipid bodies. Histochemical tests would be useful to determine their composition.

The sperm in *T. brevifolia* have no cell membrane; therefore, they are not cells. Instead the sperm are nuclei with associated cytoplasm from the spermatogenous cell. This was also reported in *T. baccata* where the nuclei are present only for a short time before fertilization (Favre-Duchartre 1958; Pennell and Bell 1986b, 1988). The sperm within *T. canadensis* and *T. cuspidata* were observed as unequal male cells (Dupler 1917; Sterling 1948a). Gianordoli (1974) explained this difference in interpretation. The spermatogenous cell divides in one hemisphere of the spermatogenous cell so the resulting nuclei have unequal amounts of cytoplasm. However, these sperm are equal nuclei once released from the spermatogenous cell membrane (Gianordoli 1974).

In *T. brevifolia*, the sperm contributes numerous plastids and mitochondria to the neocytoplasm. These organelles are primarily from the spermatogenous cell cytoplasm. The presence of a perinuclear zone containing abundant maternal mitochondria indicates that maternal mitochondria are inherited. Modified plastids are present in *T. brevifolia* so functional plastids are not present in the mature egg cell. This indicates exclusively paternal inheritance of plastids as the modified plastids are not included in the neocytoplasm. This study confirms the reports found in *T. baccata* that Taxaceae has paternal inheritance of plastids and biparental inheritance of mitochondria (Chesnoy 1987b). Molecular studies of plastid and mitochondrial DNA would be useful to determine the proportion of paternal and maternal mitochondria in the embryo.

The inclusion of the Taxaceae within the Coniferales is disputed. Three main advocates create a separate order Taxales (Sahni 1920; Florin 1948, 1954; Miller 1977). All based their decision on the presence of a solitary, terminal ovule in the taxads. The evolution of the ovulate structure has not yet been sufficiently explained and the taxonomy of *Taxus* will not be resolved until it is. However, structural evidence supports a comprehensive theory on the organization of the ovulate structure proposed by Loze (1965). The primary shoot composed of a reduced vegetative shoot is found in the axil of a leaf. This primary shoot forms three sets of decussate scales and a secondary shoot is formed in the axil of the third set. The ovule is initiated as a terminal structure on this secondary shoot. This organization explains many of the usual features found in *Taxus*, including two ovules produced in one leaf axil, a vegetative shoot produced from the same leaf axil as an ovule and ovules produced in the same leaf axil in consecutive years.

In addition, this description of the ovule has more similarities to the compound ovulate strobili of most conifers.

This chapter has provided clear evidence that the megagametophyte development, archegonial formation and fertilization in *T. brevifolia* are essentially the same as other conifers. It appears unlikely that this would be the case if *Taxus* were not a true conifer. In addition, morphological, anatomical, embryological, chemical and molecular results support the inclusion of Taxaceae within the Coniferales (Buchholz 1929; Keng 1969; Wang *et al.* 1979; Hu *et al.* 1986; Hart 1987; Raubeson and Jansen 1992; Chase *et al.* 1993; Chaw *et al.* 1993; Stefanovic *et al.* 1998). Nevertheless, the taxonomy of Taxaceae will not be resolved to the satisfaction of all until a fossil linking the uniovulate strobilus of the taxads with the compound ovulate strobilus of the conifers is discovered.

Chapter 5

Embryo development, megagametophyte storage product accumulation, and seed efficiency

Introduction

Taxus brevifolia Nutt. is a small tree or shrub found from southern Alaska to northern California and west to the Rocky Mountains (Bolsinger and Jaramillo 1990). It commonly grows in coastal Douglas-fir, coastal western hemlock and interior cedar-hemlock biogeoclimatic zones (Campbell and Nicholson 1995). *T. brevifolia* is shade tolerant, slow growing and functionally dioecious. (Bolsinger and Jaramillo 1990), though cosexual individuals have been observed (Hogg *et al.* 1996; DiFazio *et al.* 1996). Taxol® (paclitaxel), an anti-cancer drug, was originally found in the bark of *T. brevifolia* (Wani *et al.* 1971). This discovery stimulated interest in this obscure understory species.

Reproductive biology needs further research for adequate management of *T. brevifolia* in British Columbia (Campbell and Nicholson 1995). Embryogeny studies have applications in forest genetics, sivilculture and plant taxonomy (Singh 1978). As research using developing seeds to study gene expression advances, it is important to understand structural changes during development. In *Taxus*, understanding normal development is becoming even more important because cell, tissue or organ cultures are being investigated for Taxol® production (Jaziri *et al.* 1996). Reproductive rates of *T. brevifolia* are of particular interest to the BC government (Campbell and Nicholson 1995). Low pollination success has been implicated in *T. brevifolia* and may lead to poor seed efficiency (Anderson and Owens 2000). Furthermore, research into a damaging

mite, *Cecidophyopsis psilaspis* Nalepa, indicates that this has a negative impact on reproductive and vegetative buds of *T. brevifolia* in BC (Duncan *et al.* 1997). *C. psilaspis* was found in all coastal populations of *T. brevifolia* in BC, except those >700m in elevation, causing necrosis of vegetative and reproductive buds (Mitchell *et al.* 1997).

Studies of *Taxus* embryogeny cover a wide range of approaches. The unusual ovulate morphology has been described and debated in *Taxus baccata* L. and *Taxus canadensis* Marshall (Van Tieghem 1869; Aase 1915; Dupler 1920; Andre 1956; Loze 1965). Detailed anatomical research of the proembryo, embryos, and mature seeds in *Taxus cuspidata* Siebold & Zucc. has been published (Sterling 1948b, 1949). The structure of the proembryo has been described in *Taxus wallichiana* Zucc. (Sugihara 1946) and the structure and development of the proembryo and subsequent embryogenesis were examined in *T. baccata* (Robertson 1907; Brukhin and Bozhkov 1996). Several ecological studies emphasized the reproductive limitations, including pollen production, plant density, resource availability and self-fertility in *T. canadensis* (Allison 1990a, 1990b, 1990c, 1993). Recently, *in vitro* research using *Taxus* embryos for Taxol® production was comprehensively reviewed (Jaziri *et al.* 1996).

The structure and development of *T. brevifolia* strobili have been described from pollination to fertilization (Anderson and Owens 1999). Seed storage characteristics, including lipid, protein and sugar contents, in mature seeds were published (Walters-Vertucci *et al.* 1996). Ovule and male strobilus production was correlated with overstory openness and pollen limitation while female resources and vertebrate predation were explored as factors limiting seed efficiency (DiFazio *et al.* 1997; 1998).

The objectives of this research are to describe embryo development, megagametophyte storage product accumulation, and seed efficiency in two natural populations of *T. brevifolia* on southern Vancouver Island. This research will be used to evaluate the effectiveness of sexual reproduction in *T. brevifolia*.

Methods

Two natural forest sites on southern Vancouver Island were selected based on the presence of relatively large *T. brevifolia* trees. These sites were the University of Victoria (UVic) campus and Goldstream Provincial Park (Gold). Both sites were approximately 1ha in area, at an elevation of approximately 60m and within the coastal Douglas-fir biogeoclimatic zone. These were the same sites used in a related pollination and male development study (Anderson and Owens 2000). All reproductive *T. brevifolia* trees at each site were surveyed. Nine female trees at UVic and seven at Goldstream were selected based on having enough accessible branches and foliage for repeated sampling.

In 1996, three to 12 developing seeds were collected weekly from UVic from late May until late July, then once every two weeks in August. In 1997, eight to 14 developing seeds were collected monthly from UVic from early June until early September in 1997. In addition, 10 seeds were collected on June 4 from Goldstream. In 1998, 11 to 30 developing seeds were collected every two weeks from mid-May until late August from UVic. Additional collections were made on May 21 and July 16 of 10 to 33 developing seeds from Goldstream. All branches bearing the developing seeds were placed in cool, moist plastic bags then transported to the laboratory for prompt dissection.

The developing seeds were dissected, budscales removed and the seeds were sliced longitudinally on opposite sides exposing the megagametophyte tissue. Approximately, 75% of the median sections were fixed in formalin-acetic-acid alcohol (FAA) (Johansen 1940) or Navashin's fixative (Berlyn and Miksche 1976) for paraffin embedment. The remaining 25% of the specimens were more thinly sliced and fixed in 4% glutaraldehyde (Bozzola and Russell 1992) for embedment in Spurr's resin. All specimens were aspirated several times until they sank into the fixative. The procedures for embedment and sectioning of the paraffin and Spurr's resin samples have been published (Anderson and Owens 1999).

For anatomical study, the paraffin-embedded serial sections were stained with 1% aqueous safranin and 0.5% aqueous hematoxylin. The resin embedded sections were stained with either Richardson's stain (Richardson *et al.* 1960) or 0.5% toluidine blue (CI#52040) in 2.5% sodium borate (Hall and Hawes 1991). Terminology used to describe stages of embryo development are based on those used by Singh (1978) and Sterling (1948b, 1949).

Storage products accumulating in the megagametophyte and embryo were visualized using histochemical stains for starch, proteins and lipids. Each resin embedded seed was sectioned and divided onto several slides so that the same seed could be stained and observed with more than one histochemical stain. Both paraffin- and resin-embedded sections were stained with 0.5% periodic acid - Shiff's reagent (PAS) and 1% amido black B (CI#20470) in 7% acetic acid. PAS stains starch because the periodic acid oxidizes the insoluble polysaccharides, producing aldehydes. The basic fuchsin in the Schiff's reagent reacts with the aldehydes forming pink complexes (Jensen 1962). Amido

black B has been used extensively to stain for proteins. Though generally specific for proteins, it occasionally stains other macromolecules such as carbohydrates (Cawood *et al.* 1978). However, as PAS was used in combination with amido black B, the starch grains were stained preferentially by PAS. Resin embedded sections were stained with 0.3% Sudan black B (CI#26150) in 70% ethanol (Bronner 1975). Sudan black B is specific for lipids, either due to selective solubility in lipids or the formation of salt linkages between the dye and phospholipids (Gahan 1984).

Unfortunately, staining was inconsistent in the resin embedded sections, possibly due to lipid loss during the embedding process. Therefore, 25 developing seeds were collected from UVic and Goldstream on August 10 and 11, 2000. The megagametophytes were dissected from the seed coat, frozen immediately in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc.) and sectioned using a cryostat. These fresh sections were successfully stained with Ruzin's (1999) Sudan black B schedule. Cautious observations may also be made from the resin sections stained with the metachromatic stain, toluidine blue. The osmicated lipids stain a gray-blue compared with the pinkish-blue cytoplasm (Yeung 1990).

Seed efficiency was defined as the number of mature seeds that were produced divided by the number of healthy ovules at pollination. It was determined in 1997 and 1998 by tagging ovules on two large female trees at each site. One of the female trees at Goldstream produced seeds in 1997, but did not produce enough accessible ovules in 1998 so another tree was used. Otherwise, the same trees were used in both years. Branches with apparently healthy appearing ovules were tagged with small aluminum tags during pollination in late March and early April. In total, 256 ovules were tagged in

1997 and 249 ovules were tagged in 1998. The development of these ovules was assessed at the time of fertilization in early June and every two weeks from late July onwards. Developing seeds were assessed as missing, dead, unhealthy, maturing, having a red aril indicating a mature seed, or having no seed attached (i.e. a receptacle left from the budscales). In 1997, over 94% of the ovules assessed as missing were previously judged to be dead or unhealthy. Therefore missing ovules were assumed to have died and fallen off during the intervals between assessments. The presence of a receptacle indicated that a mature seed developed and dropped off or was removed by predation (Allison 1990c; DiFazio *et al.* 1998). Monitoring continued until January for the 1997 seeds and until November for the 1998 seeds. Using these data, the pre-zygotic abortion, post-zygotic abortion and seed efficiency were calculated. The pre-zygotic abortion refers to the percentage of missing, dead or unhealthy ovules before fertilization in early June, while the post-zygotic abortion refers to the percentage of missing, dead or unhealthy ovules observed after fertilization. Seed efficiency refers to the percentage of seeds that produced a red aril plus the number of receptacles indicating a mature seed was removed compared to the number of ovules present at pollination.

Although *Taxus* has no compound strobilus, the ovulate structure may produce more than one ovule over consecutive years. In both years, the tagged ovulate structures from the seed efficiency study were assessed for the presence of newly developing ovules in the same leaf axils. The tagged ovulate structures were examined in the spring when the tip of the ovule protrudes from the budscales. In 1998, 222 ovulate structures were assessed and 208 ovulate structures were assessed in 1999. These data were used to

calculate the percentage of ovulate structures reforming ovules over consecutive years. Any lost tags or dead branches were omitted from these calculations.

Observations

The *T. brevifolia* trees observed in this study typically produced a single ovule per ovulate shoot every year. Occasionally, two ovules were observed in one leaf axil. The ovulate shoots were extremely reduced such that the ovule appeared to grow directly from the leaf axil (Fig. 50). Meristematic cells at the base of the ovule began mitoses about the time of pollination (Fig. 51). This tissue grew very slowly around the developing seed forming a cup shaped aril. When the seed was mature, the aril increased dramatically in size and became fleshy and red (Fig. 52).

Embryo Development

In this study, proembryos included development from free nuclear divisions of the zygote until the primary suspensors elongated pushing the developing embryo out of the archegonial jacket. Proembryos were observed from May 26 to June 3, 1996, from June 3-10, 1997 and only on June 18, 1998. Following mitosis, two nuclei migrated to the chalazal end of the archegonium (Fig. 53). In one instance, two mitoses were observed before the four nuclei descended to the chalazal end. In total, 16 nuclei formed at the chalazal end of the archegonium (Fig. 54). The expansion of the pollen tube directly above the megagametophyte distorted many archegonia from oval to rounded (Fig. 55). The arrangement of free nuclei depended on the shape of the archegonium. Cell wall formation occurred after 16 nuclei were present. There were no clear tiers of cells but

Figures 50-56. Fig. 50. Developing seed (s) in mid-July that has expanded out of the budscales (b) and the aril (a) is just becoming visible at the base. (bar = 5mm). Fig. 51. Longitudinal section through the base of an ovule collected in mid-April. It shows a cell undergoing mitosis (mi) in the meristematic region that develops into the aril. (bar = 100μm). Fig. 52. Seeds in late July, one with an expanding aril (ea) and one with a fully formed aril. (bar = 1cm). Fig. 53. Free nuclear proembryo collected in late May. Two nuclei (Nu) are located at the chalazal end of the archegonium. (bar = 100μm). Fig. 54. Two proembryos, collected in late May. Each contained 16 nuclei counted from serial sections. There are seven nuclei visible in the free nuclear proembryo (fp). Cell walls are just beginning to form in the upper tier (tier open to the egg cell), while the lower tier has no cell walls visible yet. The cellular proembryo (cp) has four cells visible in this section all with distinct cell walls. (bar = 100μm). Fig. 55. Free nuclear proembryo, collected in early June. It contained 16 nuclei counted from serial sections but only two nuclei are visible in this section. Note the distortion of the archegonium from the pressure of the pollen tube (pt). (bar = 100μm). Fig. 56. Cellular proembryo, collected in early June, showing the lack of distinct tiers. The upper (u) region has cells that are open to the micropylar end (seen in adjacent serial sections), while the remaining cells seen here are part of the lower region that will give rise to the suspensor and embryonal cells. Vacuoles (v) are present in all cells including the embryonal cells as seen in adjacent serial sections. (bar = 100μm).

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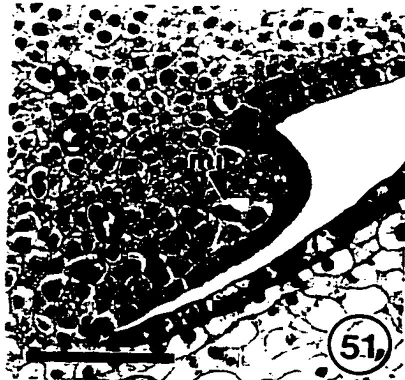
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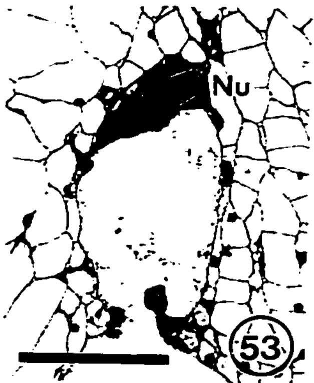
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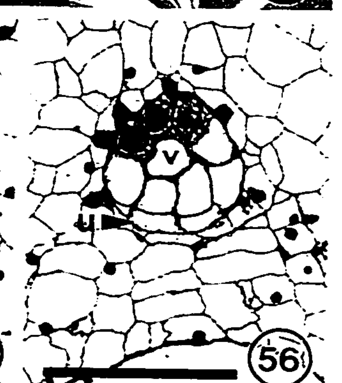
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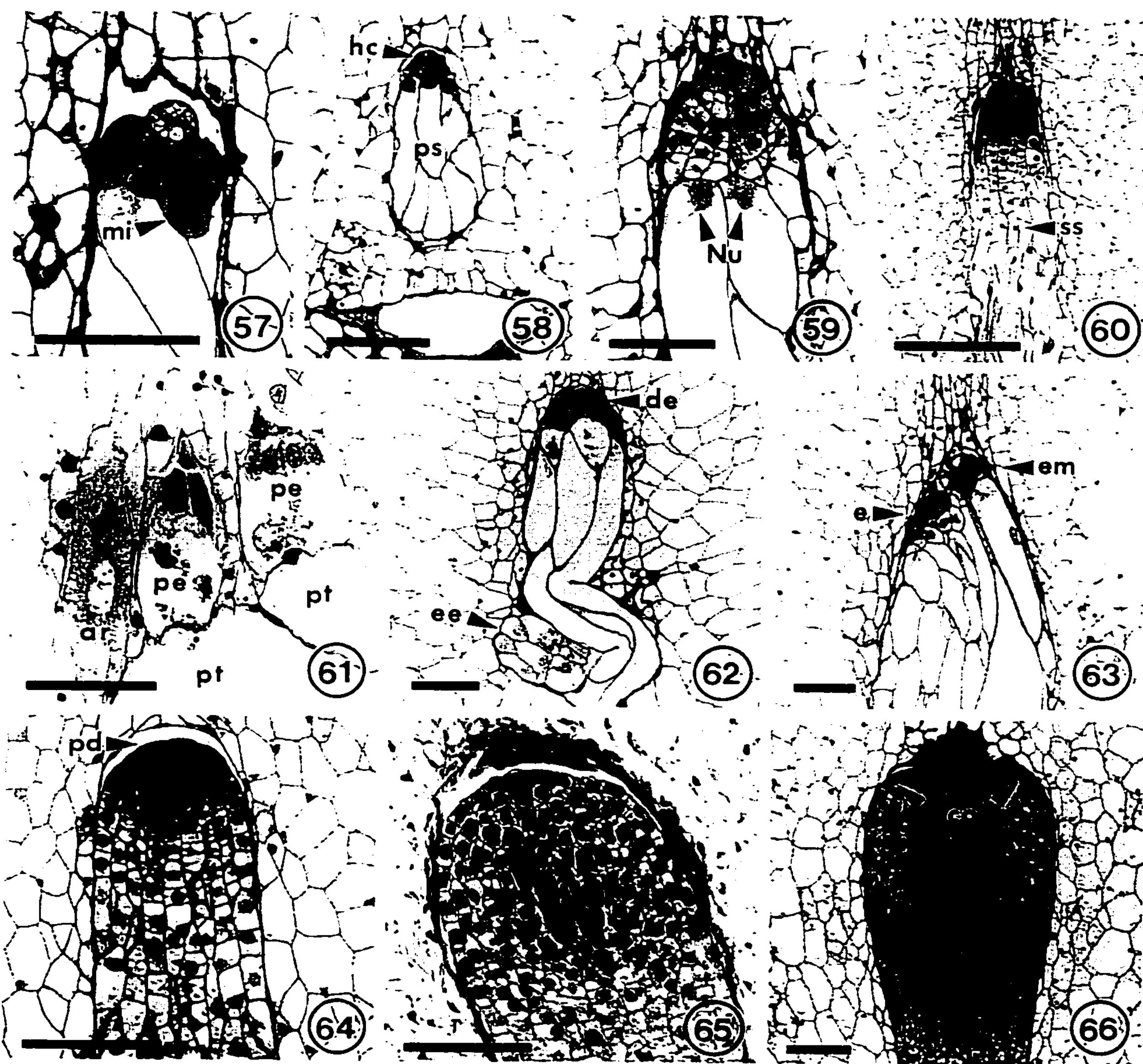
regions of cells could be observed. The number of nuclei in each region depended on how the nuclei settled into the archegonium (Fig. 56). The primary upper region was never observed to undergo further mitoses forming a suspensor and an upper (open) region. Therefore, according to Singh's (1978) terminology, the primary upper region functions directly as the upper region. The embryonal cells and suspensor cells were not distinguishable until the suspensor cells began elongating. Even then, there was no distinct boundary separating the two regions. The upper (open) region was identifiable by the lack of cell walls on the micropylar side of the nuclei.

Early embryo development began with the elongation of the primary suspensor and ended with differentiation of the embryo into distal and proximal regions. Early embryos were observed from June 3 to July 29, 1996, from June 4 to July 7, 1997 and from May 19 to August 14, 1998. The primary suspensor elongated pushing the embryonal cells into the megagametophyte. There were usually six to eight cells within the primary suspensor. At this time, simultaneous mitoses were observed in the early embryonal cells (Fig. 57). Initially, the embryonal cells formed a crescent shape (hemispherical cap) over the suspensor (Fig. 58). The nuclei of the primary suspensor cells remained at the chalazal end of the cells adjacent to the embryonal cells (Fig. 59). Soon after a massive embryo formed, the secondary suspensor cells began to elongate (Fig. 60). These embryonal tubes were distinguishable from the primary suspensor cells because their nuclei were positioned in the center of the cells, not the chalazal end, and they were shorter than the primary suspensor cells. The megagametophyte cells in the path of the embryo separated from each other but did not degenerate into a corrosion cavity until contact with the embryo.

Figures 57-66. Fig. 57. Early embryo collected in early June. The embryonal cells are undergoing mitoses (mi). (All bars = 100μm). Fig. 58. Early embryo collected in early June. The embryonal cells have formed a hemispherical cap (hc) over the primary suspensors (ps). Fig. 59. Early embryo collected in mid-June. The nuclei (Nu) of the primary suspensors are always located at the chalazal end of the cells. Fig. 60. Massive embryo collected in mid-June. The secondary suspensors (ss) are shorter than the primary suspensors and their nuclei are located at the center of the cells. Fig. 61. Simple polyembryony. There are two proembryos (pe) present, two pollen tubes (pt) and one unfertilized archegonium (ar). Fig. 62. Simple polyembryony. The dominant embryo (de) has grown around the other early embryo (ee). Fig. 63. Incomplete cleavage polyembryony. One side of the embryonal mass (em) has over grown the remaining embryonal cells (e). Fig. 64. Mid-embryo with a protoderm (pd), collected in mid-August. Fig. 65. Mid-embryo with a focal zone (f), collected in late August. Fig. 66. Late embryo initiating two cotyledons (co), collected in early September.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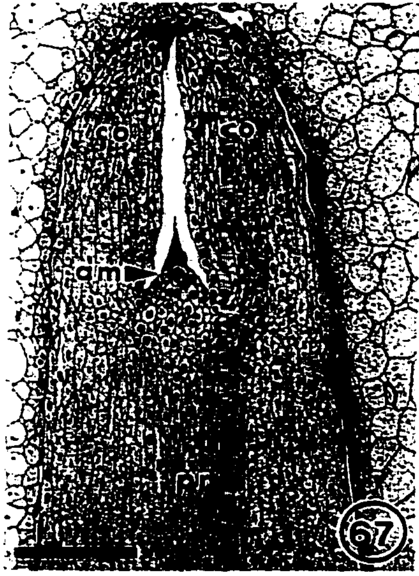
More than one developing embryo was observed until the massive embryo stage developed, indicating that simple polyembryony occurred (Figs. 61, 62). Differential growth of the embryonal cells was observed and was interpreted as incomplete cleavage polyembryony (Fig. 63). However, complete separation of the files of embryonal cells was never observed. Limited cell divisions occasionally occurred within the archegonium at the base of the suspensor system.

Mid-embryo development is the time from differentiation of the embryo into proximal and distal zones until the cotyledons are initiated. Mid-embryos were observed from June 17 to August 26 in 1996, from July 7 to August 12 in 1997 and from June 18 to August 28 in 1998. A continuous layer of cells around the embryonal cells formed the protoderm (Fig. 64). Mitoses localized in a semi-circle around the center of the embryonal cells formed a focal zone (Fig. 65). Then the apical meristem became recognizable as a slightly convex protrusion at the tip of the embryo. By this stage, numerous embryonal tubes had developed pushing the embryo into the center of the megagametophyte. The megagametophyte increased in size throughout embryogenesis and the megagametophyte cells became multi-nucleate.

Late embryo development included the formation of cotyledons until seed maturity. Late embryos were observed from August 12-26, 1996, from August 12 to September 9, 1997 and from July 16 to August 28, 1998. Individual seeds, observed as part of the seed efficiency study, remained on the trees into late fall. Two cotyledons were initiated on the flanks of the apical meristem and one procambial strand extended into each cotyledon (Fig. 66). The focal zone, first visible in the mid-embryo, elongated

Figures 67-68. Fig. 67. Distal portion of a late embryo collected in early September. Two cotyledons (co), the apical meristem (am), and procambial strands (pr) are visible. (bar for Figs. 67-68 = 200µm). Fig. 68. Basal portion of the same late embryo, showing the root cap (rc) and root generative meristem (gm).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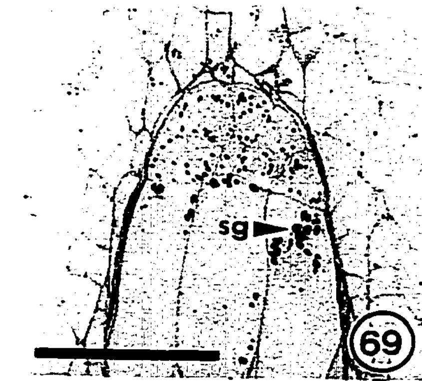
and formed the procambial strands that extended from the cotyledons to the root generative meristem in the late embryo (Figs.67, 68). As the cotyledons elongated, the apical meristem became narrower (Fig. 67). The radicle and root cap became recognizable by the cell divisions radiating out from the root generative meristem but no distinct column or pericolumn were differentiated (Fig. 68). Comparisons of late embryos with and without red arils showed no anatomical difference.

Storage Product Accumulation

Initially, starch grains were visible in the nucellar cells and the megaspore cell wall where it came in contact with the pollen tube. There was no starch within the proembryos. Starch grains became visible in early embryonal cells, especially surrounding the nuclei of the primary suspensor cells (Fig. 69). In addition, the megagametophyte cells surrounding the developing embryo accumulated starch. Starch grains were present in the proximal region of the mid-embryo and in megagametophyte cells adjacent to the mid-embryo and leading into the corrosion cavity (Fig. 70). Starch grains remained visible in the late embryo, especially in the radicle, root cap and cortex (Fig. 71).

There was no positive staining for proteins in the proembryos, early embryos or their associated megagametophyte cells (Figs. 69). Light staining of proteins began in the cytoplasm of the megagametophyte cells of mid-embryos. By the time late embryos formed, irregularly-sized protein bodies were visible in the embryo and megagametophyte cells (Figs. 72, 73). The late embryonal cells, especially in the procambium, contained fewer and smaller protein bodies compared with the

Figures 69-76. Fig. 69. Starch grains (sg) stained in an early embryo in mid-June. (All bars = 100μm). Fig. 70. Starch grains were localized within the proximal region of this mid-embryo and the surrounding megagametophyte cells in early July. Fig. 71. The boundary of a late embryo (e) and megagametophyte cells (mc) stained for starch and proteins in mid-August. Starch grains are primarily found in embryo cortex. Fig. 72. Distal portion of a late embryo, collected in early September, stained for starch and proteins. Proteins (p) are more abundant in the megagametophyte cells than in the embryo. Fig. 73. Basal portion of the same late embryo, also stained for starch and proteins. There is little staining in the root cap and procambial strands. Fig. 74. Megagametophyte cells from a seed containing a mid-embryo collected in mid-August and stained for lipids. Fig. 75. Boundary between the late embryo and megagametophyte cells from a seed with a green aril collected in mid-August and stained for lipids (l). Fig. 76. Boundary between the late embryo and megagametophyte cells from a seed with a red aril collected in mid-August and stained for lipids. An increase in lipid content within the megagametophyte cells can be seen.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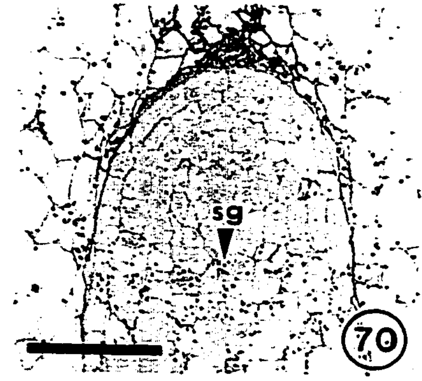
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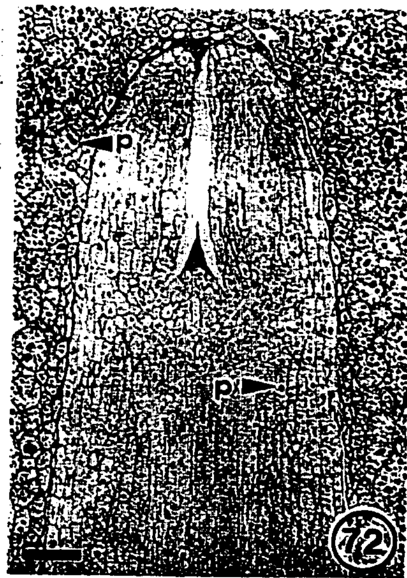
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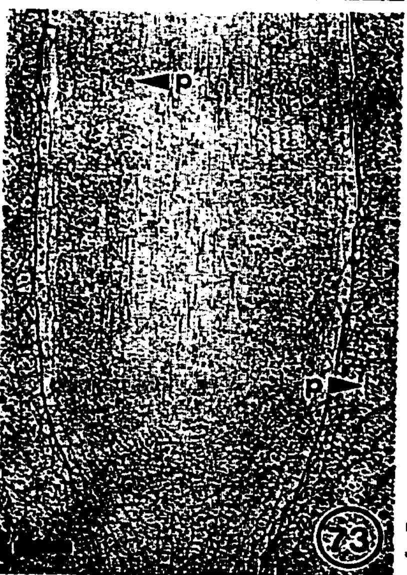
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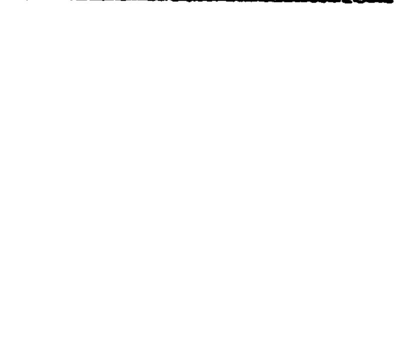

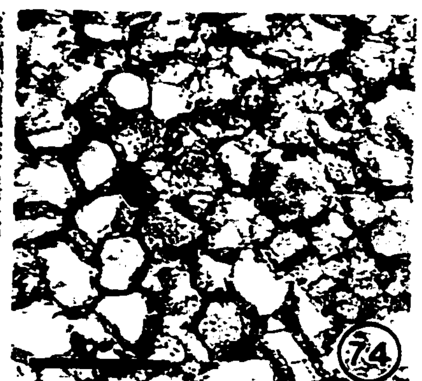
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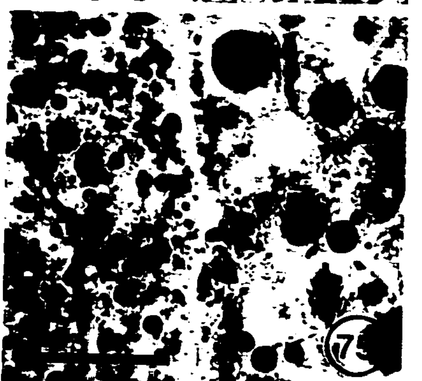


megagametophyte cells (Fig. 71). The megagametophyte cells contained numerous and conspicuous protein bodies.

There was no positive staining for lipids in proembryos, early embryos or their associated megagametophyte cells. Lipid droplets began to accumulate in the cytoplasm of the megagametophyte and embryonal cells at the mid-embryo stage (Fig. 74). The lipid droplets increased in size and abundance in maturing embryos and megagametophyte cells. The megagametophyte cells of late embryos from seeds with green arils had fewer lipids compared with the megagametophyte cells of late embryos from seeds with red arils (Figs. 75, 76). The amount of stained lipid in the megagametophyte was the major difference observed between seeds having green arils or red arils.

Seed Efficiency

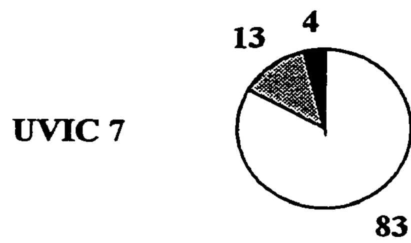
Red arils were observed on tagged branches from July 23 until October 30, 1997 and from October 27 until November 11, 1998. However, red arils were present as early as July 16, 1998 on a few ovules on untagged branches. They were not represented on the tagged branches due to the extremely high seed abortion.

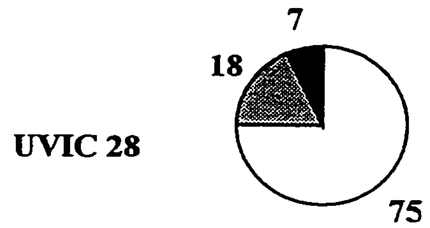
The vast majority of ovules aborted before fertilization (Fig. 77). In 1997, pre-zygotic loss ranged from 28% to 93% whereas in 1998, it ranged from 79% to 94% depending on the tree. Post-zygotic loss ranged from 5% to 56% in 1997 and from 2% to 19% in 1998. In 1997, seed efficiency ranged from 2% to 16%. It was lower in 1998 ranging from 0% to 4%.

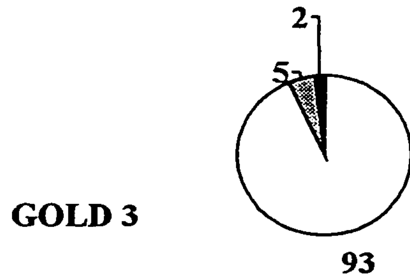
There was minimal predation by rodents or birds because mature seeds with red

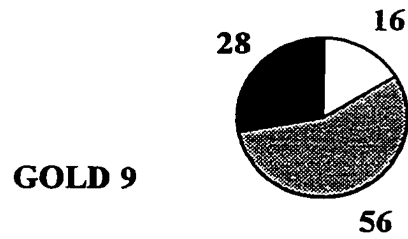
Figure 77. Fate of tagged ovules in 1997 and 1998. Pre-zygotic loss (white), post-zygotic loss (gray) and seed efficiency (black) are compared between individual trees from two sites, UVic and Goldstream. The numbers around each chart indicate the percentage of ovules within each category.

1997

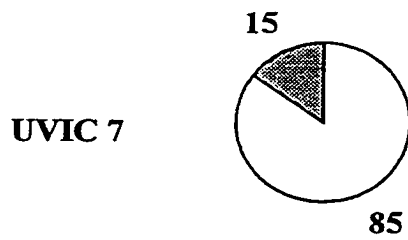


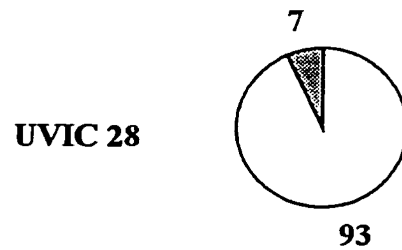


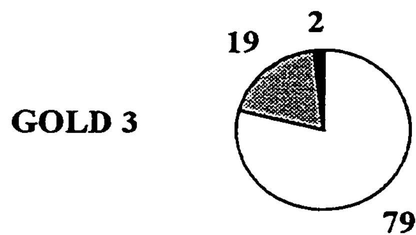




1998







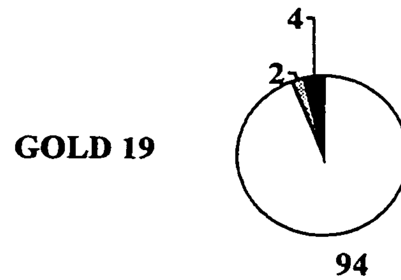


Table 5. The percentage of tagged ovulate structures that produced ovules in the following spring. The total number of ovulates structures was 222 in 1998 and 208 in 1999.

Site & Tree	**1998**	**1999**
UVic 7	23	6
UVic 28	40	11
Gold 3	25	46
Gold 9	3	-
Gold 19	-	55

Note: All missing tags or unhealthy branches were omitted.

arils remained attached for several weeks until they eventually grew moldy and dropped off. Only four of the 438 tagged ovulate structures were removed as mature seeds within the two-week interval between monitoring. There was no indication whether these four seeds were removed by predation or dropped off.

Production of new ovules in the same leaf axils (as the previous year) varied among trees in both years of study, and on some individuals occurred as a large proportion of the old axils (Table 5). In 1997, the ovulate structures reforming ovules ranged from 3% to 40%. In 1998, it ranged from 6% to 55%. Tree #9 at Goldstream had the highest seed efficiency in 1997 and the lowest number of ovules produced in the following spring.

Discussion

The unusual ovulate structure within *Taxus* has been described in *T. baccata* and *T. canadensis* (Van Tieghem 1869; Aase 1915; Dupler 1920; Andre 1956; Loze 1965). There is a primary shoot that produces budscales and a secondary shoot emerges from the axil of the last budscale. This secondary shoot bears a terminal ovule. At approximately the time of pollination, an aril is initiated from a ring of tissue at the base of the ovule. This aril remains unobtrusive until shortly before seed maturity in the summer or fall (Aase 1915; Dupler 1920; Loze 1965). This description is consistent with our observations in *T. brevifolia*. Unfortunately, there is no further evidence for the nature of the aril. It may be a special outgrowth, modified ovuliferous scale, second integument or late-appearing fleshy layer of a three layered seed coat (Dupler 1920). Further studies of other genera within Taxaceae may help determine the developmental origin of the aril.

Fertilization was observed in early June in *T. brevifolia* (Anderson and Owens 1999) and the first free nuclear division of the proembryo was observed in mid-May and late May in *T. cuspidata* and *T. canadensis*, respectively (Dupler 1917; Sterling 1948b). These latter dates are similar to those for proembryos being observed from late May to mid-June in this study. As in *T. cuspidata* (Sterling 1948b; Chowdhury 1962), the zygote nucleus of *T. brevifolia* descended to the chalazal end of the archegonium before division, or it occasionally divided while still in the center of the archegonium.

Cell wall formation occurred after 16 or 32 nuclei were present in the chalazal end of the archegonium in *T. baccata*, *T. cuspidata* and *T. wallichiana*, (Robertson 1907; Buchholz 1929; Sugihara 1946; Sterling 1948b; Brukhin and Bozhkov 1996). This fifth division forming 32 nuclei did not always occur and may not be synchronous when it is present (Sugihara 1946; Sterling 1948b). Proembryos in *T. brevifolia* never had more than 16 nuclei present so the fifth division must be rare, not occur, or be delayed until the early embryo stage.

As *T. brevifolia* had no distinct tiers, it did not follow basic conifer proembryo development (Singh 1978) exactly. The proembryo had 16 cells before suspensor elongation and the primary upper tier never directly formed the suspensor and open tiers. *T. baccata* has been described as having three distinct tiers in the proembryo (Brukhin and Bozhkov 1996), although no photographs were included to support this. Although *Taxus* embryogeny has been described using tier terminology, it is clear from drawings of different species that the proembryos are not organized into distinct tiers as in the Pinaceae (Chowdhury 1962).

The arrangement of the cells within the proembryo is quite variable in *T. cuspidata* and *T. wallichiana* (Sugihara 1946; Sterling 1948b). The mature proembryo usually had six to 14 embryonic cells, nine to 13 suspensor cells, and nine to 13 cells in the open region in *T. cuspidata*. *T. baccata*, with approximately six suspensor cells, has fewer suspensor cells than *T. cuspidata* (Sterling 1948b). *T. brevifolia* proembryos have only six to eight suspensor cells, and are most similar to *T. baccata*. Occasionally, these suspensor cells are referred to as prosuspensors to differentiate them from the primary suspensor cells derived from the primary embryonal tier in Pinaceae (Chowdhury 1962).

In *T. cuspidata*, embryonal cells remain quiescent as they are pushed into the megagametophyte by the primary suspensor (Sterling 1948b), while in *T. brevifolia* embryonal cells undergo at least one mitosis shortly after the primary suspensors began to elongate. This may be the fifth division observed in other *Taxus* proembryos, although it is delayed until the embryo penetrates through the jacket cells in *T. brevifolia*. When the early embryo in *T. cuspidata* started to divide, the secondary suspensor was formed from periclinal divisions off the proximal end of the embryo. These embryonal tubes rarely attain the same length as the individual primary suspensor cells (Sterling 1948b). This description is consistent with that observed in *T. brevifolia*.

Simple polyembryony has been observed in *T. baccata* with up to seven proembryos in one ovule (Robertson 1907). Simple polyembryony is not as common in *T. brevifolia*, possibly due to insufficient pollination. Up to two competing embryos were visible during the early embryo stages, but by the mid-embryo stage only one embryo was visible.

In this study, incomplete cleavage polyembryony or differential growth of the embryonal cells was observed. Differential growth of the embryonal cells has also been called unitary lobing (Singh 1978). Similar descriptions have been reported in *T. baccata* and *T. cuspidata* (Sterling 1949; Brukhin and Bozhkov 1996). However, the bilobed appearance of the embryo was interpreted as true cleavage polyembryony in *T. baccata* (Brukhin and Bozhkov 1996). There is some doubt as to whether these embryonal lobes actually develop into different embryos. *T. brevifolia* appears to have an intermediate form of cleavage polyembryony that may not actually result in multiple embryos. Nevertheless, true cleavage polyembryony occurred in approximately 30% of the embryos in *T. cuspidata*. Before the secondary suspensor system developed, a few primary suspensor cells separated from the main embryo and proliferated (Sterling 1948b). This was not observed in *T. brevifolia* and the primary suspensor cells remained in a tight column attached to the early embryo. Cleavage polyembryony was once considered the primitive condition (Buchholz 1929). More recent discussions have tended to consider cleavage polyembryony to be derived or to have evolved independently in different genera and this is not phylogenetically useful (Chowdhury 1962; Singh 1978).

In *T. brevifolia*, the primary suspensor system was coiled and occasionally cells proliferated near the archegonium. Historically, this proliferation is called rosette embryos. This term is misleading for two reasons. A rosette tier never forms in *Taxus* and a functional embryo has never been described from this proliferation (Singh 1978).

The mid-embryo in *T. brevifolia* resembled *T. cuspidata*, as the mid-embryo initially resembled a hemispherical cap (Sterling 1948b). The proximal region had frequent mitoses in the transverse plane that formed the secondary suspensor cells, while

the distal region had cell divisions in all planes (Singh 1978). A region of cells differentiated as the focal zone and the actively dividing cells on the lateral boundaries became the procambium (Sterling 1949). The proximal region gave rise to the secondary suspensor and the root cap, while the distal region formed the embryo (Singh 1978).

It has been reported elsewhere that two cotyledons develop in *T. baccata* while *T. cuspidata* occasionally initiates three cotyledons (Sterling 1949; Brukhin and Bozhkov 1996). In this study, only two cotyledons were observed in *T. brevifolia*. In *T. cuspidata* and *T. brevifolia*, the mature embryos have no definite pith, secretory elements, juncture zone between the epidermis and root cap, distinct column, or pericolumn (Sterling 1949). This contrasts with descriptions of mature embryos in Pinaceae (Singh 1978).

During embryogenesis the megagametophyte cells of *T. baccata* and *T. cuspidata* became quite large, multi-nucleate and filled with spherical bodies (Sterling 1949; Favre-Duchartre 1958; Brukhin and Bozhkov 1996). The megagametophyte cells in *T. brevifolia* also became quite large, multi-nucleate and stained darkly. A layer of megagametophyte cells devoid of food reserves (Singh 1978) often surrounds the embryo. This layer, one to two cells thick in *T. brevifolia*, contained fewer storage products. Throughout other megagametophyte cells, starch, lipids and proteins accumulate for use during seed germination (Singh 1978).

The dry weight of carbohydrates contained in *T. brevifolia* mature megagametophytes and embryos was 2.2% and 3.9% respectively (Walters-Vertucci *et al.* 1996). In this study, low levels of starch was found also. In *T. baccata*, starch grains were localized in the embryo cortex and the megagametophyte cells (Brukhin and Bozhkov 1996). Similarly, starch grains in mature *T. brevifolia* seeds were also localized

in the embryo cortex, megagametophyte cells and the radicle. These starch grains began to accumulate in the early embryo and became concentrated in the proximal region of the mid-embryo.

The megagametophyte of *T. brevifolia* contained 19% protein, calculated from the dry weight (Walters-Vertucci *et al.* 1996). In the study reported here, conspicuous protein bodies appeared in *T. brevifolia* during the development from mid- to late embryo. At maturity, the megagametophyte cells had accumulated more proteins than the embryo. A taxonomic study used major seed peptides to show relationships between several members of the Taxaceae. A trend from *Taxus* – *Pseudotaxus* - *Amenotaxus* – *Torreya* was proposed, with *Cephalotaxus* seed peptides being most closely related to those in *Taxus* (Hu *et al.* 1986).

Lipids compose the majority of storage products based on dry weight in *T. brevifolia*, the megagametophyte containing 71% and the embryo only 30% (Walters-Vertucci *et al.* 1996). The accumulation of lipids is difficult to visualize microscopically because the embedding process dissolves most of the lipid. Fresh sections were best and showed that the mid-embryo contained some lipid while the megagametophyte had little to no lipid. However, development of the mature embryo was accompanied by large amounts of lipid filling the megagametophyte cells and lipid droplets accumulating in the embryo.

Red arils were used as an indication of seed maturity in this study though the anatomical observations did not show any remarkable differences between seeds with developing green arils and those with red arils. There was a distinct increase in the amount of lipid present in the megagametophyte cells of seeds with red arils. This

observation is supported by the discovery of a physiological difference between *T. x media* Rehder seeds with and without red arils. *T. x media* seeds with red arils could withstand -20°C while those with green arils did not germinate following this treatment (Flores and Sgrignoli 1991). Increases in storage products such as carbohydrates or lipids have been shown to correlate with increases in abscisic acid. This hormone is thought to function in seed dormancy. Leaching mature seeds seven days in tap water to remove abscisic acid then culturing the excised embryo consistently breaks seed dormancy in *T. baccata* (Zhiri *et al.* 1994).

Seed maturation is variable in *T. brevifolia*. Maturation occurred from July through November on southern Vancouver Island. This is similar to the asynchronous seed maturation observed in *T. brevifolia* in Oregon that occurred from July through September or October (Walters-Vertucci *et al.* 1996; DiFazio *et al.* 1998). Seed maturation is also asynchronous and spans several months in *T. canadensis* and *T. x media* (Dupler 1917; Flores and Sgrignoli 1991; Wilson *et al.* 1996). Seed maturation in *T. cuspidata* is much more synchronous and the majority of arils on one individual plant turned red within a few days (Wilson *et al.* 1996).

Pre-zygotic abortion was the fate of the majority of ovules in two of the four sites in Oregon (DiFazio *et al.* 1998). The most common fate of tagged ovules on southern Vancouver Island was also pre-zygotic abortion (79%). Post-zygotic abortion had less impact for all trees except on tree #9 at Goldstream in 1997 where the reverse situation occurred. The average post-zygotic abortion in *T. brevifolia* (17%) was slightly more than in *T. cuspidata* (13%) but less than in *T. canadensis* (26%). This would be expected since

T. canadensis is the only functionally monoecious *Taxus* species and can self-pollinate (Wilson *et al.* 1996).

Seed efficiency of *T. brevifolia* in Oregon ranged from 5% to 34% depending on the site (DiFazio *et al.* 1998). Over all trees, sites, and years, seed efficiency in *T. brevifolia* on southern Vancouver Island averaged 4.5% and ranged from 0% to 16.2%. Compared with *Tsuga heterophylla* Sarg. (70%), *Pseudotsuga menziesii* Franco (39%), and *Thuja plicata* D. Don. (7%), only *T. plicata* has similarly low seed efficiency (Owens 1995). If all populations of *T. brevifolia* have similarly low seed efficiency, there may be problems in obtaining sufficient seeds for reforestation until the cause is understood. Given that low seed efficiency has been measured in 1993, 1994 (DiFazio *et al.* 1998), 1997, and 1998, it is unlikely that seed crop periodicity accounts for the low seed efficiency. More likely causes of the high seed abortion in *Taxus* are low pollination success, herbivore or insect damage, resource limitations and seed predation.

Low pollination success has been suggested for *T. canadensis* and *T. brevifolia* (Allison 1990b; DiFazio *et al.* 1998; Anderson and Owens 2000). In *T. canadensis*, pollination success was extremely variable (<5% to 100%) and was correlated with the number of male strobili produced on each monoecious tree (Wilson *et al.* 1996). In deer browsed populations, pollen limited seed efficiency due to low densities of *T. canadensis* individuals (Allison 1990b). Overall, nearest neighbor distance and pollen production explained 86% of the pollination success in *T. canadensis* (Allison 1990c). It has even been suggested that *T. canadensis* evolved monoecy because it was chronically limited by pollen (Allison 1993). Low pollination success was shown to effect *T. brevifolia*

because hand pollination of ovules doubled seed efficiency, but seed efficiency was still <15% so other factors were also limiting (DiFazio *et al.* 1998).

Browsing by ungulates decreased seed efficiency in *T. canadensis*, deer typically removed all foliage on stems less than 3mm in diameter which contained all the reproductive strobili (Allison 1990a). In BC, *T. brevifolia* is browsed by black-tailed deer, elk, moose, and caribou. However, it is likely that *T. brevifolia* is less affected than *T. canadensis* by browsing because *T. canadensis* grows exclusively in shrub form and *T. brevifolia* grows to be small to medium sized trees. The effect of browsing was not measured in this study as all tags that disappeared from the tree were omitted from further calculations.

Cecidophyopsis psilaspis is the most damaging insect pest of *T. brevifolia* (Duncan *et al.* 1997). Symptoms of infestation include bud necrosis and abnormal vegetative growth (Mitchell *et al.* 1997). Mortality of terminal vegetative buds is >20% and the reproductive buds are also affected. This mite which is found in Europe as well as in New York, occurs along the west coast of North American from Oregon to BC, but it is absent from elevations >700m in BC (Duncan *et al.* 1997). It has been hypothesized that *C. psilaspis* was introduced from Europe on horticultural *Taxus* species and may be limited by cold tolerance at higher elevations or interior climates (Mitchell *et al.* 1997). Because the mite populations peak in early spring, they may contribute to the high pre-zygotic abortion observed in *T. brevifolia* (Duncan *et al.* 1997). The site at the lowest elevation in a study in Oregon consistently had the lowest seed efficiency. Nevertheless, the presence of mites was noted at the highest elevation site of 1100-1200m (DiFazio *et

al. 1998). Both *T. brevifolia* populations in this study had *C. psilaspis* in all the vegetative and reproductive buds.

Light availability also may contribute to low seed efficiency in *T. brevifolia* in the understory environment where it usually grows. Ovule and male strobilus production of *T. brevifolia* was correlated with overstory openness in Oregon (DiFazio *et al.* 1997). Seed set in *T. canadensis* depended on ovule production which may be an indication of limited photosynthetic resources of the female tree (Allison 1990c). In Oregon, overstory openness and vertebrate predation interacted to reduce seed efficiency in *T. brevifolia*. Even though more developing ovules were observed in open environments, there was more seed predation so the seed efficiency did not increase (DiFazio *et al.* 1998).

Seed predation by the Townsend's chipmunk, *Tamias townsendii* Bachman, accounted for much of the low seed efficiency of *T. brevifolia* in two of the four sites in Oregon (DiFazio *et al.* 1998). Seed predation is also extremely prevalent in *T. canadensis* with seeds being removed within two to three days of reaching maturity (Wilson *et al.* 1996). In contrast, mature seeds of *T. cuspidata* (Wilson *et al.* 1996), as well as those of *T. brevifolia* were observed rotting on the tree until they fell to the ground. The distribution of *Tamias townsendii* borders along southwest BC and this species may not be present on Vancouver Island (Cowan and Guiguet 1973; Banfield 1974), For this reason, seed predation by this rodent was not a factor in this study.

The ovulate structure in *Taxus* can produce an ovule, produce budscales and remain dormant, or revert to a long shoot and grow a normal branch. Ovulate structures remain viable for several years and can produce new ovules on new secondary shoots in consecutive years (Dupler 1920). Over 33% of the ovules in *T. canadensis* are located on

branches greater than two-years old (Allison 1993). This corresponds with the results reported here for *T. brevifolia* in which 26% of fertile ovulate structures produced a new ovule in the following year. In coniferous trees, seed maturation occurs simultaneously with reproductive bud initiation for the following year. As the developing seeds are a stronger sink for nutrients than initiating buds, there are usually fewer reproductive buds initiated if the developing seed crop is large (Owens 1991). In the two sites and over the two years of this study, results from only one tree (Gold #9) supported this hypothesis. Perhaps *T. brevifolia* is not producing enough seed to be limited by nutrients at these sites.

Conclusions

Development of both proembryo and embryo in *T. brevifolia* was similar to that observed in *T. baccata, T. canadensis,* and *T. cuspidata*. During embryogenesis, starch began accumulating early, while proteins and lipids accumulated during late embryo development. Just prior to seed maturity, the aril turned red and the amount of lipid in the seed increased. The seed efficiency was poor in both sites over two years. Possible causes of low seed efficiency in *T. brevifolia* are poor pollination success, insect damage or light limitation. The production of ovules in the same leaf axils over consecutive years highlighted the unusual ovulate structure in *Taxus*. Research into ovulate structures in other genera within Taxaceae may yield taxonomic and evolutionary information, while research clarifying the causes of low seed efficiency may become useful to industry.

Chapter 6

Cytoplasmic Inheritance In *Taxus* Hybrids Examined Using Heterologous Probes

Introduction

Traditionally, cytoplasmic inheritance has been inferred from ultrastructural evidence (Camefort 1968; Chesnoy 1987b). In Pinaceae, Podocarpaceae, Cephalotaxaceae and Taxaceae, maternal plastids are modified and are usually excluded from the neocytoplasm (Owens and Morris 1991; Mogensen 1996). Another mechanism to exclude maternal plastids occurs in the remaining two conifer families, Cupressaceae *sensu lato* and Araucariaceae. Male cytoplasm accompanying the sperm enshrouds the egg, excluding the maternal organelles from the neocytoplasm (Chesnoy and Thomas 1971; Owens *et al.* 1995b). A mechanism that usually ensures maternal mitochondrial contribution is the formation of a perinuclear zone of mitochondria in Pinaceae, Podocarpaceae, Araucariaceae and Taxaceae (Mogensen 1996). All of these families except Araucariaceae are thought to have at least some maternal mitochondria contributed to the offspring (Owens *et al.* 1995b). Biparental contribution of mitochondria was suggested within Pinaceae, Taxaceae and Podocarpaceae when morphological differences between the paternal and maternal mitochondria were observed within the neocytoplasm (Chesnoy 1987b).

Restriction fragment length polymorphisms have become particularly important in confirming ultrastructural evidence. In addition, specific primers have been used with the polymerase chain reaction (PCR) to amplify variable regions of organelle DNA. This is becoming increasingly common as more chloroplast sequences are published. Existing

primers anchored in conserved regions of organelle DNA can be used across families as heterologous probes (Demesure *et al.* 1995). Often microsatellites are chosen as the target DNA as they show interspecific variation due to slips or extra repeats of the base unit (Vendramin *et al.* 1996). Within Coniferales, molecular techniques have only been applied to a small number of species within Pinaceae and Cupressaceae *sensu lato*. Paternal inheritance of chloroplasts was confirmed in *Pseudotsuga* (Neale *et al.* 1986), *Larix* (Szmidt *et al.* 1987), *Sequoia* (Neale and Sederoff 1988), *Pinus* (Neale and Sederoff 1989; Cato and Richardson 1996), *Picea* (Stine et al. 1989; Stine and Keathley 1990; Sutton *et al.* 1991), *Calocedrus* (Neale *et al.* 1991), *Abies* (Salaj *et al.* 1998) and *Chamaecyparis* (Kondo *et al.* 1998). Maternal inheritance of mitochondria was confirmed in *Picea* (Sutton *et al.* 1991; David and Keathley 1996), *Pseudotsuga* (Marshall and Neale 1992) and *Larix* (DeVerno *et al.* 1993). Paternal mitochondrial inheritance was shown in *Sequoia* (Neale *et al.* 1989) and *Calocedrus* (Neale *et al.* 1991). Maternal or sometimes biparental contribution of mitochondria was indicated in *Pinus* (Neale *et al.* 1988; Wagner *et al.* 1991).

The conifer family Taxaceae contains five genera, *Amenotaxus, Austrotaxus, Pseudotaxus, Taxus,* and *Torreya* (Price 1990). Research on cytoplasmic inheritance has been limited to the genus *Taxus*. Ultrastructural evidence indicates that the chloroplasts are paternally inherited and the mitochondria are biparentally inherited (Chesnoy 1987b; Pennell and Bell 1988; Anderson and Owens 1999). The objective of this research was to use molecular techniques to determine the cytoplasmic inheritance within *Taxus*.

Methods

Controlled pollinations were done between *T. brevifolia* Nutt. and *T. x media* Rehd. (*T. baccata x T. cuspidata*) to ensure genetic variation between the parent trees. Four female *T. brevifolia* trees growing in two natural forests were selected based on the presence of relatively numerous ovules. One forest site is located beside the University of Victoria (UVic), Victoria, British Columbia. The other site is located within Goldstream Provincial Park, (Gold) on southern Vancouver Island, British Columbia. Pollen cones were collected from two individual *T. x media* trees growing on the University of Victoria campus. The pollen cones were air-dried until the pollen shed and the pollen was collected and stored at -20°C. Thirty-two to thirty-seven ovules were tagged per female tree and enclosed within pollination bags before receptivity. Female receptivity was indicated by the presence of a pollination drop. Weekly from March 26 until April 30, 1999, pollen was applied to receptive ovules with a paint brush. Forty-nine of the 138 ovules were never receptive and eventually aborted. The pollination bags were removed two weeks after natural anthesis ceased. Insect bags were placed over the developing ovules at the UVic site only from April 30 until August 16, 1999. Once a month, from August 16 until November 10, 1999, seeds were accessed and collected as they matured. Maturity was indicated by the presence of a red aril, characteristic of this genus (Fig. 78). The seeds were placed in a cooler and transported immediately to the laboratory where the arils were removed. The seeds were stored in the fridge at 4°C for several months. The embryos were separated from the megagametophyte and frozen before DNA extraction.

Figure 78. Branch of *T. brevifolia* with a developing seed (s) and a mature seed with a fully expanded aril (a). (bar = 1 cm).

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Total DNA was extracted using two different methods. For the mitochondrial probes, four leaves of each parent *Taxus* tree, one leaf of *Pinus ponderosa* or one to three genetically identical hybrid embryos were ground together with liquid nitrogen. Then total DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987). The precipitated DNA was suspended in 40 µl TE (Tris-EDTA) buffer. Only vegetative tissue was included with the chloroplast probes; four *Taxus* leaves or one *P. contorta* small vegetative long shoot bud without cataphylls was immersed directly into a 30% Chelex® 100 solution (Bio-Rad, Hercules, CA) and ground with a dremel. This solution was placed in a 65°C oven for 15 minutes, a 107°C oven for 5 minutes, and centrifuged at 3000x for 15 minutes. The supernatant containing the DNA was then removed and used directly in the PCR reaction without dilution.

Four regions of mitochondrial DNA (mtDNA) (Demesure *et al.* 1995) and eight regions of chloroplast DNA (cpDNA) (Vendramin *et al.* 1996) were amplified using PCR (Table 6). The reaction mixture (10µl) for the mitochondrial probes contained 1x reaction buffer (including 2 mM Mg Cl_2), 0.2 units of Taq polymerase (Rose Scientific, Edmonton, ALB), 100 µM dNTPs, 2 mM additional $MgCl_2$, 0.2 µM of each primer, and 1 µl of extracted DNA. Amplification conditions were one cycle of 4 min at 94°C; 30 cycles of 45 sec at 92°C, 45 sec at 55°C, 4 min at 72°C; and one cycle of 10 min at 72°C. The reaction mixture (10µl) for the chloroplast probes contained: 1x reaction buffer (including 2 mM Mg Cl_2), 0.2 units of Taq polymerase, 125 µM dNTPs, 1.125 mM additional $MgCl_2$, 0.6 µM of each primer, and 1 µl of extracted DNA. Amplification conditions were one cycle of 5 min at 94°C; 30 cycles of 30 sec at 94°C, 1 min at 43°C, 1.5 min at 72°C; and one cycle of 10 min at 72°C. The PCR products from both the

Table 6. Primer sequences and observed PCR product sizes in the original species.

Primer Names	Primer Sequences	PCR Product Size
Mitochondrial Primers[a]		Size in *Q. robur*
*nad*1 exon B	5'-GCATTACGATCTGCAGCTCA-3'	1550 bp
*nad*1 exon C	5'-GGAGCTCGATTAGTTTCTGC-3'	
*nad*4 exon 1	5'-CAGTGGGTTGGTCTGGTATG-3'	1700 bp
*nad*4 exon 2	5'-TCATATGGGCTACTGAGGAG-3'	
*nad*4 exon2	5'-TGTTTCCCGAAGCGACACTT-3'	4000 bp
*nad*4 exon4	5'-GGAACACTTTGGGGTGAACA-3'	
*Rp*S14	5'-CACGGGTCGCCCTCGTTCCG-3'	1640 bp
cob	5'-GTGTGGAGGATATAGGTTGT-3'	
Chloroplast Primers[b]		Size in *P. thunbergii*
Pt1254	5'-CAATTGGAATGAGAACAGATAGG-3' 5'-TGCGTTGCACTTCGTTATAG-3'	74 bp
Pt9383	5'-AGAATAAACTGACGTAGATGCCA-3' 5'-AATTTTCAATTCCTTTCTTTCTCC-3'	87 bp
Pt36480	5'-TTTTGGCTTACAAAATAAAAGAGG-3' 5'-AAATTCCTAAAGAAGGAAGAGCA-3'	147 bp
Pt45002	5'-AAGTTGGATTTTACCCAGGTG-3' 5'-GAACAAGAGGATTTTTTCTCATACA-3'	167 bp
Pt87268	5'-GCCAGGGAAAATCGTAGG-3' 5'-AGACGATTAGACATCCAACCC-3'	165 bp
Pt102584	5'-TTCATGTAATTCCCAGATCCA-3' 5'-CATTATGTGCGCGATAATTTC-3'	129 bp
Pt107148	5'-GTTCATTCGGGATCCTTAAAA-3' 5'-GTACTTTCCTTCAGCCAATCTG-3'	122 bp
Pt109567	5'-TATTATCGAACAACGAGAATAATCC-3' 5'-TCACTGTCACTCTACAAAACCG-3'	115 bp

[a] Designed by Demesure *et al.* (1995). [b] Designed by Vendramin *et al.* (1996).

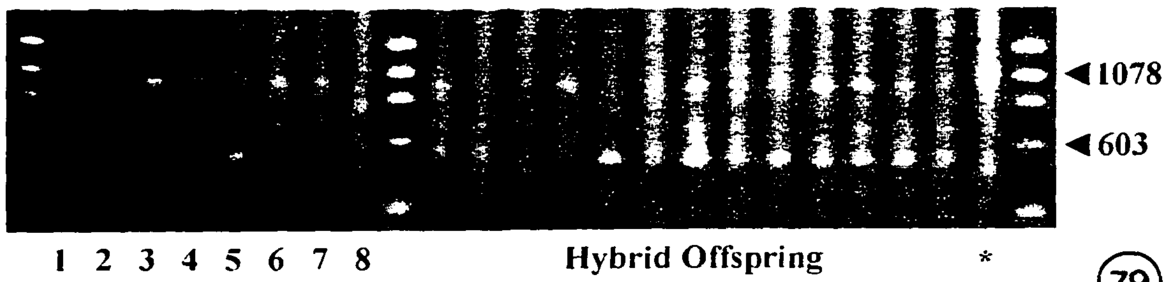
mitochondrial and chloroplast protocols were concentrated by an isopropanol/ammonium acetate precipitation, rinsed with ethanol and re-suspended for a second PCR reaction. The second PCR was necessary due to the small size of the hybrid embryos (approximately 0.5 mg each). The mitochondrial PCR products were separated on a 1% agarose gel. The chloroplast PCR products were separated on 10% polyacrylamide standard gels or pre-cast Ready gels (Bio-Rad, Hercules, CA). All gels were stained with 0.5 µg/ml ethydium bromide and visualized by exposure to UV light. Bands were measured using "Un-scan-it gel" software version 5.1 (Silk Scientific Corporation).

Observations

Primers designed by Demesure *et al.* (1995) were anchored in the conserved gene complex *rp*S14-*cob*. The primer pair *rp*S14-*cob* produced a single band in the female *T. brevifolia* trees of approximately 970 base pairs in length. The male *T. x media* trees had two bands of approximately 526 and 970 base pairs in length. The hybrid embryos all produced two bands of 526 and 970 base pairs in length (Fig. 79). Therefore, paternal contribution of mitochondria was indicated. Maternal mitochondrial inheritance could not be confirmed or refuted because the female band matched one of the male bands in size.

A negative control lane was used to visualize contamination. This lane was contaminated in all gels containing the hybrid embryo DNA. In one case, the band in the control appeared identical in size to the 526 bp male band. The female trees did not have the 526 bp band so the contamination was not present in the entire PCR cocktail. In the second case, the control contained two bands of different lengths (551 and 1009 bas pairs) compared with the *Taxus* DNA. In the final case, one control lane contained a band

Figure 79. Agarose gel showing hybrid offspring with two bands of similar size to the male *T. x media* parents (526 bp & 970 bp) with mitochondrial primer *rp*S14-*cob*. The female *T. brevifolia* parents only have one band (970 bp). Lanes 1-3: Female parents from UVic. Lanes 4-5: Male parents. Lanes 6-7: Female parents from Goldstream. Lane 8: *P. ponderosa*. Lane with asterisk: contaminated control. Remaining lanes: Hybrid Offspring. DNA Standard: X174 DNA cleaved with HAE III with bands 603 and 1078 noted (Pharmacia Biotech, Baie d'Urfré, Quebec).


1078
603
1 2 3 4 5 6 7 8
Hybrid Offspring
*
79

similar in length to the male band and the second control lane had no bands. This procedure needs to be repeated until the control lane is free from contamination.

The remaining three mitochondrial primer pairs did not produce informative bands. The *nad*1exonB-*nad*1exonC appeared promising as it produced two bands in the female parents and a single band in the male parents. Unfortunately, this result was not reproducible consistently. The remaining two primer pairs, *nad*4exon 1-2 and *nad*4exon 2-4 produced only smears of DNA even at a high annealing temperature (55°C).

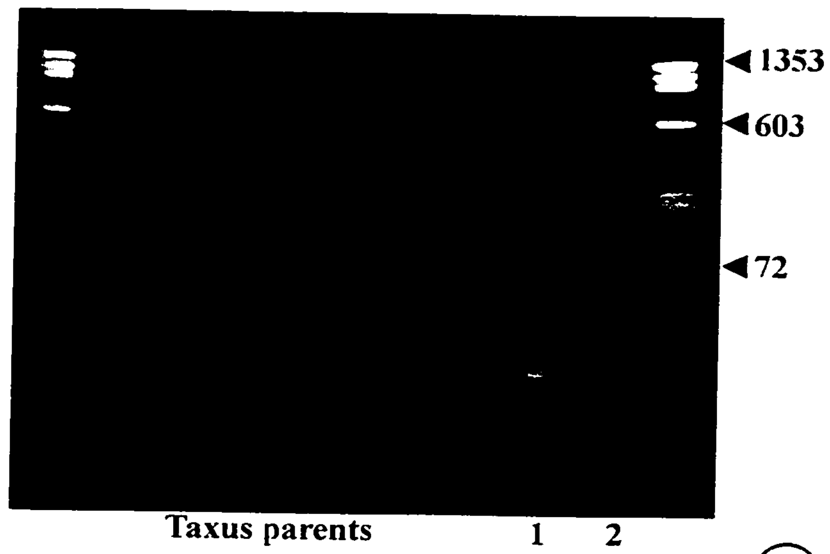
Eight of the twenty primer sequences designed for cpDNA of *Pinus thunbergii* did not produce bands in *Taxus* (Fig. 80). However, bands were produced in *P. contorta* of the appropriate size or slightly smaller. The positive results in *P. contorta* indicate the lack of bands in *Taxus* was likely due to the incompatible primer sequences.

Discussion

Paternal contribution of mitochondria was indicated in this study. In conjunction with the structural evidence of a perinuclear zone, mitochondrial inheritance in *Taxus* is likely to be biparental. Within Coniferales, *Pinus* is the only other species shown to have biparental inheritance of mitochondria (Neale *et al.* 1988; Wagner *et al.* 1991).

The uniparental inheritance of chloroplasts in conifers (paternal) or angiosperms (maternal) indicates that it is beneficial to retain only one haplotype in the offspring. Hypothesized benefits of uniparental inheritance include eliminating recombination, elimination of sperm cytoplasm to increase mobility (only applicable with maternal inheritance) and eliminating conflicts that would occur with multiple forms of a gene (Neale *et al.* 1988; Mogensen 1996). It has been assumed that mitochondria benefit from

Figure 80. Polyacrylamide gel showing negative results for all of the *Taxus* parents and a band in *P. contorta* (20 bp) for chloroplast primer Pt1254. Lane 1: *P. contorta*. Lane 2: No DNA added, bands are excess primers. DNA Standard: X174 DNA cleaved with HAE III with bands 72, 603 and 1353 noted.


1353
603
72
Taxus parents
1
2

uniparental inheritance similar to chloroplasts. However, as biparental inheritance of mitochondria occurs in *Pinus* (Neale *et al.* 1988; Wagner *et al.* 1991) and probably *Taxus,* uniparental inheritance may not be as crucial for mitochondria.

One explanation is that *Pinus* and *Taxus* exhibit a primitive form of cytoplasmic inheritance where the mechanisms to exclude organelles are not absolute. In both species, the egg donates more mitochondria than the sperm due to the concentration of mitochondria in the perinuclear zone. The participation of paternal mitochondria in the neocytoplasm may not be disadvantageous as long as the majority of mitochondria are still maternal (Mogensen 1996).

The evolutionary advantage favoring uniparental inheritance may not be as strong within mitochondria compared with chloroplasts. Chloroplasts contain numerous copies of identical circular strands of DNA. However, mtDNA has been shown to be much more complex. Within one mitochondrion, numerous variations of circular and linear DNA strands exist, many of which appear to have recombined already (Neale *et al.* 1988). Some authors assume recombination must be more detrimental to mitochondria than chloroplasts because it is more likely (Mogensen 1996). However, given the variability already present within a single mitochondrion, it may not be detrimental to inherit two versions of mitochondria. The disruption would be much greater within the plastid system where there is little to no variation within each plastid.

The finding that the *Pinus* microsatellite probes did not amplify DNA within *Taxus* suggests that this region is quite different within Taxaceae. Nevertheless, these probes have been shown to be useful in *P. leucodermis* (Vendramin *et al.* 1996) and now *P. contorta* so they may be effective within the entire *Pinus* genus and even in other

genera within Pinaceae. To transfer probes across families as different as Pinaceae and Taxaceae, they must be rooted in highly conserved regions of the DNA such as gene coding regions.

Several primers designed for *Quercus* were shown to be successful in *Pinus* (Demesure *et al.* 1995). These probes anchored in highly conserved regions may be useful in investigating *Taxus* further. Alternatively, a recent taxonomic study of Taxaceae suggested that the chloroplast gene *mat*K contains enough variability to distinguish *Taxus* species (Price 1999). Finally, a screening process used to create specific primers for *Taxus* could be applied.

Chapter 7

General Conclusions and Recommended Research

This research has covered the structure and development of the entire sexual reproductive cycle of *T. brevifolia* including microsporogenesis, pollination, male gametophyte development, megasporogenesis, megagametophyte development, archegonial development, fertilization, cytoplasmic inheritance, embryo development, storage product accumulation and seed efficiency (Fig. 81). The phenology of the entire cycle is a useful reference before undertaking any further research. Though the actual dates may vary depending on the location, the duration of each developmental stage will be similar.

One measure of successful research is the questions that it inspires others to pursue. Several questions haven arisen from this research that merit further study (Fig. 82).

The prolonged staminate strobilus development is worth investigating. This type of low light adaptation has never been previously reported in conifers. The most similar adaptation was recorded in *Chamaecyparis nootkatensis* as it can modify its seed development to fit within the short growing season in montane areas (Owens and Molder 1975). Research on early staminate bud development could easily be extended to cover bud initiation as early reproductive bud initiation has not yet been described in *T. brevifolia*.

Even with hand pollination, seed set remained low in Oregon due to vertebrate predation and light availability (DiFazio *et al.* 1998). However, supplemental pollination

Figure 81. Diagram highlighting the major observations from this research and integrating the separate research areas together within the sexual reproductive cycle of *T. brevifolia*.

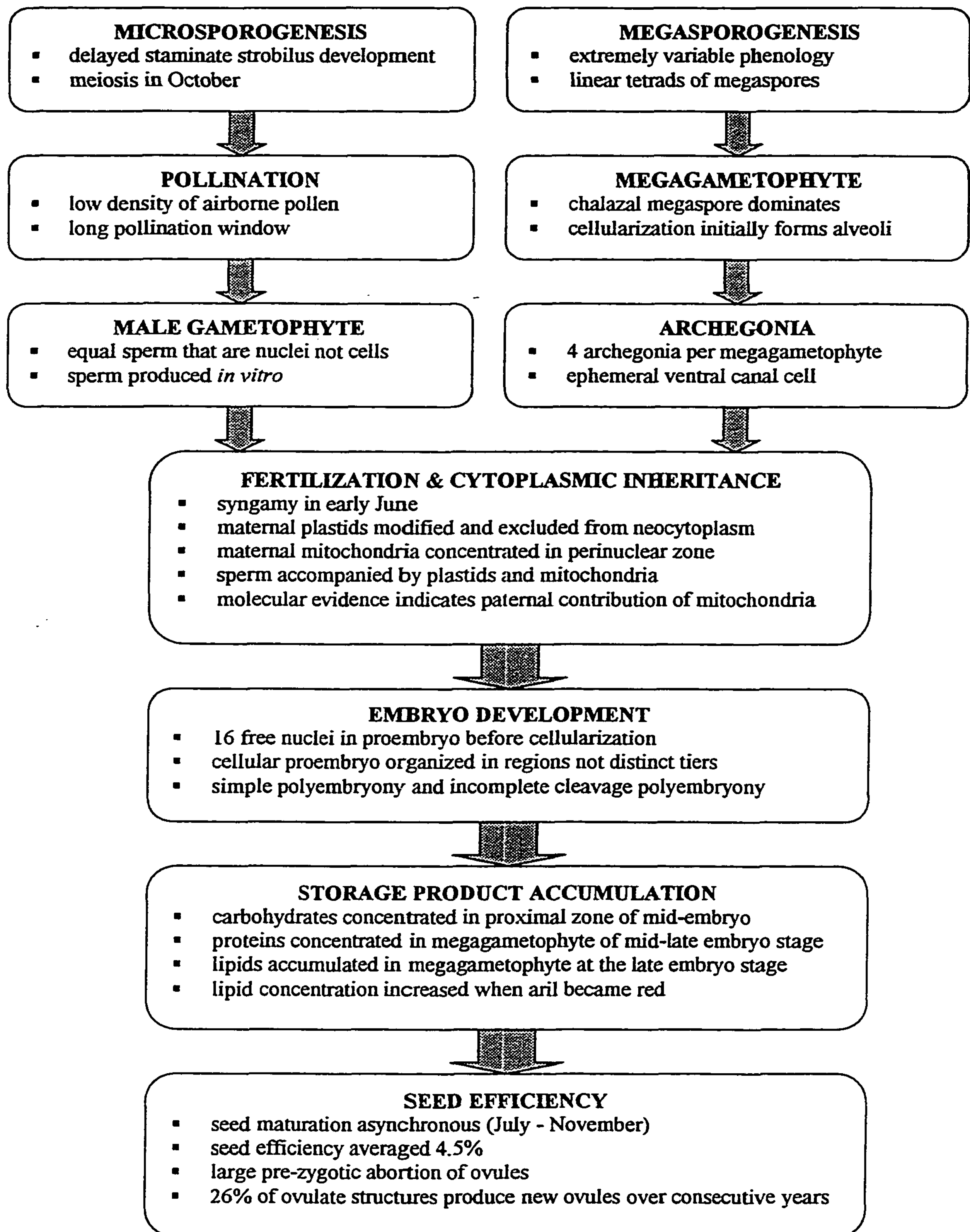
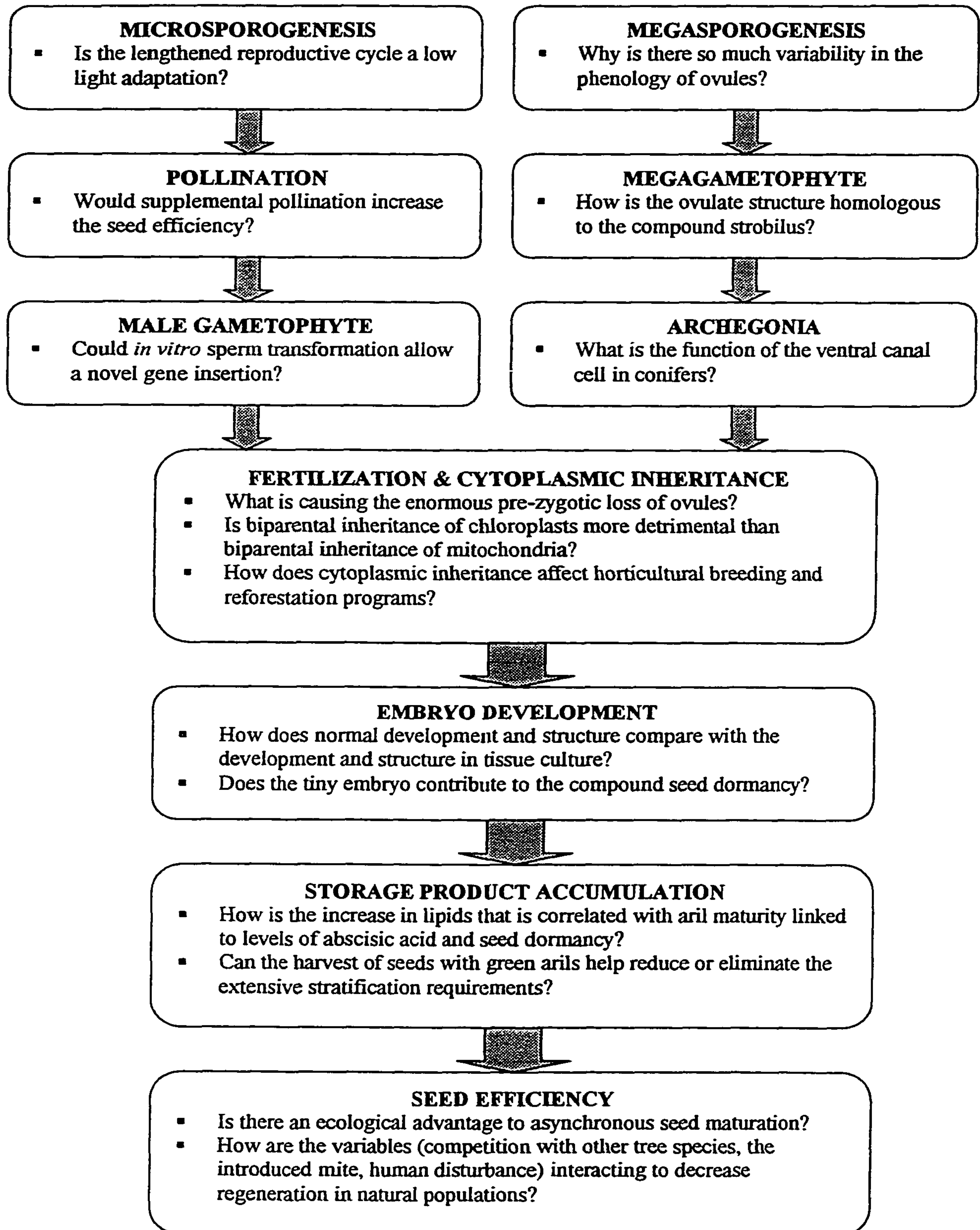

MICROSPOROGENESIS
delayed staminate strobilus development
meiosis in October
MEGASPOROGENESIS
extremely variable phenology
linear tetrads of megaspores
POLLINATION
low density of airborne pollen
long pollination window
MEGAGAMETOPHYTE
chalazal megaspore dominates
cellularization initially forms alveoli
MALE GAMETOPHYTE
equal sperm that are nuclei not cells
sperm produced *in vitro*
ARCHEGONIA
4 archegonia per megagametophyte
ephemeral ventral canal cell
FERTILIZATION & CYTOPLASMIC INHERITANCE
syngamy in early June
maternal plastids modified and excluded from neocytoplasm
maternal mitochondria concentrated in perinuclear zone
sperm accompanied by plastids and mitochondria
molecular evidence indicates paternal contribution of mitochondria
EMBRYO DEVELOPMENT
16 free nuclei in proembryo before cellularization
cellular proembryo organized in regions not distinct tiers
simple polyembryony and incomplete cleavage polyembryony
STORAGE PRODUCT ACCUMULATION
carbohydrates concentrated in proximal zone of mid-embryo
proteins concentrated in megagametophyte of mid-late embryo stage
lipids accumulated in megagametophyte at the late embryo stage
lipid concentration increased when aril became red
SEED EFFICIENCY
seed maturation asynchronous (July - November)
seed efficiency averaged 4.5%
large pre-zygotic abortion of ovules
26% of ovulate structures produce new ovules over consecutive years

Figure 82. Diagram recommending further research questions in the context of this research.


MICROSPOROGENESIS
Is the lengthened reproductive cycle a low light adaptation?
MEGASPOROGENESIS
Why is there so much variability in the phenology of ovules?
POLLINATION
Would supplemental pollination increase the seed efficiency?
MEGAGAMETOPHYTE
How is the ovulate structure homologous to the compound strobilus?
MALE GAMETOPHYTE
Could in vitro sperm transformation allow a novel gene insertion?
ARCHEGONIA
What is the function of the ventral canal cell in conifers?
FERTILIZATION & CYTOPLASMIC INHERITANCE
What is causing the enormous pre-zygotic loss of ovules?
Is biparental inheritance of chloroplasts more detrimental than biparental inheritance of mitochondria?
How does cytoplasmic inheritance affect horticultural breeding and reforestation programs?
EMBRYO DEVELOPMENT
How does normal development and structure compare with the development and structure in tissue culture?
Does the tiny embryo contribute to the compound seed dormancy?
STORAGE PRODUCT ACCUMULATION
How is the increase in lipids that is correlated with aril maturity linked to levels of abscisic acid and seed dormancy?
Can the harvest of seeds with green arils help reduce or eliminate the extensive stratification requirements?
SEED EFFICIENCY
Is there an ecological advantage to asynchronous seed maturation?
How are the variables (competition with other tree species, the introduced mite, human disturbance) interacting to decrease regeneration in natural populations?

has the potential to increase seed production in *T. brevifolia* on Vancouver Island. As in Oregon, poor pollination success is likely considering the low amounts of airborne pollen observed. However, there were few vertebrate seed predators observed at Goldstream and UVic, so vertebrate predation is not a significant factor here. Additionally, light availability reaching adjacent understory species is increased by industrial activities common in BC such as clear-cut logging. Individual trees bordering on clear-cut areas could offer a useful source of seed that are adapted to the local conditions, especially if supplemental pollen is applied.

Sperm cultured *in vitro* is a potential vector for gene transformation (Fernando *et al.* 2000). Though genetically modified organisms remain controversial, they have the potential to aid humankind if they are used ethically. *Taxus* may be a suitable model species because it easily produces sperm *in vitro*.

The phenological variability observed in developing ovules of *Taxus* is unusual. The majority of temperate conifers have less lengthy reproductive stages and are more synchronized to environmental cues (Owens and Blake 1985). The underlying cause of this asynchrony may help to explain how plants respond to environmental signals such as light, temperature, and day length.

The similarities of *Taxus* megagametophyte development to other conifers have contributed even more evidence that *Taxus* is a conifer. The vast majority of evidence available in the literature today supports the inclusion of Taxaceae within Coniferales (Table 1). The ongoing debate was most recently summarized by Pant (2000) who agreed that *Taxus* should be included in Coniferales. Nevertheless, an understanding of the

homologies between the ovulate structure in *Taxus* and a compound strobilus is necessary to end the debate over *Taxus* phylogeny.

The conserved nature of the ventral canal cell within conifers implies that it recently had or still has a function. A ventral canal nucleus or cell has been observed in Araucariaceae, Cephalotaxaceae, Cupressaceae *sensu lato*, Pinaceae, Podocarpaceae and Taxaceae (Singh 1978; Owens *et al.* 1995a; Wilson 1999; Runions and Owens 1999a). One hypothesis to be tested is that the ventral canal cell produces a signal for the pollen tube. This was suggested in *Picea* where it was observed that the pollen tube released the sperm as the tube penetrated the ventral canal cell (Runions and Owens 1999b).

Enormous pre-zygotic losses were recorded during this research. Light limitation and mite damage are the most likely causes of this ovule abortion (Mitchell *et al.* 1997; DiFazio et al. 1998). Comparisons of pre-zygotic abortion in interior populations with mite-infested populations of *T. brevifolia* would indicate the amount of ovule abortion caused by *Cecidophyopsis psilaspis*. All the evidence indicates that humans introduced the mite into British Columbia on cultivars of *T. baccata* (Mitchell et al. 1997). For this reason, we have a responsibility to mitigate its impact on native species like *T. brevifolia*.

Studies into the cytoplasmic inheritance within the conifers have indicated an advantage to uniparental inheritance of organelles (Neale and Sederoff 1988; Mogensen 1996). However, biparental inheritance of mitochondria has been found in the embryos of *Pinus* and perhaps *Taxus* (Wagner *et al.* 1991). Either uniparental inheritance is not as crucial for mitochondria compared with chloroplasts or one lineage of mitochondria is eventually eliminated. This potential for mitochondria from different lineages to recombine deserves further investigations. Furthermore, the implications of cytoplasmic

inheritance within the context of horticultural or forestry breeding programs should be addressed. Knowledge of differential organelle inheritance may explain why individual trees are often better maternal or paternal parents in reciprocal crosses.

Numerous research projects using tissue culture of *Taxus* embryos in order to produce taxanes are underway (Jaziri *et al.* 1996). In addition, the cultivation of *Taxus* is important to the horticultural industry (Chadwick and Keen 1976). The normal *in vivo* development should become a useful reference in understanding how the *in vitro* procedures affect the embryo tissue. This is necessary as *in vitro* procedures can cause developmental abnormalities (Singh 1978).

The elimination of lengthy seed stratification techniques would save the forest and horticultural industries time and money; therefore, basic research on seed dormancy is warranted. This study has shown a correlation between aril maturity and lipid content in the megagametophyte. In addition, previous research has implicated abscisic acid as an important hormone in seed dormancy. If abscisic acid levels are shown to correspond to the presence of a red aril, germinating seeds with green arils may help decrease the stratification requirements (Le Page-Degivry 1973; Flores and Sgrignoli 1991).

Asynchronous seed maturation has now been observed in *T. canadensis, T. x media*, and *T. brevifolia* (Dupler 1917; Flores and Sgrignoli 1991; Wilson *et al.* 1996). Conversely, synchronous seed maturation was observed in *T. cuspidata* (Wilson et al. 1996). Maturing seeds synchronously may be advantageous for species with seed dispersers that also function as seed predators (Herrera *et al.* 1998). Research comparing seed production within *T. cuspidata* with other *Taxus* species may illuminate variable evolutionary pressure from seed predators in the different *Taxus* species.

Recent research on age classes of *T. baccata* in a Himalayan region has indicated that there is no seedling regeneration due to a combination of factors including human disturbance, biotic influences and low seed germination (Rikhari *et al.* 2000). Similar research is needed to assess the population structure of *T. brevifolia* in British Columbia. Given the low seed production, it is probable that there will be few seedlings found. Major detrimental factors to be investigated include the post-glaciation competition from *Thuja plicata*, the introduced mite *C. psilaspis*, and human disturbances due to harvesting and habitat modification. The capacity of *Taxus* to reproduce asexually will help ensure its long-term survival (Bolsinger and Jaramillo 1990). Nevertheless, *Taxus* may eventually require functional sexual reproduction in order to adapt more quickly to the globally changing climate through recombination and long distance transport of seeds.

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