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The Reproductive Biology of *Podocarpus totara* (Podocarpaceae).

by

Vivienne Ruth Wilson
B.Sc., University of Auckland, 1993
M.Sc., University of Auckland, 1995

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

Dr. J.N. Owens, Supervisor (Department of Biology)

Dr. G.A. Allen, Departmental Member (Department of Biology)

Dr. B.J. Hawkins, Departmental Member (Department of Biology)

Dr. S. Misra, Outside Member (Department of Biochemistry and Microbiology)

Dr. D. Southworth, External Examiner (Department of Biology, Southern Oregon State College)

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University of Victoria

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Supervisor: Dr. John N. Owens

ABSTRACT

A reproductive cycle of *Podocarpus totara* in New Zealand was complete within two years. After initiation of the male and female strobili in September, there was a nine-month period of dormancy until emergence in July-August of the following year. The period from pollination through to proembryo growth was continuous, and mature seed was shed in April.

A peak of pollen release from male trees was recorded in early October. Mature pollen contained six nuclei: three prothallial nuclei, tube and sterile nuclei and the body cell. Pollen release and ovule receptivity were synchronous, and an average of 4.52 pollen grains was observed within the micropylar canal of the ovule. Pollen germination occurred soon after entrance to the micropyle, and the pollen tube had penetrated through the nucellus by late November. The body cell entered the pollen tube after all the other nuclei, and was accompanied by prothallial nuclei until gamete formation. Once in contact with the megagametophyte, branching of the pollen tube created a disk-shaped area in which the body cell rested. Mitosis of the body cell resulted in male gametes which were unequal in nuclear size and apportionment of body cell cytoplasm. Only the large gamete was functional.

Female strobili consisted of one or two ovules attached to a pair of fused bracts (the receptacle). Ovules were pollinated at the megaspore tetrad stage, and by the time the pollen tube had emerged from the nucellus, archegonial initials had formed. A megagametophyte contained four to six archegonia, each of which had seven to nine neck

cells and an individual jacket cell layer. The egg nucleus was surrounded by a perinuclear zone containing abundant mitochondria, and all maternal plastids were transformed into large inclusions. Fertilization occurred in early December, and produced a fusion nucleus with a neocytoplasm containing paternal plastids and mitochondria from both parents.

Four free nuclear divisions occurred prior to cell wall formation in the proembryo. The embryonal tier consisted of a single binucleate cell. Thickening of the chalazal wall of the binucleate embryonal cell, and production of a network of vesiculate material at the chalazal tip of the cell happened just prior to suspensor cell elongation. These cell modifications are thought to facilitate embryo movement through the megagametophyte by release of degradative enzymes. No cleavage polyembryony was observed in totara ovules, and the first embryo to emerge from the egg cell appeared to have an advantage in the simple polyembryony mechanism. Small secondary embryos are likely to be the product of suspensor-cell proliferation. Mature embryos had two vascular strands in each cotyledon.

This study documents the first ultrastructural evidence of cytoplasmic inheritance in a member of the Podocarpaceae. Many features of gametophyte and embryo development described in this study are unique to the Podocarpaceae, and suggest that *Podocarpus totara* is a highly-derived species within the family.

Examiners:

~~Dr. J.N. Owens~~, Supervisor (Department of Biology)

Dr. G.A. Allen, Departmental Member (Department of Biology)

Dr. B.J. Hawkins, Departmental Member (Department of Biology)

Dr. S. Misra, Outside Member (Department of Biochemistry and Microbiology)

Dr. D. Southworth, External Examiner (Department of Biology, Southern Oregon State College)

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Dedicated to my grandmother, Rose Phyllis Wilson,
whose love of plants and trees inspired the same in me.

Chapter 1

General Introduction

The Podocarpaceae is the second largest family contained within the order Coniferales. Despite the large number of species, distributed throughout the Southern Hemisphere and tropics, there has been comparatively little study of the morphological and anatomical traits of the members of this family. Within the Coniferales, analysis of cytoplasmic inheritance mechanisms are currently incomplete because no data has been collected for the Podocarpaceae.

Fossil podocarps have been positively identified from as early as the beginning of the Triassic (Miller, 1977) and there is a remarkable similarity in vegetative and reproductive features between proposed ancestral conifer species and fossil podocarps. This has led to speculation that features of podocarp species may indicate an evolutionary progression within the Coniferales. The “extreme” (Kelch, 1997) range of features of reproductive and vegetative anatomy observed within the Podocarpaceae has resulted in ambiguous and frequently re-worked taxonomic states within the family. Confusion over function and derivation of reproductive anatomies in podocarps has made it difficult to carry out cladistic analysis or interpret evolutionary progressions within the family.

The focus of this study is on examining and describing reproductive features in a podocarp species for which we currently have only limited information, but which has silvicultural potential. *Podocarpus totara* (totara) is a forest tree species native to New Zealand. It has been characterised in taxonomic studies as exhibiting many highly

derived features compared to other members of the Podocarpaceae. Totara wood has been used extensively in the construction of Maori war canoes and meeting houses, and as exterior joinery, building foundations and wharf pilings (Cheeseman, 1925; Bergin and Kimberley, 1992). Conservation regulations governing the logging of native species in New Zealand have severely reduced the current supply of totara lumber, but the high dollar value of the wood has led to new interest in the silviculture of totara and other native podocarp species.

The Forest Research Institute in New Zealand is developing a number of research proposals to assess the viability of native conifer species for commercial forestry (FRI website, 1999). Assessments of provenance variation, growth form, wood quality and plantation design are currently being carried out for totara and other potential plantation forestry species. In the short-term, these native species are seen as high-value crops for use in the “farm-forestry” sector, and in private and Maori-owned forests. FRI assesses the use of totara in large-scale commercial forests as an alternative timber species to be developed in addition to current plantations of *Pinus radiata*.

Totara is distributed throughout New Zealand in lowland and subalpine (up to 600 m) forests (Cheeseman, 1925 ; Bergin and Kimberley, 1992). Provenance variation in frost hardiness (Hawkins et al., 1991), height growth and stem form (Bergin and Kimberley, 1992) has been identified in totara seedlings collected from stands throughout New Zealand. This suggests considerable genetic variation within the totara population, and potential for development of a superior stock for forestry purposes. One of the major

hurdles to successful silviculture of totara is the current lack of information about the phenology of the reproductive cycle, and potential influences on the quality of seed.

This study focuses on three main objectives:

1. To determine the phenology of the reproductive cycle in totara from cone initiation to seed maturity, including assessments of pollination and fertilization success.
2. To determine the course of male and female gametophyte and embryo development by examining anatomical and ultrastructural features from pre-fertilization stages to seed maturity.
3. To determine the ultrastructure of male and female gamete formation, the process of fertilization, and the mechanism of cytoplasmic inheritance in totara zygotes.

Chapter 4 describes the sequence of events occurring between strobilus initiation and late embryo development. The processes of male and female gametophyte development (Chapters 5 and 6), fertilization and cytoplasmic inheritance (Chapter 7) and proembryo to early embryo development (Chapter 8) are examined in ultrastructural detail.

Measurement of levels of reproductive potential such as pollen release, pollination success, and fertilization success are presented in Chapter 9. Chapter 10 summarises the results of this study and discusses the implications of these results with respect to the evolutionary position of the Podocarpaceae within the Coniferales.

Note on format

Chapter 4 has been published as Wilson and Owens (1999), and is cited in subsequent chapters as a journal publication. To prevent repetition, we have moved the “Material

and Methods” section to Chapter 3, which describes methods for all experimental work contained in this dissertation. No other significant changes have been made to the content of Chapter 4. Chapters 5, 6, 7 and 8 are currently being submitted for publication in different journals, and therefore contain repeats of some information in the introductory sections.

Chapter 2

The Podocarpaceae: phylogeny, taxonomy and reproductive anatomy

Ancestral podocarps

Podocarps were likely to have diverged from the ancestral Voltziales in the Late Paleozoic (Miller, 1977). Doyle (1945) concluded that the Lebachiaceae were ancestral to the podocarps, due to the inverted ovule found in *Walchia* and *Ullmania*, however Stockey (1981) identified *Ullmania* as a more likely ancestor for the Araucariaceae. Stiles (1912) predicted a primitive podocarp as a “tree bearing spirally-arranged leaves, with reproductive shoots bearing male and female cones with spirally arranged sporophylls...microsporophylls have two microsporangia... megasporophyll has a single erect ovule with a single integument, situated axillary.” Kelch’s (1998) 18s rDNA analysis of members of the Podocarpaceae concluded that the earliest podocarps had bifacial leaves and multiovulate cones with large epimatia. Miller (1977) examined Mesozoic conifers and found that the Podocarpaceae were well represented by the Lower Triassic, and some fossil podocarps looked remarkably like these predictions.

The earliest podocarp fossils were found in southern Africa, Antarctica, Australia and New Zealand (Miller, 1977; 1982; Greenwood, 1987; Hill and Pole, 1992). *Rissikia* and *Mataia* had spirally arranged leaves, peltate microsporophylls bearing two microsporangia on a stalked pollen cone, and terminal seed cones made up of 15-20 bract-scale complexes, with stalked seeds borne on the adaxial surface of the scale. In *Mataia* the apical portion of the scale folded back on itself to partially cover the inverted,

rounded seeds. Fossils such as *Nipaniostrobus* and *Nipanioruha* found in India, also had a folded-back scale. *Sitholyea* combined a folded scale with a cone made up of a single bract-scale complex positioned terminally on the shoot, much like some *Podocarpus* or *Dacrydium* species today (Miller, 1977). *Nothodacrium* had pinnately-branched shoots with helically arranged leaves, and spike-like seed cones with 10-15 bract-scale complexes. The bract and scale were free from each other (the bract simple and the scale 3-lobed) and the seed was situated centrally on a stalk.

Stiles (1912) suggested that there may have been two lines of development in early podocarps. One line underwent a reduction in leaf size, number of megasporophylls in the strobilus, and number of archegonia, and also underwent development of a second integument around the seed, like the modern genera *Microstrobus* and *Phyllocladus*. The other line underwent intercalary growth of the ovule stalk (lifting the ovule from the cone axis) and the ovule was inverted and developed an epimatium, like the modern genera *Saxegothaea*, *Microcachrys*, *Dacrydium* and *Podocarpus*. This theory appears to be supported by the fossil record.

The position of the Podocarpaceae within the Coniferales

The fossil record of the Coniferales dates back to the Permian, and coniferous species are known to have dominated forest vegetation during the Mesozoic era (Chamberlain, 1966; Miller, 1977; Hart, 1987). The identity of ancestral species is uncertain, and there are equivocal relationships among conifers, cordaites, cycads and some seed ferns (Chase et al., 1993; Rothwell, 1994), however, the Coniferales is considered to form a

monophyletic group, descended from the Mesozoic Voltziales (Miller, 1977). Kelch (1997) described the Podocarpaceae as having “distinctive combinations of autapomorphies and characters that occur only singly in other conifer taxa”. An autapomorphy is defined as an inherited condition resulting from convergent evolution of a character (Futuyma, 1986). Page (1990) considered it likely that the podocarps were separated geographically from other conifer families early in their development. Millers (1988) analysis of morphological characters in the Coniferales suggested that the Podocarpaceae form a highly derived clade with the Pinaceae, Araucariaceae and the Cephalotaxaceae, although Hart’s (1987) analysis found that the Podocarpaceae was a sister group to all other conifer families with the exception of the Pinaceae. Cladistic DNA analysis supports the position of the Podocarpaceae as sister to all other families except the Pinaceae, although results differed as to which families were most closely related (Bousquet et al., 1992; Chase et al., 1993; Chaw et al., 1993; Chaw et al., 1995; Stefanovic, 1998).

Sinnott (1913) and Stiles (1912) suggest that *Podocarpus* had features derived from the Abietoideae (Pinaceae); specifically the presence of prothallial cells in the male gametophyte, the distribution and arrangement of archegonia, and winged pollen. Affinities between the Podocarpaceae and Taxaceae have been based on the presence of a fleshy aril and abaxial microsporangia (Page, 1990; Keng, 1975; 1978). Florin (1958) and Stiles (1912) did not support a relationship between the Taxaceae and the Podocarpaceae due to differences in ovule position, morphology of the female strobilus and the absence of prothallial cells. The only affinities to be borne out by cladistic analysis are those between Podocarpaceae and the Cephalotaxaceae and Araucariaceae.

Sinnott (1913) noted the “striking similarity” between the strobilus structure, megagametophyte and embryo development of *Prumnopitys* and *Cephalotaxus*. He concluded that *Cephalotaxus* (later raised to family rank), Araucariaceae and Podocarpaceae were likely to have arisen from ancestral abietinous stock. Miller (1988) placed Podocarpaceae in a clade with both the Cephalotaxaceae and Araucariaceae, and both Young (1910) and Page (1990) commented that there was a strong resemblance between *Saxegothaea* and members of the Araucariaceae, particularly in the anatomy of the pollen grain. Affinities with other families of conifers are likely to be very ancient ones due to the early geographic isolation of podocarp species. Cooler temperatures in the Oligocene forced podocarp species south, and the subsequent breakup of Gondwana isolated them from more northern conifer species (Page, 1990; Kelch, 1997).

The taxonomy of the Podocarpaceae

The Podocarpaceae are the second largest conifer family, with c.f. 125 species organised into 19 genera (Page, 1990; Molloy, 1995; Kelch, 1997). Analyses of genera and characteristics found in the Podocarpaceae have been revised many times (Pilger, 1926; Buchholz, 1951a; 1951b; Buchholz and Gray, 1948a; 1948b; Hair and Beuzenberg, 1958; De Laubenfels, 1959; 1969; 1985; 1987; Gray, 1953; 1955; 1956; 1958; 1960; 1962; Tengner, 1967; Quinn, 1982; Page, 1988). Taxonomic analysis was limited by a lack of material and analysis of abnormal material in the first part of this century (Aase, 1915), and subsequent reorganisations are listed in Table 1. The heterogeneity of characteristics in the Podocarpaceae have been described as “extreme” in comparison to

Table 1. Changes to traditional and proposed taxa in the Podocarpaceae, based on taxonomic studies.

Stiles (1912) Pilger (1926)	Buchholz and Gray (1948a,b)	Dallimore (1966) De Laubenfels (1969) Quinn (1970)	Page (1990) Molloy (1995)
<i>Acmopyle</i>		<i>Acmopyle</i>	<i>Acmopyle</i>
<i>Dacrydium</i>		<i>Dacrydium</i> group A	<i>Falcatifolium</i>
		<i>Dacrydium</i> group B	<i>Dacrydium</i>
		<i>Dacrydium</i> group C	<i>Halocarpus</i>
		<i>Dacrydium</i> group C	<i>Lagarostrobos</i>
		<i>Dacrydium</i> group C	<i>Manoao</i>
		<i>Dacrydium</i> group C	<i>Lepidothamnus</i>
<i>Microcachrys</i>		<i>Microcachrys</i>	<i>Microcachrys</i>
<i>Phaerosphera</i>		<i>Microstrobos</i>	<i>Microstrobos</i>
<i>Phyllocladus</i>		<i>Phyllocladus</i>	<i>Phyllocladus</i>
<i>Podocarpus</i> sect. <i>Eupodocarpus</i>	<i>Podocarpus</i> sect. <i>Eupodocarpus</i>	<i>Podocarpus</i> sect. <i>Podocarpus</i> subsect. A,C,D,E	<i>Podocarpus</i> subgroup <i>Podocarpus</i>
		<i>Podocarpus</i> sect. <i>Podocarpus</i> subsect. B,F	<i>Podocarpus</i> subgroup <i>Foliolatus</i>
<i>Podocarpus</i> sect. <i>Nageia</i>	<i>Podocarpus</i> sect. <i>Nageia</i>	<i>Podocarpus</i> sect. <i>Nageia</i>	<i>Nageia</i>
	<i>Podocarpus</i> sect. <i>Afrocarpus</i>	<i>Podocarpus</i> sect. <i>Afrocarpus</i>	<i>Nageia</i> sect. <i>Afrocarpus</i>
	<i>Podocarpus</i> sect. <i>Polypodiopsis</i>	<i>Podocarpus</i> sect. <i>Polypodiopsis</i>	<i>Nageia</i> sect. <i>Polypodiopsis</i>
<i>Podocarpus</i> sect. <i>Microcarpus</i>	<i>Podocarpus</i> sect. <i>Microcarpus</i>	<i>Podocarpus</i> sect. <i>Microcarpus</i>	<i>Parasitaxis</i>
<i>Podocarpus</i> sect. <i>Dacrycarpus</i>	<i>Podocarpus</i> sect. <i>Dacrycarpus</i>	<i>Podocarpus</i> sect. <i>Dacrycarpus</i>	<i>Dacrycarpus</i>
	<i>Podocarpus</i> sect. <i>Sundacarpus</i>	<i>Podocarpus</i> sect. <i>Sundacarpus</i>	<i>Sundacarpus</i>
<i>Podocarpus</i> sect. <i>Stachycarpus</i>	<i>Podocarpus</i> sect. <i>Stachycarpus</i>	<i>Podocarpus</i> sect. <i>Stachycarpus</i>	<i>Prumnopitys</i>
<i>Saxegothaea</i>		<i>Saxegothaea</i>	<i>Saxegothaea</i>

other conifer families (De Laubenfels, 1969; Hart, 1987; Page, 1990), due in part to the mixture of actively evolving genera (e.g. *Podocarpus* and *Dacrydium*) and isolated relicts (e.g. *Microcachrys* and *Saxegothaea*) in the family (Kelch, 1997).

Each genus in the Podocarpaceae has a unique set of both primitive and advanced features, and this is expected if each genus represents the end of an independent line evolved from a common ancestor (Quinn, 1970). Each genus appears as a terminal taxon in the cladistic diagrams of Hart (1987) and Kelch (1997) (Figs. 1 and 2). It should be noted that the taxa identified as least highly derived in Kelch's (1997) analysis (*Sundacarpus*, *Saxegothaea*, *Prumnopitys*) are identified as highly derived in the Hart (1987) analysis. This difference is due to the method of cladistic analysis used, the number of characters evaluated (53 in Kelch (1997); 24 in Hart (1987)), and differences in character coding states chosen for each genus.

Some workers have advocated elevating specific podocarp genera to the family level. Fu (1992) stated that *Nageia* formed a monotypic family on the basis of its parallel-veined leaves, and Woltz (1986, from Stockey and Ko, 1988) placed *Saxegothaea* and *Microcachrys* into separate families due to their mode of seedling growth. Keng (1975; 1978) elevated *Phyllocladus* to the Phyllocladaceae on the basis that the phylloclad was a link between the branches of ancient progymnosperms and the leaves of extant conifers. However, Hart (1987) and Kelch (1997) identified five characters which unite all podocarp genera:

1. bi-nerved or multi-nerved cotyledons in the embryo
2. binucleate embryonal tier cells

3. microsporophylls bearing two sporangia
4. uniovulate cone-scale complexes
5. root nodules

The epimatium is considered typical for the family, but it is missing in two genera (*Phyllocladus* and *Microstrobos*) and very reduced in others. Fleshiness of some part of the female strobilus is found in 18 out of the 20 taxa in the family, which suggests that there has been a strong selection or predisposition for fleshy seeds (Kelch, 1997). Kelch (1997) identified affinities between genera in his cladistic analysis. The only taxa which did not show affinity for other members of the family were those described as “isolated, relict” taxa (*Saxegothaea*, *Sundacarpus* and *Prumnopitys*) (Kelch, 1997). *Phyllocladus*, *Microstrobos*, *Microcachrys*, *Manoao*, *Lagarostrobos*, *Halocarpus* and *Parasitaxus* formed a so-called “scale-leaved” clade. The remaining genera formed a “tropical” clade (Kelch, 1997). Cladistic study of morphological features favours retaining all 19 genera (20 taxa) within the Podocarpaceae.

Podocarpus totara D. Don. ex Lambert is a member of *Podocarpus* L’Her. ex Pers. subgroup *Podocarpus* (see Table 1). Characteristics such as the high degree of fusion and reduction of the female strobilus (one or two ovules per strobilus, fusion of the epimatium to the integument), amalgamation of the bracts into the fleshy receptacle (Sinnott, 1913; Page, 1990; Tomlinson, 1992), lateral position of the whorl of female strobili (Tomlinson, 1992) and single binucleate embryonal tier cell in the early embryo (Konar and Oberoi, 1969b; Buchholz, 1941) are recognised as highly derived features within the Podocarpaceae.

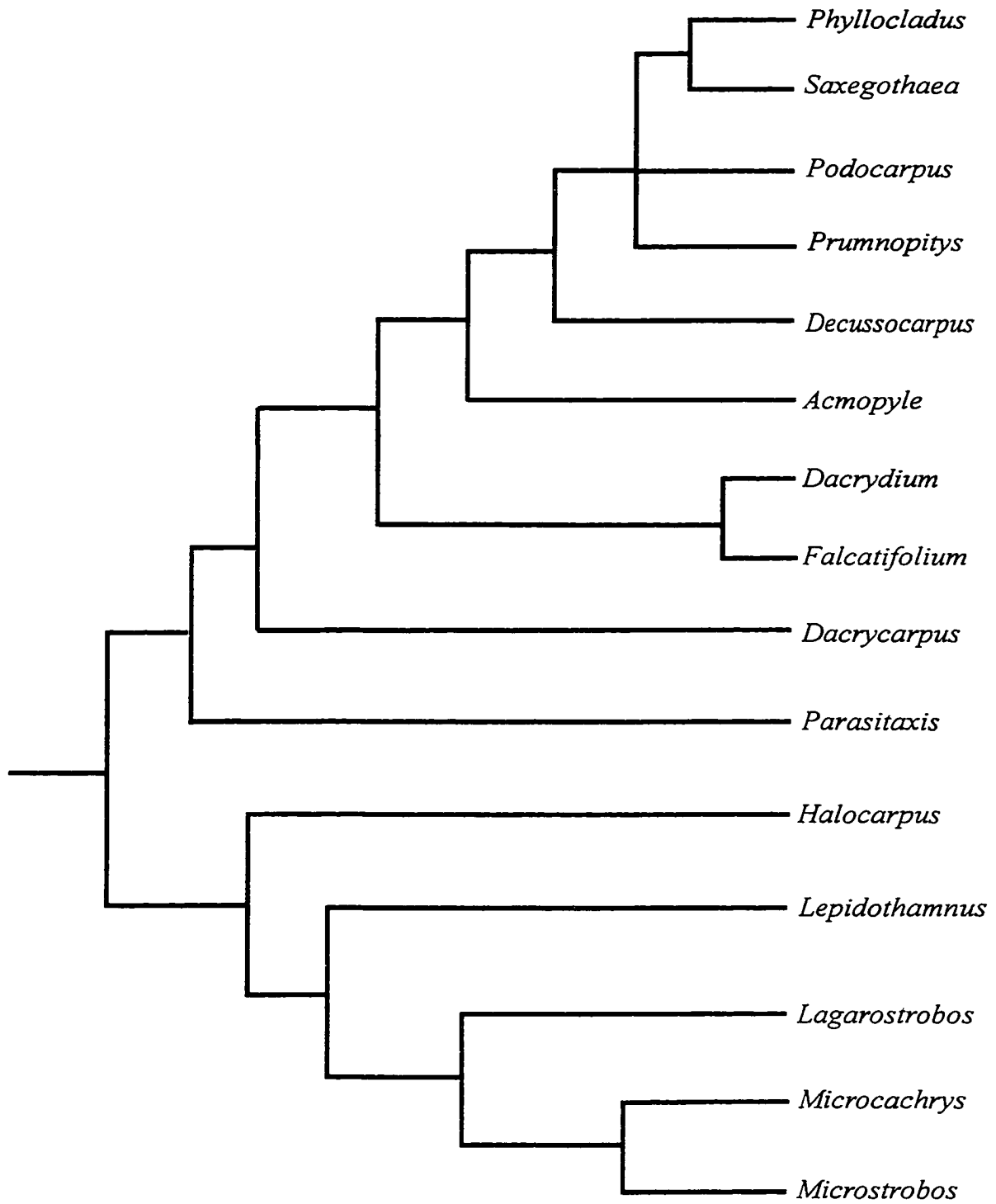


Figure 1. Phylogenetic relationships of Podocarpaceae. Adapted from Hart (1987).

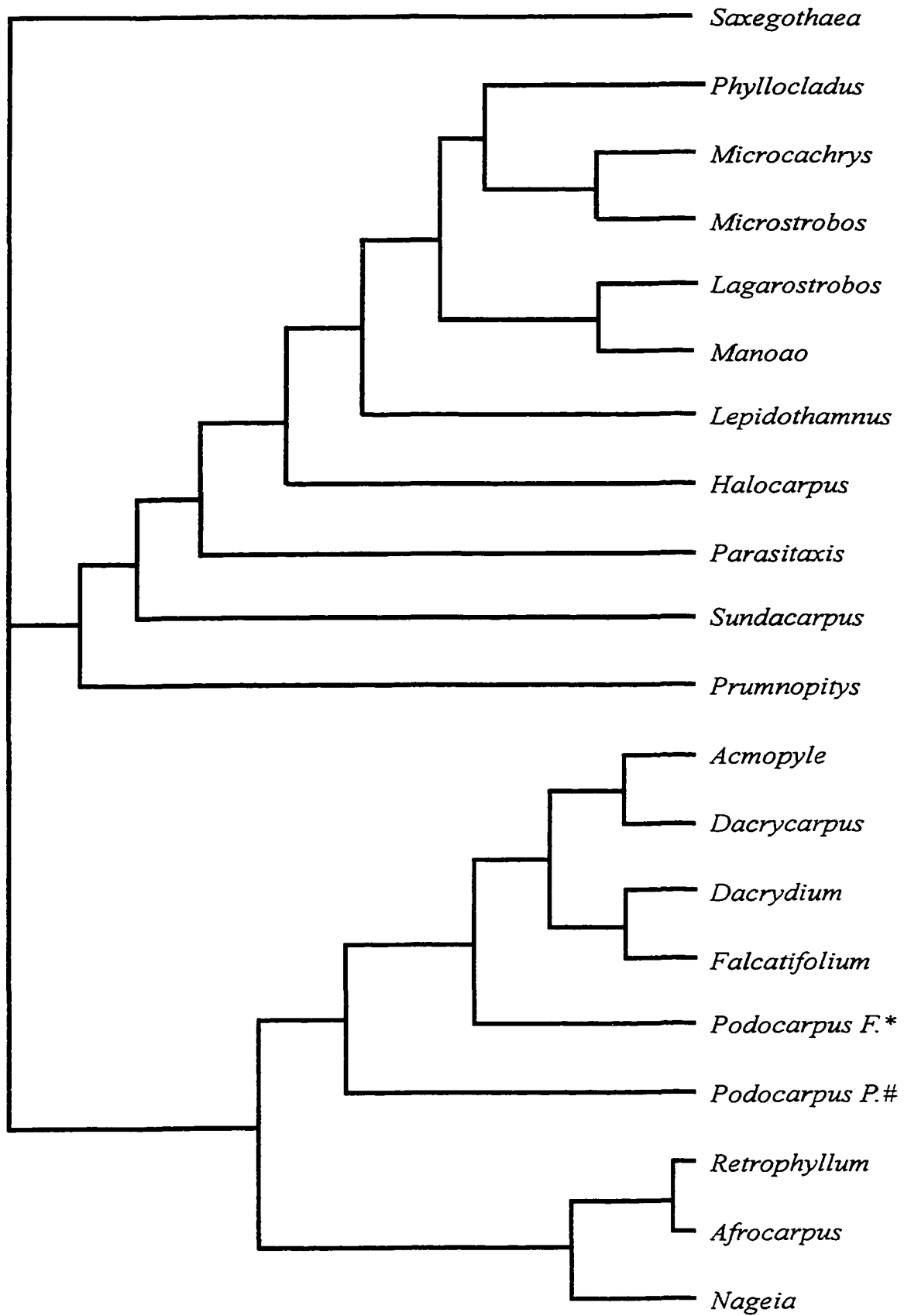


Figure 2. Phylogenetic relationships of Podocarpaceae. Adapted from Kelch (1997).

* - *Podocarpus* subgroup *Foliolatus*. # - *Podocarpus* subgroup *Podocarpus*.

Development of the male gametophyte in the Podocarpaceae

The male cones of all podocarp genera are very similar, consisting of bisporangiate, peltate microsporophylls arranged around a central axis (Sinnott, 1913; Page, 1990). They are borne singly or in loose spikes amongst the leaves of growing shoots, and either terminally or laterally, depending on genus (Page, 1990). The male cone of *Podocarpus totara* consists of 100-120 microsporophylls (Coker, 1902; Burlingame, 1908; Sinnott, 1913).

Microsporogenesis has been reviewed by Jeffrey and Chrysler (1907) (*Podocarpus polystachya*), Sinnott (1913) (*Podocarpus sp.* and *Dacrydium sp.*), Konar and Oberoi (1969b), Singh (1978) and Del Fueyo (1996). Vasil and Aldrich (1970; 1971) and Aldrich and Vasil (1970) completed extensive ultrastructural studies of microspore differentiation and pollen wall formation in *Podocarpus macrophyllus*. The sporangium wall is four to seven cells thick. It consists of a thick-walled epidermal layer, three to five layers of thin-walled cells which collapse before the sporangium dehisces, and one or two innermost layers of binucleate tapetal cells (Coker, 1902; Burlingame, 1908; Sinnott, 1913; Konar and Oberoi, 1969b; Del Fueyo, 1996). Sporogenous cells are distinguished by their thin cell wall and dense cytoplasm.

All podocarp genera except *Saxegothaea* have saccate pollen; *Microcachrys*, *Microstrobos* and *Dacrycarpus* are trisaccate, and all remaining genera are bisaccate (Konar and Oberoi, 1969b; Pocknall, 1981; Page, 1990). Podocarp pollen is filled with lipid globules, starch grains and proteinaceous deposits at maturity (Vasil and Aldrich, 1970; 1971). Exine texture depends on genus, but all podocarp pollen is noted for a slit-

like “region of weakness” found between the sacci, and extremely thick exine development at the cap region (Pocknall, 1981). Mature pollen is multinucleate when shed.

All podocarp pollen grains contain one or more prothallial nuclei, with those of most genera containing three to six (Konar and Oberoi, 1969b; Kelch, 1997). The first two mitotic divisions in the pollen grain produce two primary prothallial cells (Fig. 3) (Coker, 1902; Jeffrey and Chrysler, 1907; Burlingame, 1908; Sinnott, 1913; Hodcent, 1964). Anticlinal mitoses of primary prothallial cells form a cluster of two to eight secondary prothallial cells depending on species. The generative cell divides anticlinally to form the body cell and sterile cell (Fig. 3). At pollen maturity the cell walls surrounding the sterile and prothallial nuclei disintegrate, and the prothallial, sterile and tube nuclei lie free in the cytoplasm. The body cell nucleus is contained within a distinct cell wall (Jeffrey and Chrysler, 1907; Sinnott, 1913; Konar and Oberoi, 1969b; Del Fueyo, 1996). The tube nucleus can sometimes be distinguished by its comparatively large size.

All podocarps are wind-pollinated. In most genera, ovules produce a pollination drop to scavenge pollen grains from the surface of the ovule. Exudation of the pollination drop is continuous over a period of about a week in *Podocarpus* (Konar and Oberoi, 1969b; Singh, 1978). *Saxegothaea* is the only genus which does not produce a pollination drop; pollen grains land on the ovuliferous scale and develop a long pollen tube which eventually contacts the nucellus (Tomlinson, 1991). Pollen germination occurs immediately (Konar and Oberoi, 1969b) or within a few weeks (Looby and Doyle, 1944a) of the grains coming into contact with the nucellus within the micropylar canal.

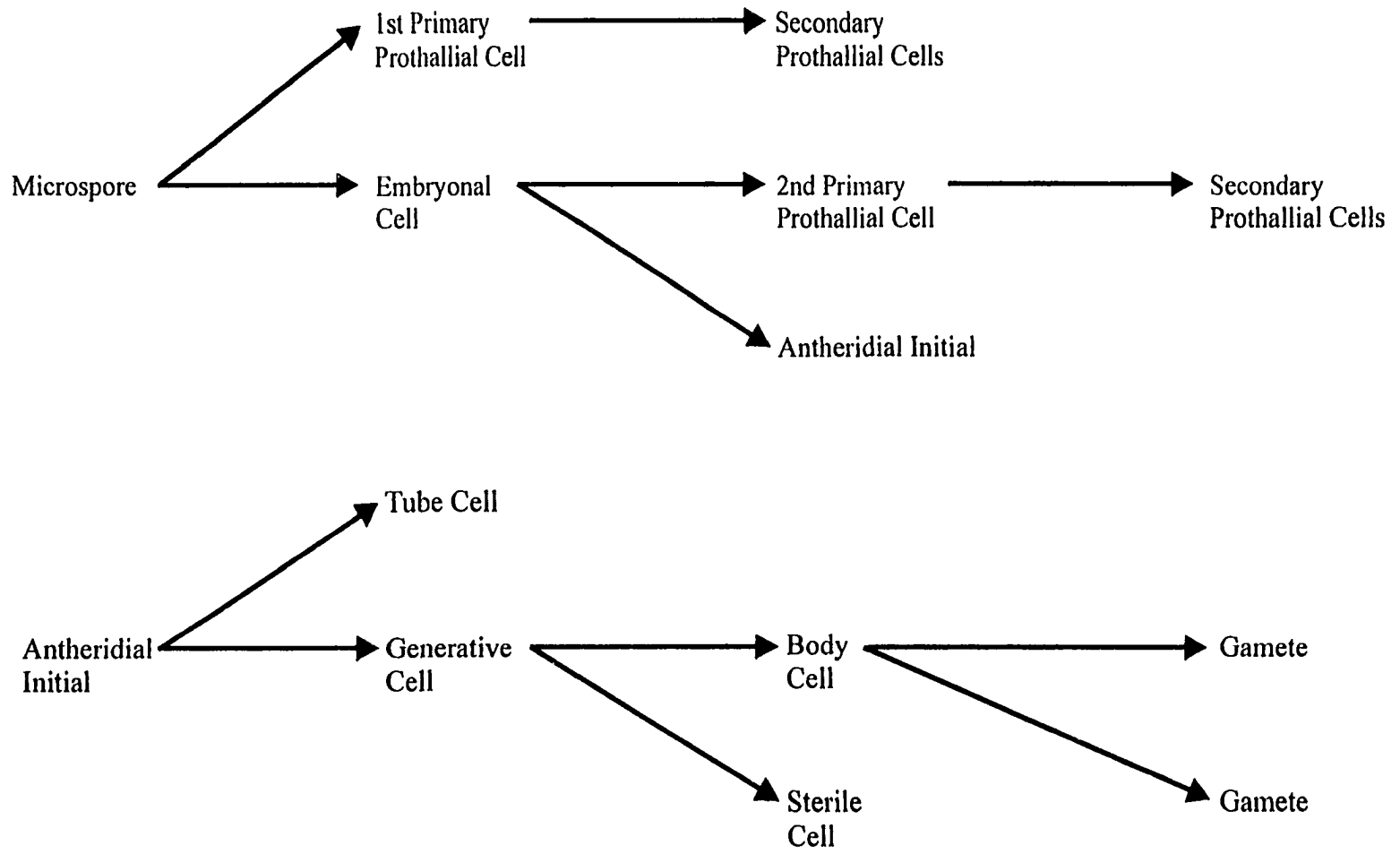


Figure 3. Pollen grain development in the Podocarpaceae.

The exine of podocarp pollen splits in the “region of weakness” (Pocknall, 1981) between the sacci, and the pollen tube extends into the nucellar tip. The exine of the pollen grain remains intact throughout germination and often persists in the micropyle after fertilization (Looby and Doyle, 1944a; Singh, 1978). As the pollen tube penetrates the nucellus, the prothallial and sterile nuclei follow the tube nucleus, but the body cell lags behind and is last to leave the pollen grain (Sinnott, 1913; Looby and Doyle, 1944a; Boyle and Doyle, 1953; Singh, 1978). Konar and Oberoi (1969a) report that in *Podocarpus gracilior* the body cell usually overtakes some of the prothallial nuclei, and continues through the pollen tube in close association with them.

The pollen tube reaches the micropylar end of the nucellus while the megagametophyte is still in the free nuclear stage (Coker, 1902; Looby and Doyle, 1944a; Singh, 1978). At this point the pollen tube splays out into a thin disc covering the apex of the megagametophyte, and branches down between the megaspore wall and the nucellus. The body cell enlarges within the pollen tube disc, and divides to form male gametes just prior to fertilization (Sinnott, 1913; Konar and Oberoi, 1969a; 1969b; Singh, 1978). Looby and Doyle (1944a) observed that the body cell of *Podocarpus andinus* divided soon after the pollen tube had penetrated through the nucellus, and the gametes then remained in the pollen tube until fertilization. Konar and Oberoi (1969b) reported that division of the body cell forms three unequal male gametes in most podocarp genera, but most studies agree that only two gametes are formed. In *Phyllocladus*, *Saxegothaea*, *Microcachrys* and *Podocarpus andinus* the gametes are reported to be equal cells (Young, 1910; Lawson, 1923; Looby and Doyle, 1939; Looby and Doyle, 1944a). Unequal male nuclei are reported in other species (Coker, 1902; Stiles, 1912; Boyle and Doyle, 1953;

Osborn, 1960; Quinn, 1965; 1996a; 1966b; Konar and Oberoi, 1969a). Singh (1978) tried to make a distinction between formation of male cells or male nuclei by noting that male cells are reported for species whose archegonia are arranged in one or more complexes, and that male nuclei are reported for species whose archegonia are placed singly. In most cases, division of the body cell initially results in male nuclei of the same size, but the more centrally-placed nucleus enlarges and presses the other smaller nucleus into a lenticular area to one side of the body cell cytoplasm (Konar and Oberoi, 1969a; Singh, 1978). The smaller nucleus persists, and although some studies suggest that it is extruded into the pollen tube (Coker, 1902; Sinnott, 1913) it usually remains close to the functional male nucleus, and enters the egg cell at fertilization.

Development of the megagametophyte in the Podocarpaceae

The megagametophyte is initiated as a small area of “poorly differentiated” sporogenous tissue deep in the nucellus of the young ovule (Looby and Doyle, 1944a; Singh, 1978). The megaspore mother cell is distinguished by its large size in comparison to other sporogenous cells, and divides to form a linear tetrad of megaspores in *Podocarpus totara* (Sinnott, 1913), *P. gracilior* (Konar and Oberoi, 1969a), *Microcachrys*, *Dacrydium* and *P. falcatus* (Osborn, 1960). A triad of megaspores (the micropylar-most cell being binucleate) has been observed in *P. andinus* (Looby and Doyle, 1944a) and *P. nivalis* (Boyle and Doyle, 1953). The three (or two) megaspores closest to the micropylar end abort. A tapetum of densely cytoplasmic, multinucleate cells forms around the remaining megaspore (Young, 1910; Gibbs, 1912; Sinnott, 1913;

Looby and Doyle, 1944a; Singh, 1978). Coker (1902) did not observe a tapetal layer around the megaspore of *P. macrophyllus*. The tapetal cells closest to the megaspore deposit lipid droplets onto the megaspore cell wall (Singh, 1978), but eventually the tapetum becomes indistinguishable from surrounding nucellar tissue (Sinnott, 1913).

The megaspore undergoes a series of free nuclear divisions, enlarges, and develops a large vacuole (Sinnott, 1913; Looby and Doyle, 1944a; Singh, 1978). Vesicular and lipidic material deposited on the megaspore wall increases its surface area, and two layers develop - an inner cellulose-pectinaceous layer, and a suberized outer layer (Gibbs, 1912; Singh, 1978). The megaspore wall is usually thickest at the chalazal end of the megagametophyte. The free nuclei, positioned at the periphery of the vacuole, undergo a series of mitoses, and are eventually connected by spindles to six adjacent nuclei (Singh, 1978). Cell walls form across the spindles, and are laid down centripetally from the megaspore cell wall to the centre of the megagametophyte. Transverse walls are then laid down until the megagametophyte becomes a body of thin-walled uninucleate cells (Sinnott, 1913). Sinnott (1913) reports that the development of two megagametophytes is common in the ovule of many podocarp species, although it is rare for both to mature.

Archegonial initials are not apparent until late in cell wall formation. The total number of archegonia varies among species, but ranges from one or two in *Phyllocladus* (Young, 1910) to 20-25 in *Podocarpus nivalis* (Boyle and Doyle, 1953). Archegonial development, fertilization and embryogeny have been observed in many species of *Podocarpus* (Coker, 1902; Gibbs, 1912; Stiles, 1912; Sinnott, 1913; Looby and Doyle, 1944b; Boyle and Doyle, 1953; Osborn, 1960; Konar and Oberoi, 1969a). The position

of archegonia has been suggested to be influenced by contact of the pollen tube with the megagametophyte as cell walls are laid down (Konar and Oberoi, 1969a).

Archegonial initials divide periclinally to form a large central cell, and a small primary neck cell. Periclinal divisions in cells adjacent to the archegonia form layers of binucleate jacket cells (Looby and Doyle, 1944a; Singh, 1978). In some species, archegonia are grouped within a single layer of jacket cells (e.g. *Podocarpus andinus* (Looby and Doyle, 1944a)), and in others, each archegonium has a separate jacket layer (e.g. *P. totara* (Sinnott, 1913), *P. gracilior* (Konar and Oberoi, 1969a), *P. nivalis* (Boyle and Doyle, 1953)). The primary neck cell divides to form a variable number of neck cells (dependent on species) arranged radially in one or two tiers.

The central cell enlarges, the nucleus lies close to the neck cells and the cytoplasm becomes vacuolate and “foamy” (Sinnott, 1913; Looby and Doyle, 1944a; Singh, 1978). Once the archegonium has reached its full size, the central cell nucleus divides obliquely and to one side of the neck cells, forming the egg nucleus which migrates to a central region of granular cytoplasm, and the ventral canal nucleus which remains appressed to the archegonial wall to one side of the neck cells (Sinnott, 1913; Looby and Doyle, 1944a; Boyle and Doyle, 1953; Konar and Oberoi, 1969a). The ventral canal nucleus may persist until fertilization, but is described as “ephemeral” in most species. Coker (1902) observed that in unfertilized archegonia, the ventral canal nucleus sometimes detaches from the archegonial wall and migrates towards the egg nucleus. The egg-cell cytoplasm becomes vacuolar and granular, with one large vacuole at the chalazal end. The egg nucleus moves to the centre of the egg cell, and is surrounded by a thin perinuclear layer of cytoplasm.

Just prior to fertilization, the megagametophyte has reached two-thirds of its final size. Active periclinal divisions in the area around the archegonia produce a 'cone' of small multinucleate cells extending into the middle of the megagametophyte (Sinnott, 1913).

Fertilization and proembryogeny

Fertilization in the Podocarpaceae has only been observed in *Podocarpus sp.* (Coker, 1902; Sinnott, 1913; Looby and Doyle, 1944b; Boyle and Doyle, 1953; Konar and Oberoi, 1969a). The pollen tube has been reported as entering "laterally" to the neck cells in *Podocarpus gracilior* and *P. nivalis*, leaving them intact but degenerative, and as entering directly through the neck cells in *P. totara* and *P. andinus*. Looby and Doyle (1944b) observed that in *P. andinus*, some of the egg cytoplasm is discharged into the pollen tube as the neck cells rupture. The functional male gamete is the first to enter the egg cell, accompanied by a portion of the body cell cytoplasm. The non-functional gamete and prothallial nuclei have been observed degenerating outside the archegonium (Konar and Oberoi, 1969a), but usually follow the functional male gamete into the archegonium. The non-functional and prothallial nuclei remain at the micropylar end of the archegonium, and may persist for some time after fertilization (Sinnott, 1913; Singh, 1978).

The functional male gamete migrates to the egg nucleus, and flattens against it. The two gametes are initially separated by their nuclear membranes, but these gradually break down, and the nuclei fuse (Sinnott, 1913; Singh, 1978). Boyle and Doyle (1953) describe the male gamete as "slipping out of its cytoplasm" to fuse with the egg nucleus.

However, Sinnott (1913) and Singh (1978) report that the male cytoplasm coalesces with the egg cytoplasm around the egg nucleus, and the resulting neocytoplasm contains organelles from male and female parents. This dense neocytoplasm then accompanies the fusion nucleus as it migrates towards the chalazal end of the archegonium (Sinnott, 1913).

Coker (1902) observed that in *Podocarpus macrophyllus* the fusion nucleus moves to the chalazal end of the egg cell before dividing. However in most other podocarps, the first division takes place at the point of gamete fusion, and the resulting two nuclei migrate (Sinnott, 1913; Konar and Oberoi, 1969a; Singh, 1978). Once at the chalazal end of the egg cell, the nuclei undergo three or four more rounds of free nuclear division producing 16 or 32 nuclei respectively, depending on species. In all species, the free nuclei arrange into two tiers before cell-wall formation; a primary embryonal tier and a primary upper tier (Singh, 1978). After cell-wall formation, the cells divide again, but embryonal tier cells do not form another cell wall and become binucleate. The embryonal tier of *Podocarpus gracilior* (Konar and Oberoi, 1969a) consists of 9-12 binucleate cells, and that of *P. totara* (Sinnott, 1913) and *P. macrophyllus* (Coker, 1902) consists of a single binucleate cell. Division of the primary upper tier results in formation of the suspensor tier and the open tier. As the suspensor cells begin to elongate, the open tier cells form a “plug” and degenerate (Singh, 1978). Some studies have commented on the presence of “rosette” nuclei which may proliferate towards the micropylar end of the egg cell (Coker, 1902; Sinnott, 1913). Buchholz (1941) did not find evidence of rosette nuclei, and Singh (1978) suggests that they are likely to be the degenerating prothallial and supernumerary nuclei which have persisted since fertilization.

Embryogeny

The binucleate embryonal cell(s) remain quiescent until suspensor cell elongation has pushed the embryo deep into the megagametophyte tissue. The suspensor cells are anchored at the micropylar end of the megagametophyte by a “hardened plug” of tissue, thought to be the remains of the archegonium (Brownlie, 1953). Not all of the suspensor cells elongate - some can be seen as small rounded cells at the top of the coiled suspensor region (Boyle and Doyle, 1954). At this stage the cytoplasm of the embryonal cell(s) appears to retract from the chalazal pole of the cell (Brownlie, 1953; Boyle and Doyle, 1954; Osborn, 1960). The embryonal cell cytoplasm stains extremely intensely, and several studies have noticed a tendency for it to plasmolyze. The chalazal area of the cell wall becomes thickened (labelled a “cap-like structure” by Sinnott (1913) and Osborn (1960)). Boyle and Doyle (1954) observed that this cap region was separated from the rest of the cell by a membrane, but acknowledged that due to destaining procedures, it was difficult to confirm the structure of this area. Species which have not been observed to have this cap-like structure in the embryonal cells, e.g. *Podocarpus gracilior* (Konar and Oberoi, 1969a) and *Nageia sp.* (Buchholz, 1941) typically have more than six embryonal tier cells arranged into two groups; an outer layer of large, thick-walled embryonal cells enclosing a group of two to four smaller embryonal cells. This outer layer is considered to function as a cap region.

Binucleate cells are not found after early embryo stages (Buchholz, 1941; Brownlie, 1953) except in *Microstrobos* (Elliott, 1948). Once suspensor elongation is nearly complete, the binucleate embryonal cell(s) undergo mitosis, and longitudinal wall formation produces four uninucleate embryonal cells (Buchholz, 1941; Looby and Doyle,

1944b; Boyle and Doyle, 1954; Osborn, 1960; Quinn, 1965; 1966a; 1966b; Konar and Oberoi, 1969a). Mitosis and wall formation do not necessarily occur simultaneously in all embryonal cells (Boyle and Doyle, 1954). Buchholz (1941) considered that the binucleate nature of the embryonal cells up to this point may be a way of holding the embryo in a state of delayed differentiation.

Suspensor cells are still slowly elongating as the embryonal tier cells divide (Brownlie, 1953). The cellulose cap region is still recognisable in embryonal masses as large as 16 cells (Boyle and Doyle, 1954). Embryonal tier cells are very small, as cell multiplication is more rapid than cell size increase at this point. Embryonal tube cells form, and Brownlie (1953) considers that this may be part of a mechanism to ward off competing embryos; smaller embryos become tangled in the mass of embryonal tubes, and appear to be physically pushed back towards the micropylar end of the megagametophyte by embryonal tube growth. As the embryonal cells proliferate, surrounding megagametophyte cells become multinucleate, and fill with starch and lipid (Brownlie, 1953; Singh, 1978).

Late embryo development has been reviewed by Sinnott (1913), Buchholz (1933; 1941), Brownlie (1953), Doyle, (1957), De Laubenfels (1962), Chowdhury (1962), Konar and Oberoi (1969b) and Singh (1978). The first cell differentiation in the embryo occurs as periclinal division forms a dermatogen layer around the periphery of the embryonal mass (Brownlie, 1953). Anticlinal and periclinal divisions at the centre of the mass produce a core of elongated cells which will become the column. After the root apex is delineated, the division of many pericolumn layers forms a "root cap" area (Brownlie, 1953). After an increase in length, anticlinal divisions on the flanks of the apical region

form two cotyledons in all podocarp genera except *Saxegothaea* (where there are four cotyledons (Morvan, 1991). Each cotyledon has at least two vascular strands (*Saxegothaea* has a single vascular strand in each cotyledon) (Brownlie, 1953; Konar and Oberoi, 1969b; Kelch, 1997). The apex of podocarp embryos is slightly domed, but not easy to differentiate from surrounding tissue until the cotyledons have formed (Brownlie, 1953).

Simple and cleavage polyembryony are typical of different podocarp genera (Chowdhury, 1962). Simple polyembryony occurs in *Podocarpus andinus* (Looby and Doyle, 1944b), *P. falcatus* (Osborn, 1960) and *Phyllocladus alpinus* (Buchholz, 1941). Simple polyembryony occurs in *Podocarpus totara* and *P. nivalis*, but is often mistaken as cleavage polyembryony due to the proliferation of detached suspensor cells (Buchholz, 1941; Konar and Oberoi, 1969b). True cleavage polyembryony has been documented in *Podocarpus gracilior* (Konar and Oberoi, 1969b) and *Dacrydium sp.* (Quinn, 1965; 1966a; 1966b).

Seed maturation and germination

Prest (1963) described the seeds of podocarps as “moist and heavy when ripe, and filled with oily food reserves”. In *Podocarpus henkelii*, the mature embryo sits within a small corrosion cavity formed by the collapse of megagametophyte cells (Dodd et al., 1989a). Dodd et al. (1989a) did not observe the presence of any type of transfer cell between the embryo and surrounding megagametophyte, or in embryo tissue itself. At fertilization, the moisture content of *P. henkelii* seeds has been recorded at 82%, and the

seed is shed with a moisture content of 62% (Dodd and Van Staden, 1981). Artificial desiccation of podocarp seed to a water content lower than 62% caused a loss in viability.

Podocarp seed has been described as “starch-storing” (59% of major storage reserves in mature *P. henkelii* seed) (Dodd et al., 1989a), but also stores protein (8%), lipid (4%) and free sugars (2.5%). Protein levels in the megagametophyte and embryo of *P. henkelii* are high at fertilization, decline during early embryo development, and then rise again in the last stages of embryo development (Dodd et al., 1989a). In combination with the high moisture level at seed shed, this has been interpreted as a strategy of ongoing development without the usual intervention of drying and developmental arrest seen in other conifer species. Storing podocarp seeds for longer than 18 months has proved to be a problem. Dodd and Van Staden (1981) measured a decrease in moisture content from 62% to 54% in podocarp seeds stored at 4° C for 18 months. Dissection of the seeds showed that there had been continuous movement of reserves to the embryo after seed fall, supporting the theory that podocarp seeds do not undergo a developmental arrest after seed fall.

Dodd et al (1989b) observed “slow and sporadic” germination in *Podocarpus henkelii* seeds. Their observations suggest that podocarp seeds do not require rehydration to germinate (not having undergone maturational desiccation), but seed dormancy is imposed by the seed coat. A similar dormancy is observed for *Araucaria* (Tompsett, 1984). The seed coat of some podocarps consists of the leathery epimatium and integument layers, and in other podocarps consists of the stony sclerotesta (Dodd et al., 1989a; Page, 1990; Geldenhuys, 1993). Dodd et al. (1989b) and Geldenhuys (1993) have increased the speed of germination by scarifying podocarp seed - specifically by removing

a portion of the epimatium layer at the micropyle in *Podocarpus henkelii* and *P. falcatus* seed.

Bird dispersal of seed is common for many podocarp species, as most genera have a fleshy portion of the female strobilus (receptacle, epimatium, or aril in *Phyllocladus*) (Page, 1990; Kelch, 1997). This is an important seed dispersal mechanism, as there is no wind dispersal of seed, and most falls to the ground directly beneath the parent tree (Preest, 1963; Ogden, 1985; Norton, 1991; Geldenhuys, 1993). Wardle (1963), Ogden (1985) and Norton (1991) have observed that podocarp seed do not germinate well under parent or other mature podocarp trees. There is a “regeneration gap” in New Zealand podocarp forests, created by a scarcity of young podocarp seedlings under mature trees.

Most New Zealand podocarp seeds (e.g. *Dacrydium cupressinum* (Wardle, 1963)) germinate in the summer following seed fall. Only a few seeds remain viable after 12 months, and after 18 months, only those buried in deep leaf litter are still viable (Wardle, 1963; Norton, 1991). Lipid storage products in the megagametophyte, and embryo storage products are used in the early stages of germination (Dodd et al., 1989b). The hypocotyl extends and forms a characteristic hooked “U” shape as the root system develops but the cotyledons remain in contact with the megagametophyte (Dodd et al., 1989b; Woltz et al., 1993). The cotyledons can remain in this position for up to two months in *P. henkelii*, as starch and protein stored in the megagametophyte are slowly transferred to the seedling. The resulting slow, sustained seedling growth is considered to be an advantage for podocarp seedlings growing in deep shade, allowing them to take rapid advantage of canopy breaks (Wardle, 1963; Ogden, 1985; Dodd et al., 1989b).

Predation of podocarp seeds is a significant problem for many species, as the oil-rich seeds attract animals such as bushpigs, bats, rats and insects (Ogden, 1985; Geldenhuys, 1993). In New Zealand, podocarp species have been observed to have “mast” years in which there is a particularly heavy seed crop (Ogden, 1985). This is not the same as the cyclical pattern of cone-bearing observed for Northern Hemisphere conifer species, as mast years may occur in successive years, or years apart. Mast years are thought to have two functions; satiating seed predators and therefore allowing some seed to remain intact, and maintaining the level of podocarp seed in the forest “seed bank” (Ogden, 1985). There is some evidence that high levels of seed viability may coincide with mast years in podocarp species (Wardle, 1963).

The study of cytoplasmic inheritance in conifers

Evidence of a cytoplasmic mode of inheritance was first discovered in flowering plants in 1909, when it was discovered that some heritable traits were transmitted to progeny in a non-Mendelian distribution (Szmidt et al., 1987; Mogensen, 1996). In members of the Abietoideae and Cupressaceae, male gametes and the egg cell were found to contain organelles distributed in specialized areas of cytoplasm, and transformed maternal plastids and small inclusions were identified (Chesnoy, 1969; 1973; 1977; Chesnoy and Thomas, 1971). Willemse (1974) found that the neocytoplasm of the *Pinus* fusion nucleus excluded maternal plastids. Ohba (1971) studied the inheritance of mutant chloroplasts, providing evidence of male plastid inheritance in *Cryptomeria*.

Ultrastructural studies have provided detail of plastid modification into large inclusions, and differences between male and female cytoplasm contributions to zygote neocytoplasm (Chesnoy, 1987b; Owens et al, 1995b). DNA fluorochromes such as DAPI (4,6-diamidino-2-phenylindole dihydrochloride) have allowed identification and quantification of organelle DNA complements. This has confirmed the presence of paternal organelles in 'strictly' maternally-inheriting angiosperms (Corriveau and Coleman, 1988) and has shown that male plastids in such angiosperms have rudimentary structures and may have degenerated (Connett, 1987).

Molecular techniques have allowed analysis of parent and progeny DNA markers to confirm contributions of maternal and paternal organelles. Two types of markers have been used: restriction fragment length polymorphisms (RFLP) which use enzyme digestions to differentiate between DNA complements, and simple sequence repeat (SSR) markers which use a PCR reaction to amplify differences in numbers of markers between maternal and paternal organelles. RFLP studies on members of the Pinaceae show paternal plastid inheritance (Neale et al., 1986; Szmidt et al., 1987; Wagner et al., 1987; Neale and Sederoff, 1989; Stine et al., 1989; Furnier and Stine, 1995; David and Keathley, 1996) and maternal mitochondrion inheritance (Neale and Sederoff, 1989). RFLPs of members of the Cupressaceae and Taxodiaceae show strictly paternal inheritance of organelles (Neale and Sederoff, 1989; Neale et al., 1991; David and Keathley, 1996). SSR studies have demonstrated maternal leakage in *Pinus* plastid inheritance (Cato and Richardson, 1996). Confirmation of cytoplasmic inheritance requires both molecular and microscopy techniques (Sewell et al., 1993); genetic results

verify the mode of inheritance in the progeny, and microscopy establishes the mechanism of maternal or paternal inheritance.

Within the conifers, there appear to be two main mechanisms of neocytoplasm formation as the male and female gametes fuse. In the first, clusters of male organelles from cytoplasm around the male nucleus combine with the perinuclear cytoplasm of the egg nucleus, resulting in organelle contributions from both parents, e.g., paternal plastids and maternal mitochondria in the Pinaceae, and paternal plastids and biparental mitochondria in *Taxus* (Pennell and Bell, 1988). In the second mechanism, cytoplasm from the male cell enshrouds the egg nucleus, effectively pushing the maternal organelle complement away from the neocytoplasm. Organelles are also found within the male nucleus in some species (Singh, 1978; Owens et al., 1995b). This results in paternal plastid and mitochondrion inheritance in the Cupressaceae, Taxodiaceae and Araucariaceae, and paternal plastid and biparental mitochondrion (unconfirmed) inheritance in the Cephalotaxaceae. Features of these two mechanisms vary in five areas: (1) whether the male gametes are cells or nuclei; (2) whether or not the two male gametes are of equal size and cytoplasmic volume; (3) whether one or both male gametes enter the egg cell; (4) whether the maternal organelles are arranged in a perinuclear zone or scattered throughout the egg cell; and (5) whether or not the maternal plastids have been modified and are non-functional. The only family in which there is no genetic or microscopic information on cytoplasmic inheritance is the Podocarpaceae.

Kuroiwa and Uchida (1996) comment that primitive cytoplasmic inheritance is that which mixes male and female organelles in the zygote, whereas a more advanced state exhibits a uniparental inheritance. This may be too generalized given the inherent

'leakiness' of cytoplasmic inheritance in conifers. A continuum of inheritance mechanisms would explain the range of parental organelle contributions observed in conifer species (Connett, 1987; David and Keathley, 1996).

Chapter 3

Material and Methods

Strobilus collection and dissection

The field site consisted of approximately 80 year old totara trees growing between 0-40 m elevation near Drury, 40 km south of Auckland, New Zealand. Collections of female strobili from four female trees and male strobili from four male trees were made weekly from September 1, 1995, to May 18, 1996, and twice-weekly from November, 1995 through to March, 1996. Strobili were collected, wrapped in wet paper towels, placed in a plastic bag inside a chilled cooler and taken immediately to laboratory facilities provided by the University of Auckland. Strobili were dissected within two to three hours of collection.

Thin longitudinal slices were removed from either side of the ovule(s) on early pre-fertilization female strobili, to allow greater penetration of the fixative.

Megagametophytes at fertilization and post-fertilization were dissected from the epimatium and integument layer, and longitudinal slices were cut from the megagametophyte. Longitudinal slices were removed from male strobili, and the whole median slice or a portion of it was fixed according to the size of the strobilus. No further male strobili were fixed after dehiscence.

Fixation of specimens for paraffin embedding

The 1-2 mm thick median portions of all strobili were fixed in Navashin's fixative (5% glacial acetic acid, 0.5% chromium trioxide, 20% formaldehyde)(Berlyn and Miksche, 1976). Specimens were aspirated for 3 hours, and then remained in the fixative for 2 days prior to rinsing. After dehydration through an ethanol and tertiary butanol series (Johansen, 1940), specimens were embedded in Tissue Prep 2 (Fisher Scientific, New Jersey). Embedded specimens were softened at 37°C for 12 days in Gifford's solution (19% glacial acetic acid, 45% ethanol, 4% glycerine)(Gifford, 1950), and serially sectioned on a Spencer 820 microtome. After dewaxing in Hemo-De (Fisher Scientific, Pittsburgh), sections were stained with safranin and hematoxylin, and mounted in Entellan (Merck, Darmstadt, Germany).

Fixation of specimens for resin embedding

Specimens used for resin embedding were dissected as above, except that the fixed median portion was only 1 mm thick. Specimens were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 0.15 M sucrose. After 2 h of aspiration, the specimens were stored for 2 d at 4°C. Specimens were rinsed in 0.1 M cacodylate buffer and then postfixed in 1% osmium tetroxide for 1 h. Following dehydration through an ethanol series, the specimens were rinsed in propylene oxide, infiltrated in Spurr's resin (Spurr, 1969) and hardened at 60°C for 24 h.

Semithin sections (0.5µ) were stained with Toluidine Blue O in 1% borate buffer (Roland and Vian, 1991) and viewed with a light microscope. Ultrathin sections were cut

at 0.06 μ m and placed on uncoated 150- or 200-hexagonal-mesh copper grids. After staining with 2% aqueous uranyl acetate and 0.2% lead citrate (Reynolds, 1963), grids were viewed with an Hitachi H-7000 electron microscope at 75 kV.

Pollen germination and DAPI staining

Samples of 20 mL of pollen from three of the four male totara trees used for strobili collections were first air-dried, then dried over a silica gel desiccant and frozen in scintillation vials. The fourth tree aborted most pollen cones after an insect infestation and pollen was therefore not collected.

Germination medium consisted of a Murashige and Skoog nutrient mixture following the method of Fernando et al. (1997) (1 mgL⁻¹ Ca(NO₃)₂, 3 mgL⁻¹ H₃BO₃, 2 mgL⁻¹ MgSO₄, 1 mgL⁻¹ KNO₃ were added to the stock solution). The medium stock was diluted 1:10 with distilled water, and 10% sucrose, 10% polyethylene glycol and 0.4% phytigel were added. The pH was adjusted to 5.5.

Pollen grains were cultured on the germination medium and incubated in the dark at 24°C. At two, three and four days after initial culturing, elongating pollen grains were examined under a dissecting microscope and fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. After being rinsed in PBS, pollen grains were stained with DAPI (4',6-diamidino-2-phenylindole) and examined using a fluorescence photomicroscope.

Pollen monitoring

Mature pollen carried in the air was monitored from 6 October to 28 November 1995, using two weather-vane pollen monitors. The pollen monitors were mounted on 3 m poles situated near two of the female trees used in this study. Each monitor was fitted with a glass microscope slide and a stainless-steel scanning electron microscopy (SEM) stub which were changed twice a week during the pollination period. The glass slide was marked with a 1 cm² grid, and covered in melted petroleum jelly. The SEM stub was covered with a section of double-sided tape. An estimation of the pollen landing per mm² was made by counting the number of grains within the 1 cm² area. The presence of pollen from sources other than totara on the slide was noted. SEM stubs were stored for use in the event that identification of pollen types on the slide was difficult.

Measurement of pollination success

One week after the pollen receptivity period of the female strobili (i.e. one week after the pollination drop disappeared from the micropylar area), 20 strobili were collected from each of the four female totara trees used in this study. Ovules were sliced in half to expose the micropylar canal, however exudation of oil and fluid from the cut surface obscured attempts to count pollen grains in this area.

To overcome this problem, a further collection of 20 strobili from each female tree was made a week later, once the micropyle had closed and pollen could not be washed out of the micropylar canal. Ovules were dissected from the receptacle, fixed in FAA (Johansen, 1940) and stored in scintillation vials. After rinsing in distilled water, the

ovules were sliced in half, and the number of pollen grains within the micropyle was determined using a dissecting microscope.

Measurement of fertilization success

Four weeks after the fertilization period (mid-January, 1996), 50 strobili were collected from each of the four female trees used in this study. Ovules were dissected from the receptacle, fixed in FAA and stored in scintillation vials. After rinsing in distilled water, the ovules were sliced down the middle and a count of the number of thread-like embryos was made using a dissecting microscope.

Chapter 4

The reproductive biology of totara (*Podocarpus totara*) (Podocarpaceae).

Introduction

The conifer family Podocarpaceae is made up of at least 17 genera and 125 species (Page, 1990). A lack of information available for many of its genera means that the family is still under taxonomic revision. The Podocarpaceae are predominantly distributed in the Southern Hemisphere with the genus *Podocarpus* widespread throughout all of the southern continents and extending northwards to southern China and Japan (Page, 1990). *Podocarpus totara* (D. Don ex Lambert) is one of five New Zealand *Podocarpus* species, and is known by the Maori name, totara. Totara is a dioecious, lowland and subalpine species found throughout New Zealand, and typically grows to a height of 30 m (Salmon, 1982).

Previous studies of the reproductive biology of *Podocarpus* have focused on aspects of morphology and embryology (Coker, 1902; Jeffrey and Chrysler, 1907; Young, 1910; Osborn, 1960; Konar and Oberoi, 1969a), with examinations of gamete development and fertilization by Looby and Doyle (1944a, 1944b) and Boyle and Doyle (1953, 1954). Studies of *P. totara* have been limited to those carried out prior to 1914 on the gross morphology of male and female strobili (Burlingame, 1908; Gibbs, 1912; Stiles, 1912; Sinnott, 1913), and Buchholz's (1941) survey of embryogeny in the Podocarpaceae, including totara. These studies are characterized by: (a) incomplete collections of specimens (Sinnott, 1913); (b) poor fixation resulting in loss of cellular detail (Jeffrey and

Chrysler, 1907; Burlingame, 1908); and, (c) specimens from trees grown in the Northern Hemisphere (Looby and Doyle, 1944a, 1944b) showing possible abnormal development and abortion of embryo(s). These problems have resulted in a confusing and often contradictory body of literature on reproduction in podocarps. There is a lack of ultrastructural information on female gametophyte development, fertilization and embryology for any podocarp species (Singh, 1978).

This is a light microscopy study of a reproductive cycle in *Podocarpus* as the basis for a thorough ultrastructural study of reproduction in *Podocarpus*, and to address areas of dispute concerning anatomical features of the reproductive cycle. In the results and discussion, the structure fused to the outside of the integument is called the epimatium. The epimatium is interpreted as a novel structure peculiar to the Podocarpaceae, and is thought to function in ovule support and inversion (Tomlinson, 1992). The fused layer is referred to as the seed coat in the mature seed. The fused sterile bract complex subtending the ovule(s) is called the receptacle, in accordance with the nomenclature used by Gibbs (1912) and Page (1990).

Observations and Results

Totara has a two-year reproductive cycle from strobilus initiation through to seed maturation and dispersal (Fig. 4). Strobili are initiated in September and October of the first year, after which they undergo a 9-month period of dormancy. The first collections began at the end of this dormant period, in early September of the second year. At this point, the male strobili are ca. 15 mm long and have not yet shed pollen (Fig. 5). Male

strobili are borne in the axils of one-year-old leaves or occasionally as the terminal bud. At maturity (late September - October), male strobili elongate to 40-50 mm, and shed pollen after drying (Fig. 6). Female strobili are borne near the base of the current year's flush of growth, and although ovules do not become evident until mid-September of the second year, collections of terminal buds were made in early September to study ovule primordia (Fig. 7). At pollen receptivity in late September of the second year (Fig. 8), female strobili are 2-3 mm long. Female strobili at maturity in March have ovules approx. 4-5 mm long, subtended by a pair of fused swollen bracts (receptacle) ca. 5-6 mm long (Fig. 9).

Male strobilus

The male strobilus consists of 100-120 peltate microsporophylls borne in a helical arrangement (Fig. 5). Each microsporophyll bears two abaxial microsporangia. Male strobilus buds probably undergo dormancy at or before the sporogenous cell stage, as this was the earliest condition seen in collected specimens. The sporogenous cells (Fig. 10) are characterized by large nuclei, with one or more nucleoli, and dense cytoplasm. The sporangial wall is four to five cells thick (Fig. 10) and the inner two cell layers differentiate into a tapetal layer later during sporogenous cell differentiation. Microspore development is extremely rapid after pollen mother cell formation. Within 3 weeks of male strobilus emergence, the mature pollen grains complete mitoses and lie free in the sporangium (Fig. 11).

Male gametophyte

The mature pollen grain is bisaccate, spheroidal, 40-50 μm in diameter with a thick sculptured exine and heavily thickened cap region (Figs. 12 and 13). Each pollen grain contains three prothallial cells, and the tube, generative and sterile cells. The prothallial and sterile cell walls disappear soon after pollen maturity, and the nuclei lie free in the tube cell cytoplasm.

Pollen grains are captured in a pollination fluid produced by the female strobili in late September. The average number of pollen grains found in the micropyle is 4.52, although up to 30 grains were present in some ovules. Pollen germination occurs in late October near the nucellar tip of the ovule (Fig. 13). The intine of the pollen tube is thick and distinct. The pollen tube grows through nucellar tissue, and once it reaches the megaspore cell wall, branches to form a disc-like structure over the neck cells of the archegonia (Fig. 14). The body cell follows the tube nucleus in the pollen tube, and frequently has a number of prothallial nuclei and the sterile nucleus clustered around it. The body cell is released into the archegonial chamber (Fig. 14). The body cell nucleus is large and spheroidal, and the cytoplasm is distinct and well-organized. Mitosis of the body cell occurs in early December, in the area of the pollen tube close to the neck cells. Mitosis results in unequal male nuclei - one nucleus is large and associated with the bulk of the body cell cytoplasm (Fig. 15). The other smaller nucleus remains on the periphery of the larger gamete.

Female strobilus

The female strobilus consists of one or two inverted ovules, subtended by a pair of fused sterile bracts (receptacle) (Figs. 4, 8, and 9). One ovule is at an earlier stage of development than the other in strobili with two ovules, and frequently abscises prior to strobilus maturation. The integument of the ovule is fused to the epimatium, and this fused layer remains green until dehydration of the seed after seed dispersal. At maturity, the ovules elongate to 4-5 times their initial size (about 5-6 mm), and the receptacle swells (Fig. 9) and becomes orange or red.

Pollination

The 1-2 mm long ovules become receptive to pollen about 12 days after their emergence from the terminal bud (October) (Fig. 8) and appear to be receptive for about one week. All ovules in the same whorl produce pollination drops at the same time. As not all terminal buds flush at the same time on a tree, the pollen receptivity period within a tree is staggered over about two weeks. The pollination drops of ovules on the same strobilus tend to coalesce, and scavenge pollen from a wide area surrounding the micropyle and strobilus apex.

At the time of pollination drop secretion, the megaspore mother cell forms within the nucellus. After meiosis, a linear tetrad of megaspores develops, the most chalazal of which becomes the functional megaspore (Fig. 16). Megaspores are surrounded by darkly staining sporogenous cells (Fig. 16). The functional megaspore enlarges, and undergoes free nuclear division at the end of October (Fig. 17). As the free nuclear megagametophyte enlarges to a cylindrical shape, three to four layers of surrounding

sporogenous cells become densely cytoplasmic (Fig. 17). The surrounding nucellar cells have a thin parietal layer of cytoplasm and are highly vacuolate, while nucellar cells at the micropylar end are densely cytoplasmic (Fig. 17).

In mid-November, radial cell walls begin to form in the free nuclear megagametophyte, producing long thin prothallial cells (Fig. 18). Many periclinal and limited anticlinal cell divisions form many small prothallial cells, which then elongate and divide further to fill in the vacuole of the megagametophyte (Fig. 19). At this stage, the pollen grains in the micropyle have germinated, but the pollen tubes have not completely penetrated the nucellus.

The megagametophyte of unpollinated ovules degenerates soon after becoming cellular. These ovules abscise at the beginning of December.

Archegonial development

Archegonial initials are long vacuolate cells with large nuclei formed at the micropylar end of the cellular megagametophyte. At the beginning of December, the megagametophyte cells surrounding the initials divide and form a layer of small, binucleate, densely-cytoplasmic jacket cells (Fig. 19). The 4-6 archegonia per ovule each have a separate layer of jacket cells (Fig. 20). Two archegonia were occasionally observed to share a common jacket layer. Unequal division of the archegonial initial results in a small primary neck cell at the micropylar end, and a larger central cell (Fig. 19). The primary neck cell undergoes anticlinal and periclinal divisions to form two tiers of neck cells (Figs. 20 and 21). The number of neck cells in each tier varies between two and five. The central cell enlarges, and divides to form the egg cell and the ventral canal

cell, however, the ventral canal cell was not observed in this study. The egg cell cytoplasm stains darkly and is filled with small vacuoles (Fig. 21).

Archegonia mature in mid-December, at which point the egg nucleus migrates to the micropylar end of the egg cell, and a large vacuole occupies the chalazal end. The egg cell cytoplasm becomes densely granular, and is arranged in centripetal strands around the nucleus (Fig. 21). A thin perinuclear zone forms around the egg nucleus (Fig. 21). By this time, the pollen tubes have penetrated through the nucellus, and the body cell remains central in the archegonial chamber (Fig. 14).

Fertilization

Fertilization and proembryo development occurs during two weeks in mid-December (Fig. 4). Entry of the pollen tube into the archegonium may or may not destroy the neck cells and surrounding micropylar jacket cells (Fig. 15). Both male gametes enter the egg cell, and the larger gamete migrates toward the egg nucleus (Fig. 21). Cytoplasm associated with the large male nucleus, recognizable by its dense and non-granular appearance, follows closely behind (Fig. 21). The smaller male nucleus and prothallial nuclei also enter the egg cell, but remain towards the micropylar end (Fig. 21). The egg cell cytoplasm undergoes a rapid change and possibly degradation after the male gametes enter the egg cell. The cytoplasm becomes darkly-staining and condensed in appearance (Fig. 21). The number of fertilized archegonia varies from one to four per ovule. Unfertilized archegonia degenerate within two weeks of the fertilization period. The cytoplasm of these archegonia becomes dark and densely granular, and the jacket cells quickly degrade.

Proembryo formation

The cytoplasm of the zygote becomes dense and darkly-staining. A vacuole forms between the zygote cytoplasm and the rest of the degrading egg cell cytoplasm (Fig. 22). The zygote undergoes free nuclear division during the early part of January (Fig. 4). Proembryo nuclei are large, granular, and have distinct chromatin and nucleoli (Fig. 22). The infrequency of specimens found prior to embryo emergence through the egg cell wall suggests that proembryo growth is rapid. The four free nuclei migrate to the chalazal end of the egg cell (Fig. 22). Further mitosis and cell-wall formation result in a three-tier proembryo (Fig. 23), with one binucleate embryonal-tier cell, seven to nine suspensor-tier cells and a variable number of open-tier cells. The binucleate nature of the embryonal tier cell was confirmed with resin sections, which showed that both nuclei were proximal to a heavily-thickened cell wall area at the chalazal end of the cell (Figs. 24 and 25).

Embryo growth

By mid-January, the megagametophyte cells in a cone-shaped area around the archegonia become multinucleate and accumulate starch granules (Fig. 26). The suspensor-tier cells elongate and force the proembryo through the jacket of the archegonium (Fig. 26). No noticeable corrosion cavity forms around the embryo. As the suspensor cells coil behind the embryonal tier, the binucleate cell undergoes a series of mitoses to produce an embryonal mass (Fig. 27). One or two suspensors may cleave away from the embryo, forming small secondary embryos (Fig. 27). Frequently more than one egg cell is fertilized, but the largest embryo usually becomes dominant. Growth

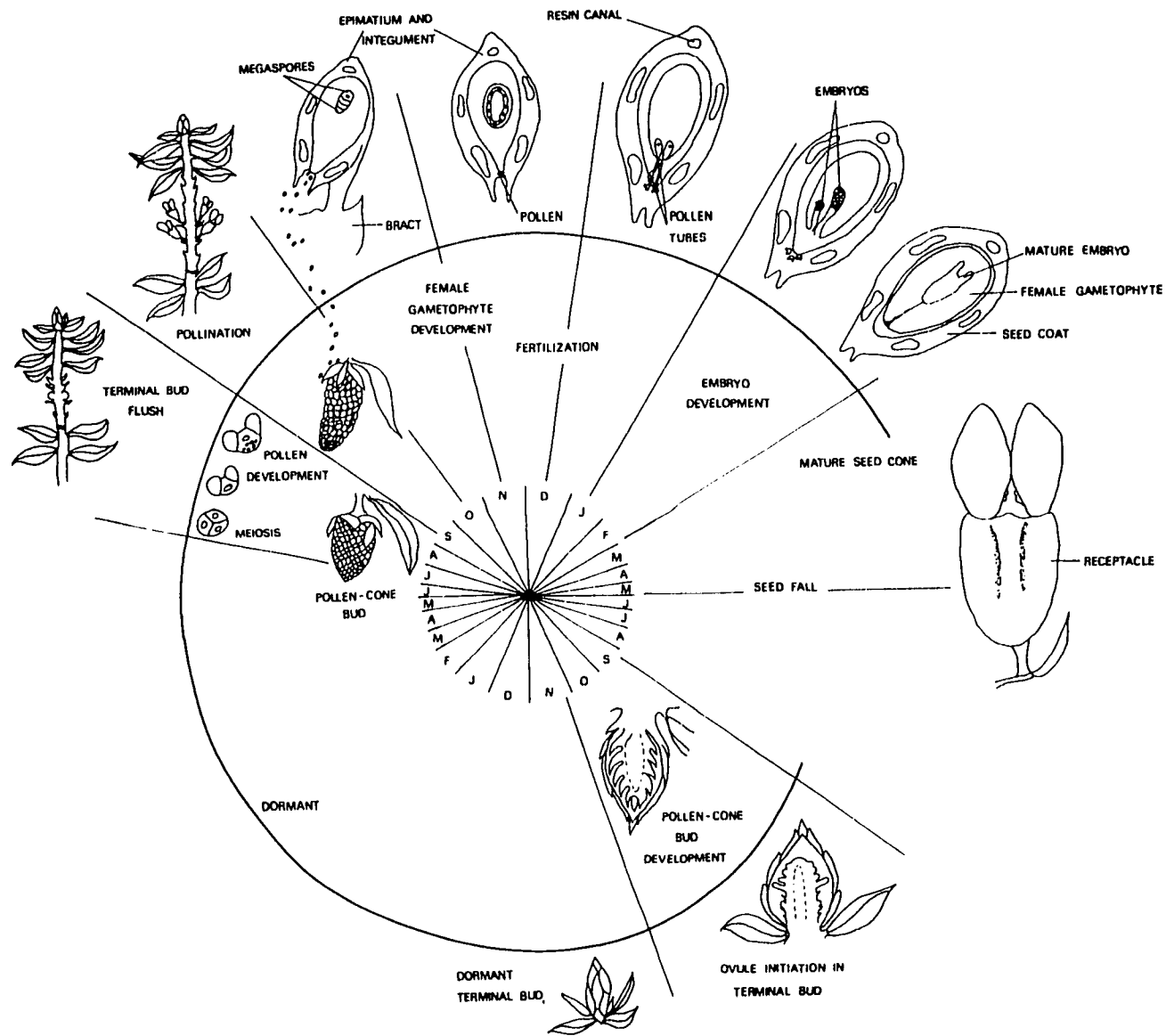
of the embryonal tube cells in the dominant embryo appears to physically prevent further elongation of smaller embryos.

Proximal embryonal mass cells divide to form the rib meristem, and the base of the rib meristem forms embryonal tubes. By the end of January, the transition to late embryo stages occurs as the root generative initials appear (Fig. 28). After the two cotyledons form, the suspensor cells degenerate leaving a small space proximal to the embryo (Fig. 29). Late embryo stage development occurs during February and March as the ovules mature. The root generative initials, column, pericolumn and stele meristems form within the embryo (Fig. 29). The embryo is positioned in the upper half of the megagametophyte, and occupies about one-third of the megagametophyte at maturity. At maturity the lengths of embryo organs are as follows : whole embryo 1.38 ± 0.019 mm, cotyledons 0.39 ± 0.051 mm, root cap 0.205 ± 0.028 mm, suspensor system 0.6 ± 0.094 mm. The contents of megagametophyte cells at the mature embryo stage stain darkly.

After seed fall, the integument and epimatium dehydrate and discolour, and become resinous. Germination occurs as early as one month after seed fall.

Figure 4

Fig. 4 Totara reproductive cycle. Letters near centre indicate months of the year.



Figures 5-9

Figs. 5-6 Male strobili.

Fig. 5 Immature male strobili collected in early September.

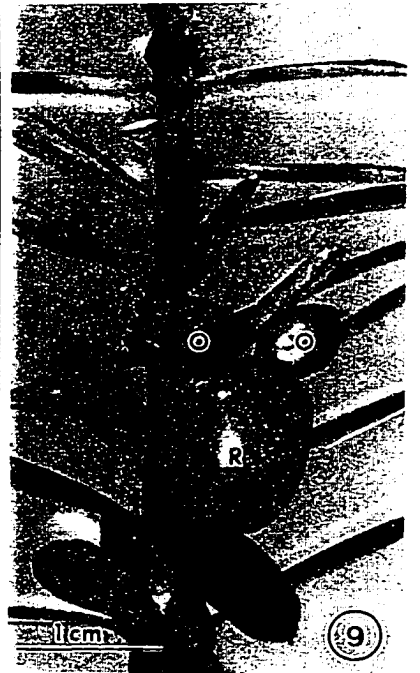
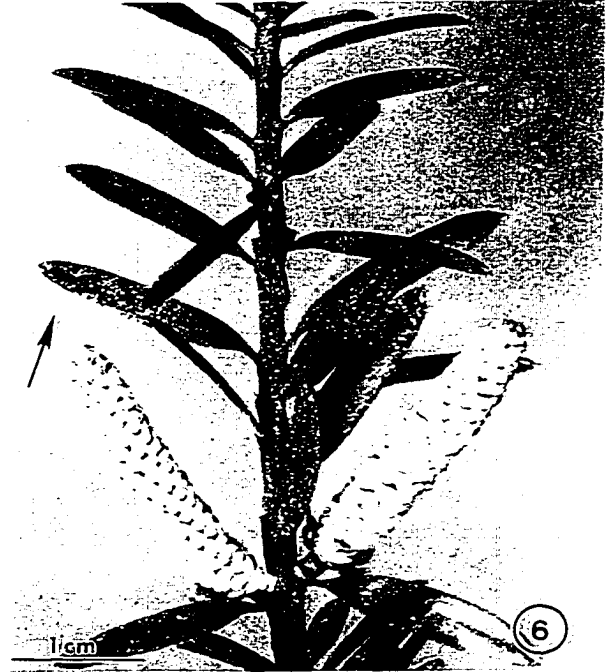
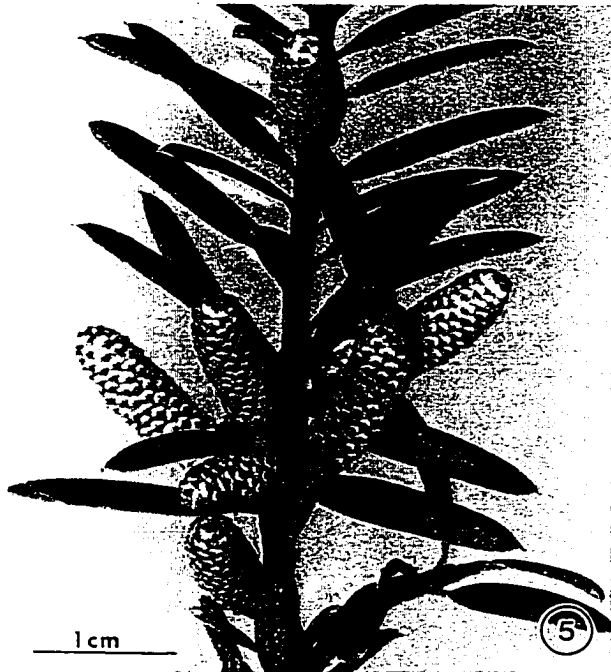
Fig. 6 Mature male strobili collected at pollen (arrow) shed in late September.

Figs. 7-9 Female strobili.

Fig. 7 Median longitudinal section of paraffin-embedded female strobilus collected in September, showing ovule primordium (OP), bract primordium (BP) and epimatium (E).

Fig. 8 Immature female strobili collected at pollination in October, showing ovule (O) and receptacle (R).

Fig. 9 Mature female strobilus collected prior to seed fall in March, with two ovules and mature swollen receptacle.



Figures 10-15

Figs. 10-12 Longitudinal sections of paraffin-embedded male strobili.

Fig. 10 Microsporangium showing sporogenous cells (Sp) and surrounding tapetal layers (T).

Fig. 11 Microsporangium at mature pollen grain stage with degenerating tapetal layers.

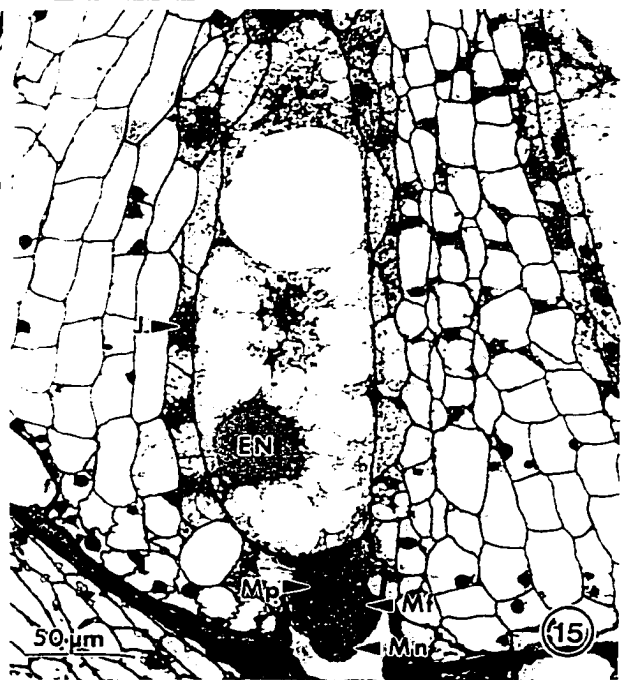
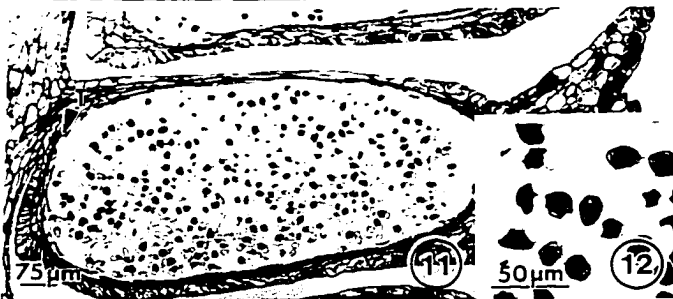
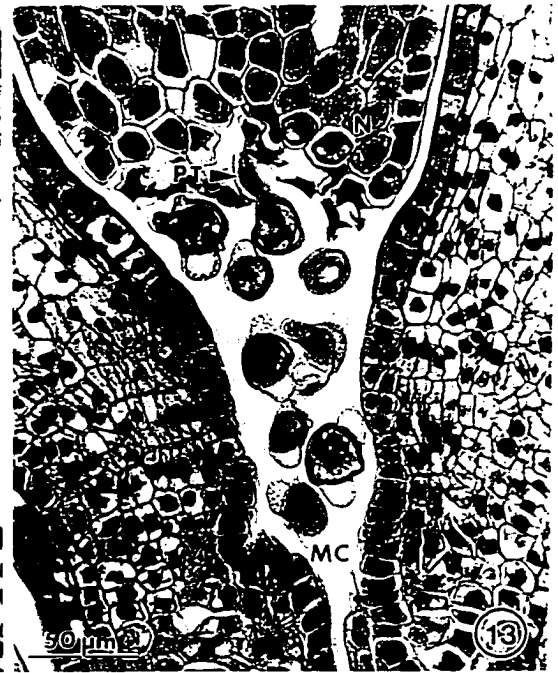
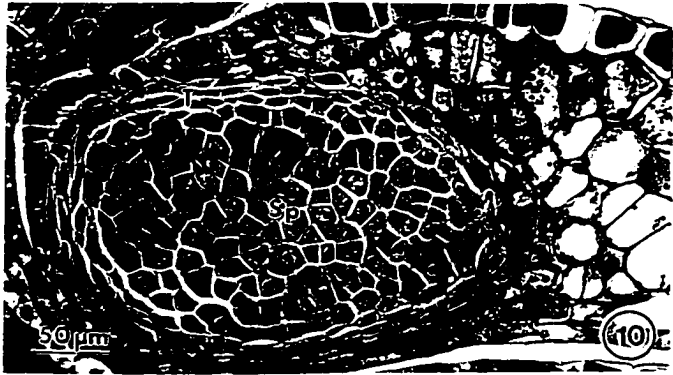
Fig. 12 Portion of microsporangium enlarged to show bisaccate pollen grains.

Figs. 13-14 Longitudinal sections of paraffin-embedded ovules.

Fig. 13 Germination of pollen tubes (PT) near the nucellar tip (N) within the ovule micropylar canal (MC).

Fig. 14 Branched growth of the pollen tube between the nucellus and the megagametophyte (M). Note the body cell (BC) prior to gamete formation resting within the pollen tube.

Fig. 15 Section of resin-embedded ovule. Male gametes near the neck cells of an archegonium. Large functional male gamete (Mf) and surrounding cytoplasm (Mp) precedes the small non-functional gamete (Mn) into the egg cell; egg nucleus (EN), jacket cells (J).



Figures 16-21

Figs. 16-21 Longitudinal sections of paraffin-embedded ovules.

Fig. 16 Ovule collected in October showing functional megaspore (FM) within the sporogenous cells (Sp) of the nucellus.

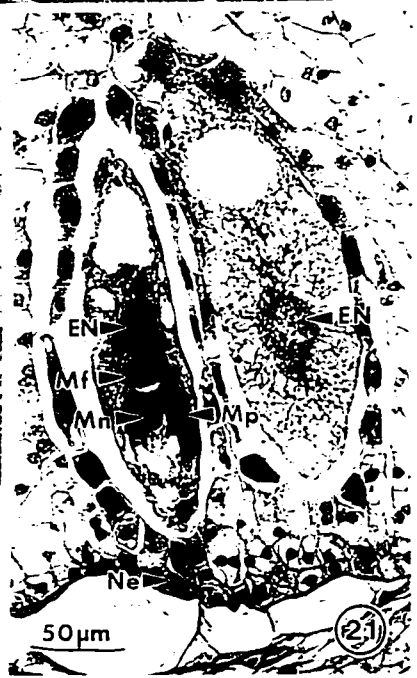
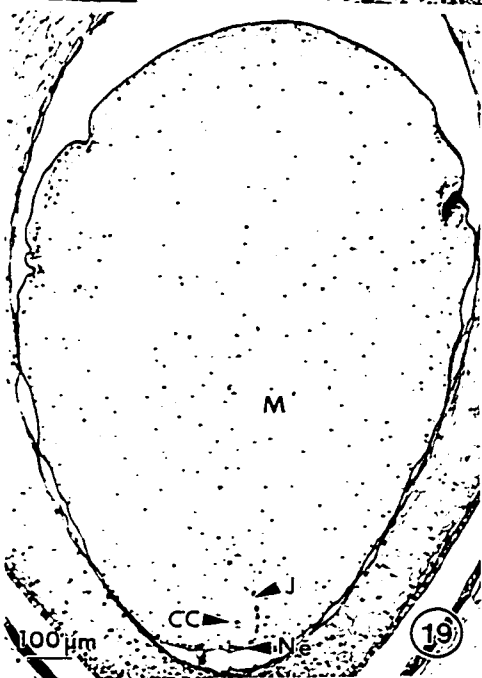
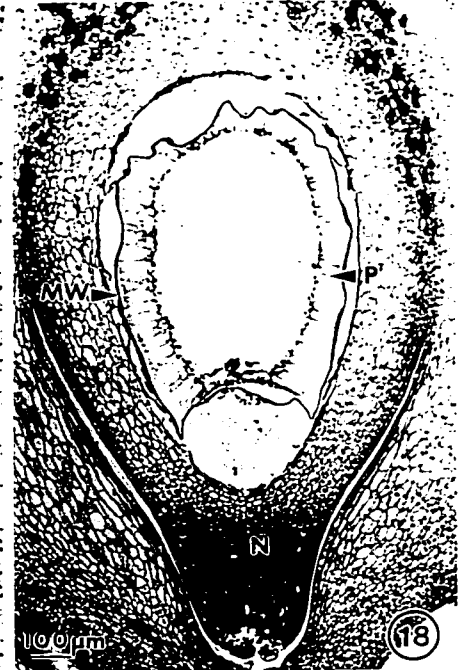
Fig. 17 Early free nuclear megagametophyte (FN) and surrounding sporogenous cells within the nucellus (N). Collected in early November.

Fig. 18 Formation of prothallial cells (P) in early cellular megagametophyte, collected mid-November; megaspore wall (MW).

Fig. 19 Cellular megagametophyte (M) with archegonial initial at the central cell (CC) stage; neck cells (Ne), jacket cells (J).

Fig. 20 Functional archegonia with egg nucleus (EN), neck cells, separate jacket cell layers, large chalazal vacuole (VC) and branched pollen tube (PT).

Fig. 21 Fertilization of the egg nucleus in the left-most archegonium by the functional male gamete (Mf). Male gamete cytoplasm (Mp) and non-functional gamete (Mn) trail behind the functional gamete. Note degradation of egg cell cytoplasm. Unfertilized archegonium (right-most) contains granular, dispersed egg cytoplasm.



Figures 22-29

Figs. 22-29 *Totara* ovules during proembryo and embryo growth.

Figs. 22-23 Longitudinal sections of paraffin-embedded proembryos.

Fig. 22 Proembryo showing three of the four free nuclei (FP), degenerating egg cytoplasm (EC) and jacket cell layer (J).

Fig. 23 32-cell proembryo arranged in three tiers; nucellus (N), megagametophyte (M).

Figs. 24-25 Resin-embedded longitudinal sections of same binucleate embryonal-tier cell (BE) and suspensors (S).

Fig. 24 Thickened chalazal cell wall (*).

Fig. 25 Two embryonal cell nuclei (arrows) appressed tightly together, occupying the micropylar half of the cell.

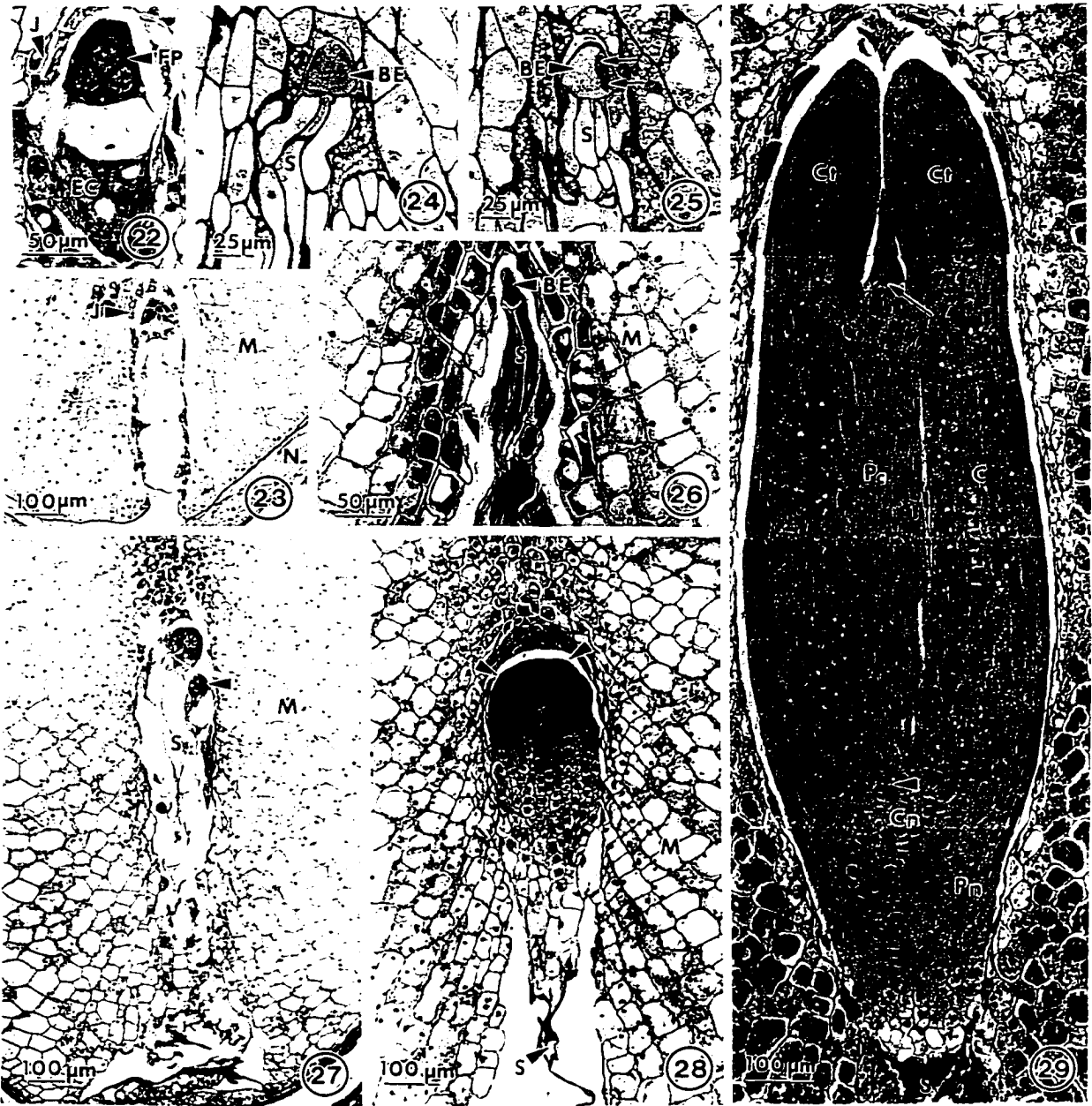
Figs. 26-29 Paraffin-embedded longitudinal sections of embryos.

Fig. 26 Early embryo showing start of suspensor growth through the megagametophyte. Note the single embryonal-tier cell.

Fig. 27 Secondary embryo (arrowhead) formed as a result of suspensor cleavage. Primary embryo (*) is at the top of the figure.

Fig. 28 Late embryo at cotyledon initiation (arrowheads) stage.

Fig. 29 Mature embryo with cotyledons (Ct), shoot apex (arrow), hypocotyl-shoot axis showing procambium (Pc), embryonic cortex (C), and embryonic root showing root initials (arrowhead), column (Cn), pericolumn (Pn) and suspensor.



Discussion

A reproductive cycle of totara lasts about 18 months from cone initiation to seed maturity, which is similar to other *Podocarpus* species (Vasil and Aldrich, 1970), but is shorter than that of other New Zealand genera in the Podocarpaceae such as *Prumnopitys*, *Halocarpus*, and *Dacrydium* (Tomlinson, 1992). The only period of dormancy in the totara cycle is nine months occurring between cone initiation and emergence. Pollination occurs in late September, about two weeks after female cone emergence, and fertilization occurs in early December. Embryo development occurs over the next three months. The embryo does not appear to undergo further development after seed dispersal, in contrast to the incomplete embryo development at seed fall in *Podocarpus andinus* reported by Looby and Doyle (1944b).

All of the female trees selected for the study produced large numbers of strobili, although about 20% of the ovules aborted soon after pollination (frequently the less developed second ovule on a strobilus), and a further 10% of ovules were lost by the end of December. The difference in developmental maturity between the two ovules in a strobilus may result in one not being receptive at pollination. Levels of seed production in totara do not follow a discernible yearly cycle as in members of the Pinaceae (Owens and Blake, 1985), but Ogden (1985) found evidence of periodic massive seeding in totara. This was suggested to be an adaptation to predation of seed stocks by animals (Ogden, 1985).

Podocarpus seeds have been described as recalcitrant because they have a high moisture content at seed fall and do not appear to undergo a dehydration period prior to

germination (Dodd, Van Staden and Smith, 1989). This high moisture and lipid content, coupled with the tendency of the epimatium to prevent water uptake but allow rapid water loss has been suggested as a cause for the limited storage life of podocarp seeds (Dodd and Van Staden, 1981).

Morphological characteristics in the Podocarpaceae have been described as covering an “extreme range” by De Laubenfels (1969) and Hart (1987), due to the mixture of actively evolving genera and isolated relicts found within the family. Isolated relict genera such as *Saxegothaea*, *Microstrobos* and *Microcachrys* have multiovulate female strobili (Page, 1990), but the remaining 14 genera in the family (including *Podocarpus*) have female strobili bearing one or two ovules at maturity. Genera within the Podocarpaceae display differing levels of integument fusion and incorporation of a fleshy component in the mature strobilus depending on how highly derived they are within the family. Reduction to one or two ovules per strobilus, fusion of the integument and epimatium layers around the ovule, and amalgamation of the bracts into the fleshy receptacle of totara identify it as a highly derived member of the Podocarpaceae (Sinnott, 1913; Page, 1990; Tomlinson, 1992). Cladistic analyses of the Podocarpaceae support this conclusion (Hart, 1987).

Pollen grains of totara are similar to those of other *Podocarpus* species: large, bisaccate, and coarsely sculptured, with a heavily thickened cap (Vasil and Aldrich, 1970; Pocknall, 1981). Development of the pollen grain in the Podocarpaceae, and the Araucariaceae, differs from that of other conifers by the proliferation of the prothallial cells soon after their formation in the maturing pollen grain. In totara, 6-8 prothallial cells have been observed in mature pollen (Burlingame, 1908; Sinnott, 1913). Preliminary results from our study suggest that there are three prothallial nuclei present in addition to the sterile

and tube nuclei and the body cell. Three prothallial nuclei have been noted for *P. nivalis* (Boyle and Doyle, 1953) and *P. macrophyllus* (called *P. coriaceus* in the original paper, but later re-identified)(Coker, 1902). The number of prothallial nuclei can vary among totara pollen grains depending on whether one or both of the primary prothallial cells undergoes division (Jeffrey and Chrysler, 1907; Burlingame, 1908; Hodcent, 1964). The function of prothallial nuclei in germination and pollen tube growth is unclear, but all prothallial nuclei appear to exit the pollen grain, and are often clustered close to the body cell as it enters the pollen tube. They persist in their association with the body cell until fertilization, remaining at the micropylar end of the archegonium.

Our study shows that growth of other pollen tubes is severely limited by the expansion of the first one to reach the megagametophyte. The pollen tube branches laterally as it emerges from the nucellus, and progressively enlarges to form a disk-like structure over the neck cells of the archegonia. Small branches penetrate between the megaspore membrane and nucellus. This type of growth has also been noted in *P. macrophyllus* (Coker, 1902), *P. andinus* (Looby and Doyle, 1944a) and *P. nivalis* (Boyle and Doyle, 1953). Looby and Doyle (1944a) hypothesized that this branching may prevent the megagametophyte from elongating and obscuring the neck cells of the archegonia. Prothallial nuclei have been suggested to have a role in directing this extensive branching (Stiles, 1912), but their position close to the body cell at all times makes this unlikely.

We were unable to find evidence of the ventral canal nucleus in any totara archegonia. Sinnott (1913) reported the ventral canal nucleus as appressed to the egg cell wall, at the micropylar end to one side of the neck cells in totara. The ventral canal nucleus has been observed in a similar position in *P. nivalis* (Boyle and Doyle, 1953) as a result of the

oblique lateral division of the central cell, but it tended to degenerate before fertilization. Reports of the appearance and activity of the ventral canal nucleus in different *Podocarpus* species vary widely (Sinnott, 1913; Looby and Doyle, 1944a; Boyle and Doyle, 1953). Maturation of the archegonium from the archegonial initial to the egg and neck cells occurs in two weeks in totara, so if the ventral canal cell is ephemeral, it may not be observed. The ventral canal nucleus does not appear to participate in fertilization events (Coker, 1902), although it was observed to move from the archegonial wall and migrate close to the egg nucleus in the unfertilized archegonia of *P. macrophyllus*.

There is much debate over the details of male gamete formation in the Podocarpaceae, but little evidence. Most studies agree that the male gametes are unequal in nuclear size and in allocation of body-cell cytoplasm, as we confirmed in this study. However there is disagreement over whether the gametes are cells or nuclei. In totara, as in many other *Podocarpus* species, the body cell nucleus divides to form gametes which are initially the same size. The functional nucleus then increases in size, and the other gamete is either extruded and degenerative (Coker, 1902; Stiles, 1912; Looby and Doyle, 1944a) or is moved to one side of the functional gamete by an area of cytoplasm (Boyle and Doyle, 1953; Konar and Oberoi, 1969b). We found no evidence of membrane formation between the two gametes, which agrees with Konar and Oberoi (1969a), however Boyle and Doyle (1953) asserted that there must be some membrane system to hold the gametes in close position during entry into the egg cell, and Looby and Doyle (1944a) claimed to have seen a membrane between the male gametes of *P. andinus*. Only an ultrastructural study can confirm the presence of such a membrane.

Other studies have reported the egg nucleus to be four to five times larger than the male nucleus (Looby and Doyle, 1944a). However, we found the two nuclei to be of similar size. Relative gamete size may depend on species. We found evidence that a large quantity of cytoplasm enters the egg cell with the functional gamete, and contacts the egg nucleus during fusion. The egg cell cytoplasm is granular and sparsely organized around the nucleus. There did not appear to be the well-defined perinuclear zone characteristic of the Pinaceae (Owens et al., 1995b). In contrast, the male gamete cytoplasm is dense, finely granular and trails behind the nucleus in a well-organized mass. Coker (1902) and Sinnott (1913) identified a dense mass of cytoplasm which surrounded the fusion nucleus and accompanied it during migration to the chalazal end of the egg. However, Looby and Doyle (1944a) and Boyle and Doyle (1953) commented that although the male cytoplasm remained distinct, it never forms a 'special sheath' around the fusion nucleus, and the male nucleus 'slips' out of the male cytoplasm to fuse with the egg. Given the lack of organization of egg cytoplasm, the dense cytoplasm associated with the fusion nucleus may be composed of all or part of the male gamete cytoplasm. Ultrastructural work will determine if large or small inclusions are present in the egg cytoplasm, and allow identification of gamete cytoplasm contributions to the zygote. There is not currently any molecular marker information to identify such contributions.

We found that the zygote undergoes four free nuclear divisions, the first completed at or near the point of gamete fusion, and all subsequent divisions at the chalazal end of the egg. Wall formation occurs at the 16-nucleate stage, after the nuclei become arranged into three tiers. The embryonal tier consists of one cell, the suspensor tier of 7-9 cells, and the remaining nuclei are in an open tier with incomplete cell walls. The numbers of

nuclei making up each tier vary among proembryos examined. Boyle and Doyle (1954) hypothesized that some nuclei are excluded from the proembryo and lie beyond the open tier ('relict nuclei'). Doyle and Brennan (1971) and Buchholz (1941) found that the embryonal tier nucleus subsequently underwent mitosis to produce a binucleate cell. We confirmed the presence of two nuclei with resin sections. The chalazal end of the cell is occupied by a thickened cell wall area referred to as the terminal cap region (Looby and Doyle, 1944b). This terminal region is thought to facilitate penetration of the embryo through the archegonial wall and starch-filled megagametophyte cells (Looby and Doyle, 1944b). Our preliminary observations suggest that this binucleate cell is highly vesicular and may therefore be involved in secretory activity. The two nuclei are positioned in the micropylar half of the cell, and have a rounded shape.

There is disagreement on the fate of embryonal tier cells during early embryogeny. We found no stages between the binucleate embryonal tier cell and its rapid division into four uninucleate cells. Most previous studies found that this transformation is direct after a resting period while the suspensors elongate (Buchholz, 1941; Looby and Doyle, 1944b). Boyle and Doyle (1954) claimed that two binucleate embryonal tier cells are common in the totara proembryo, and used this to explain apparent cleavage polyembryony. We did not observe cleavage polyembryony in any of the ovules we studied. Doyle and Brennan (1971) found that the four uninucleate cells formed from the binucleate embryonal cell remain in contact, but divide as distinct 'lobes'. We observed formation of secondary embryos, but due to their position and morphology, they are more likely the result of suspensor cell separation and proliferation during early embryo growth, as seen in *P.*

andinus (Looby and Doyle, 1944b) and *P. nivalis* (Boyle and Doyle, 1954). Simple polyembryony was observed in many ovules.

The purpose of this study has been to document a complete reproductive cycle of *Podocarpus totara* from cone emergence to seed fall. This light microscopy study of totara male and female strobili and gametophytes is intended as a basis for future articles examining in more detail the ultrastructure of male and female gametes, cytoplasmic inheritance, fertilization and proembryo formation

Chapter 5

Development of the male gametophyte in *Podocarpus totara* : ultrastructure of germination to male gamete stages.

Introduction

Examination of male gamete formation and the distribution of organelles in different areas of the tube and body cell, provides important information about the contribution of the paternal parent to the zygote and embryo. In members of the Podocarpaceae, there is currently no ultrastructural information available to draw conclusions about the process of cytoplasmic inheritance. This has led to a gap in our current knowledge concerning fertilization in conifers. This study looks at body cell development and male gamete formation in *Podocarpus totara*, a podocarp species indigenous to New Zealand. Thorough observation of organelle morphology and distribution may make it possible to trace the fate of male gamete cytoplasm during fertilization.

Several studies of microsporogenesis and microgametogenesis in members of the family Podocarpaceae were carried out early this century (Coker, 1902; Jeffrey and Chrysler, 1907; Burlingame, 1908; Brooks and Stiles, 1910; Young, 1910; Stiles, 1912; Sinnott, 1913), but this work was limited to light microscope (LM) examination of material shipped from the Southern Hemisphere. Incomplete collections and deterioration of specimens have resulted in missing information for many species. Conflict over details which are difficult to distinguish under LM, such as the presence and number of prothallial cells in the mature pollen grain, and the anatomy of male gametes

has made interpretation of trends within the Podocarpaceae extremely difficult. Later LM work by Looby and Doyle (1944a), Boyle and Doyle (1953), Osborn (1960) and Konar and Oberoi (1969a) provided extensive anatomical descriptions of various *Podocarpus* species. Ueno (1960), Hodcent (1964) and Pocknall (1981) concentrated on pollen morphology and prothallial cell numbers and distribution. Ultrastructural study has been limited to aspects of microsporogenesis and wall formation in the pollen grain (Vasil and Aldrich, 1970; 1971; Del Fueyo, 1996). There are still many areas of male gametophyte development and gamete formation which require ultrastructural investigation.

This is the first in a series of articles which reinvestigate gametophyte development, fertilization and cytoplasmic inheritance, and proembryo development in a podocarp species, using LM and TEM. This article describes the processes of germination, pollen tube development and male gamete formation.

Observations and Results

Male strobili (Fig. 30) released pollen in late September - October as described in Chapter 4. Pollen collected in the pollination drop produced by the female strobilus underwent germination within one week of being taken up into the micropylar canal (Fig. 31). The quantity of pollen grains collected by the pollination drop was commonly so large that only a portion came into contact with the nucellus; the remainder was held far down in the micropylar arms and did not appear to rehydrate and germinate (Fig. 31).

Mature pollen grain

The totara pollen grain was bisaccate and 40-50 μm in diameter. The exine was about 0.6 μm thick and the intine was unevenly thickened, reaching a maximum thickness (0.4 μm) at the rugulate cap region of the exine (Fig. 32). Projections of the ectexine formed irregularly-shaped lumina within the sacchi (Fig. 32). The intine and exine layers ruptured in the furrow between the sacchi as the pollen grain germinated (Fig. 32). The cytoplasm of the tube cell fixed poorly with glutaraldehyde and osmium tetroxide, however clusters of mitochondria and Golgi bodies could be seen in the area adjacent to pollen tube emergence through the exine (Fig. 32). Lipid globules and the remains of starch grains were present in the cytoplasm (Figs. 32 and 33).

The body cell was positioned close to the cap region of the pollen grain, and was surrounded by three prothallial nuclei (Fig. 32). Each prothallial nucleus had a large nucleolus and dispersed chromatin (Fig. 32), and the nuclear membrane was intact and continuous (Fig. 34). The body cell consisted of a large granular nucleus with a well-defined nucleolus, and cytoplasm containing large numbers of mitochondria and plastids packed tightly together (Fig. 33). The interface between the body cell and tube cell cytoplasm was undulating (Fig. 34). Small granular vesicles were distributed evenly around the periphery of the body cell membrane (Fig. 34). Body cell mitochondria were ca. 0.45 μm in diameter, ovoid, and had distinct cristae (Fig. 35).

Nucellus

Nucellar cells adjacent to the developing pollen tubes were small and densely cytoplasmic. The nuclei were comparatively large and filled with condensed masses of chromatin (Fig. 36). The cytoplasm consisted of a series of small vacuoles containing fine osmophilic granules, lipid droplets and many Golgi bodies and mitochondria. Cup-shaped plastids with layers of internal membrane and lipid droplets and occasionally enclosing the remains of mitochondria, were observed in nucellus cells closest to the pollen tube (Fig. 36). Contact of the pollen tube to the nucellus cell wall resulted in the cytoplasm becoming densely staining and filling with lipid bodies and layered membrane systems arranged close to the plasma membrane (Fig. 37). The nucellar cell wall nearest the pollen tube became dilated and fibrous (Fig. 37).

Pollen tube development

Serial sections showed that the tip of the pollen tube consisted of a wide area between the endexine and the intine, filled with an irregular matrix of fine, fibrous material (Fig. 37). Large numbers of small secretory vesicles, produced by Golgi bodies packed into the nearby tube cell cytoplasm, were embedded in this matrix (Fig. 37). The vesicle contents could be seen accumulating on both the exterior of the intine, and the wall of nucellus cells (Fig. 38). After contact with the pollen tube, nucellar cell walls collapsed, the cytoplasm became extremely electron-dense (Fig. 36), and the cells were pushed out of the way by the extending pollen tube.

Once the pollen tube had reached a length of ca. 2mm, the nuclei moved away from the cap region and clustered close to the pollen tube entrance. The first to move into the

pollen tube was the tube nucleus, which migrated close to the tip and remained there for the duration of pollen tube growth (Figs. 39, 40 and 41). The prothallial nuclei did not proliferate at any time during germination or pollen tube growth (Fig.39). The three prothallial nuclei and the sterile nucleus followed the tube nucleus into the pollen tube (Fig. 40), and the body cell was the last to enter (Fig. 41). The body cell was identifiable in the microscope by its slightly larger nucleus and cytoplasm concentrated close to the nucleus (Fig. 41). *In vitro*, all nuclei had entered the pollen tube within five days of culture. *In vivo* pollen tubes had penetrated the nucellus, and reached a length comparable to that of five-day-old cultured pollen tubes, within one week of pollination.

Once the pollen tubes had penetrated through the nucellus, they branched laterally to form a disc-like structure covering the neck cells of all or most of the archegonia (Fig. 42). The dissociation of the megagametophyte from the megaspore wall in Figure 42 is an artifact of the fixation and dehydration process. The intine of the pollen tube was thick and remained distinct from surrounding nucellar and megaspore cell walls (Figs. 42 and 43). The prothallial nuclei and body cell remained central in the disc-like branched area, with only traces of tube cell cytoplasm present in the branches of pollen tube which grew laterally up the sides of the megagametophyte (Fig. 42). The prothallial nuclei included a granular nucleolus contained within a continuous nuclear membrane (Fig. 43). Prothallial nuclei stayed close to the body cell in small areas of tube cell cytoplasm. The tube cell cytoplasm contained abundant plastids, mitochondria, sections of rough ER and small lipid droplets (Fig. 43).

Body cell division

The body cell had a finely granular nucleus and unevenly-staining cytoplasm as seen in LM (Fig. 42). The uneven appearance of the body cell cytoplasm was resolved in TEM as extremely dense aggregations of mitochondria and plastids interspersed by highly vesiculate and “frothy” areas of cytoplasm (Figs. 44 and 45).

Once the archegonia had progressed to the central cell stage, the body cell divided to form two equal male nuclei. Although nuclear size was equal at division, the apportionment of body cell cytoplasm was not; one nucleus remained central in the body cell cytoplasm, and the other nucleus migrated or was extruded to one side and was surrounded by a very thin layer of cytoplasm which did not contain any organelles (Fig. 44). The body cell membrane remained around both nuclei, however it was discontinuous (Fig. 44). The number of vesicles in the body cell cytoplasm increased, especially near the membrane (Figs. 44 and 45). Some vesicles had fine granular contents, others appeared to contain condensed areas of cytoplasm (Fig. 45). The cell membrane was indented with a large number of exocytotic vesicles releasing material from the body cell cytoplasm into the pollen tube (Fig. 46).

The spherical, central male nucleus increased in size to a maximum of 35 μm diameter, with an evenly granular nucleoplasm (Figs. 44 and 47). There did not appear to be any organelles included into the nucleoplasm. Organelles in the cytoplasm surrounding this large nucleus were different in shape and size from those found in the body cell prior to germination and in the tube cell cytoplasm. The plastids increased in size (from 0.5 μm to 2 μm), became more densely-staining, and developed large starch granules (Fig. 48).

The mitochondria changed from ovoid to a vermiform shape, increased in length (from 0.45 μm to 2-3 μm) and developed more prominent cristae (Figs. 48 and 49).

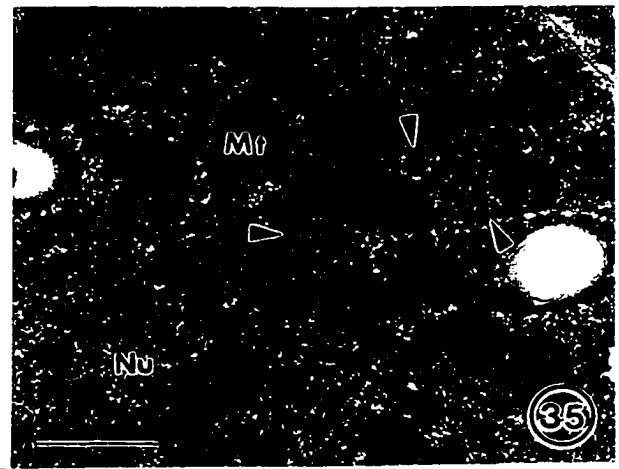
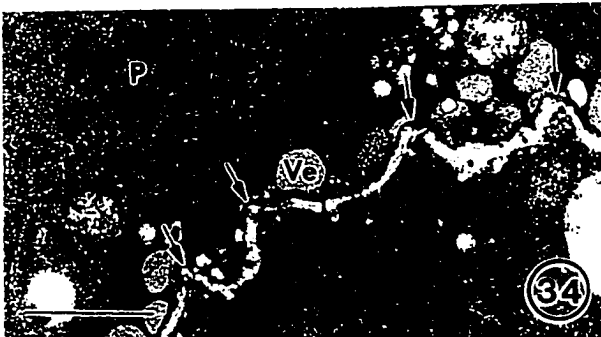
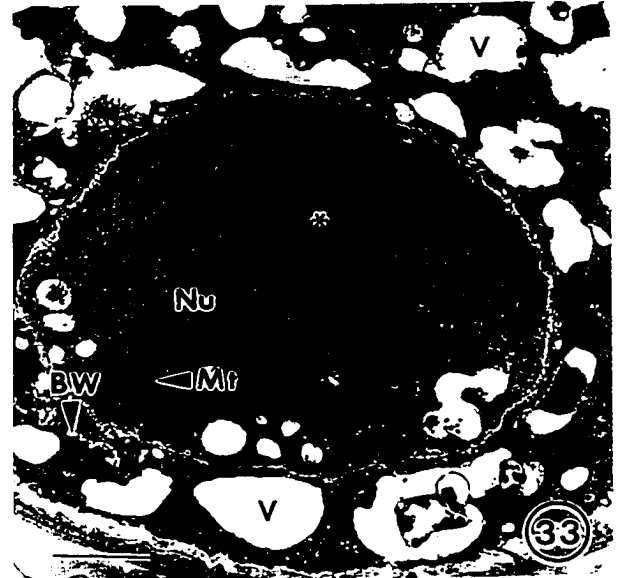
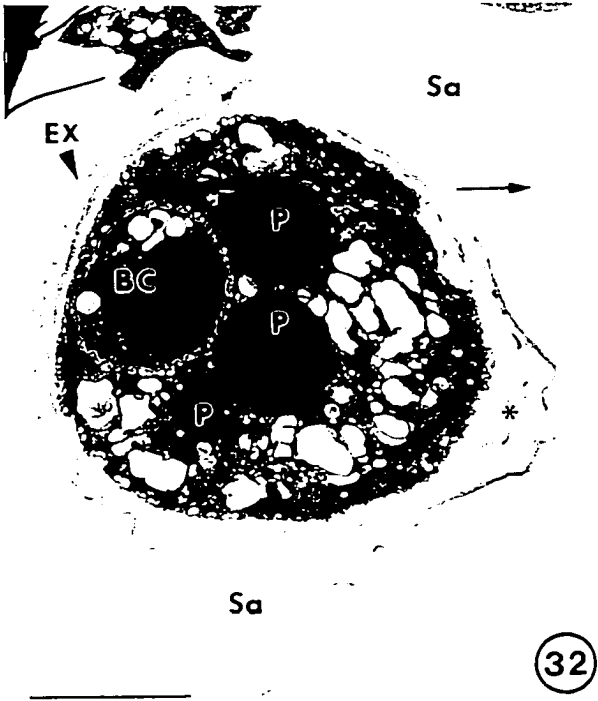
The small male nucleus (16 μm in diameter) was flattened on the side nearest the large male nucleus (Fig. 44). The nucleoplasm was more finely granular than that of the large male nucleus (Fig. 47), and contained darkly-staining areas towards the periphery (Figs. 44 and 47).

Pollen tube contact with the archegonium

As the egg nucleus moved to the centre of the archegonium, and the ventral canal nucleus appressed to the egg cell wall near the neck cells, the pollen tube penetrated through the neck cells and made contact with the egg cell (Figs. 50 and 51). There did not appear to be a preference of the pollen tube for archegonia in a particular position, as the disc-like branched pollen tube usually covered the necks of all available archegonia. Passage of the pollen tube either left the neck cells intact (Fig. 50) or collapsed them completely, forming a wide chamber at the entrance to the egg cell (Fig. 51). Tube cell cytoplasm degraded at this point, becoming electron-dense and darkly-staining (Fig. 50). The male gametes were arranged so that the large nucleus and the accompanying mass of cytoplasm was closest to the egg cell, and the smaller nucleus followed behind (Fig. 51). The smaller nucleus remained in close association with the large nucleus until the pollen tube breached the egg cell.

Figures 30-35

- Fig. 30 Mature male strobili collected prior to pollen shed in late September. Bar = 10mm.
- Fig. 31 Longitudinal section of paraffin-embedded ovule. Germination of pollen grains (Po) at the micropylar tip of the nucellus (N). Note the pollen grains (arrow) accumulated outside the micropylar canal (MC). LM. Bar = 100 μ m.
- Figs. 32-35 Resin-embedded sections of a pollen grain in the micropylar canal.
- Fig. 32 Entire pollen grain showing rugulate exine layer (Ex), sacci (Sa), body cell (BC) and prothallial nuclei (P). Note the projections of the ectexine layer inside the sacci (arrow) and early germination of the pollen tube from the furrow between the sacci (*). TEM. Bar = 10 μ m.
- Fig. 33 Body cell within the ungerminated pollen grain; nucleus (Nu), nucleolus (*), mitochondria (Mt), body cell wall (BW). Small vacuoles (V) fill the tube cell cytoplasm. TEM. Bar = 2 μ m.
- Fig. 34 Close-up of the body cell wall, showing undulating surface (arrows) and vesicles (Ve) clustered around the external surface. TEM. Bar = 1 μ m.
- Fig. 35 Body cell mitochondria and aggregations of ribosomes (arrowheads) near the nucleus. TEM. Bar = 0.5 μ m.



Figures 36-38

- Fig. 36 Cells at the tip of the nucellus, showing nucleus (Nu), vacuoles (V), aggregations of mitochondria (Mt), lipid droplets (L) and cup-shaped plastids (arrows). Cells degenerate at the tip of the nucellus (DN). TEM. Bar = 4 μ m.
- Fig. 37 Contact of the pollen tube (PT) with a nucellar cell (N). Vesiculate matter (arrow) from the tube cell cytoplasm (Tu) is embedded in the fibrous matrix (*) developed between the intine and endexine of the pollen grain. Densely-osmophilic membranes (arrowheads) accumulate at the periphery of the nucellus cell, and the cell wall (NW) becomes dilated. TEM. Bar = 2 μ m.
- Fig. 38 Densely-osmophilic vesicles (arrowheads) produced by Golgi bodies (arrow) in the tube cell cytoplasm passing across the fibrous matrix (*) and accumulating on the tip of the pollen tube and adjoining nucellus cell wall. TEM. Bar = 1 μ m.



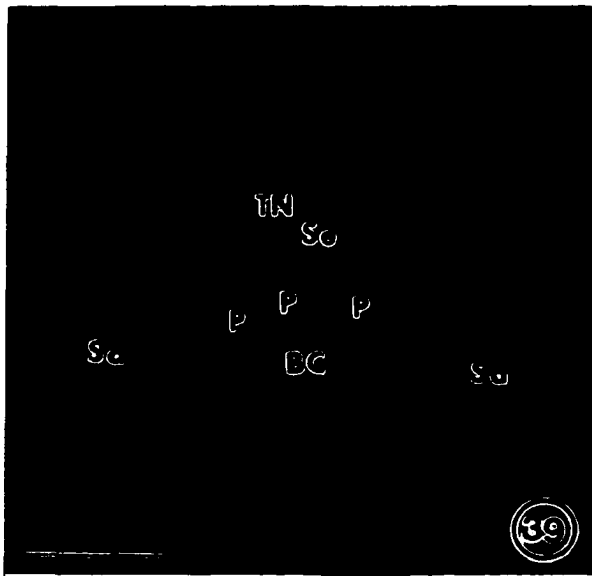
Figures 39-41

Figs. 39-41 DAPI fluorescence preparations of rehydrated and germinating pollen grains.

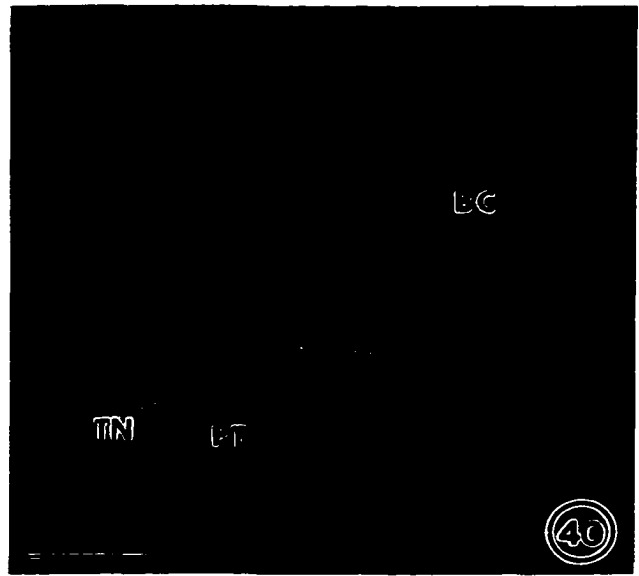
Fig. 39 Pollen grain after 2 days germination. The body cell (BC), prothallial (P), sterile (Se) and tube (TN) nuclei lie within the pollen grain. Pollen tube growth has not yet started. Sacci, (Sa). LM. Bar = 20 μ m.

Fig. 40 Pollen grain after 3 days germination. The tube nucleus has moved from the pollen grain to the tip of the pollen tube (PT). The prothallial and sterile nuclei are clustered near to the entrance of the pollen tube, but the body cell remains back within the pollen grain. LM. Bar = 20 μ m.

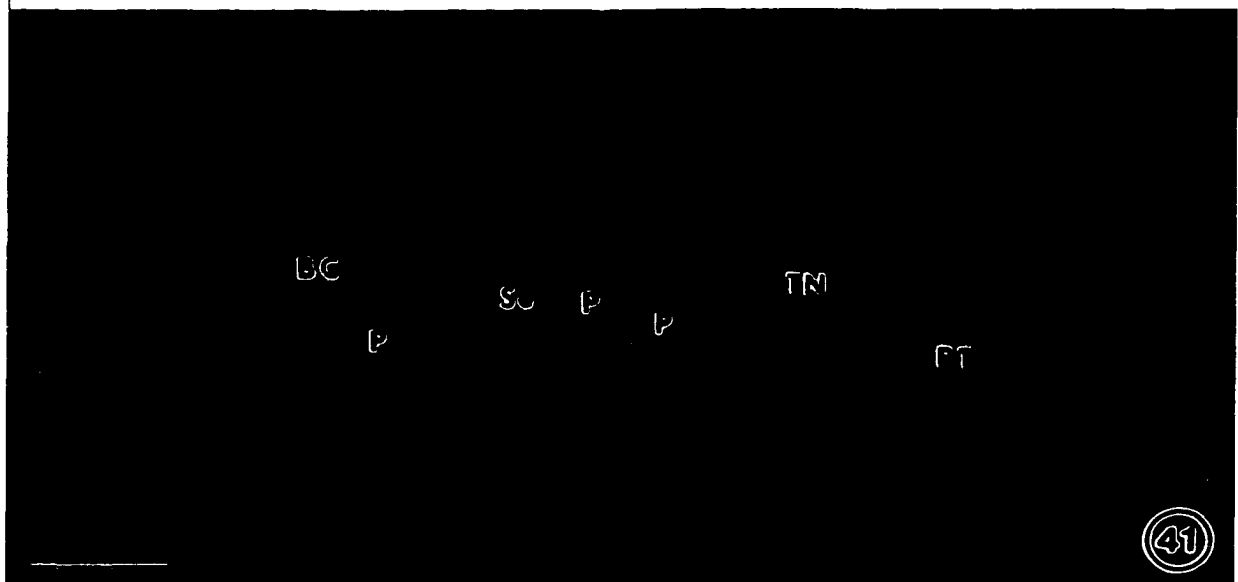
Fig. 41 Pollen grain after 4 days germination. The tube nucleus remains near the growing tip of the pollen tube. The prothallial and sterile nuclei follow the tube nucleus through the pollen tube, but the body cell still lags behind in the pollen grain. LM. Bar = 20 μ m.



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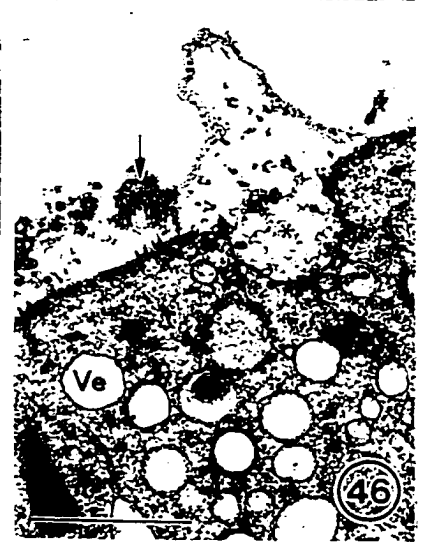
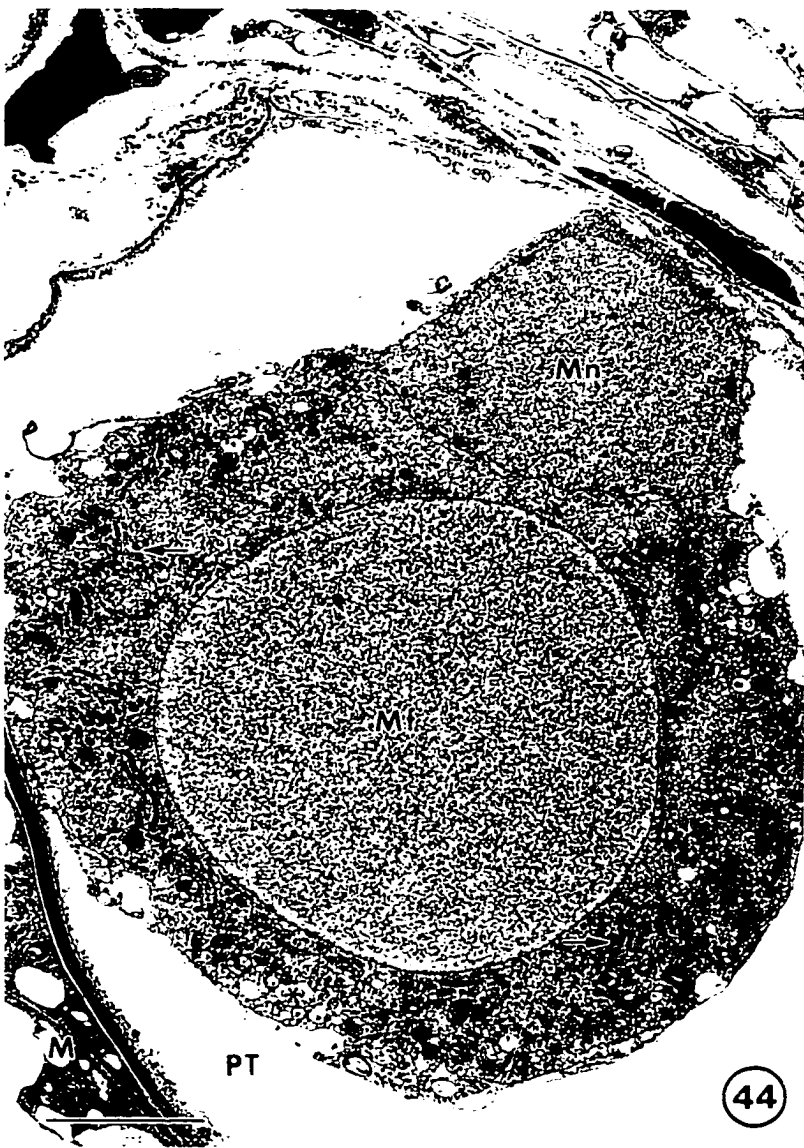
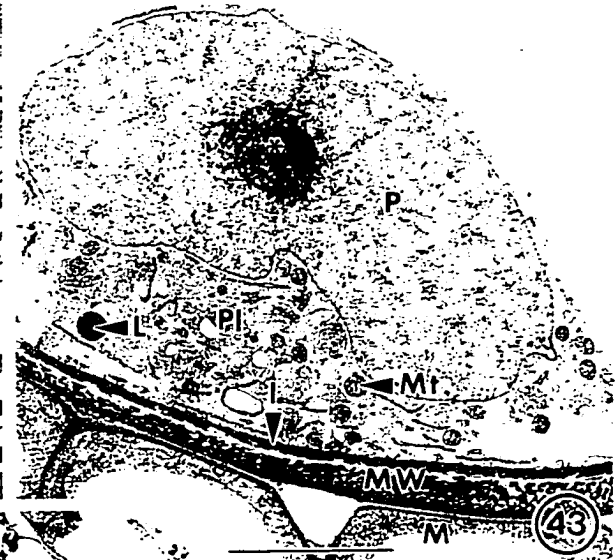
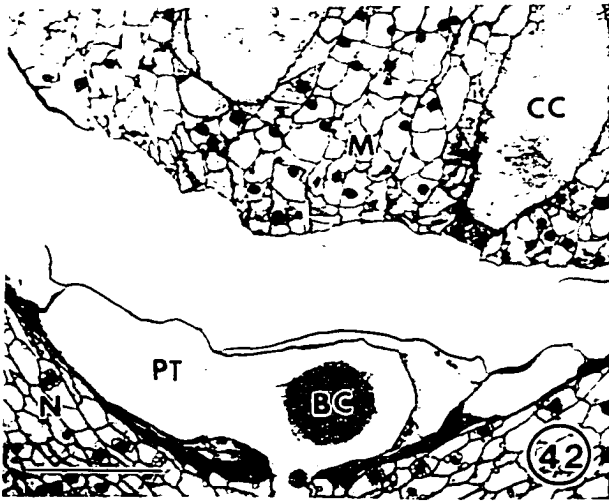
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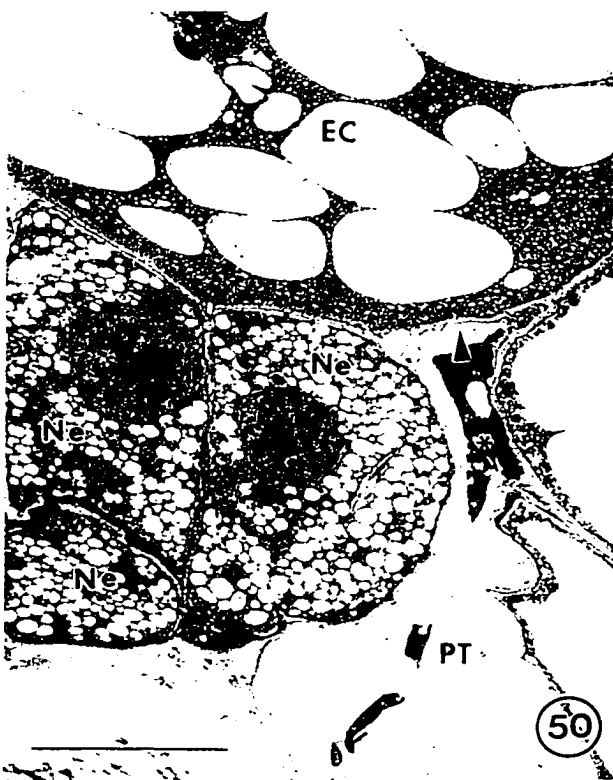
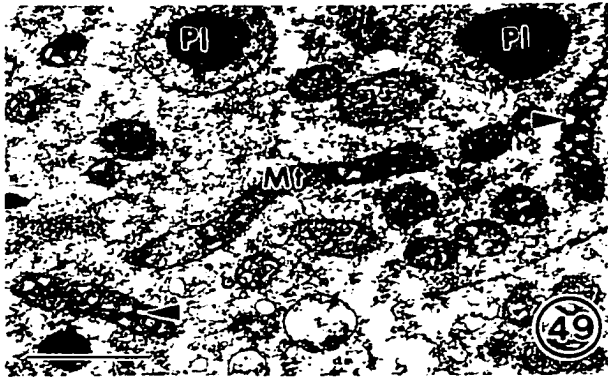
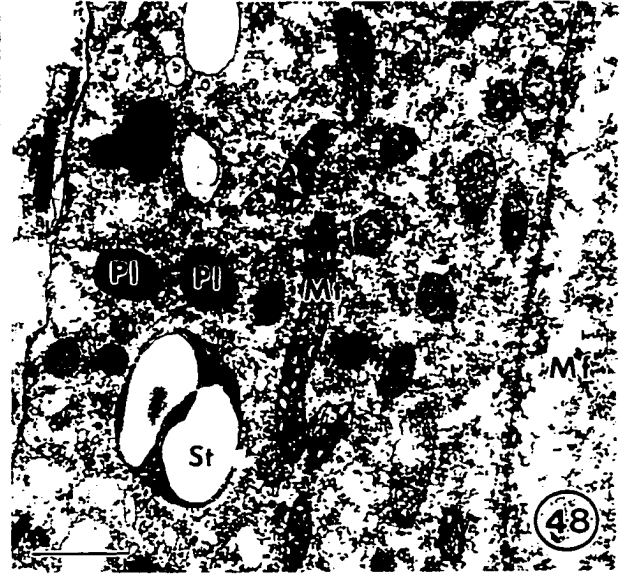
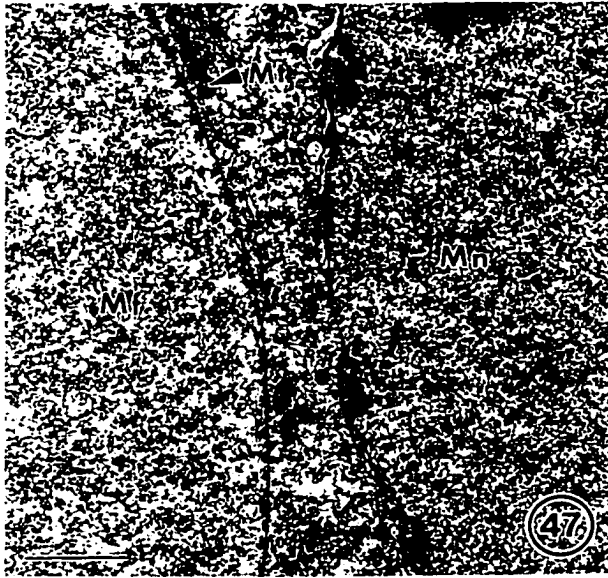
Figures 42-46

- Fig. 42 Pollen tube (PT) branched out between nucellus (N) and megametophyte (M), containing body cell (BC) prior to division and prothallial nucleus (arrowhead). The archegonia are at central cell stage (CC). LM. Bar = 100 μ m.
- Fig. 43 Prothallial nucleus (P) in pollen tube close to megaspore wall (MW). Note the granular nucleolus (*). Pollen tube intine (I) is appressed tightly to the megaspore wall. Tube cell cytoplasm contains lipid droplets (L), plastids (Pl) and mitochondria (Mt). TEM. Bar = 4 μ m.
- Fig. 44 Functional (large) male nucleus (Mf) and non-functional (small) male nucleus (Mn) in the pollen tube between the megagametophyte and nucellus. Note the clusters of mitochondria (arrows) and vesicles (*) within the body cell cytoplasm. TEM. Bar = 10 μ m.
- Fig. 45 Cluster of vesicles (Ve) within the body cell cytoplasm, surrounded by plastids. TEM. Bar = 1 μ m.
- Fig. 46 Cluster of vesicles releasing contents from body cell plasma membrane (arrow) by process of exocytosis (*). TEM. Bar = 1 μ m.



Figures 47-51

- Fig. 47 Area between functional (large) male nucleus (Mf) and non-functional (small) male nucleus (Mn), with a small group of mitochondria (Mt) close to the large male nuclear membrane. TEM. Bar = 2 μ m.
- Fig. 48 Cytoplasm close to the functional (large) male nucleus, showing plastids (Pl) containing starch grains (St) and vermiform mitochondria in longitudinal and cross sections. TEM. Bar = 1 μ m.
- Fig. 49 Vermiform mitochondria with tubular cristae (arrowheads) and plastids in cytoplasm near large male nucleus. TEM. Bar = 1 μ m.
- Fig. 50 Pollen tube (PT) penetration past the neck cells (Ne) to the egg cell (EC). Darkly-staining and degenerated tube cell cytoplasm (*) lies near the contact point of the pollen tube and the egg cell wall (arrowhead). TEM. Bar = 10 μ m.
- Fig. 51 Male gametes in pollen tube making contact with the egg cell wall. Remains of the neck cells can be seen on the left-hand side of the pollen tube; nucellus (N), egg cell nucleus (EN), jacket cells (J), megagametophyte (M). LM. Bar = 50 μ m.



Discussion

Podocarpus totara had a reproductive cycle lasting two years from cone initiation to seed maturity. There was no dormancy period during this cycle, which is similar to that reported for *P. macrophyllus* (Coker, 1902), *P. nivalis* (Boyle and Doyle, 1953), *P. falcatus* (Osborn, 1960) and *P. gracilior* (Konar and Oberoi, 1969a). *P. andinus* (Looby and Doyle, 1944a) and *Dacrydium sp.* (Sinnott, 1913) have a three-year reproductive cycle which includes an overwintering break between male gamete development in the pollen tube and fertilization. The period from pollination to division of male gametes occurred between October and December of the second year in totara, which agrees with reports from Sinnott (1913) and Burlingame (1908).

Development of the male gametophyte in totara and other podocarp species was differed from that of other conifer families in many aspects such as the number of prothallial nuclei, the inequality of the male nuclei and the branching pattern of the pollen tube.

The pollen grain of totara was bisaccate, 40-50 μm diameter and had an irregularly rugulate exine surface. The bisaccate pollen grain is typical of the genus *Podocarpus* and most other genera in the Podocarpaceae, but no sacci are found in the genus *Saxegothaea*, and three sacci are found on the pollen grains of *Microstrobos* and *Microcachrys* (Page, 1990; Kelch, 1997). Totara pollen is distinguished from other bisaccate-pollen podocarps native to New Zealand by the perfectly rounded sacci of hydrated pollen, and by the large irregular lumina formed by reticulations of the exine inside the sacci (Pocknall, 1981).

Podocarps appear to be highly variable in the number of prothallial nuclei proliferated during pollen grain development. Four prothallial nuclei are reported in *Podocarpus ferruginea* (Jeffrey and Chrysler, 1907), and in three Argentinian *Podocarpus* species (Del Fueyo, 1996). Burlingame (1908) originally reported *Podocarpus nivalis* to have six prothallial nuclei, but Boyle and Doyle (1953) found only three. Previous studies of totara have reported a range of prothallial nuclei numbers, with the most common being six (Burlingame, 1908; Sinnott, 1913). Our examination of totara confirmed the presence of three prothallial nuclei free in the tube cell cytoplasm of the mature pollen grain, clustered close to the body cell. Germination experiments showed that there was no proliferation of prothallial nuclei prior to pollen tube development. The close association of prothallial nuclei with the body cell and sterile nucleus make it extremely difficult to make an accurate count under the light microscope as previous studies have done. The use of DNA-specific fluorochromes such as DAPI in this study, and the similarity between *P. nivalis* and *P. totara* (Boyle and Doyle, 1953) confirm three prothallial nuclei as a more accurate figure. Most studies agree that at pollen maturity, the prothallial cell walls break down and the nuclei lie free in the tube cell cytoplasm (Burlingame, 1908; Stiles, 1912; Sinnott, 1913; Konar and Oberoi, 1969a), but Boyle and Doyle (1953) observed that cell walls did not dissolve until after germination in *P. nivalis*. This study did not show any vestige of a cell wall around the prothallial nuclei in the mature pollen grain, nor was any cytoplasm associated with them.

In contrast, the body cell wall in mature totara pollen was distinct and had an unusual configuration. The body cell wall stained extremely lightly with osmium tetroxide, was made up of a series of undulating projections and had membrane-bound vesicles filled

with a fine granular material clustered close to the exterior of the wall. Although the body cell wall has been described as “distinct” in a number of studies, the only further comment has been made by Vasil and Aldrich (1970) who observed “thin and conspicuous cell walls traversed by numerous plasmodesmata” around the body and prothallial cells. They suggest that as prothallial cell walls dissolve, the body cell is demarcated only by a plasma membrane and the cell wall does not exist (Vasil and Aldrich, 1970). The contents of the vesicles in the area between the body cell and tube cell cytoplasm are unknown, but consist of osmophilic particles distributed in a finely granular matrix. These vesicles do not appear around the body cell of *P. macrophyllus* (Vasil and Aldrich, 1970). Owens and Morris (1990) reported lobed projections of the tube cell cytoplasm penetrating into the body cell of *Pseudotsuga menziesii*. They suggested that this may be a mechanism by which the tube cell pulls the body cell into the pollen tube. The projections of the body cell wall in totara were not as deep as the body cell wall invaginations seen in *P. menziesii*, nor was there extensive contact between tube cell cytoplasm and body cell membrane. It is possible that the body cell wall in totara is undergoing dissolution prior to pollen tube emergence from the nucellus, as later stages reveal only a thin plasma membrane surrounding the body cell cytoplasm as it rests in the archegonial chamber. It is therefore possible that these vesicles contain degradative enzymes or breakdown products from the cell wall, however further histochemical investigation would be required to confirm this.

Fluorescence microscopy confirmed that the last nucleus to move into the pollen tube in totara is that of the body cell. It lagged behind the tube, prothallial and sterile nuclei by about two days in cultured pollen tubes. This agrees with studies by Sinnott (1913) and

Konar and Oberoi (1969a), but Boyle and Doyle (1953) observed that in *Podocarpus nivalis* another nucleus (hypothesized to be the sterile nucleus) remained behind and accompanied the body cell into the pollen tube. Similarly in species which have an overwintering dormancy period, the body cell and one other nucleus remained in the pollen grain during dormancy, while the other nuclei overwintered in the pollen tube embedded in the nucellus of the ovule (Looby and Doyle, 1944a; Elliot, 1950). This study found that in totara, all other nuclei had moved down the pollen tube before the body cell joined them.

The pollen tube in totara branched laterally as it penetrated through the nucellus and contacted the megaspore wall. This occurred just after archegonial initial formation in the megagametophyte. Pollen tube contact with the megagametophyte was not a requirement for archegonial initiation. The branched, disk-like pollen tube covered the neck cells of all or most of the archegonia as they developed in the ovule. This “splaying” of the pollen tube over the archegonial region appears to be characteristic of most podocarp species, and the position of the enlarged pollen tube area is said to influence the shape and distribution of archegonia in *P. gracilior* (Konar and Oberoi, 1969a) and *P. falcatus* (Osborn, 1960). There are many theories about the formation of the “archegonial chamber” or enlarged area between the nucellus and megaspore wall directly beneath the archegonia. Konar and Oberoi (1969a) and Boyle and Doyle (1953) report that the pollen tube “burrows” into the megagametophyte tissue, and forces separation between the archegonial wall and neighbouring cells, creating a depression in the megagametophyte. Both of these studies suggested that the burrowing action of the pollen tube was haustorial. There is no evidence to support the pollen tube in totara

having an haustorial function, and megagametophyte cells do not appear to be damaged by enlargement of the archegonial chamber. The “burrowing” action described by Boyle and Doyle (1953) appears to be characteristic of pollen tubes in species where the archegonia are initially at the micropylar surface of the megaspore membrane, such as *totara*. In species such as *P. andinus* (Looby and Doyle, 1944a) where the archegonia are deeply sunken into the megagametophyte before the pollen tube has penetrated through the nucellus, it has been observed that the pollen tube grows into the sunken area and expands by crushing the surrounding megagametophyte cells.

Konar and Oberoi (1969a) observed that the pollen tube of *P. gracilior* branched extensively during growth through the nucellus and next to the megagametophyte. This study did not observe branching of the pollen tube in *totara* until contact with the megagametophyte. It did not appear that the prothallial nuclei participated in directing the branching growth of the pollen tube in *totara*, unlike that observed in *Agathis australis* where the prothallial nuclei were located at the tips of branched areas (Owens et al., 1995b). The prothallial nuclei of *totara* remained central in the archegonial chamber, close to the body cell throughout division and male gamete formation.

Male gamete development in *totara* appeared to be similar to that described for *P. nivalis* (Boyle and Doyle, 1953) and *P. gracilior* (Konar and Oberoi, 1969a). The body cell did not divide until after a short “resting” period in the archegonial chamber. The body cell cytoplasm became darkly-staining, and the nucleus very prominent just before division. Other studies have observed that the body cell nucleus was eccentrically-placed prior to division, and that the more centrally-placed nucleus after division became the functional male gamete (Boyle and Doyle, 1953; Konar and Oberoi, 1969a). This study

did not observe a significant displacement of the body cell nucleus in totara, and the two male nuclei appeared to be equally central immediately after division. Konar and Oberoi (1969a) claimed that three unequal male nuclei are the product of body cell division in most podocarp species, but this and all other studies have found only two. One nucleus rapidly increased in diameter and the body cell organelles became organised in dense clusters around this nucleus. The other smaller nucleus was displaced to one side, usually the side furthest away from the archegonia. There were no body cell organelles in the thin layer of cytoplasm surrounding the smaller nucleus, and it was further excluded from the body cell cytoplasm just prior to pollen tube entrance to the egg cell. Inequality of male gametes in size and cytoplasm distribution appears to be typical of the Podocarpaceae (Singh, 1978). Chesnoy (1987b) comments that male gametes are often unequally-sized in genera with individual archegonia, as both gametes are liberated directly to the archegonium but only one is required for fertilization. It is always the larger of the two gametes which fertilizes the egg cell (Gianordoli, 1978 in Chesnoy, 1987b).

In totara, after enlargement of the functional male nucleus, and exclusion of the smaller male nucleus, the two nuclei remained within the body cell cytoplasm surrounded by the discontinuous body cell membrane. At no time in this study was cell plate formation observed between the two gametes, or incomplete cell plate structures as observed in *Picea glauca* (Dawkins and Owens, 1993) and *Taxus baccata* (Pennell and Bell, 1986). The body cell membrane persisted around the two male nuclei until just prior to fertilization, but at the time of fertilization was reduced to small lengths of disorganised membrane adhering to the surrounding cytoplasm.

The nature of male gametes in podocarps has been debated in many studies, and observations vary (Table 2). Singh (1978) concluded that male cells were typical of taxa whose archegonia were arranged in complexes (i.e., more than one archegonium sharing a set of jacket cells), and cited Looby and Doyle's (1944a) study of *P. andinus* as an example in the podocarps. However *P. andinus* was not observed to have archegonial complexes. In contrast, male nuclei are typical of taxa where archegonia are placed individually (Singh, 1978). Podocarp genera listed in Table 2 have individually placed archegonia, yet five studies claim to have observed male gametes to be cells. Three factors which may influence the validity of observations of male cells in podocarp genera include: (1) these studies were completed in fixation conditions which frequently did not permit clear observation of a membrane system of any sort around the gametes, and this has been acknowledged in some studies (Sinnott, 1913; Boyle and Doyle, 1953); (2) the study by Sinnott (1913) has been intensively critiqued by Boyle and Doyle (1953), who concluded that the male gametes shown in Sinnott's (1913) figures were in fact the body cell with either the sterile or a prothallial nucleus in close association; (3) studies by Coker (1902), Young (1910) and Kildahl (1908) show two male gametes enclosed in the remains of the body cell membrane, but without a cell plate formed between the two nuclei.

Three of the four podocarp genera acknowledged as basal in cladistic analyses of the Podocarpaceae (*Phyllocladus*, *Microcachrys* and *Saxegothaea*; *Microstrobos* has not been studied in great detail) (Kelch, 1997; 1998), are identified as having male cells rather than male nuclei. Both morphological and 18S RNA analyses consistently place

Table 2. Male gamete type in podocarps and other conifer species.

Species / Family	Male Gamete Type		Study Cited
	Cells	Nuclei	
<i>Dacrydium sp.</i>		X	Quinn (1965; 1966a; 1966b)
<i>Podocarpus andinus</i>	X		Looby and Doyle (1944a)
<i>Podocarpus sp.</i>	X		Sinnott (1913)
<i>P. macrophyllus</i>	X		Coker (1902)
<i>P. macrophyllus</i>		X	Stiles (1912)
<i>P. falcatus</i>		X	Osborn (1960)
<i>P. nivalis</i>		X	Boyle and Doyle (1953)
<i>P. gracilior</i>		X	Konar and Oberoi (1969a)
<i>P. totara</i>		X	This study
<i>Phyllocladus</i>	X		Kildahl (1908) Young (1910)
<i>Microcachrys</i>	X		Lawson (1923)
<i>Saxegothaea</i>	X		Looby and Doyle (1939)
Araucariaceae		X	Owens et al. (1995b)
Cephalotaxaceae		X	Singh (1961)
Pinaceae		X	Dawkins and Owens (1993)
Taxaceae		X	Pennell and Bell (1986)
Cupressaceae / Taxodiaceae	X		Chesnoy (1975)

these genera earliest in the podocarp lineage (Kelch, 1997; 1998). Although observations for *Phyllocladus* are questionable given the condition of the material studied, it is possible that male gametes in the form of nuclei is a more derived condition in the Podocarpaceae. Observations of other key genera such as *Microstrobos* and *Prumnopitys* which are also derived early in cladistic analyses would be necessary to confirm this.

Chesnoy (1987a, 1987b) commented that the male gametes of conifers “are not limited by a true wall” but only by a plasma membrane. Singh (1978) describes two male nuclei enclosed in the body cell plasma membrane but without an intervening cell plate, as male nuclei. Dawkins and Owens (1993) described the male gametes of *Picea glauca* as nuclei, even though cell plate components were present, as did Pennell and Bell (1986) in *Taxus baccata*. As the crucial factor distinguishing male cells from male nuclei appears to be the presence/absence of a cell membrane between the two nuclei, the male gametes of totara are interpreted as male nuclei. They are enclosed in the discontinuous remains of the body cell plasma membrane until entrance to the archegonium. This may be a condition typical of podocarp male gametes, but ultrastructural investigation of the species listed in Table 2 is the only method of confirming this. For the reasons listed above, the presence of male cells rather than nuclei in *Podocarpus* (Coker, 1902; Sinnott, 1913) and *Phyllocladus* (Kildahl, 1908; Young, 1910) is questionable.

Arrangement of the body cell cytoplasm and the organelles within it was different in the body cell at pollen germination, compared to just prior to division in the archegonial chamber. Plastids and mitochondria were evenly distributed around the body cell nucleus at pollen germination. The plastids were small, and predominantly without starch granules. The mitochondria were oval-shaped, with few widely-spaced cristae and a

lightly-staining granular matrix. In contrast, the organelles were grouped in tight clusters surrounding the large male nucleus after division, and none were arranged around the small male nucleus. The plastids increased in diameter, and frequently contained large starch granules. The mitochondria changed from oval-shaped to vermiform or cup-shaped, with many cristae and a darkly-staining granular matrix. The mitochondria were packed very tightly together, in an area close to the nuclear membrane. The body cell cytoplasm also contained large numbers of Golgi bodies, and vesicles concentrated into small densely-packed areas. Before division, the body cell has been described as having a dense and homogeneous cytoplasm (Boyle and Doyle, 1953; Konar and Oberoi, 1969a). Coker (1902), Sinnott (1913), Looby and Doyle (1944a) and Konar and Oberoi (1969a) described a dense body of cytoplasm surrounding the large male gamete after division, as distinct from a less darkly-staining cytoplasm around the smaller gamete and in the pollen tube. The range of organelles present in the cytoplasm is similar to that of other species (Willemse and Linskens, 1969; Chesnoy, 1971; Dawkins and Owens, 1993; Owens et al, 1995b). Chesnoy (1971) identified *Biota* male gamete cytoplasm as having distinct “zones” ; an organelle-free zone directly next to the nucleus, a middle zone dense with organelles and an outer zone containing mainly Golgi bodies and ER. Such zones were not observed in totara, or in other podocarp species, although Boyle and Doyle (1953) and Konar and Oberoi (1969a) noted that the cytoplasm was irregularly darkly-staining around the large gamete, and *P. nivalis* was briefly suggested to have more than two male nuclei due to the size of large darkly-staining areas close to the gametes (Boyle and Doyle, 1953). The light microscopy techniques used were not capable of resolving these

areas to be the dense groups of organelles this study observed in totara body cell or male gamete cytoplasm.

The morphological change in the mitochondria of the totara body cell has not been described in other podocarps, or other conifer species. Tzagoloff (1982) noted that the inner membrane of a mitochondrion can assume different spatial arrangements depending on the physiological state of the cell. In rat liver cells, the mitochondria have a “condensed” morphology relative to that of other cells, in which the cristae surfaces separate and appear like long tubules, and the granular matrix can stain very darkly (Tzagoloff, 1982). The appearance of mitochondria in the totara male gamete complex was similar to the condensed morphology of rat liver mitochondria. The body cell cytoplasm undergoes a rapid period of redistribution and condensation as the gametes form, and a change in mitochondrial morphology accompanying this physiological change is possible. Although Willemsse (1971) found that mitochondria do not change morphologically during microsporogenesis, Camefort (1966; 1968) found that male gamete mitochondria in *Larix* and *Pinus nigra* had an “electron-dense matrix criss-crossed with numerous elongated tubules” similar to that seen around totara male gametes.

A morphological change in plastids is routinely described, as proplastids differentiate into amyloplasts or leucoplasts in the male gametes, and later in the embryo. In totara, this change in plastid structure resulted in larger plastids containing starch granules being present around the male gametes, and at fertilization. The observations of this study suggest that a similar change in structure occurs in mitochondria, and that this is a response to a change in physiology of the body cell cytoplasm as gametes are formed. It

is also possible that such a structural change has a bearing on which organelles are passed on to the zygote during fertilization and cytoplasmic inheritance. This will be discussed in the Chapter 7.

Chapter 6

Development of the female gametophyte in *Podocarpus totara* : ultrastructure of the central cell to mature egg cell stages.

Introduction

Investigation of development in the female gametophyte of podocarps and other conifers provides detail about the integrity and distribution of egg cell organelles, and organisation of the egg nucleus. Thus far, information about podocarp female gametophyte development has been limited to light microscope studies, the resolution of which does not allow observation of most cytoplasmic organelles. This study will look in detail at the structure of the egg cell cytoplasm and nucleus, jacket cells, neck cells and the megaspore membrane of *Podocarpus totara* using LM and TEM techniques.

Ultrastructural studies of female gametophyte development have been undertaken for conifer species such as *Pinus nigra* (Camefort, 1966), *Larix decidua* (Camefort, 1968), *Biota orientalis* (Chesnoy, 1975), *Pseudotsuga menziesii* (Owens and Morris, 1990), *Picea glauca* (Dawkins and Owens, 1993; Runions, 1997), and *Agathis australis* (Owens et al., 1995a; 1995b). Detailed light microscope studies have been carried out on a number of podocarp species: *Podocarpus andinus* (Looby and Doyle, 1944a), *P. nivalis* (Boyle and Doyle, 1953) and *P. gracilior* (Konar and Oberoi, 1969a).

Observations of the period between megasporogenesis and megagametophyte development in podocarp development have been thoroughly investigated at the light microscope level, and so this study focuses on areas of development for which fine detail

is currently lacking : cytoplasmic contents of egg, jacket and neck cells, evidence of morphological change or degradation of plastids or mitochondria in the egg cell, and position and nature of the ventral canal cell/nucleus.

The ventral canal product of central cell division has not been well documented for podocarps, and many studies have not reported it. Evidence for a ventral canal cell or nucleus was not found in the light microscope study presented in Chapter 4, and some studies have suggested that it may be rapidly broken down (Coker, 1902; Stiles, 1912). Other reports describe it as persistent and free in the egg cell cytoplasm, and even capable of spontaneous mitoses (Coker, 1902; Sinnott, 1913; Looby and Doyle, 1944a). Runions (1997) hypothesized that the position of the ventral canal cell or nucleus may influence the growth of the pollen tube, and direct its entry into the egg cell. This study seeks to confirm the presence or absence of a cell wall around the ventral canal cell or nucleus in *Podocarpus totara*, and its position relative to the neck cells and/or pollen tube.

Observations and Results

Central cell

Archegonial initials appeared soon after cell wall formation in the megagametophyte (see Chapter 4). The initials divided unequally to form a small neck cell and a large, elliptical central cell (Fig. 52). The central cell consisted of an ovoid nucleus with a distinct and compact nucleolus (Figs. 52 and 53), and thin strands of cytoplasm separating large vacuolate, electron-transparent areas (Fig. 52). The cytoplasm contained large numbers of mitochondria and plastids dispersed throughout the cell. The vacuolate nature of the central cell resulted in poor fixation in paraffin and resin preparations.

Megaspore wall and megagametophyte(s)

The megaspore wall was thick and extended into short, densely-osmophilic baculae at the micropylar end of the megagametophyte (Fig. 54). The baculae were most abundant in the micropylar portion where the intine of the pollen tube contacted the megaspore cell wall (Fig. 54). Towards the chalazal end of the megagametophyte, the megaspore wall became thinner and the projections shorter and less abundant. The cell walls of adjacent megagametophyte cells were thickened on the outer surface in contact with the megaspore wall. Megagametophyte cells contained a thin, parietal layer of cytoplasm, one or two nuclei and a large central vacuole.

In 16 out of 310 ovules sectioned, there was more than one megagametophyte. Each megagametophyte had a separate and distinct megaspore wall, and appeared to function independently (Fig. 55). In most cases, each megagametophyte formed archegonial initials, and mature egg cells were observed in some specimens (Fig. 55). The megagametophytes were arranged either with one taking up the chalazal half of the space inside the nucellus and one taking up the micropylar half, or more often the megagametophytes formed side by side (Fig. 55). Archegonia formed in both megagametophytes regardless of how far removed they were from the nucellar tip and pollen tubes. Two separate megaspore walls were observed at the point where the megagametophytes were appressed, however one wall usually did not form baculae, and small densely-cytoplasmic cells were tightly enclosed between them (Fig. 56).

Jacket cells

At formation of the archegonial initials, surrounding megagametophyte cells divided to form a jacket layer around each developing archegonium (Fig. 52). At the central cell

stage, the jacket layer was composed of small cells with large nuclei and dense cytoplasm. The jacket layer completely encased the central cell except for a small area near the neck cells in which the central cell was in direct contact with the megagametophyte.

At egg cell maturity, the jacket cells had a large number of small vacuoles in the cytoplasm (Fig. 57). Jacket cells were typically uninucleate, with a distinct nucleolus and dense granules of chromatin dispersed throughout the large nucleus (Fig. 57). Some jacket cells were bi- or multinucleate. The cytoplasm contained ovoid mitochondria, large numbers of lipid bodies, cup-shaped modified plastids and small inclusions (Fig. 57). The small inclusions contained quantities of cytoplasm, whorls of membrane and small lipid droplets. The jacket cell wall adjacent to the egg cell was thickened (Figs. 57 and 58). Plasmodesmatal connections between the egg cell and jacket cells were distributed evenly along the shared cell wall area (not illustrated).

Each archegonium usually had a separate jacket cell layer, however two archegonia occasionally shared jacket cells but had separate neck cells (Fig. 58). Shared jacket cells were long and thin, and the cell wall was thickened in areas adjacent to both egg cells.

Neck cells

The number of neck cells varied among archegonia, but seven to nine cells were common. Neck cells were elongate to isodiametric, and arranged in one or two tiers (Figs. 59 and 60). The cell wall of neck cells was thickened in areas adjoining the egg cell and megaspore membrane (Fig. 60). Neck cells were uni- or binucleate, and the large nuclei took up most of the volume of the cell (Figs. 60 and 61). The nucleolus was

dispersed and the chromatin condensed and granular (Fig. 61). The cytoplasm of neck cells looked much like that of jacket cells, except that fewer membrane-bound structures were present. In comparison to surrounding megagametophyte cells (Fig. 62), both neck and jacket cells had extremely dense and darkly-staining cytoplasm and contained a higher number of mitochondria and other organelles.

Central cell division

Division of the central cell occurred after pollen tube contact with the megagametophyte. The central cell nucleus was located close to the cell wall at the micropylar end (Fig. 52). Mitosis of the central cell produced a small, lens-shaped ventral canal nucleus, and a slightly larger ovoid egg nucleus (Figs. 63 and 64). A cell wall did not form between these two nuclei. The ventral canal nucleus remained at the periphery of the egg cell, either appressed to the cell wall close to the neck cells (Fig. 63) or free in the cytoplasm at the micropylar end of the egg cell (Fig. 64). The ventral canal nucleus had an intact nuclear membrane, and finely granular nucleoplasm containing small darkly-staining areas (Figs. 63 and 64).

Egg cell

The egg nucleus increased in size and migrated to the centre of the egg cell cytoplasm (Figs. 64 and 65). It was enclosed by a thin perinuclear zone of densely-staining cytoplasm, surrounded by 5-25 μm diameter vacuoles which filled the micropylar half of the egg cell (Fig. 65). The area of egg cell immediately chalazal to the egg nucleus contained small vacuoles, cytoplasm in fine strands and aggregations of small electron-

dense granules. A large vacuole occupied the chalazal end of the egg cell, except for the very tip, which was filled with smaller vacuoles and fine strands of cytoplasm (Fig. 65).

The egg nucleus was bounded by a distinct bilayered membrane, interrupted by numerous nuclear pores (Fig. 66). The perinuclear zone was composed of dense aggregations of mitochondria, lipid droplets and rough and smooth ER systems (Fig. 66). There were also many small electron-transparent vacuoles. The mitochondria were ovoid, with a densely granular matrix and few cristae spaced widely apart (Fig. 67). Plastids were not apparent, nor was there any evidence of starch grains.

In the area of cytoplasm immediately chalazal to the egg nucleus four types of membrane-bound structures occurred: (1) cup-shaped modified plastids which had engulfed quantities of cytoplasm (Fig. 68); (2) lipid droplets enclosed in bilayered membranes (Fig. 69); (3) densely osmophilic structures incorporating cisterna-like membrane systems with small circular structures arranged in single rows between the cisternae (Fig. 70); (4) small inclusions which contained dense granular cytoplasm or sometimes cellular structures such as mitochondria (Fig. 71).

Smooth ER was found in all areas of egg cell cytoplasm, and appeared as discontinuous cisternae created by the plane of section (Figs. 66, 67 and 71). Around the egg nucleus, smooth ER was arranged into long continuous strands running parallel to the nuclear membrane (Fig. 72). The strands of ER were not observed to make contact with the nuclear membrane or nuclear pores, but there was a high concentration of ribosomes between the strands (Fig. 72).

The micropylar end of the egg cell was filled with dense cytoplasm, containing abundant smooth ER, but few mitochondria or other organelles (Fig. 73). This cytoplasm

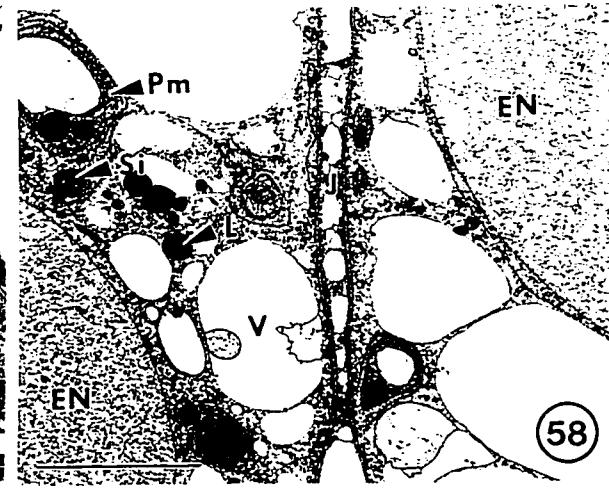
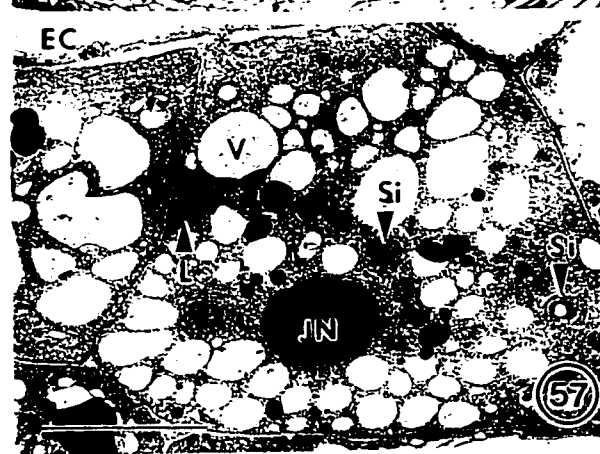
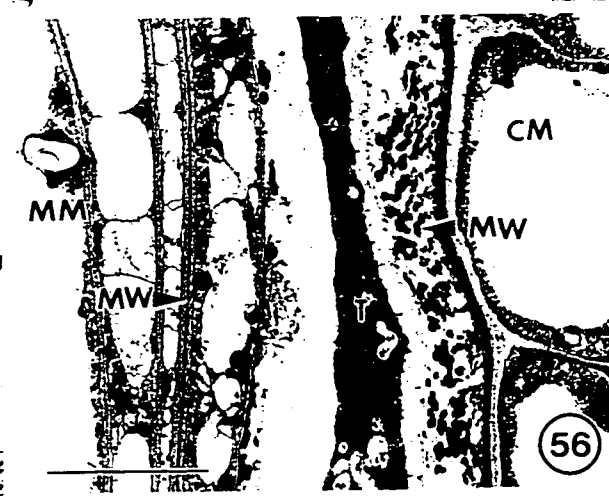
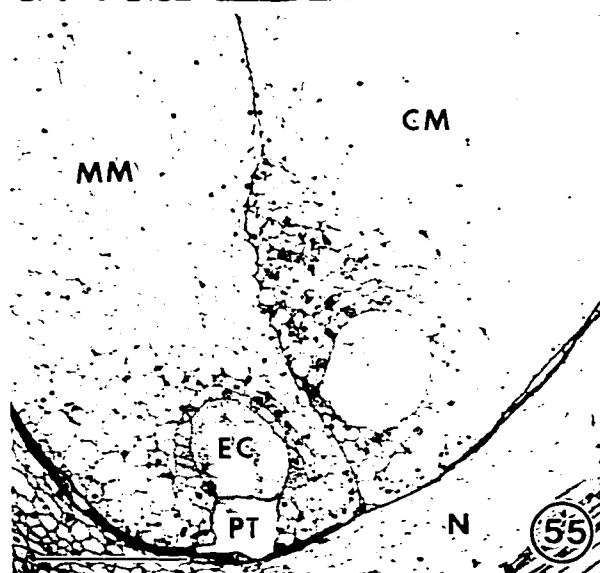
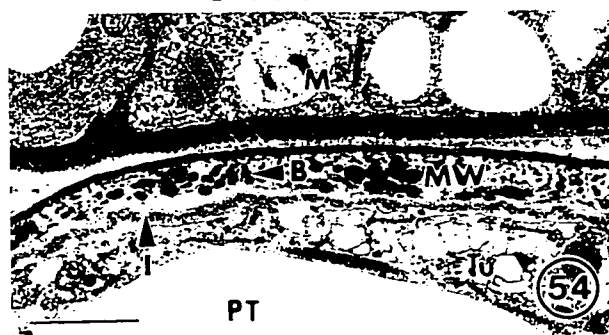
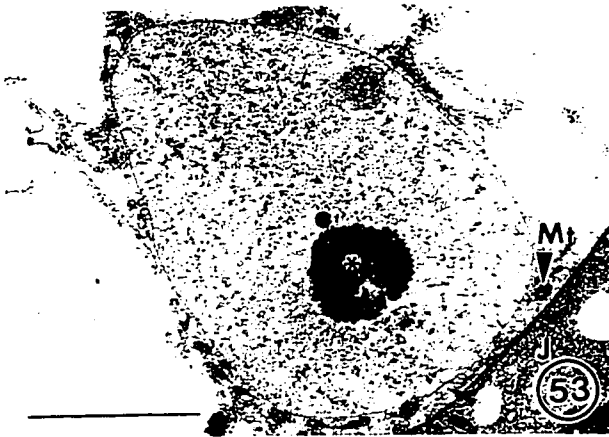
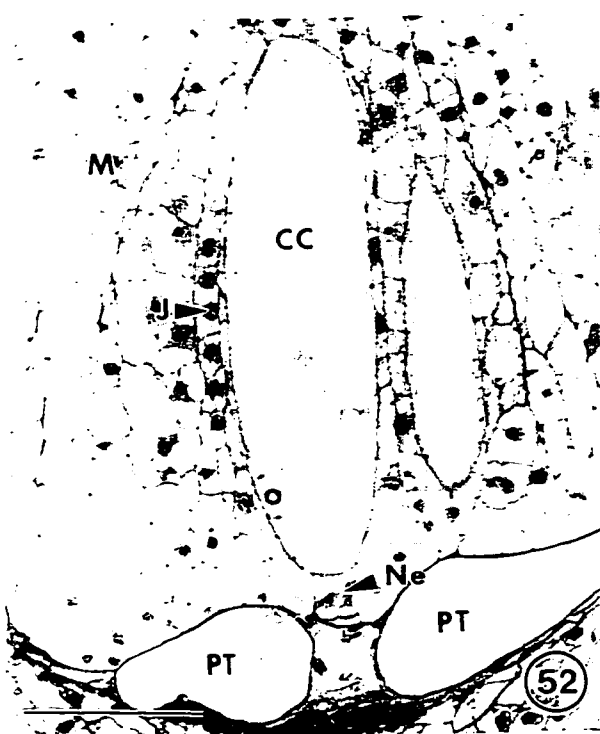
covered the area of egg cell wall in contact with the neck cells, and surrounding the ventral canal nucleus (Figs. 63 and 73). Some small inclusions, apparent as slightly more darkly-staining areas, were present in this cytoplasm (Fig. 73).

In the cytoplasm at the chalazal tip of the cell, 6-8 μm structures circular in profile were suspended in fine granular cytoplasm. These structures were either electron-dense, coarsely granular material contained within a bilayered membrane (Fig. 74) or large concentric whorls of bilayered membrane which stained intensely in the centre and had lipid droplets interspersed among the membrane layers (Fig. 75).

Figures 52-58

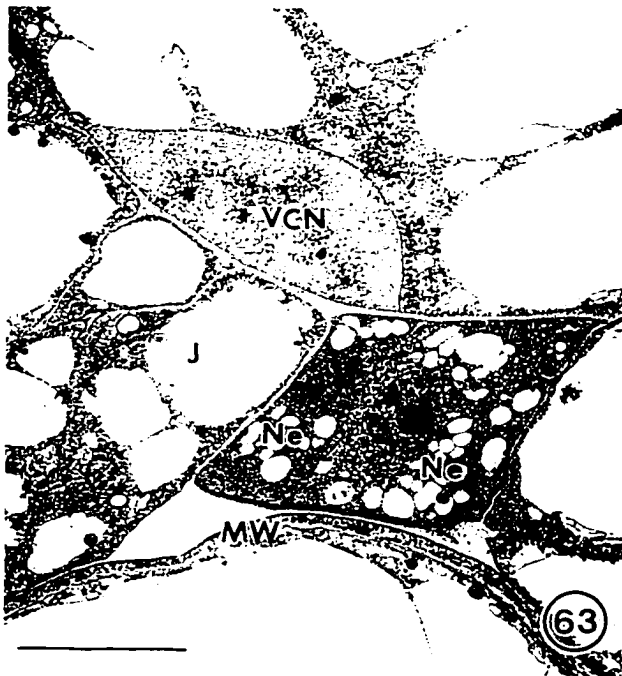
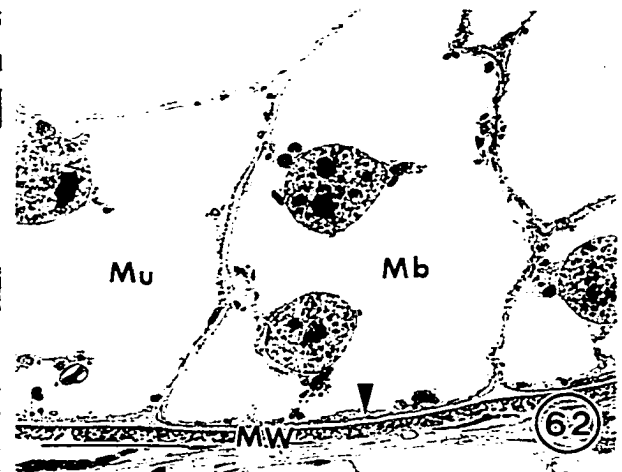
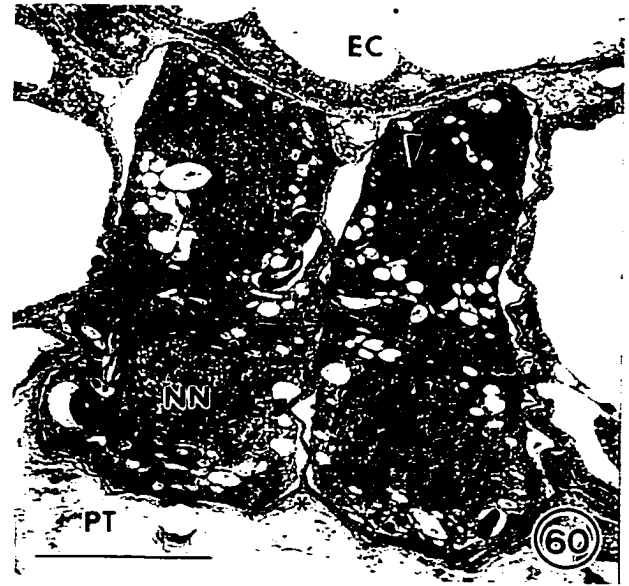
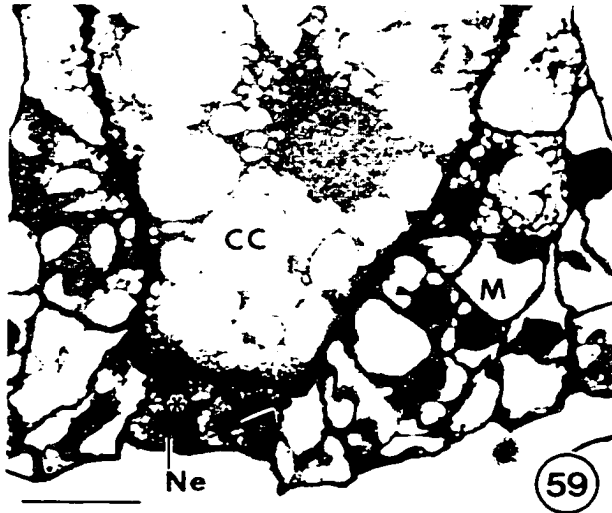
- Fig. 52 Archegonium at the central cell (CC) stage of development. Jacket (J) and neck cells (Ne) have divided. Pollen tube (PT); megagametophyte (M). LM. Bar = 100 μ m.
- Fig. 53 Central cell nucleus, with prominent nucleolus (*) adjacent to the cell wall prior to division. Surrounding cytoplasm is rich in mitochondria (Mt). TEM. Bar = 10 μ m.
- Fig. 54 Megaspore wall (MW) interposed between the megagametophyte and the intine (I) of the pollen tube. The megaspore wall is extended into osmophilic baculae (B). Tube cell cytoplasm (Tu) is aggregated close to the pollen tube intine. TEM. Bar = 3 μ m.
- Fig. 55 Two megagametophytes contained in a single ovule. Both the micropylar-most (MM) and chalazal-most (CM) megagametophyte have developed archegonia. Archegonia are more rounded than in ovules with a single megagametophyte, and a pollen tube has contacted an egg cell (EC) in the micropylar-most megagametophyte. Nucellus (N). LM. Bar = 200 μ m.
- Fig. 56 Megaspore walls between two megagametophytes in the same ovule. The wall of the chalazal-most megagametophyte has developed osmophilic baculae, but that of the micropylar-most one has not. The remains of the tapetal nucellus cell layers (T) are trapped between the two megaspore walls. TEM. Bar = 4 μ m.

- Fig. 57 Jacket cell at egg cell maturity. The nucleus (JN) appears small due to the plane of section. The cytoplasm is filled with small vacuoles (V), small inclusions (Si) and lipid droplets (L). The cell wall (*) adjoining the egg cell is thicker than those adjoining other jacket and megagametophyte cells. TEM. Bar = 8 μ m.
- Fig. 58 Single layer of jacket cells shared by two archegonia. The jacket cells are narrow with thickened cell walls bordering both egg cells. Cup-shaped modified plastids (Pm), small inclusions, lipid droplets and vacuoles are clustered close to the egg nucleus (EN). TEM. Bar = 10 μ m.



Figures 59-64

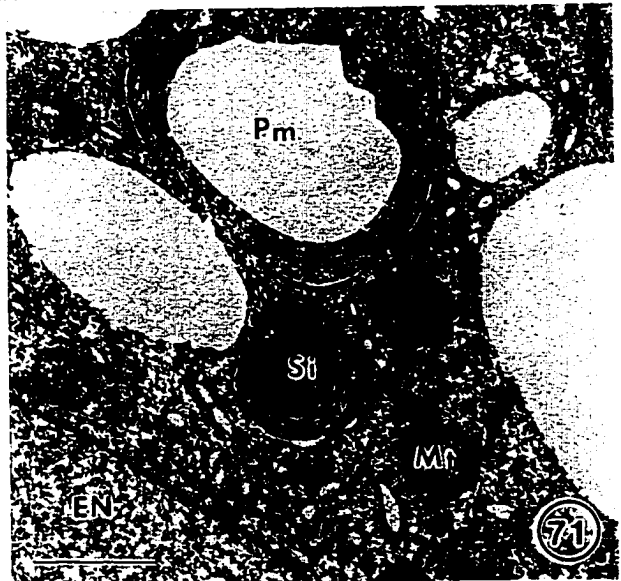
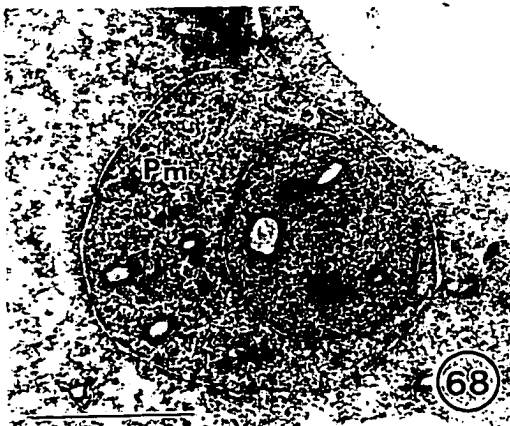
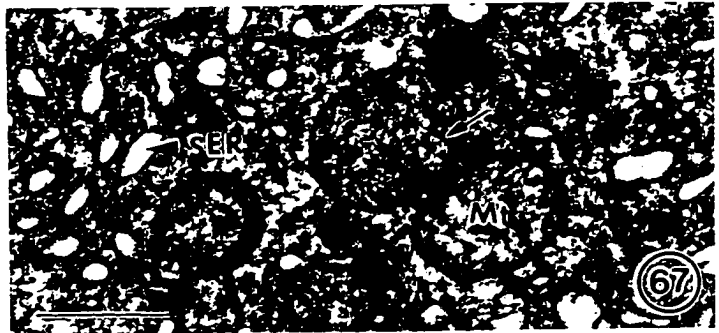
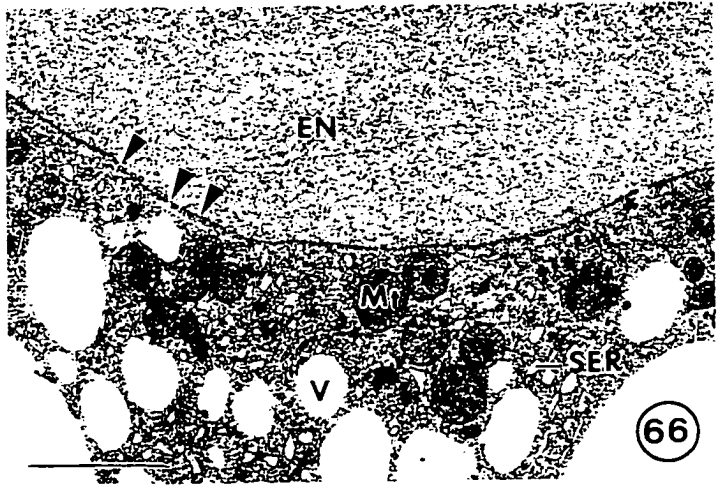
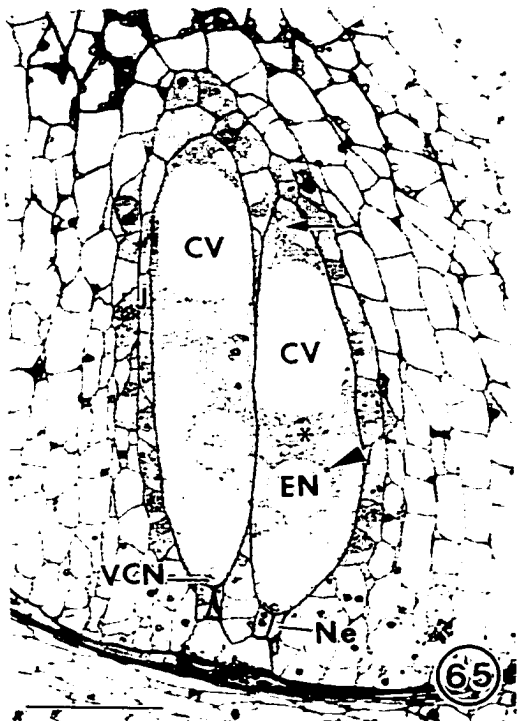
- Fig. 59 Neck cells (Ne) in the process of division (*) at the central cell (CC) stage of development. An oblique division in neighbouring neck cells (arrowhead) creates a mixture of one and two tiers of cells. Megagametophyte (M). LM. Bar = 25 μ m.
- Fig. 60 Two tiers of neck cells between the pollen tube (PT) and egg cell (EC). The neck cell nucleus (NN) takes up much of the volume of the cell. The neck cell walls adjoining the egg cell and pollen tube (*) are thickened. Neck cell cytoplasm contains many small vacuoles, lipid droplets and mitochondria. Note the presence of attenuated plastids (arrowhead). TEM. Bar = 10 μ m.
- Fig. 61 Binucleate neck cell, showing mitochondria (Mt), plastids (Pl) and small vacuoles clustered around the two nuclei. Two uninucleate neck cells are immediately to the left. TEM. Bar = 6 μ m.
- Fig. 62 Megagametophyte cells can be binucleate (Mb) or uninucleate (Mu). A thin, parietal layer of cytoplasm (arrowhead) contains mitochondria and plastids. Megaspore wall (MW). TEM. Bar = 10 μ m.
- Fig. 63 Ventral canal nucleus (VCN) appressed to the egg cell wall near the neck cells. No cytoplasm separates the ventral canal nucleus from the cell wall. Surrounding egg cell cytoplasm (*) contains few organelles. Jacket cell (J). TEM. Bar = 10 μ m.
- Fig. 64 Ventral canal nucleus lies free in the egg cell cytoplasm prior to male gamete entry, but remains chalazal to the egg nucleus (EN). LM. Bar = 100 μ m.



Figures 65-71

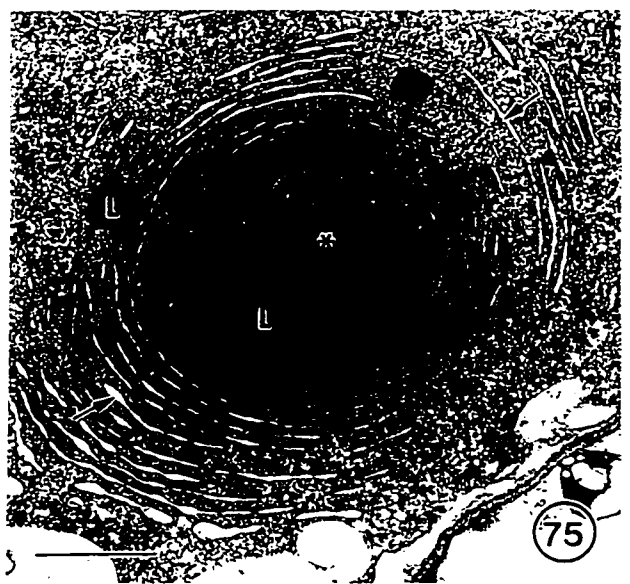
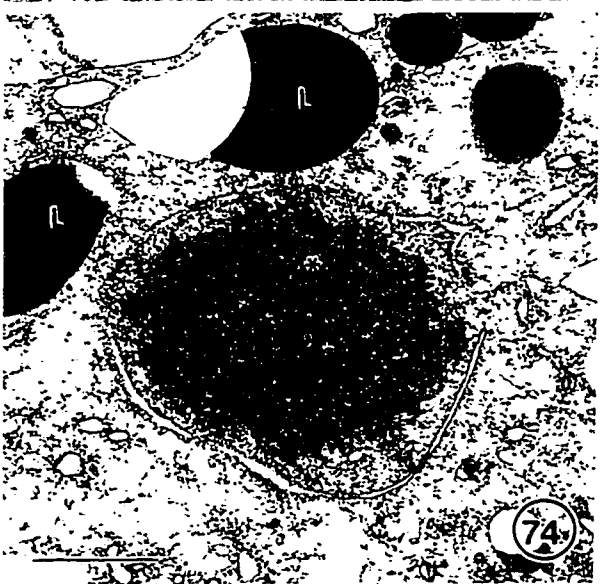
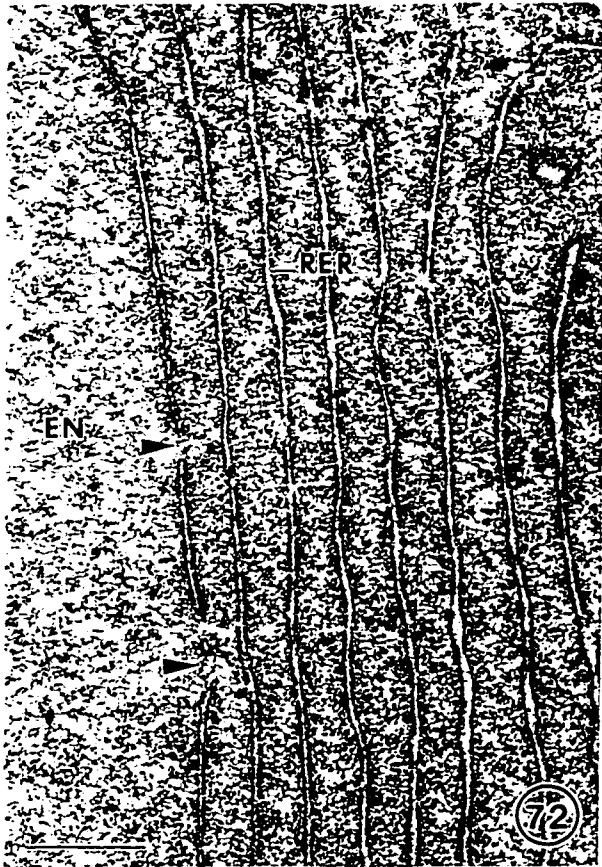
- Fig. 65 Mature archegonia prior to male gamete entry. The egg nucleus (EN) is surrounded by a perinuclear zone of darkly-staining cytoplasm (arrowhead). The ventral canal nucleus (VCN) and a small area of egg cell cytoplasm containing few organelles and extensive smooth ER remain at the micropylar end of the egg cell, near the neck cells (Ne). Egg cell cytoplasm is rich in cup-shaped plastids (*) and lies between the egg nucleus and the chalazal vacuole (CV). Cytoplasm at the chalazal tip of the egg cell (arrow) has large membrane whorls and protein bodies. The jacket cell layer (J) does not entirely encase the egg cell. LM. Bar = 100 μ m.
- Fig. 66 The perinuclear zone surrounding the egg nucleus is filled with small vacuoles (V), mitochondria (Mt) and smooth ER (SER). The nuclear membrane is interrupted by numerous pores (arrowheads). TEM. Bar = 2 μ m.
- Fig. 67 Close-up of mitochondria in the perinuclear zone. Cristae (arrow) are widely-spaced and difficult to see. TEM. Bar = 1 μ m.
- Fig. 68 Cup-shaped transformed maternal plastid (Pm) showing typical double membrane and enclosed areas of cytoplasm. TEM. Bar = 2 μ m.
- Fig. 69 Small inclusion (Si) enclosing lipid droplets (L). Egg cell wall (*) adjoins a jacket cell. TEM. Bar = 4 μ m.
- Fig. 70 Membrane structures found in cytoplasm surrounding the perinuclear zone. Annular cisternae (AC) enclose a darkly-staining matrix. Layers of small circular structures (arrows) are layered between the cisternae. TEM. Bar = 2 μ m.

Fig. 71 Cup-shaped modified maternal plastids in cytoplasm bordering the perinuclear zone. Mitochondria in the perinuclear zone have developed undulating membranes and cristae are difficult to locate. TEM. Bar = 1 μ m.



Figures 72-75

- Fig. 72 Strands of rough ER (RER) arranged parallel to the nuclear membrane of the egg nucleus (EN). The nuclear membrane contains many pores (arrowheads). TEM. Bar = 0.5 μ m.
- Fig. 73 The micropylar tip of the egg cell (EC) contains cytoplasm with very few organelles. Small inclusions (Si) are rare, but smooth endoplasmic reticulum (SER) is abundant. Neck cells (Ne). TEM. Bar = 6 μ m.
- Fig. 74 Cytoplasm at the chalazal tip of the egg cell contains membrane-bound areas with densely-staining, granular contents (*), and large lipid droplets (L). TEM. Bar = 1 μ m.
- Fig. 75 Also found at the chalazal tip of the egg cell are large whorls of membranes (arrows), with densely-staining interiors (*) and interspersed lipid droplets. TEM. Bar = 2 μ m.



Discussion

The time between archegonial initiation and egg cell maturation in totara lasts for ca. one month (beginning of November to early December, see Chapter 4). Many stages of central and egg cell development were similar to those observed in other podocarp and conifer species. Details of central and egg cell morphology, and conformation of the megaspore membrane are presented in this study in greater detail and higher resolution than has previously been available for a member of the Podocarpaceae.

Pollination and pollen tube growth did not appear to be a requirement for initiation of archegonia or division of the central cell. The position and number of archegonia was similar to members of the Pinaceae (Owens and Blake, 1985): typically four to six archegonia arranged at the micropylar tip of the megagametophyte. It was unlikely that pollen tube contact with the megagametophyte influenced the position of the archegonia as has been observed in *Podocarpus falcatus* (Osborn, 1960), because the archegonial initials formed before the pollen tube has fully penetrated through the nucellus in totara (see Chapter 6).

The megaspore wall of totara ovules was similar in structure to that of other conifer species (Singh, 1978). The baculate exine layer was thickest in the micropylar region of the megagametophyte, especially in areas where the pollen tube intine was in contact. This agrees with studies of other conifer species such as *Pseudotsuga menziesii* (Owens and Morris, 1990) and *Agathis australis* (Owens et al., 1995a), but not with observations of *Picea glauca* (Runions, 1997; Dumont-Beboux et al., 1998) in which the megaspore wall was reported to be thickest at the chalazal end of the ovule.

The occurrence of multiple megagametophytes in a single ovule has not been reported for many conifer species, and appears to be rare. Approximately 5% of totara ovules contained two megagametophytes, of approximately equal size. A similar occurrence was noted in totara by Sinnott (1913). Each megagametophyte cellularized, although the orientation of cell wall development after the free nuclear stage often differed between the two. Examination of the boundary between the two megagametophytes showed that megaspore wall development appeared normal in the chalazal-most megagametophyte, which developed a baculate exine layer. In the micropylar-most megagametophyte, the megaspore wall did not develop an obvious exine layer, and was bordered by the remains of what were perhaps tapetal cells of the nucellus trapped between the two megaspore walls as free nuclear development occurred. The micropylar megagametophyte did develop a baculate exine layer on surfaces not in contact with the chalazal megagametophyte. Multiple megagametophytes have been reported in *Podocarpus macrophyllus* (Coker, 1902), *Sequoia* (Arnoldi, 1899 in Coker, 1902), and in members of the Taxaceae (Cecchi Fiordi and Tani, 1991; Cecchi Fiordi et al., 1996; Anderson and Owens, 1999). Typically, organelles concentrate in the chalazal-most megaspore during tetrad formation (Singh, 1978). The uniform distribution of cytoplasm, storage reserves and mitochondria during division of the megaspore mother cell is thought to facilitate survival of more than one megaspore (Pennell and Bell, 1987; Cecchi Fiordi et al., 1996). In *Taxus*, 1% of ovules have been reported to contain three megagametophytes (Anderson and Owens, 1999).

Archegonia formed at the micropylar end of each megagametophyte. Apart from being a more rounded shape, possibly due to the pressure exerted by megagametophyte growth

within such a confined space, each archegonium appeared functional. In the few specimens found late in development, pollen tubes penetrated the nucellus and branched around the tip of the micropylar-most megagametophyte. Normal megagametophyte development is a result of abortion of the three micropylar megaspores, and free nuclear development of the chalazal megaspore. If multiple megagametophyte development is the result of growth of more than one megaspore (i.e., abortion of only two of the four megaspores), then the chalazal-most megagametophyte is most likely the product of the chalazal-most megaspore. The two functional megaspores would have different haploid genetic makeup. The genetic complement of fertilized egg cells would differ depending on which megagametophyte was fertilized. This study did not observe stages past pollen tube contact with the megaspore membrane in such ovules, and therefore cannot confirm whether the archegonia of the micropylar-most megagametophyte were viable. In *Taxus brevifolia*, the pollen tube diverts around the micropylar-most megagametophyte(s) and contacts the chalazal-most (Anderson and Owens, 1999). This is the opposite of that observed for totara, and raises the question of how much effect the behaviour of pollen tube growth has on inheritance of maternal chromosome complements, when there is more than one megagametophyte in an ovule.

The number of neck cells developed by each archegonium in totara is variable, but seven to nine neck cells is most common. A large variation in neck cell number has been recorded for members of the Podocarpaceae: 2-25 in *Podocarpus macrophyllus* (Coker, 1902), 10-15 in *P. andinus* (Looby and Doyle, 1944a), 5-6 in *P. nivalis* (Boyle and Doyle, 1953) and 4-6 cells in *P. gracilior* (Konar and Oberoi, 1969a). There is also wide variation in the number of tiers these neck cells form. In totara neck cells were usually

arranged in two tiers, however binucleate neck cells in one tier were common. Boyle and Doyle (1953) commented on “the difficulty of getting a clear picture of the condition of the neck cells throughout the genus” in *Podocarpus*, as studies have recorded differing patterns of cell division in the neck cell area leading to variable arrangements. As was noted in Chapter 5, the pollen tube of totara often penetrated to the egg cell leaving the neck cells intact.

The ultrastructure of neck cells has not been examined in many conifer species. In totara, the neck cells were densely cytoplasmic, with large distinct nuclei. The cytoplasm was vacuolate, and contained large numbers of mitochondria, lipid droplets and small inclusions. Owens and Morris (1990) described the neck cells of *Pseudotsuga menziesii* as secretory, and observed deposition of material in the archegonial chamber apparently from the neck cells. This was not observed in totara, although in cases where the pollen tube penetrated through the neck cells to the egg cell, the contents of the neck cells were dispersed through the archegonial chamber. The number of small inclusions within the neck cells appeared to increase, and the cytoplasm became denser and more difficult to preserve with age. It is likely that the cytoplasm of these cells is undergoing degradation, and release of substances from the cells may be a result of a decline in the integrity of the cell wall and plasma membrane. In older neck cells, the cytoplasm often appeared plasmolysed, and the cell wall in contact with the archegonial chamber appeared fibrous and stained unevenly. Konar and Oberoi (1969a) observed that the neck cells of *Podocarpus gracilior* archegonia degenerated completely soon after they developed, and presented no obstacle to pollen tube entry to the egg cell. Although the integrity of neck

cell cytoplasm declined with age in totara, the cells did not collapse prior to pollen tube contact.

The jacket cell layer around the archegonia of totara encompasses the entire egg cell, except for a gap two to three cells wide where the neck cells are located. An incomplete jacket layer has been observed in *Araucaria* (Haines, 1983) and *Agathis* (Owens et al., 1995a), and has been suggested to provide an alternative entry point to the neck cells as the pollen tube penetrates the egg cell. In totara, the pollen tube bypassed the neck cells in many cases. The jacket layer is hypothesized to mediate transfer of storage products from surrounding megagametophyte cells, by translocating soluble protein and starch through primary pit fields on the walls in contact with the egg cell (Singh, 1978). The wall between the jacket and egg cells in totara was not typified by the extent of cell wall thickening and large primary pit fields seen in *Picea* (Runions, 1997) and *Pseudotsuga* (Owens and Morris, 1990). Plasmodesmata were distributed evenly between jacket and egg cells, but were often difficult to see. The cell wall appeared evenly thickened. Coker (1902) and Stiles (1912) commented on the difficulty of identifying plasmodesmatal connections in *Podocarpus sp.* Although direct connections between jacket and egg cell may not be as concentrated as in other species, podocarps are noted for the densely-cytoplasmic, multinucleate, thick-walled cone of cells extending from the jacket layer into the centre of the megagametophyte (see Chapter 4). This has been observed in *Saxegothaea* (Looby and Doyle, 1939), *Podocarpus andinus* (Looby and Doyle, 1944a), *P. nivalis* (Boyle and Doyle, 1953) and *P. gracilior* (Konar and Oberoi, 1969a). It has been described as an extension of the jacket layer (Singh, 1978), and the entire cone of cells fills with starch and lipid immediately after fertilization (see Chapter 8).

The jacket cells around totara archegonia are frequently multinucleate or have extremely large nuclei. This has also been observed in *Podocarpus macrophyllus* (Coker, 1902) and *P. gracilior* (Konar and Oberoi, 1969a). Singh (1978) cited studies which measured the DNA content of uninucleate jacket cells in *Ginkgo*, and found them to be diploid, tetraploid and even octaploid. Konar and Oberoi (1969a) found that the nuclei of the jacket cells occasionally escaped into the archegonium, however this study did not observe this happening unless the archegonium remained unfertilized and began to degrade. At this point, the egg cell wall collapsed and the jacket cell contents were released. Chesnoy (1987b) reported that the jacket cells degenerated at the time of fertilization, but in totara they persisted until well after the proembryo had penetrated through the archegonial wall (see Chapter 8).

The ventral canal product of central cell division was confirmed to be a nucleus, with an intact nuclear membrane, dispersed nucleolus, a very small amount of cytoplasm and no plasma membrane. Although mitosis was not observed, the position of the ventral canal and egg nuclei immediately afterwards showed that division was oblique and lateral, as reported in Looby and Doyle, (1944a) and Boyle and Doyle, (1953). The ventral canal nucleus was usually appressed to the micropylar end of the egg cell wall, close to the neck cells. No cytoplasm separated the ventral canal nucleus and the egg cell wall. In a few cases, the ventral canal cell was detached from the wall, and lay free at the micropylar end of the egg cell cytoplasm. It did not approach the egg nucleus, and by the time the pollen tube had made contact with the egg cell wall, it had usually disintegrated.

The ventral canal nucleus has been variously reported as evanescent, persisting up until fertilization, or enlarging and lying free in the egg cell cytoplasm (Coker, 1902; Stiles,

1912; Sinnott, 1913; Looby and Doyle, 1944a; Osborn, 1960). In *totara*, the ventral canal nucleus did not persist after pollen tube contact with the egg cell, and became extremely difficult to identify just prior to fertilization. Owens et al. (1995b) observed that the ventral canal nucleus of *Agathis* also became difficult to locate prior to fertilization. This appears to be the case for most podocarp species, but reports of the fate of the ventral canal nucleus after its division from the egg nucleus are few. Stiles (1912) commented that just prior to fertilization, all that could be seen of the ventral canal nucleus was “a safranin-staining mass” at the micropylar end of the archegonium. Coker (1902) reported that the ventral canal nucleus left its lateral position against the egg cell wall, increased in size and began to divide amitotically. He suggested that it performed a role in the nourishment of the embryo. Analysis of Coker’s (1902) diagrams, shows that most probably he was looking at either a collection of supernumerary nuclei (the small male nucleus, prothallial and perhaps sterile) at the micropylar end of the egg cell, or, later in development, at a “rosette” of nuclei commonly formed by the proliferation of relict nuclei produced during proembryo formation (see Chapter 8).

Runions (1997) hypothesized that the ventral canal nucleus in *Picea glauca* had a role in direction of pollen tube growth, as abnormal pollen tube entry to the egg cell appeared to correspond to abnormal placement of the ventral canal nucleus. In *totara*, the ventral canal nucleus was located either just to one side of the neck cells against the cell wall, or just chalazal to the neck cells, free in the cytoplasm. This study did not observe other placements of the ventral canal nucleus, but pollen tube contact with the egg cell varied considerably. The pollen tube sometimes bypassed the neck cells completely and entered the archegonium above and to one side even when the ventral canal nucleus was near the

neck cells. It appears that the splayed branching pattern of the pollen tube allows it access to entry points other than the neck cells. As Owens and Morris (1990) commented for *Pseudotsuga*, it is unlikely that the neck cells are a passive portal to the egg cell, and likely that they positively influence pollen tube growth by secretory activities. This study suggests that in totara, attraction of the pollen tube to the archegonia occurs much earlier than just prior to fertilization. The morphology of the megagametophyte around the archegonia, with the neck cells flush with the surface, and the cone-shaped, extended jacket-layer area of multinucleate megagametophyte cells, make it possible that both the neck and megagametophyte cells secrete pollen tube attractants, and this likely happens just as the pollen tube penetrates through the nucellus. This would account for a lone pollen tube branching in such a manner that it covers the neck cells of all archegonia at the same time. Takaso and Owens' (1994) study of *Pseudotsuga* observed secretory products released from the egg cell and megagametophyte cells which affect pollen tube growth. Analysis of the contents of neck and megagametophyte cells at this precise stage in totara would be necessary to confirm such a process.

Archegonia had reached a mature form once the egg nucleus had migrated to the middle of the cell and the perinuclear zone of maternal mitochondria had formed. An area of small vacuoles surrounded the perinuclear zone, with a large vacuole occupying most of the chalazal end, and small areas of cytoplasm clustered at the micropylar and chalazal poles of the egg cell. The nature of the three main zones of cytoplasm (the perinuclear, micropylar and chalazal) varied in staining density, membranous systems and organelles.

The egg nucleus was large with granular nucleoplasm and a distinct nucleolus, and the nuclear membrane had numerous nuclear pores. The perinuclear zone around the egg

nucleus consisted of clusters of mitochondria, lipid droplets and an extensive ER system aligned parallel to the nuclear membrane. The mitochondria in this zone stained lightly, and had a coarsely granular matrix; the cristae were barely visible and widely-spaced. This change in mitochondrial morphology has also been noted in *Pseudotsuga* (Camefort, 1968; Owens and Morris, 1990), *Larix* (Camefort, 1968) and other conifer species (Chesnoy, 1987b). The extent to which mitochondria are concentrated in the perinuclear zone of the egg cell depends on the conifer species. In most members of the Pinaceae (Camefort, 1968; Owens and Morris, 1990; Runions, 1997), Taxaceae (Pennell and Bell, 1986; Brukhin and Bozhkov, 1996; Anderson and Owens, 1999) and *Agathis* (Owens et al., 1995b) many mitochondria are localized within the perinuclear zone. In contrast, in the Cupressaceae, Taxodiaceae (Chesnoy, 1971; 1987a) and possibly the Cephalotaxaceae (Gianordoli, 1974), the egg cells lack a distinct perinuclear zone and mitochondria and other organelles are scattered throughout the egg cytoplasm. A perinuclear zone has been observed in other podocarp species (Coker, 1902; Looby and Doyle, 1944b; Boyle and Doyle, 1953; Konar and Oberoi, 1969a), however the use of light microscopy limited description of the contents of the dense cytoplasm around the egg nucleus. The significance of an organised perinuclear zone in totara will be examined in greater detail in Chapter 7.

The arrangement of parallel rough ER membranes around a nucleus has been noted during cell wall formation in the coenocytic female gametophyte of *Taxus baccata* (Pennell and Bell, 1986) and the dividing central cell of *Pinus* (Camefort, 1966). In this case, the encircling ER is thought to be responsible for the spatial organisation of the protoplast. Pennell and Bell (1986) comment that terminal connections between the ER

membranes and the nuclear envelope may be involved in the “accurate siting” of microtubules. Although this study did not identify connections between the ER and the nuclear membrane, it is entirely possible that these were present, but out of the plane of sections examined. Microtubule arrays have been identified as being responsible for a radial arrangement of mitochondria and ribosomes in the central cell of conifer species such as *Juniperus* (Singh, 1978; Chesnoy, 1987b), and it is possible that the ER system around the nucleus forms a framework for an “asteroid-like” arrangement of microtubules holding mitochondria close to the nuclear membrane. Runions (1997) commented on the number of microtubules oriented with the long axis pointing towards the egg nucleus of *Picea*, and this can be seen in Figures 79 and 80 of Chapter 7 for totara. The ribosome density within the layers of ER cisternae is high in the perinuclear zone, and it is possible this facilitates rapid post-fertilization protein manufacture.

The micropylar end of the egg cell contained the ventral canal nucleus and a large quantity of cytoplasm devoid of organelles except for extensive smooth ER. This is the area into which the pollen tube will discharge the male gametes at fertilization. Above this organelle-free zone, and surrounding the perinuclear zone was an area of cytoplasm containing small vacuoles and strands of cytoplasm. A similar cytoplasmic organisation was seen at the micropylar ends of egg cells in *Podocarpus ferrugineus* and *P. spicatus* (Sinnott, 1913), *P. andinus* (Looby and Doyle, 1944a) and although not commented on in the text, in the figures of *P. nivalis* drawn by Boyle and Doyle (1953). Camefort (1968) described this area as made up of two parts: an “included cytoplasm” contained within small inclusions and cup-shaped transformed maternal plastids, and a “fundamental cytoplasm” which forms a very fine web around these structures. Ultrastructural

examination of this area in the totara egg cell showed two types of inclusion: small inclusions clustered close to the perinuclear zone, and larger more darkly-staining inclusions towards the periphery of the cytoplasm. The small inclusions were similar to those observed in a number of other conifers, and consisted of small areas of cytoplasm which contained mitochondria, ER or lipid droplets enclosed in a bilayered membrane (Chesnoy, 1987b). Runions (1997) reported that small inclusions were visible in the cytoplasm of *Picea* egg cells because they did not contain any organelles. In contrast, the small inclusions of totara egg cells contained cytoplasm which appeared slightly more condensed than its surroundings, and often one or more of the organelles listed above.

The transformed maternal plastids seen at the periphery of totara egg cytoplasm have also been seen in light microscope studies of other podocarps, where they stain very darkly compared with the surrounding cytoplasm and vacuoles. Looby and Doyle (1944a) commented on the occurrence of densely-staining “protein vacuoles” of an irregular shape, and Konar and Oberoi (1969a) commented on darkly staining “rounded or irregular masses” in the vicinity of the egg nucleus. Transformed maternal plastids are considered to be cup-shaped plastids with an electron-transparent matrix and bound by a double membrane (Camefort, 1963; 1968; Singh, 1978; Chesnoy, 1987b). Plastids elongate, the lamellae disperse and invagination encloses areas of egg cell cytoplasm (Singh, 1978). The darkly-staining granules seen in totara egg cells are the remains of plastids which have been transformed in this way. No intact plastids with normally-appearing lamellae or starch grains were seen in the cytoplasm of the egg cell. The importance of this transformation during cytoplasmic inheritance is discussed in Chapter 7.

Some of the darkly-staining granules in this area did not look like cup-shaped maternal plastids, rather they appeared as densely-staining cytoplasm contained within U-shaped or circular cisternae. Between these cisternae were layers of circular structures lined up in rows. A similar structure was observed in the egg cell cytoplasm of *Sciadopitys* by Gianordoli (1969), who called them “annular lamellae”. The circular structures were interpreted as hexagonal pores arranged on the surface of the lamellae. Gianordoli (1969) commented that the only other reference to such structures has been for animal cells, and that they are the product of the fusion of vesicles produced by the ER. They do not persist in the egg cell of totara, and in later stages are associated with lipid droplets. The ends of the lamellae become distended and appear to pinch off vesicles containing dark granular material. It is suggested that they function as a part of the “maturation process of the egg” (Gianordoli, 1969), and it is likely that they function as complex dictyosomes in totara.

The dense area of egg cytoplasm contained at the chalazal tip of the totara egg cell lies above the vacuole which takes up most of the cell volume chalazal to the egg nucleus. Two types of darkly staining bodies occurred in this cytoplasm: small membrane-bound aggregations of dark granular material which may be protein bodies, and whorls of membranes surrounding dense osmophilic cytoplasm and lipid droplets. Such membrane whorls have not been mentioned, except by Willemse and Linskens (1969) who described the ER of *Pinus silvestris* male gametes to be in the form of “a watch-spring”. Similar structures called “myelin figures” are seen in the rat kidney cell, and are interpreted to be lysosomes full of partially broken down membranes (de Duve, 1965). de Duve (1965) comments that lysosomes in animal cells play vital roles in fertilization, often in the

breakdown of structures too chemically complex for dissolution directly in the cell cytoplasm. The breakdown of bilayered membranes such as ER is a complex process, the final products of which are lipid and protein. Some studies have assumed that membrane whorls within the cytoplasm of the egg cell are solely an artifact of fixation, however this study suggests that it is possible that they are ephemeral lysosomal bodies created for the purpose of membrane dissolution. The presence of lipid droplets interspersed among the membrane layers supports this. The presence of membrane-bound protein bodies is also a possible indication of lysosomal activity. By the time the free nuclei of the proembryo have migrated to the chalazal tip of the egg cell, there is no egg cytoplasm left in the area and the process of cytoplasmic degradation and breakdown appears to be complete at this time.

The detailed account of organelle distribution and anatomy in the totara egg cell is intended as a background for the description of fertilization and cytoplasmic inheritance in Chapter 7. Information identifying organelles from a maternal or paternal source are essential for an understanding of the mechanism of fertilization and inheritance of maternal and paternal cytoplasm into the zygote.

Chapter 7

Ultrastructure of fertilization and cytoplasmic inheritance in *Podocarpus totara*.

Introduction

Details of fertilization and cytoplasmic inheritance in most conifer families have been examined ultrastructurally (Mogensen, 1996). In the Pinaceae, Cupressaceae and Taxodiaceae, the parentage of organelles in the zygote has been further confirmed with restriction fragment length polymorphism (RFLP) analysis (Neale et al., 1986; Szmidt et al., 1987; Neale and Sederoff, 1989; Neale et al., 1991; Cato and Richardson, 1996; David and Keathley, 1996). According to Mogensen's (1996) review of cytoplasmic inheritance in both angiosperms and gymnosperms, the Podocarpaceae was the only conifer family in which the pattern of organelle inheritance was unknown. No ultrastructural or molecular evidence is yet available for a podocarp species, and Owens and Morris (1991) commented that there is not sufficient information available at this time to predict a mode of cytoplasmic inheritance.

Light microscope studies have observed events of fertilization in *Podocarpus andinus* (Looby and Doyle, 1944b), *P. nivalis* (Boyle and Doyle, 1953), and *P. gracilior* (Konar and Oberoi, 1969a). Details of these studies were extremely limited, but it was generally agreed that the entire contents of the pollen tube were expelled into the egg cell after which the large male nucleus and a body of male cytoplasm migrated towards the egg nucleus. After this point, observations were divided as to whether the male cytoplasm integrated into the neocytoplasm of the zygote (Looby and Doyle, 1944b) or whether it

remained at the micropylar end of the egg cell and was not involved in zygote development (Boyle and and Doyle, 1953). This study presents the first ultrastructural examination of fertilization in a podocarp, *Podocarpus totara*. Ultrastructural detail of organelle appearance, distribution and behaviour during fertilization are presented for maternal and paternal cytoplasm, as well as details of nuclear fusion. Incorporation of male and female cytoplasm in the zygote and ultimately the proembryo are presented here, and in Chapter 8 which covers events after nucleus fusion.

Observations and Results

After male gamete division and maturation of the egg cell, the pollen tube pushed against the megaspore wall, and broke through it to make contact with the neck cells of the archegonium. The neck cells had become extremely vacuolate by this time, and the nuclei disintegrated. Contact with the pollen tube destroyed central neck cells and/or megagametophyte cells, but peripheral neck cells often remained intact even after male gamete discharge into the egg cell (Fig. 76). The pollen tube occasionally avoided the neck cells altogether and entered the egg cell from the side, where the jacket cell layer ended and the egg cell was in direct contact with the megagametophyte. The archegonial chamber became filled with the degenerating remains of neck cells, pieces of pollen tube intine and darkly-staining tube cell cytoplasm.

After initial contact between the pollen tube and the egg cell wall (Fig. 76), invaginations created by projections of pollen tube distorted the micropylar tip of the egg cell (Figs. 77 and 78). The pollen tube projections contained darkly-staining tube cell cytoplasm, and the egg cell wall was noticeably thinner at the apex of the invaginations

(Fig. 78). The ventral canal nucleus was no longer visible at this stage. Both male gametes were released into the egg. The large male gamete entered the egg cell first, accompanied by the bulk of the male cytoplasm. The infrequency of obtaining specimens at this stage indicates that the process of male gamete migration through the egg cell was very rapid. A large vacuolate area appeared in the wake of male gamete movement through the micropylar region of the egg cell (Figs. 79 and 80). Egg cytoplasm did not appear to be expelled from the egg cell by pollen tube contact or by the force of male gamete entry, and that which remained at the micropylar end of the cell began to darken and degenerate. The smaller male gamete did not migrate close to the egg nucleus, but could be seen lying at the micropylar end of the egg cell until well after nuclear fusion (Fig. 80).

The nuclear envelopes of the male and female gametes fused at several points, creating islands of intermingled male and female cytoplasm between areas in which the nucleoplasm was continuous (Fig. 81). A depression in the egg nucleus formed to accommodate the large male gamete as the fusion plate developed (Figs. 79,80 and 81). The accompanying male cytoplasm was distinguishable from the perinuclear zone of the egg nucleus by its denser consistency and more granular appearance in light microscope sections (Fig. 79), and by differences in organelle content described below. The perinuclear zone of the egg cell appeared to be structured with a "halo" of microtubules connected to the nuclear membrane of the egg nucleus (Figs. 79 and 80). The male nucleus (23 μm diameter) was approximately one quarter the volume of the egg nucleus (36 μm diameter), and had a more densely granular appearance (Fig. 79).

Electron micrographs of fertilization confirmed a difference in granularity and size of the male and egg nuclei (Fig. 82). The male cytoplasm accompanying the large male nucleus was packed with mitochondria and plastids containing starch granules (Figs. 82, 83 and 84), and was clustered close to the nucleus. Dense accumulations of organelles in small areas stained darkly and appeared as dark spots (Figs. 79 and 84). The perinuclear cytoplasm surrounding the egg nucleus contained mitochondria and a series of large lipid droplets (Figs. 82 and 85). The egg nucleus appeared slightly less dense than the male, and contained darkly-staining condensed areas of chromatin (Fig. 82).

The vacuolate area in the egg cytoplasm left by the passage of the male gamete contained plastids approximately three times the size of those found in the male cytoplasm (Fig. 82). Several small nuclei were visible in this area, some of which had dense nucleoli similar to those of pollen prothallial nuclei (Fig. 82). Also in this micropylar area were dense clusters of lipid droplets (Fig. 82). The small male gamete remained at the micropylar end of the large vacuolate region throughout fertilization (Figs. 82 and 86).

Once fusion of the nuclei had begun, the small inclusions in egg cytoplasm areas surrounding the perinuclear zone became rounded and their membrane layers separated (Fig. 87). The number of lipid droplets in the egg cell cytoplasm increased extremely quickly (Fig. 87). The male cytoplasm from the body cell settled around the fusion nucleus, and appeared to enclose much of the circumference (Figs. 79 and 88). The physical mechanism by which the male cytoplasm displaced the perinuclear zone was not observed. The male cytoplasm was still distinguishable from the egg cytoplasm by its comparative density and darkly-staining groups of organelles (Fig. 88). The majority of

the neocytoplasm was, therefore, made up of body cell cytoplasm accompanying the large male nucleus, and the remainder from the perinuclear zone of the female nucleus. Details of neocytoplasm ultrastructure and zygote division are presented in Chapter 8.

Variable numbers of archegonia were fertilized in a megagametophyte depending on how many pollen tubes penetrated the nucellus, but one was usually well ahead in development by the time other pollen tubes had made contact with surrounding archegonia. There did not appear to be a pattern to which archegonium was fertilized first. The branching of one pollen tube excluded all others until it began to degenerate after fertilization. Typically two to three archegonia were fertilized, and remaining unfertilized archegonia began to degenerate. Details of this degeneration are described in Chapter 8, as the process did not usually begin until fertilized archegonia had reached proembryo stages.

The cell wall at the chalazal tip of the egg cell increased in thickness and developed a fibrous matrix similar in appearance to the tip of the pollen tube during early germination (Fig. 89). Egg cytoplasm near the thickened cell wall contained large numbers of mitochondria, ribosome clusters and ER (Fig. 89).

Figures 76-81

- Fig. 76 Pollen tube (PT) in contact with the egg cell (EC). The pollen tube has destroyed most of the neck cells (arrows), but peripheral neck cells (Ne) remain intact. Megagametophyte, (M). TEM. Bar = 20 μ m.
- Fig. 77 Pollen tube contact with the egg cell has caused invagination (arrowheads) of the cell wall. Note the darkly-staining tube cell cytoplasm (*) near an intact neck cell. TEM. Bar = 20 μ m.
- Fig. 78 Magnification of invaginations in the egg cell wall (arrowhead) caused by contact of the pollen tube. Membranes around the degenerating tube cell cytoplasm (*) organelles are no longer intact. TEM. Bar = 6 μ m.
- Figs. 79-80 Two sections from the same egg cell during fertilization.
- Fig. 79 Fusion of the male nucleus (Mf) and egg nucleus (EN). The egg nucleus is surrounded by microtubules radiating from the nuclear membrane (arrowheads). The denser male cytoplasm (Mp) which has accompanied the male nucleus is clustered close to the fusing nuclei. The interface between male cytoplasm and egg perinuclear zone is denoted by the dashed line. LM. Bar = 40 μ m.
- Fig. 80 The next section from this egg cell shows the smaller male nucleus (Mn) trailing behind the larger male nucleus and male cytoplasm fusing with the egg nucleus. The vacuolate area left in the wake of male gamete entry and migration to the egg nucleus lies at the micropylar end of the egg cell (*). LM. Bar = 40 μ m.

Fig. 81 Fusion plate between the egg nucleus and male nucleus. Bridges of nucleoplasm between the fusing nuclei (arrowheads) created islands of intermingled paternal (Me) and maternal (Mm) mitochondria and other organelles. (TEM). Bar = 2 μ m.

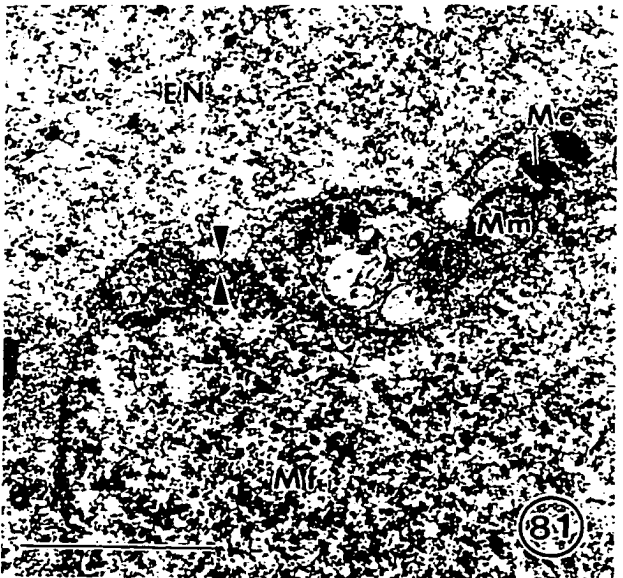
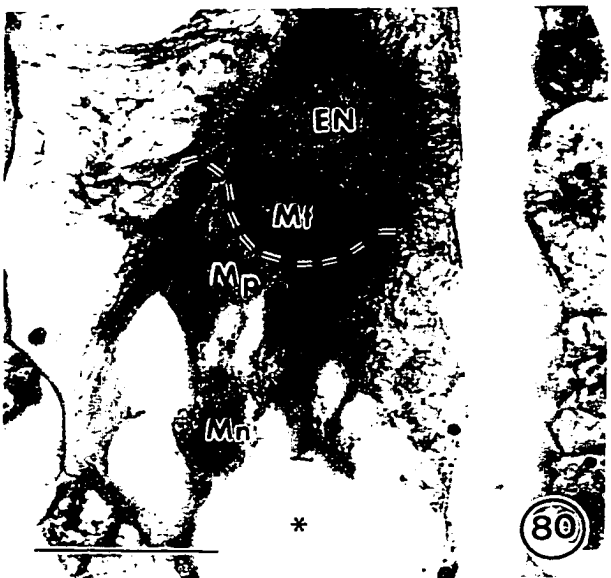
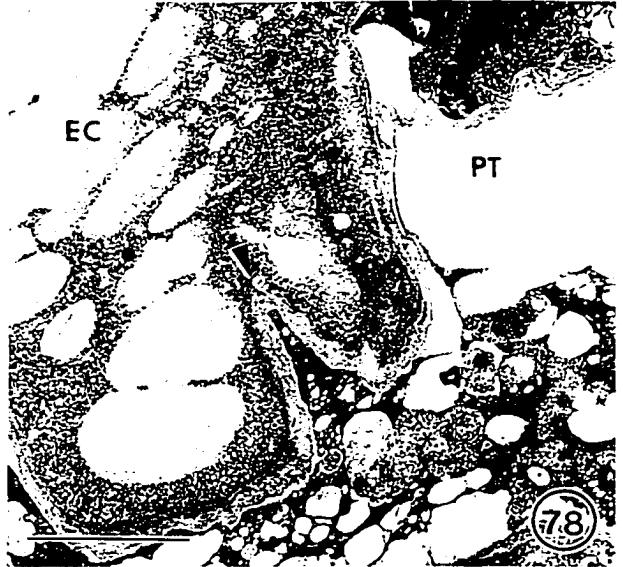
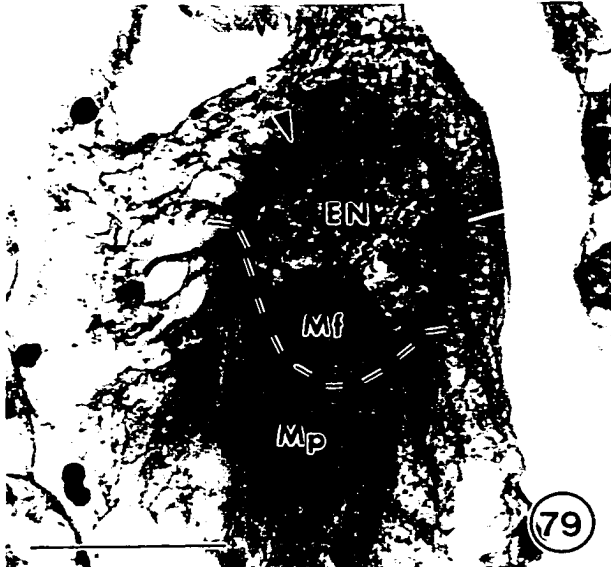
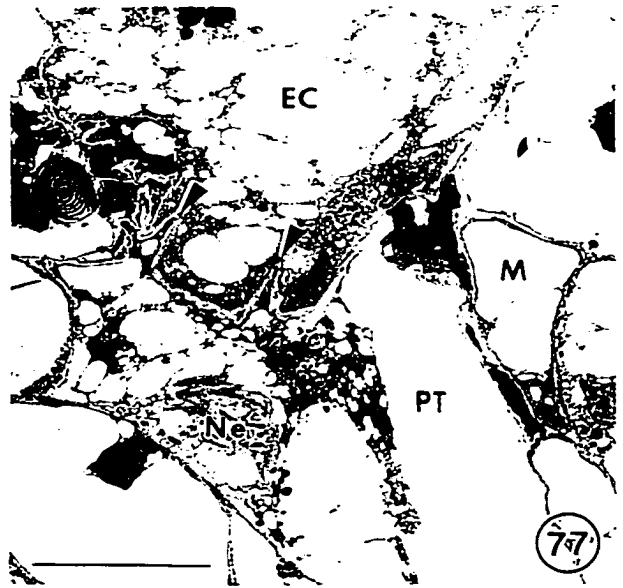
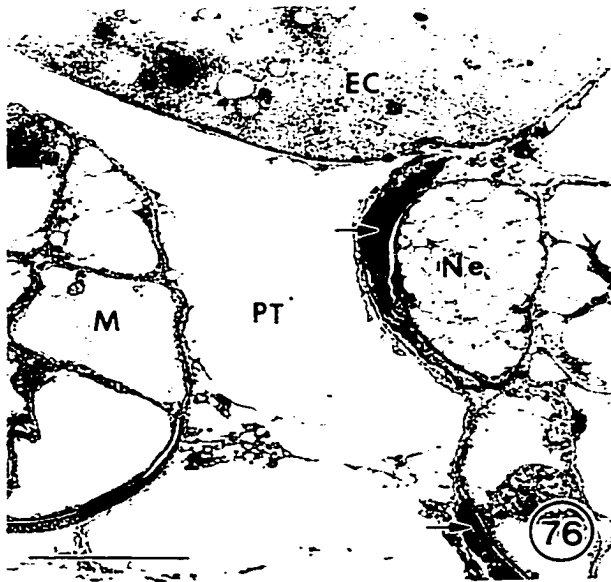


Figure 82

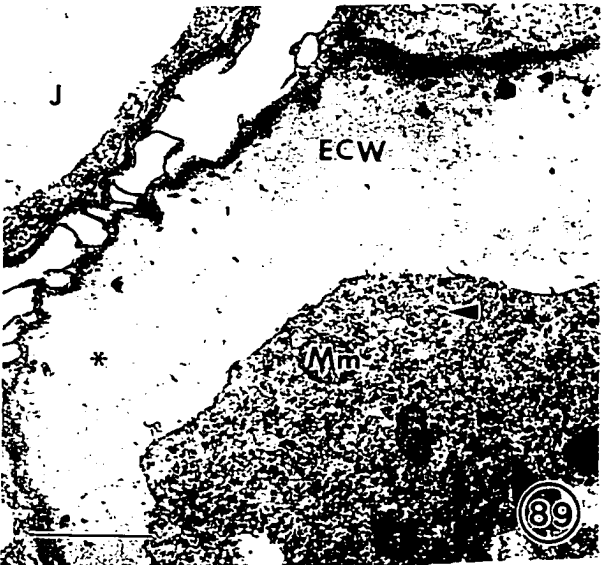
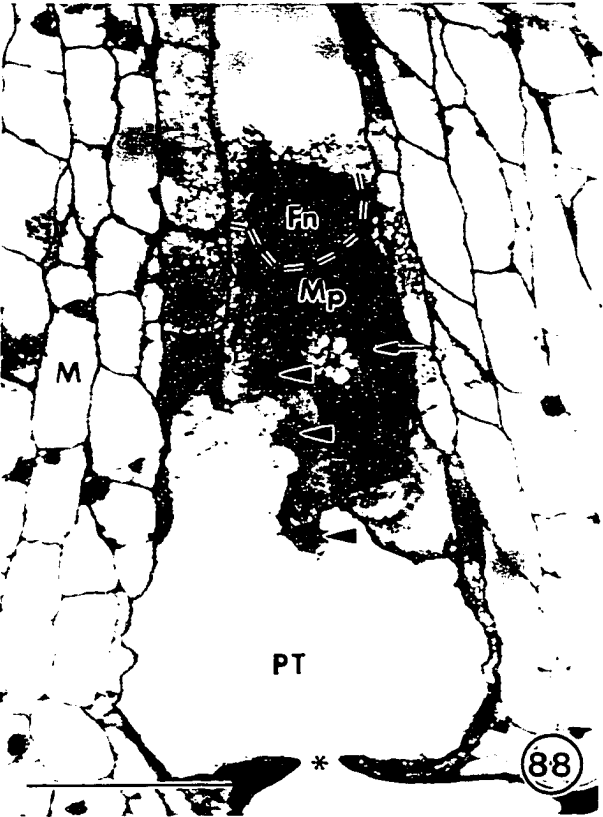
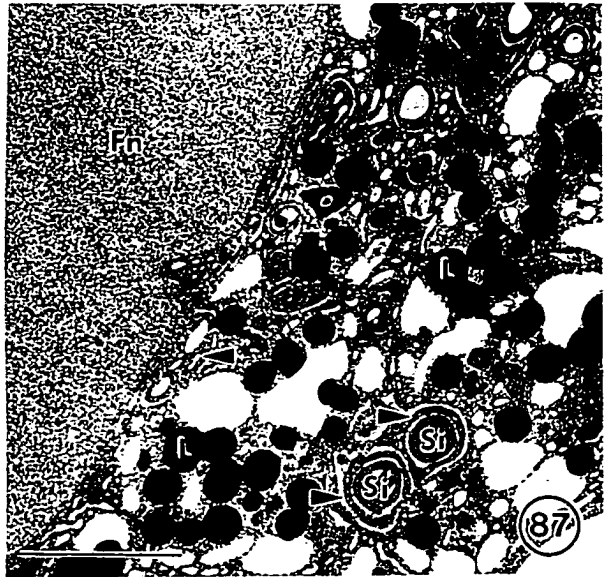
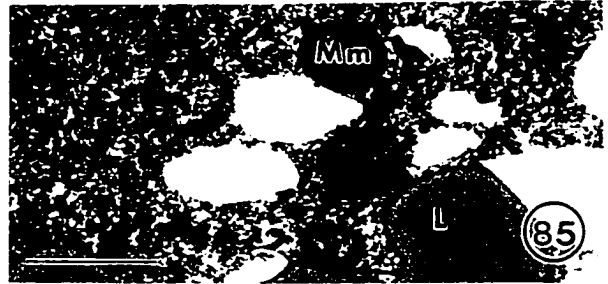
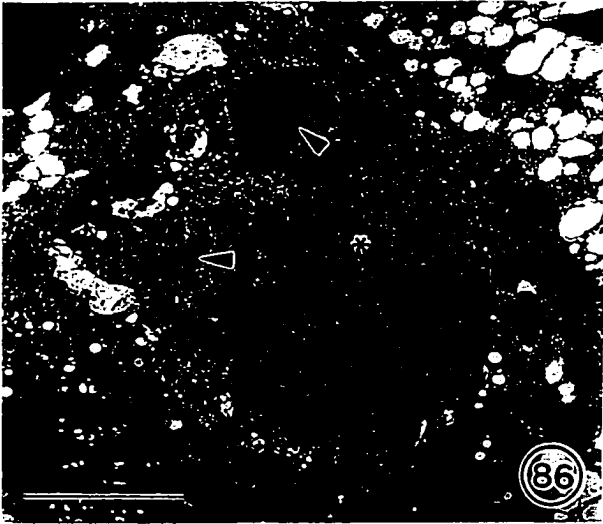
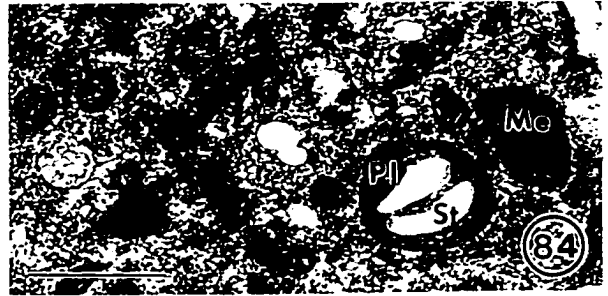
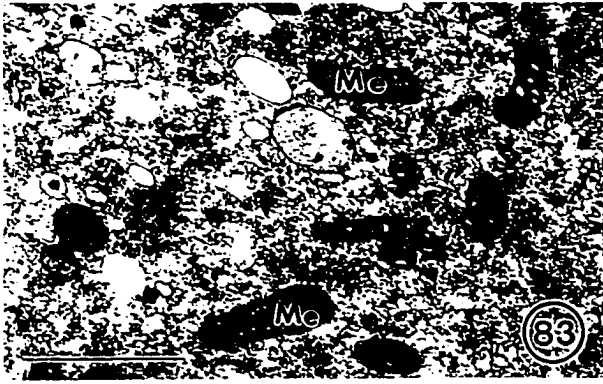
Fig. 82 Fertilization of the egg cell. A fusion plate (*) has formed between the large male nucleus (Mf) and egg nucleus (EN). Maternal mitochondria (Mm) are clustered close to the egg nucleus in the perinuclear zone. The male cytoplasm from the body cell (arrowheads) is filled with dense clusters of plastids (Pl) and mitochondria (Me) and follows closely behind the male nucleus. At the micropylar end of the egg cell lies a vacuolate area created by passage of the male nuclei and body cell cytoplasm (arrows) in which large plastids with starch grains and other organelles from the male cytoplasm are retained. The small non-functional male nucleus (Mn) lies at the micropylar end of this vacuolate area. To one side lies a nucleus (P) similar in structure to a prothallial nucleus from the pollen tube. Jacket cell (J). TEM. Bar = 8 μ m.



Figures 83-89

- Fig. 83 Male cytoplasm in the egg cell is filled with elongate mitochondria (Me) with a darkly-staining matrix. TEM. Bar = 1 μ m.
- Fig. 84 Male cytoplasm also contained large plastids (Pl) with starch grains (St). TEM. Bar = 1 μ m.
- Fig. 85 The perinuclear zone of the egg nucleus contained ovoid mitochondria (Mm) in which cristae were not observed. Lipid droplets (L) also accumulated in this area. TEM. Bar = 1 μ m.
- Fig. 86 The non-functional male nucleus (*) remained in an area of male cytoplasm at the micropylar end of the egg cell. It was recognisable by the darkly-staining structures accumulated near the nuclear membrane (arrowheads). The nuclear membrane does not appear to be continuous. TEM. Bar = 6 μ m.
- Fig. 87 During formation of the fusion nucleus (Fn), the membranes of cup-shaped plastids and small inclusions (Si) dilate and separate (arrowheads). Osmophilic lipid droplets accumulate in large numbers at this time. TEM. Bar = 6 μ m.
- Fig. 88 The male cytoplasm from the body cell (Mp) settles around the fusion nucleus. A number of small nuclei (arrowheads) with prominent nucleoli lie at the micropylar end of the egg cell. Dark spots (arrow) in the male cytoplasm are clusters of mitochondria and plastids. Entry of the pollen tube (PT) to this egg cell has destroyed all neck cells (*) and some of the jacket layer. LM. Bar = 60 μ m.

Fig. 89 The egg cell wall (ECW) at the chalazal end of the egg cell increased in thickness as the male and egg nuclei fused. A thick, fibrous matrix (*) formed and resembled the tip of the growing pollen tube. Egg cytoplasm in this area contained maternal mitochondria and clusters of ribosomes (arrowhead). Jacket cell, (J). TEM. Bar = 1 μ m.



Discussion

Maturation of male and female gametes in totara was complete in the first half of December, ca. two months after pollination. Fertilization of most ovules on a tree was synchronous, and the entire process took place within one week. The high lipid content and vacuolate nature of the egg cytoplasm made fixation and sectioning of material, and localization of this stage extremely difficult. The best results were obtained in ovules where the previous fertilization and proembryo development of one archegonium “stabilized” the tissue enough for the later fertilization of surrounding archegonia to fix adequately. The micropylar end of archegonia at the proembryo stage thickened and formed a “plug” of tissue which prevented the separation of the megagametophyte and megaspore wall, and preserved the integrity of archegonia at earlier stages.

The pollen tube of totara did not always break through the megaspore membrane and enter the egg cell through the neck cells. In 50% of fertilized archegonia observed, all or some of the neck cells were left undamaged and intact after pollen tube entry. Pollen tubes often entered through areas at the micropylar end of the egg cell, where there were no jacket cells and the egg was in direct contact with megagametophyte cells. Similar pollen tube behaviour occurred in *Podocarpus gracilior* (Konar and Oberoi, 1969a), where lateral entry of the pollen tube left the neck cells intact, but slightly displaced and exhibiting a degenerated appearance. In *P. nivalis* (Boyle and Doyle, 1953), pollen tubes also penetrated the egg cell wall to one side. Most studies did not comment extensively on pollen tube entry to the egg cell, but noted that a branch of the pollen tube entered and broke through the neck cells of the archegonium (Stiles, 1912; Sinnott, 1913; Quinn,

1965; 1966a; 1966b). Boyle and Doyle (1953) make the point that these stages may not have been available in material studied (as appears to be the case for most fertilization stages) and that remarks were made “on the general grounds that entry of a pollen tube...is to be expected through the neck cells”. In most conifer species, this appears to be the case (Singh, 1978). Runions (1997) found that pollen tube entry through the side of an archegonium was abnormal in *Picea*, and was attributable to the misplacement of the ventral canal cell. In *totara*, the ventral canal nucleus was not visible after pollen tube contact with the egg cell wall, and was not noted to have a direct influence over pollen tube growth.

In *totara*, a peg-like extension of the pollen tube made contact with the egg cell wall after the megaspore membrane had been broken. Subsequent invagination of the egg cell wall appeared to facilitate pollen tube entry into the egg cell. Looby and Doyle (1944b) reported a surge of egg cell cytoplasm into the pollen tube after the egg cell wall had been breached, but this was not seen in *totara* or in *Podocarpus gracilior* (Konar and Oberoi, 1969a). The egg cell is turgid and the egg cytoplasm dilute and rich in lipid at this time. It seems more likely that egg cytoplasm escape into the pollen tube is an artifact of poor fixation. Formation of a receptive vacuole in the egg cell prior to male gamete entry has been described in a number of conifer species e.g. *Pseudotsuga* (Owens and Morris, 1991), but such a vacuole did not form in *totara*. In *totara*, passage of the male gametes through the egg cell wall and micropylar cytoplasm left a vacuolate area containing tube cell cytoplasm, supernumerary nuclei and male organelles.

Both male nuclei and a large amount of male cytoplasm entered the egg, but only the bulk of the cytoplasm and the large male nucleus migrated towards the egg nucleus. The

large male gamete was about one quarter the size of the egg nucleus. A similar difference in nucleus size was noted in *P. nivalis* (Boyle and Doyle, 1953) and *Podocarpus* species examined by Sinnott (1913). A much larger inequality in relative gamete size exists for *P. andinus* (Looby and Doyle, 1944) and three *Dacrydium* species (Quinn, 1965; 1966a; 1966b), where the egg nucleus appears to be 10-12 times the size of the male nucleus. Boyle and Doyle (1953) considered it possible that relative gamete size was a trait related to evolutionary position within the Podocarpaceae, with a large inequality being a primitive condition. It is possible that this is the case, however information about fertilization in podocarp species is extremely rare, and usually incomplete. Working from drawings in early studies is difficult, due to the fact that nuclear appearance changes quickly during and after fertilization, and comparisons are therefore of doubtful quality. Inequality of male and female gamete size is common to all members of the Podocarpaceae studied so far, but the degree of this inequality depends on species.

The male cytoplasm stayed in close contact with the large male nucleus throughout pollen tube entry to the egg cell, and during migration and fusion with the egg nucleus. Male cytoplasm was distinguished from surrounding egg cell cytoplasm by its relatively dense consistency, and darkly-staining areas of clustered mitochondria and plastids. The morphology of these organelles, as discussed in Chapter 5, was somewhat modified from plastids and mitochondria of the body cell prior to division. The mitochondria were elongated, with many cristae and a darkly-staining matrix. They retained this morphology during fertilization, and so could be distinguished from the maternal ovoid mitochondria, which had a lightly-staining matrix and very few cristae. The paternal mitochondria and plastids of *Larix decidua* (Camefort, 1968) and *Pseudotsuga menziesii* (Owens and

Morris, 1991) showed similar modifications. As the egg cell matured, the maternal mitochondria swelled and developed an undulating outer membrane. Cristae were infrequent and difficult to see. Paternal plastids had a darkly-staining matrix, and usually contained large starch granules. Maternal plastids were transformed into a cup-shaped structure, as described in Chapter 6, and did not contain starch granules. Further modification of maternal plastids included the dilation and lysing of most of these close to the egg nucleus as the nuclei fused. The membranes around small inclusions also separated and lysed, and the contents were released into the egg cytoplasm.

It was therefore possible to distinguish paternal from maternal organelles during fertilization in totara. The paternal cytoplasm settled around the fusion nucleus, and enclosed most of its circumference. Organelles in this area included paternal plastids, and a mixture of maternal and paternal mitochondria. In Chapter 8, it will be shown that this pattern of organelle inheritance can be recognised in proembryo stages.

Fertilization and cytoplasmic inheritance mechanisms vary among conifer families. The content of the neocytoplasm (the cytoplasm surrounding the fusion nucleus formed from contributions of maternal and paternal organelles (Camefort, 1966)) differs between conifer families depending on factors outlined in Chapter 2. There appear to be two main patterns of cytoplasmic inheritance within the Coniferales: paternal plastid and mitochondrial inheritance as found in the Cupressaceae, Taxodiaceae and Araucariaceae, or paternal chloroplast and biparental mitochondrial inheritance as found in the Pinaceae, Taxaceae, Cephalotaxaceae and now in the Podocarpaceae.

In the Cupressaceae, Taxodiaceae and Araucariaceae, plastids within the egg cytoplasm do not change in morphology (Chesnoy 1973; 1975; 1987; Owens et al., 1995b). In the

Cupressaceae and Taxodiaceae, the egg nucleus is not surrounded by a perinuclear zone; organelles are distributed throughout the egg cytoplasm (Chesnoy, 1987b). In *Agathis* (Araucariaceae), the egg nucleus is surrounded by a perinuclear zone containing abundant mitochondria (Owens et al., 1995b). In these three families, paternal cytoplasm accompanying one male gamete settles around the fusing nuclei and effectively excludes maternal cytoplasm from the neocytoplasm of the zygote. RFLP studies of *Calocedrus decurrens* (Neale et al., 1991) and *Sequoia sempervirens* (Neale et al., 1989) have confirmed that plastid and mitochondrial inheritance is strictly paternal. No RFLP studies have yet been carried out on a species of the Araucariaceae.

The ultrastructural observations of this study suggest that the Podocarpaceae has a cytoplasmic inheritance mechanism most similar to that of the Pinaceae, Taxaceae and Cephalotaxaceae (Gianordoli, 1974; Owens and Morris, 1991; Dawkins and Owens, 1993; Chesnoy, 1987b; Pennell and Bell, 1988). In the Pinaceae, male gametes are nuclei and enter the egg cell with closely associated clusters of male organelles. As the leading male gamete fuses with the egg nucleus, the male cytoplasm blends with the perinuclear zone around the egg cell (Camefort, 1968; Bruns and Owens, 1989; Owens and Morris, 1991; Dawkins and Owens, 1993) and therefore maternal and paternal cytoplasm make up the neocytoplasm of the zygote. Transformation of egg cell plastids to a cup-shaped structure, and the exclusion of these structures from the neocytoplasm results in paternal inheritance of plastids. Mitochondria were initially thought to be exclusively from the maternal source, but in-depth ultrastructural analysis, and the use of RFLP techniques to track maternal and paternal DNA complements has shown that about 10% of mitochondria in the neocytoplasm and proembryo are of paternal origin (Neale et al.,

1986; Chesnoy, 1987b; Szmidt et al., 1987; Neale and Sederoff, 1989; Owens and Morris, 1991; Wagner et al., 1991; David and Keathley, 1996).

In the Taxaceae, RFLP analysis has not yet been carried out, but ultrastructural studies show that the male gametes are nuclei, and enter the egg cell with closely associated male cytoplasm. The egg cell has a defined perinuclear zone around the nucleus, and the morphology of the plastids has changed to a cup-shaped structure (Pennell and Bell, 1988; Anderson and Owens, 1999). The male cytoplasm and perinuclear zone combine to form neocytoplasm with biparentally-inherited mitochondria and paternally-inherited plastids (Pennell and Bell, 1988; Anderson and Owens, 1999). The maternal and paternal mitochondria of *Taxus* have a similar morphology, which made it impossible to track the contribution of either parent in the neocytoplasm and proembryo (Anderson and Owens, 1999).

There is contradictory information about cytoplasmic inheritance in the Cephalotaxaceae, but evidence so far suggests that the egg nucleus lacks a perinuclear zone and plastids are transformed in structure (Singh, 1961; Gianordoli, 1974). Paternal cytoplasm associated with the male gametes (variously reported as cells or nuclei) settles around the fusing nuclei, and the resulting neocytoplasm is thought to contain paternal plastids and mitochondria, and maternal mitochondria (Singh, 1961).

In comparison, *Podocarpus* male gametes are nuclei, as they are in the Pinaceae and Taxaceae (and perhaps the Cephalotaxaceae). The main difference at this point is the inequality in size and inequality of distribution of body cell cytoplasm around the two nuclei. In the Pinaceae, there have been reports of unequal male gametes, but it is agreed that the nuclei are only slightly dissimilar if at all (Singh, 1978; Chesnoy, 1987a). Both

male nuclei enter the egg cell in these four conifer families, and the leading male gamete fuses with the egg nucleus. In totara, the leading gamete is always the larger of the two. The egg cell exhibits a defined perinuclear zone around the egg nucleus, and the plastids have been transformed into cup-shaped structures. Combination of the male cytoplasm clustered around the large male gamete and the perinuclear zone of the egg nucleus provide the neocyttoplasm for the zygote. In totara, it appears that plastid inheritance is solely paternal, on the basis that lysis of membranes around cup-shaped plastids happens soon after nuclear fusion, and they are subsequently absorbed into the egg cytoplasm. Mitochondria in totara appear to be biparentally inherited, and the morphological differences between maternal and paternal mitochondria have made it possible to distinguish between them in proembryo stages. This will be examined more fully in Chapter 8. Exact relative mitochondrial contribution from paternal and maternal parents will require RFLP analysis.

Chapter 8

Proembryogeny and early embryogeny in *Podocarpus totara*.

Introduction

The majority of studies describing the reproductive biology of members in the Podocarpaceae have concentrated on their embryogeny. In 1902, Coker gave the first account of embryo development in *Podocarpus macrophyllus*, and this was followed by observations of *P. nivalis* and *P. totara* (Sinnott, 1913), *P. spicatus* and *P. ferrugineus* (Buchholz, 1936), *P. andinus* (Looby and Doyle, 1944b), *P. hallii* and *P. totara* (Brownlie, 1953), *P. nivalis* (Boyle and Doyle, 1954) and *P. gracilior* (Konar and Oberoi, 1969a). Embryogeny in other podocarp genera has been documented for *Dacrydium sp.* (Buchholz, 1941; Quinn, 1965; 1966a; 1966b), *Phyllocladus* (Buchholz, 1941) and *Pherosphaera* (= *Microstrobos*) (Elliot, 1950). These studies provide detailed and comprehensive observations of early through late embryo stages, but information about proembryo stages is incomplete or missing entirely from many studies. Successfully fixing and sectioning the large, vacuolate and highly lipidic egg cells of podocarp species proved difficult in this study, and was doubtless an even more serious problem in earlier studies in which fixatives and embedding agents produced many tissue artifacts. The purpose of this study is to clarify the processes of proembryo and early embryo formation, and to provide ultrastructural information about the composition of proembryo cytoplasm and organelle distribution, and the nature of the embryonal tier cell in *Podocarpus totara*.

Coker (1902) was the first to identify the binucleate nature of embryonal tier cells in *P. macrophyllus*. This has since been observed in all other podocarp species studied, and is acknowledged as a characteristic feature of the family (Singh, 1978). Other features of proembryo and embryo development appear to be highly variable within the family, and in particular in the genus *Podocarpus* (Konar and Oberoi, 1969b). The fusion nucleus has been described as dividing once (Sinnott, 1913; Looby and Doyle, 1944b; Brownlie, 1953; Boyle and Doyle, 1954; Konar and Oberoi, 1969a), twice (Tahara, 1941; Osborn, 1960) or not at all (Stiles, 1912) before migrating to the chalazal end of the egg cell. The number of nuclei present in the proembryo prior to wall formation is variously reported as 32 (Buchholz, 1941; Looby and Doyle, 1944b; Quinn, 1965; Konar and Oberoi, 1969a) or 16 (Buchholz, 1941; Brownlie, 1953; Boyle and Doyle, 1954). The number of nuclei included in the proembryo after wall formation appears to be a function of how many nuclei are excluded as “relicts” during formation of the primary embryonal and primary upper tiers (Buchholz, 1941).

The binucleate embryonal tier cell has only been portrayed in diagrams, and very little is known about its function or anatomy. Boyle and Doyle (1954) described a “resting phase” of the embryonal tier cell(s) during suspensor cell elongation, after which a retraction of the cytoplasm and development of a “membrane” at the chalazal tip of the cell(s) produced a thickened “cap” region. Boyle and Doyle (1954) suggested that this had a protective function, but were not able to confirm its structure using light microscopy. This study examines the ultrastructure of the “cap” region in the early embryo, to ascertain whether its function is similar to cap cells in the embryos of other conifer species.

Observations and Results

Proembryo and early embryo development occurred in the second half of December, and continued into January. By the beginning of March, the embryos were almost mature, and seed dispersal began in April.

The fusion nucleus, formed after fertilization of the egg nucleus by the large male nucleus, was positioned towards the micropylar end of the egg cell. The first division of the fusion nucleus occurred at this position (Fig. 90), and the two free nuclei subsequently migrated to the chalazal end of the archegonium. The infrequency with which this stage was seen in specimens suggests that this division occurred within 24 hours of fertilization. The two free nuclei were ca. 33 μ m in diameter with lightly-staining fine granular contents (Fig. 91), and a dispersed nucleolus. The nuclear membrane surrounding each nucleus was continuous and punctuated by numerous pores (Fig. 91). A small nucleus situated just below the two free nuclei appeared to be one of the supernumerary nuclei in the process of disintegration, as the nuclear membrane was incomplete, and the granular nucleoplasm unevenly distributed and concentrated near the membrane (Figs. 90 and 92). Organelles in the cytoplasm surrounding the two free nuclei included cup-shaped maternal plastids, maternal and paternal mitochondria, lipid droplets and darkly-staining granular masses not bound by a membrane (Fig. 93). Mitochondria and plastids were concentrated in the cytoplasm lying between the free nuclei and the chalazal vacuole (Fig. 90).

As the first division of the fusion nucleus occurred, megagametophyte cells near the chalazal tip of the archegonium filled with starch grains, and the nuclei became large and prominent (Fig. 94). Megagametophyte cells frequently became multi-nucleate (see

Chapter 6), and the nuclei contained darkly-staining granular nucleoli and condensed areas of chromatin (Fig. 95). The cells contained abundant mitochondria (Fig. 95). The plastids containing the starch grains increased in size until they consisted of only a thin layer of stroma appressed to the membrane, surrounding starch granules ranging from 1.5 μm to 4.5 μm diameter (Fig. 95). This area of starch-rich megagametophyte tissue was continuous from the tip of the archegonium to the centre of the megagametophyte.

Surrounding megagametophyte cells were uninucleate, with only a thin, parietal layer of cytoplasm containing plastids with comparatively small starch granules (Fig. 96).

Once the two free nuclei had migrated to the chalazal end of the egg cell, the nuclear membrane became indistinct and disintegrated at the onset of metaphase (Figs. 97 and 98). Chromatin condensed into separate darkly-staining chromosomes which were scattered throughout the nucleoplasm (Fig. 97). The neocytoplasm of the proembryo was electron-dense, and contained mitochondria and lipid droplets distributed evenly throughout (Fig. 97). Plastids containing starch granules were concentrated at the chalazal tip of the neocytoplasm (Fig. 97). The egg cell wall thickened rapidly during this time, until at the four free-nuclear-stage it was ca. 10 μm thick. After the second division, the resulting four free nuclei were ovoid with a distinct nuclear membrane. Plastids and mitochondria were distributed evenly around them (Fig. 99). The nucleoplasm was mottled with condensed chromatin and a darkly-staining nucleolus (Figs. 99).

Two further mitotic divisions occurred rapidly, resulting in a 16-free-nuclear proembryo (Figs. 100 and 101). It appears that all free nuclear divisions occurred within three to four days as proembryos were rarely found at free nuclear stages. The nuclei became arranged into two tiers: one ovoid nucleus in a pyramid-shaped area at the chalazal tip of the

proembryo (the primary embryonal tier), and seven to nine slightly elongated nuclei in a tier directly beneath the tip area (the primary upper tier). Between five and seven relict nuclei lay in an area of sparse cytoplasm at the base of the proembryo (Figs. 101 and 102). These relict nuclei often completely detached from the cytoplasm and lay free in the egg cell. The remainder of the egg cell was empty of cytoplasm, except at the micropylar tip where the supernumerary nuclei persisted for some time (Fig. 101).

Wall formation was first visible as collections of cell wall components arranged between the nuclei of the primary embryonal and upper tiers (Fig. 100). The discrete bundles of wall material soon formed continuous cell walls, first in the most peripheral cells and then in more central ones. Wall formation around the nuclei in the primary upper tier created elongated cells which were slightly narrower at the chalazal end (Fig. 102). Plastids stained very darkly, and were concentrated at the chalazal tip of these cells (Fig. 102). Relict nuclei which floated free in the egg cell away from the proembryo cytoplasm did not develop a cell wall. Division of the primary upper tier cells yielded two tiers of uninucleate cells (the prosuspensor and open tiers). Cell walls thickened rapidly after the final internal division, and partial walls formed between nuclei in the open tier (Fig. 102). In the pyramid-shaped embryonal cell at the tip of the proembryo, no cell wall formed after division, and this cell became binucleate.

As the binucleate embryonal cell developed, open tier or "relict" nuclei were further excluded from the proembryo cytoplasm (Fig. 103), along with clusters of cup-shaped maternal plastids (Fig. 104). Maternal plastids were not seen in the embryonal cell or suspensor tier cells. Relict nuclei were ovoid, and were similar to the small male nucleus

in appearance, as they often had darkly-staining areas clustered close to the nuclear membrane (Fig. 103).

The mature proembryo filled the top quarter of the egg cell (Fig. 101). The pyramid-shaped embryonal cell (at the chalazal tip of the proembryo) began to elongate, and the two nuclei were located at the base of the cell (Fig. 106). The embryonal cell wall in contact with the egg cell wall thickened, and electron-transparent vesicles were produced by Golgi bodies clustered on the inside surface (Figs. 106 and 107). The cytoplasm of the embryonal cell stained darkly compared to that of the suspensor tier cells beneath in light microscope preparations (Fig. 105), and contained abundant plastids, mitochondria, ER and clusters of ribosomes (Fig. 106). At this stage it became difficult to distinguish paternal from maternal mitochondria, although paternal mitochondria sometimes retained a slightly vermiform shape and stained more darkly.

The nucleus of suspensor tier cells stained more darkly than the two embryonal cell nuclei, and had a prominent nucleolus (Fig. 108). Suspensor cell nuclei migrated to the base of the cell, and mitochondria accumulated close to the nuclear membrane (Fig. 108). The suspensor cells began to elongate ca. two weeks after wall formation in the proembryo (Fig. 109). Suspensor cell walls stained very darkly, and increased in thickness towards the base of the proembryo (Fig. 109). A “plug” of extremely darkly-staining wall material and cytoplasm was excluded from the proembryo and remained in the egg cell as the suspensors elongated (Fig. 109). The proembryo burst through the egg cell wall and jacket cell layer, and grew rapidly through the starch-filled megagametophyte cells. The corrosion cavity around the embryonal cell was very small, and confined to an area ca. 8 μ m thick towards the tip of the cell (Figs. 109 and 110). The

cytoplasm of megagametophyte cells in contact with the embryonal cell tip darkened, and the cell wall collapsed (Fig. 109).

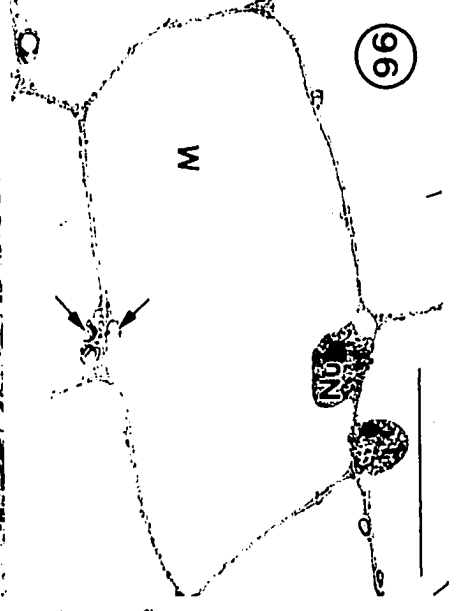
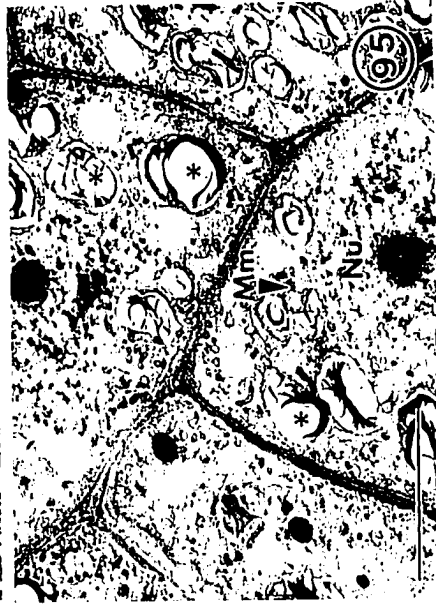
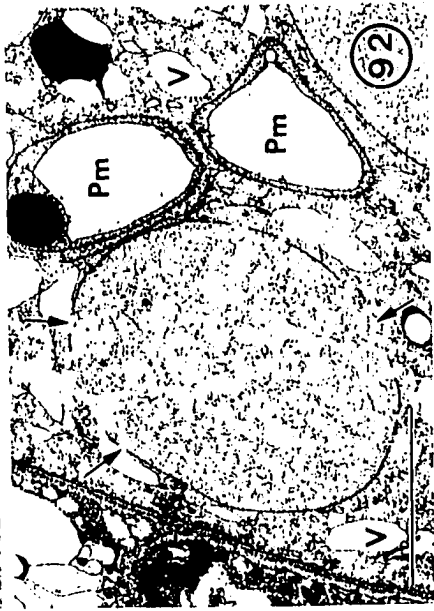
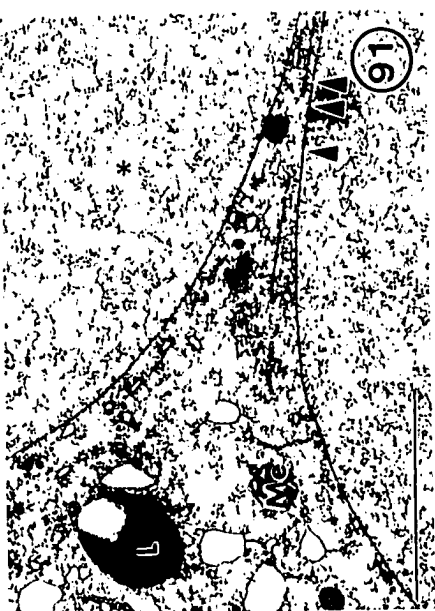
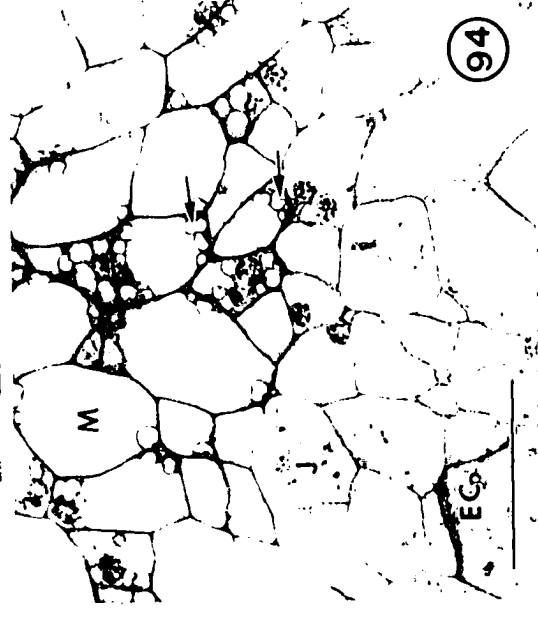
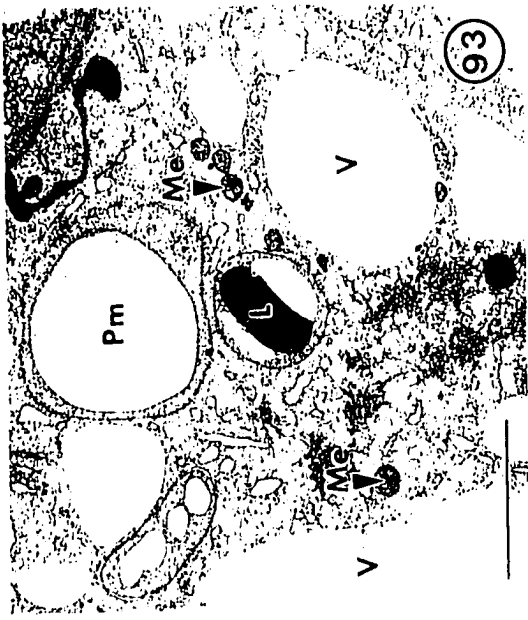
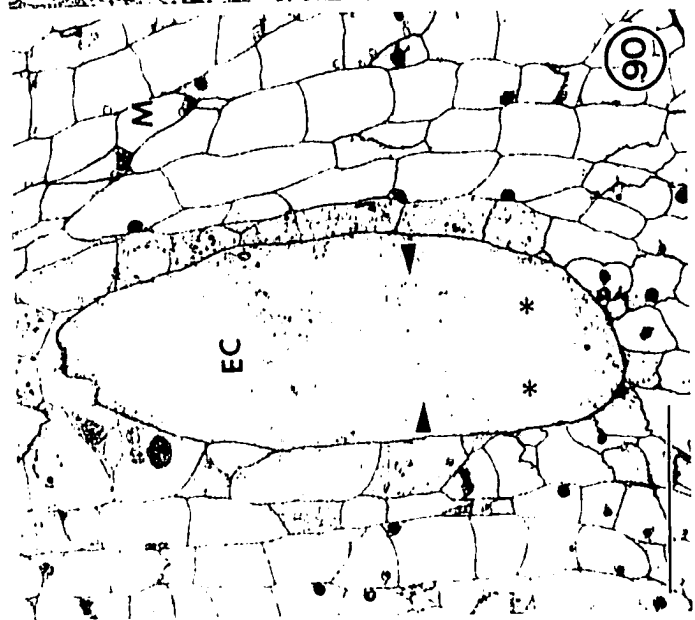
Once suspensor growth had begun from one archegonium, nearby unfertilized egg cells degenerated. This appeared to happen earliest in unfertilized archegonia which shared a jacket layer with a fertilized egg cell (Fig. 109). The cytoplasm darkened and plasmolysed, and the egg nucleus became unevenly granular and decreased in size (Fig. 111). Egg cytoplasm was filled with degenerating membranes, tiny vacuoles and lipid droplets (Fig. 111). Organelles were no longer distinguishable. The jacket cell layer collapsed and the contents of jacket cells were released into the egg cell cavity (Fig. 109).

The tip of the embryonal cell wall increased in thickness as the embryo penetrated through the megagametophyte. In light microscope sections, it appeared as a distinct, darkly-staining region (Fig. 110). Ultrastructurally, it was made up of a thickened, fibrous membrane layer, with a network of large and small electron-transparent vesicles appressed to the inner surface (Fig. 112). The vesicles were separated by thin strands of cytoplasm which contained clusters of ribosomes, but no other organelles (Fig. 112). Each vesicle was membrane-bound, and vesicles often coalesced to form large reticulated areas (Fig. 113). Golgi bodies were abundant close to the thickened wall area, and were observed to produce vesicles with electron-transparent contents (Fig. 114). The area of cytoplasm adjacent to the thickened wall was also rich in mitochondria, ER and lipid bodies, although plastids remained towards the centre of the cell (Fig. 115).

Figures 90-96

- Fig. 90 Near median section of egg cell (EC) just after division of the fusion nucleus. The two free nuclei (*) lie towards the micropylar end of the cell. Abundant mitochondria and plastids are found in the cytoplasm chalazal to the nuclei (arrowheads). Megagametophyte (M). LM. Bar = 80 μ m.
- Fig. 91 Cytoplasm between the two free nuclei (*). Both nuclei have membranes punctuated by many nuclear pores (arrowheads). The surrounding cytoplasm contains paternal mitochondria (Me) and numerous lipid droplets (L). TEM. Bar = 4 μ m.
- Fig. 92 The small nucleus lying slightly micropylar to the two free nuclei has an incomplete membrane (arrows). Small vacuoles (V) and modified maternal plastids (Pm) are found in the egg cytoplasm. TEM. Bar = 4 μ m.
- Fig. 93 Cytoplasm surrounding the free nuclei contains cup-shaped maternal plastids, many small vacuoles, paternal mitochondria and darkly-staining crystalline areas (*). TEM. Bar = 4 μ m.
- Fig. 94 Megagametophyte cells above the archegonia become multinucleate and fill with starch granules (arrows) as the fusion nucleus divides and the free nuclei migrate to the chalazal end of the archegonium. These starch-filled cells form a cone-shaped area extending from the archegonia into the middle of the megagametophyte. Jacket cell layer (J). LM. Bar = 40 μ m.
- Fig. 95 Starch-filled megagametophyte cells, with large nuclei (Nu), large and small starch granules (*) and abundant mitochondria in the dense cytoplasm. TEM. Bar = 10 μ m.

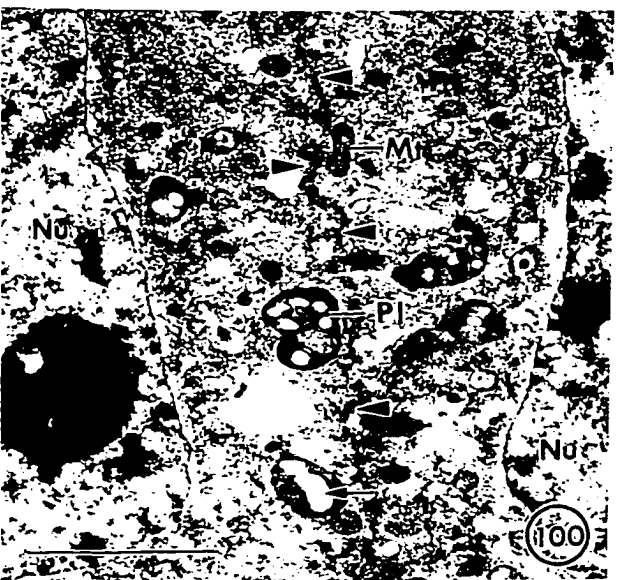
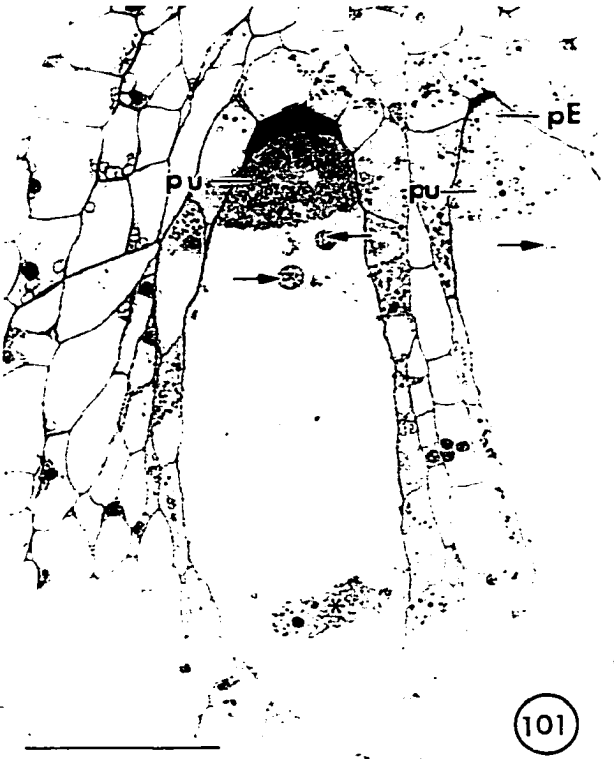
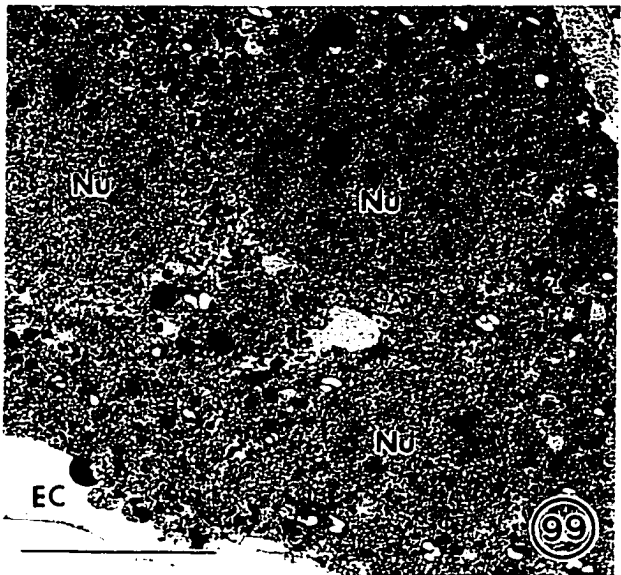
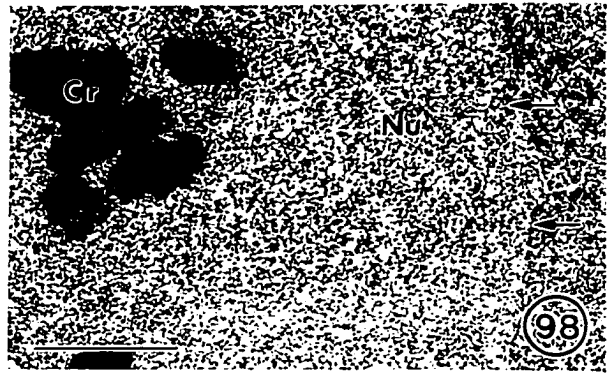
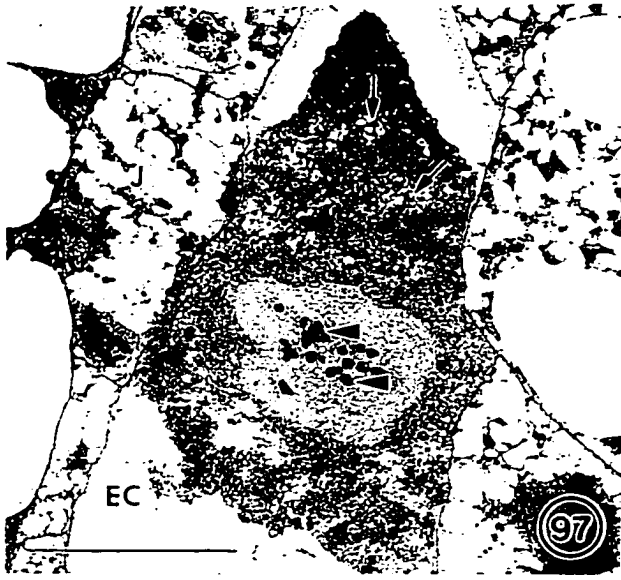
Fig. 96 Megagametophyte cells in areas outside the cone of starch-filled cells have small nuclei, very small starch granules (arrows) and only a thin parietal layer of cytoplasm. TEM. Bar = 20 μ m.



Figures 97-102

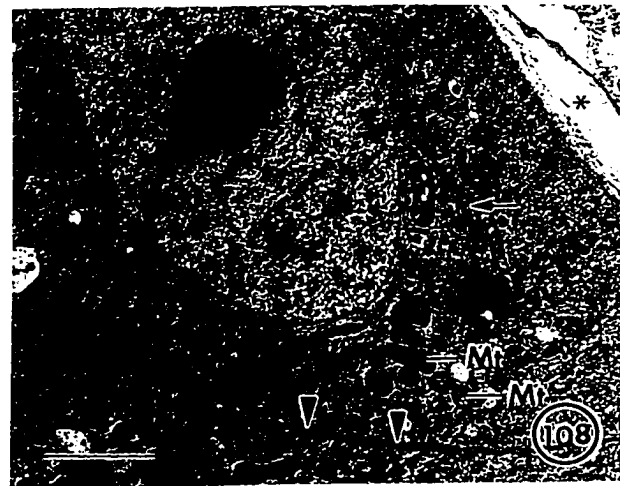
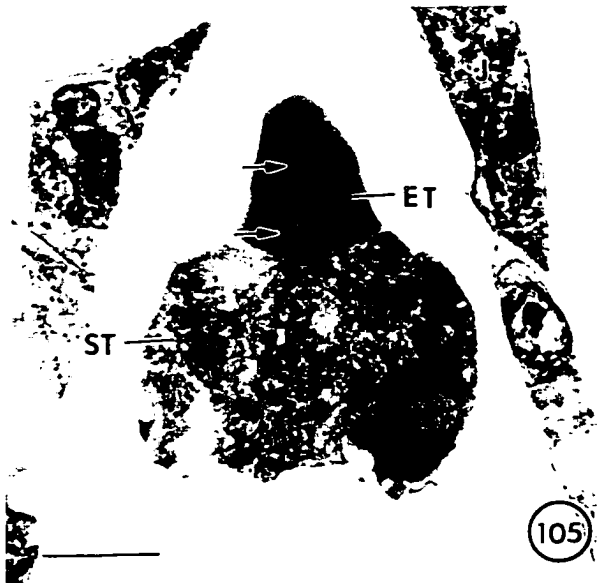
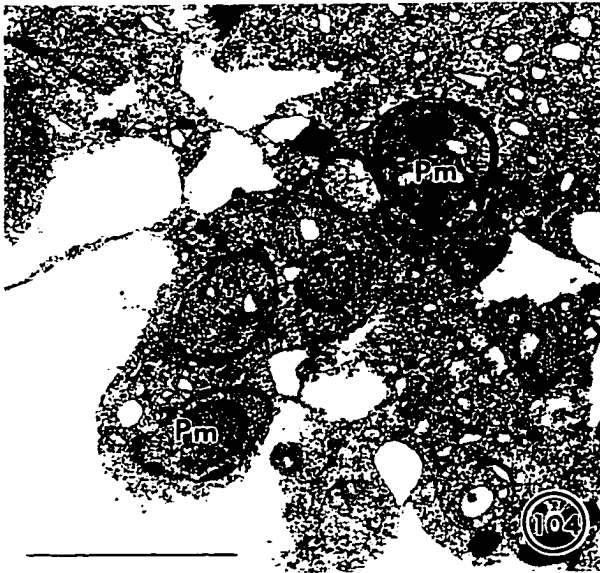
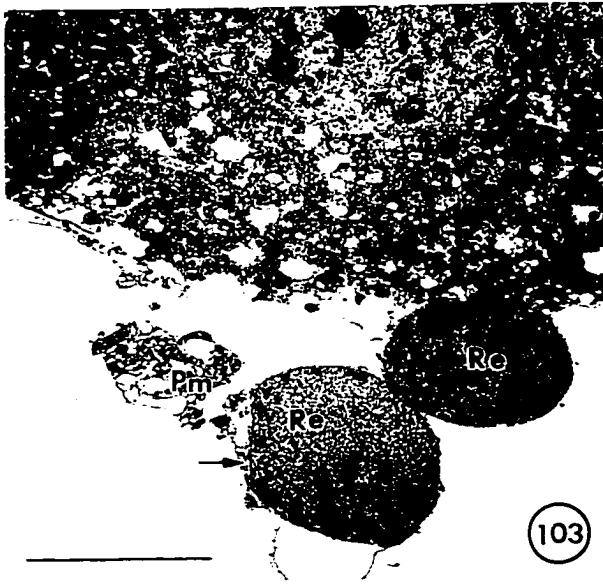
- Fig. 97 Once the two free nuclei have migrated to the chalazal tip of the egg cell (EC), the nuclear membrane breaks down, and chromosomes condense (arrowheads). The densely-staining neocytoplasm accumulates plastids at the chalazal end (arrows), and is filled with mitochondria and lipid droplets. Jacket cell (J). TEM. Bar = 20 μ m.
- Fig. 98 A portion of the proembryo as seen in Fig. 97 showing condensed chromosomes (Cr), and small fragments of membrane (arrows) at the boundary of the low density nucleoplasm (Nu) and the more dense surrounding cytoplasm (*). TEM. Bar = 2 μ m.
- Fig. 99 Four-free-nuclear proembryo (only three nuclei (Nu) are seen in this plane of section). The nuclei differ in appearance from the two-free-nuclear stage, by having a large nucleolus, and darkly-staining granular chromatin. The four nuclei lie in one tier. The chalazal end wall of the egg cell is thickened (*). Plastids are evenly distributed throughout the cytoplasm. TEM. Bar = 16 μ m.
- Fig. 100 Wall formation in the 16-free-nuclear proembryo begins as discrete bundles of wall material (arrowheads) lying between the nuclei. Surrounding cytoplasm contains plastids (Pl), starch grains (arrow) and mitochondria (Mt). TEM. Bar = 4 μ m.
- Fig. 101 Sixteen-free-nuclear proembryo. Relict nuclei (arrows) are excluded from the proembryo, and the nuclei are arranged into the primary embryonal (pE) and primary upper (pu) tiers. Note the accumulation of cytoplasm and supernumerary nuclei at the micropylar end of the egg cell (*). LM. Bar = 80 μ m.

Fig. 102 Two stages of wall formation in neighbouring proembryos. In the left proembryo, wall formation has only just started. The walls of the embryonal tier (ET) and suspensor tier (ST) are indistinct and undulating, and the partial walls of the open tier (OT) have just begun to form. In the right proembryo, wall formation is complete and the cells of the suspensor tier have become vacuolate. LM. Bar = 60 μ m



Figures 103-108

- Fig. 103 Relict nuclei (Re) excluded from the proembryo cytoplasm are ovoid with unevenly staining nucleoplasm. Shreds of cytoplasm adhere to the nuclear membrane (arrow). Modified maternal plastids (Pm) also excluded from the cytoplasm lie nearby. TEM. Bar = 10 μ m.
- Fig. 104 Modified maternal plastids are all excluded from the proembryo cytoplasm before wall formation begins. TEM. Bar = 2 μ m.
- Fig. 105 Light microscope preparation of the proembryo showing the difference in cytoplasm density between the embryonal cell (ET) and the suspensor tier (ST) cells. Both nuclei of the embryonal cell can be seen in this plane of section (arrows). Jacket cells (J). LM. Bar = 20 μ m.
- Fig. 106 The nuclei of the binucleate embryonal cell (ET) are ovoid, with prominent nucleoli and unevenly granular nucleoplasm (*). The cytoplasm contains abundant plastids (Pl) and mitochondria (Mt). The vesiculate area at the chalazal tip of the cell has already begun to form (arrow). TEM. Bar = 8 μ m.
- Fig. 107 Golgi bodies (G) situated near the chalazal end wall of the embryonal cell produce large numbers of vesicles (Ve) which migrate to the inner surface of the cell wall (CW). TEM. Bar = 0.5 μ m.
- Fig. 108 Mitochondria cluster close to the nucleus of suspensor cells. The cytoplasm is rich in ER (arrow) and ribosomes. The suspensor cell wall in contact with the archegonial wall (*) is much thicker than that between adjacent suspensor cells (arrowheads). TEM. Bar = 3 μ m.

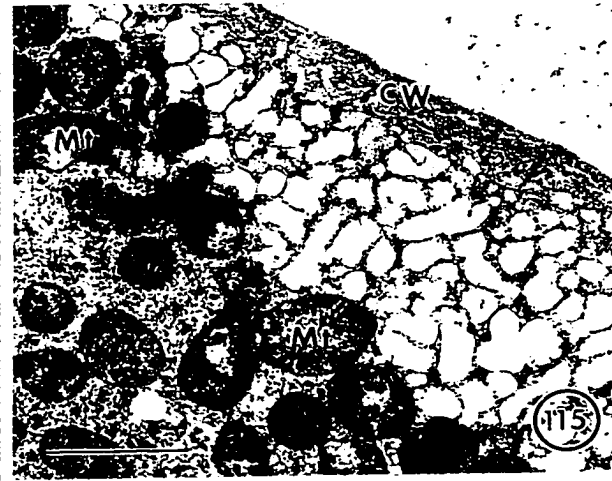
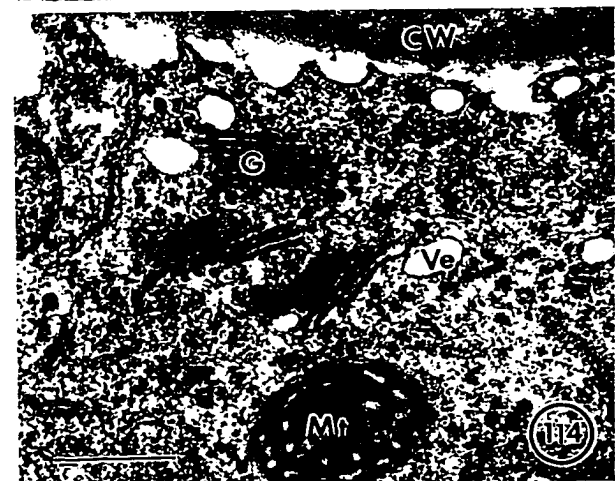
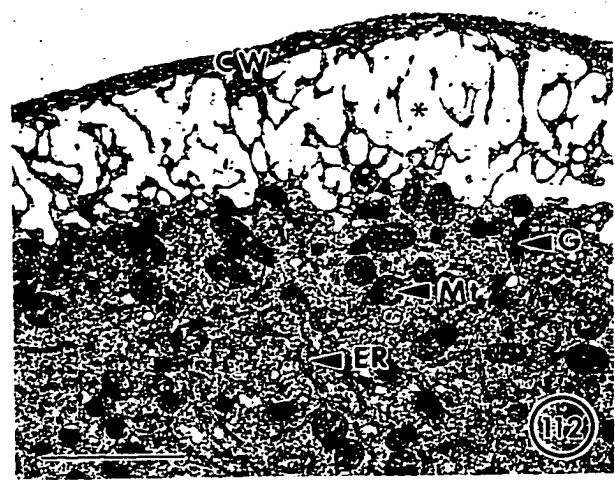
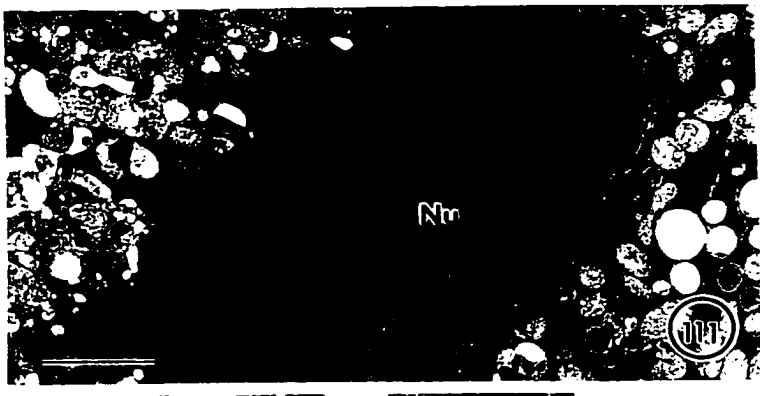
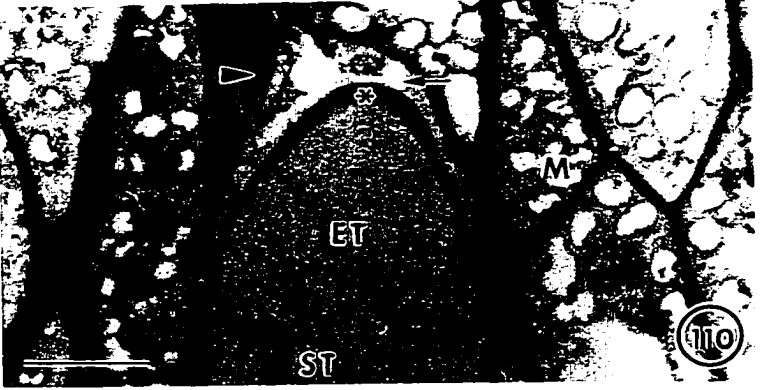
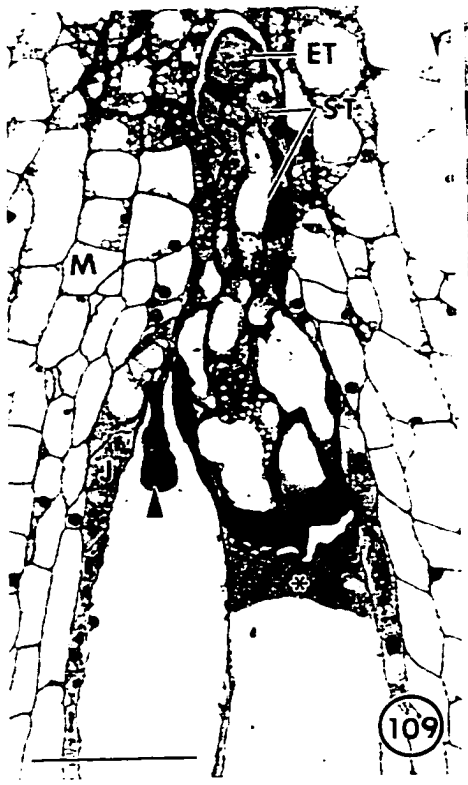


Figures 109-115

- Fig. 109 Suspensor cell (ST) elongation pushes the embryo through the jacket cells and into the megagametophyte tissue. The embryonal cell (ET) has only a small corrosion cavity surrounding it. A plug of dense cytoplasm and wall material (*) has formed below the suspensors. The archegonium to the left of the growing embryo has degenerated, and the cytoplasm stains very darkly (arrowhead). The jacket layer (J) cells are also degenerating and will soon release their contents into the remains of the egg cell. Megagametophyte, (M). LM. Bar = 80 μ m.
- Fig. 110 Close-up of the region around the embryonal tier cell, showing the small corrosion cavity (arrow), and the cap region of the cell wall (*). Surrounding megagametophyte cells are filled with starch granules, but ones close to the embryo darken and degenerate after contact with the embryonal tier cell (arrowhead). LM. Bar = 20 μ m.
- Fig. 111 Darkly-staining cytoplasm inside a degenerating archegonium contains the remains of the egg nucleus (Nu) and granular, vacuolate areas which are probably degenerating organelles. TEM. Bar = 2 μ m.
- Fig. 112 The cap region at the chalazal tip of the embryonal cell is made up of a network of coalescing vesicles (*) situated beneath the cell wall (CW). The cytoplasm near this area contains abundant Golgi bodies (G), mitochondria (Mt) and ER. TEM. Bar = 2 μ m.
- Fig. 113 As in Fig. 112. Individual vesicles (Ve) coalesce into a reticulated network (*) on the inner side of the cell wall. Polysome, (arrowhead). TEM. Bar = 0.5 μ m.

Fig. 114 Golgi bodies are concentrated near the vesiculate cap region. TEM. Bar = 0.5 μ m.

Fig. 115 As the embryonal cell increases in maturity, the cap region becomes wider, and the number of mitochondria packed against the cap region increases. TEM. Bar = 1 μ m.



Discussion

Doyle and Brennan (1971) commented that although podocarps show the same “general plan” of embryo development in three stages typical of other conifer species (proembryo, early embryo and late embryo), “considerable variation within the limits of the general plan is characteristic of the family”. The progression of free nuclear divisions and wall formation observed in this study agrees with observations of totara proembryos carried out by Buchholz (1941) and Brownlie (1953). The structure of proembryo formation and organisation differs between podocarp species, and some authors have suggested evolutionary trends within the family.

In totara, the fusion nucleus divides only once before the free nuclei, accompanied by a dense mass of neocytoplasm, migrate to the chalazal tip of the archegonium.

Ultrastructural observations showed that the cytoplasm in direct contact with the free nuclei contained few organelles. Most organelles were located in an area of neocytoplasm just chalazal to the free nuclei, until they migrated to the chalazal end of the archegonium with the nuclei. While most studies record one mitosis before nucleus migration, two mitoses are recorded for *Dacrydium sp.* (Quinn, 1965; 1966a; 1966b), *Podocarpus falcatus* (Osborn, 1960) and *P. gracilior* (Konar and Oberoi, 1969a). The observations of Coker (1902) who reported that the fusion nucleus migrated to the chalazal area prior to any mitoses, has been refuted by Boyle and Doyle (1954) who found it likely that Coker did not have a wide enough range of specimens and therefore missed the migration period altogether.

The neocytoplasm of the totara proembryo contained maternal and paternal mitochondria, and paternal plastids with large starch granules. In early free nuclear stages it was possible to distinguish between paternal and maternal mitochondria, but by the time of wall formation, all mitochondria were ovoid, with numerous cristae and a granular matrix. Camefort (1968) described a similar situation in *Larix*, where a morphological difference between maternal and paternal mitochondria was noticeable until late in proembryo development. In totara, the paternal mitochondria have a more densely-staining matrix and a larger number of cristae than the lightly-staining maternal mitochondria in which cristae are difficult to distinguish. In *Pseudotsuga* (Owens and Morris, 1991) the paternal organelles remained in a discrete cluster until the free nuclei had migrated to the chalazal end of the archegonium, and in *Agathis* (Owens et al., 1995b) the mainly-paternal neocytoplasm was distinguished by its relatively high density compared to surrounding egg cytoplasm. In totara, the paternal organelles did not remain discrete from the maternal complement, and intermingling of the two occurred soon after fertilization. Cup-shaped modified maternal plastids from the egg cell cytoplasm were briefly included in the neocytoplasm of the proembryo, but these were excluded to the micropylar edge as free nuclear mitoses progressed. It is therefore likely that functional plastids found in the proembryo cytoplasm originated from the paternal cytoplasm.

There appeared to be some polarity of plastid distribution during free nuclear and early wall formation periods, as they remained clustered in the chalazal cytoplasm of the free nuclear proembryo, and at the chalazal tip of the newly-formed suspensor and embryonal cells. A similar polarity of plastid distribution was observed by Runions (1997) in *Picea*. Plastids were not found in the open tier of totara proembryos. In the densely cytoplasmic

embryonal cell, the plastids were arranged near the nuclei at the micropylar end, and mitochondria were clustered close to the thickening cell wall at the chalazal tip. In suspensor cells, the mitochondria were clustered close to the nucleus, and there appeared to be far fewer plastids than in the embryonal cell. This distribution of organelles makes some sense given the fact that the embryonal cell is the sole precursor of all tissue areas in the mature embryo, including the cotyledons. It is therefore necessary to have a complement of plastids in the embryonal cell, but not at such great density in the suspensor cells where the primary function is to push the embryo into the megagametophyte, and therefore a higher abundance of mitochondria is of more immediate benefit. Owens et al. (1995b) observed ultrastructural differences in *Agathis* between the cap and central cells of the embryonal tier, and suspensor cells as mainly to do with the relative thickness of cell walls. Plastids and mitochondria appeared to be equally abundant in each cell type. In totara, cell wall thickness was initially similar for cells in the embryonal and suspensor tiers, but subsequent elaboration of the embryonal cell wall made it far thicker than any other wall structure in the proembryo.

The number of nuclei in the embryonal, suspensor and open (or relict) tiers of the proembryo varies greatly among podocarp species. In totara, the sixteen free nuclei (product of four mitoses) initially formed an embryonal tier of one cell, and a primary upper tier of seven to nine cells. One further mitosis resulted in a variable number of suspensor cells, depending on how many nuclei were excluded into the open or “relict” tier, and one binucleate embryonal cell. These observations agree with those of Buchholz (1941) and Brownlie (1953) for totara. In the closely-related species, *P. nivalis*, proembryo tiers have variously been reported as made up of seven to ten suspensor cells

and one binucleate embryonal cell (like totara) (Brownlie, 1953) or 8-14 suspensor cells and one to three (but usually two) binucleate embryonal cells (Boyle and Doyle, 1954). Having compared all studies made to date concerning embryo development in podocarps, it appears that not only does the constitution of proembryo cell tiers differ between closely-related podocarp species, it is also variable amongst different samples of the same species. It appears to be a general rule that among the members of *Podocarpus*, subgroup *Podocarpus* and subgroup *Foliolatus* (Kelch, 1997) there are 7-14 suspensor cells and one to three binucleate embryonal cells. In members of the genus *Prumnopitys* (formerly *Podocarpus* subgroup *Stachycarpus*) and other podocarp genera, there are 9-25 suspensor cells and 3-15 binucleate embryonal cells (Chowdhury, 1962). The significance of the difference in number of embryonal tier cells with regard to formation of a “cap” region in the embryo will be discussed below.

The number of nuclei excluded from the proembryo cytoplasm into the “relict” tier appears to be the major cause of variation in the number of suspensor cells in specimens of totara and other podocarps. In totara, there appeared to be two times when nuclei were excluded; some nuclei lay apart from the proembryo cytoplasm as free nuclei began to form into the embryonal and primary upper tiers, and some were excluded as the open tier formed after cell wall formation and internal division of suspensor cells. These relict nuclei had an ovoid shape, and unevenly-stained nucleoplasm in which the nucleolus appeared granular and dispersed. No vestiges of cytoplasm were associated with them. Some studies (Coker, 1902; Young, 1910) have claimed that relict nuclei have proliferated and formed “rosette embryos” towards the micropylar end of the archegonium, however this was not seen in totara and it appears more likely that the

rosettes were clusters of relict and perhaps the remains of supernumerary nuclei.

Buchholz (1926; 1936) found that rosette nuclei sometimes formed at the base of the elongating suspensors due to the proliferation of non-elongating suspensor cells.

Many studies have described a “resting” or quiescent stage of embryonal cell development which occurs as the suspensors elongate and push the embryo deep into the megagametophyte. In totara, this was noticeable in light microscope preparations as a period when the embryonal cell stained extremely darkly and cellular detail was difficult to interpret. This is undoubtedly why there has been debate about the morphology of the embryonal cell prior to its proliferation. Ultrastructurally, there does not appear to be a reason for the exceptionally dark staining of the embryonal cell, except that it is densely cytoplasmic, and cell organelles are abundant and grouped in tight clusters especially around the nuclei and near the chalazal tip of the cell. Some authors (Boyle and Doyle, 1954; Konar and Oberoi, 1969a; Doyle and Brennan, 1971) have referred to the embryonal cell(s) as having a degenerated appearance, presumably as they stain similarly to the cytoplasm of degenerating egg cells, but there is no sign of any breakdown of the cell or cell wall.

Brownlie (1953) described the binucleate embryonal cell as having a “thickened cellulose cap”, and Boyle and Doyle (1954) reported the formation of “ a small apical area...cut off by formation of a membrane” from the remainder of the cell cytoplasm. The formation of this cap region was described as concurrent with the return of the quiescent embryonal cell cytoplasm to a lighter-staining state. This study observed that the cap region consisted of a thickened wall (ca. 15 μ m) with a densely-vesiculate area immediately beneath. This is the area Boyle and Doyle (1954) referred to, however it is

not separated from the rest of the cytoplasm by a membrane. Any detail of this region is impossible to ascertain in light microscope sections, but its ultrastructure suggests that the embryonal cell is actively and profusely secreting material across the chalazal cell wall. It is possible that this secretion mechanism has a function in the breakdown of megagametophyte cells in the small corrosion cavity surrounding the embryo. Evidence of a similar secretory area in the cap cells of the *Agathis* embryo (Owens et al., 1995b; 1995c) was shown to be a network of dilated smooth ER reticulae, which contained osmophilic contents. The same area in the totara embryonal cell was made up of membrane-bound vesicles (formed by the abundant Golgi bodies situated in the cytoplasm nearby) which had coalesced into a network. These vesicles did not have as darkly-osmophilic contents as the ER in *Agathis* (Owens et al., 1995b), but osmophilic material could be seen crossing the cell wall of the totara embryonal cell.

The purpose of such a well-defined secretory area and zonation of organelles within the totara embryonal cell, likely has to do with the reduction of the embryonal region to a single binucleate cell. In other podocarp species with more than three embryonal cells, and in a few other conifer species, some of the embryonal tier differentiates as cap cells (Chowdhury, 1962; Singh, 1978). Many functions have been attributed to cap cells, but Owens et al. (1995b) found that it was most likely that the cap protected the meristematic embryonal cells and secreted enzymes causing formation of the corrosion cavity. Boyle and Doyle (1954) commented that “there is of course no evidence that the cap function is protective...it does not develop until suspensor growth has slowed markedly or stopped.” This was not the case in totara, where the cap region was found to develop even before the proembryo had emerged from the archegonium. Species such as *Podocarpus andinus*

(Looby and Doyle, 1944b), *P. spicatus* and *P. ferrugineus* (Buchholz, 1936) and *Dacrydium sp.* (Quinn, 1965; 1966a; 1966b) have a storied embryonal cell arrangement. All of these species have a terminal cell or cells which have been designated a cap region. In *P. andinus*, the terminal cell of the embryonal tier is uninucleate (Looby and Doyle, 1944b) and in *P. spicatus* and *P. ferrugineus* the terminal cell(s) are binucleate (Buchholz, 1941). Quinn (1965) recognised up to three cell types in the embryonal tier of *Dacrydium*, some of which were binucleate and some uninucleate. The proportions of these types appeared to depend on species (Quinn, 1965; 1966a; 1966b). The cap cells of these species do not have the thickened cell wall area typical of the totara embryonal cell, and often collapse during the course of embryo penetration into the megagametophyte. *P. totara* is considered to demonstrate a more derived condition than the former species, as a progression towards fewer cells in the embryonal tier is thought to have occurred within the Podocarpaceae (Buchholz, 1941; Chowdhury, 1962; Konar and Oberoi, 1969a; Doyle and Brennan, 1971). Totara, a member of *Podocarpus* subgroup *Podocarpus*, has the most derived condition with only a single embryonal cell. Confirmation that the tip of this cell is secretory reinforces the hypothesis that this is a derived condition within the Podocarpaceae; there has been a reduction from multiple cap cells in a storied embryonal tier to a cap region within a single embryonal cell.

Chapter 9

Pollination and reproductive success of *Podocarpus totara*.

Introduction

A reproductive cycle in totara lasts two years from cone initiation through to seed fall, as has been documented in Chapter 4 of this study. Although we now know something of the timing of reproductive events, there is currently no information available describing measures of reproductive success or how much effect the relative timing of pollen release and ovule receptivity has on seed yield. Examination of totara thus far in New Zealand has included assessments of provenance variation in seedling growth (Bergin and Kimberley, 1992) and frost hardiness (Hawkins et al., 1991), studies of regeneration of totara and other podocarp species in natural forest (Wardle, 1963; Beveridge, 1973) and details of seedling and shoot morphology (Philipson and Molloy, 1990).

It appears likely that totara will be used for silvicultural and reforestation purposes in the near future in New Zealand, so it would therefore be helpful to know of any pre-zygotic or post-zygotic factors which might affect seed production. The purpose of this study is to provide quantitative data on three main areas : pollen release and concurrent receptivity of ovules, pollination success as measured by the quantity of pollen contained in the micropyle, and fertilization success as measured by the number of embryos developed in the ovule.

Observations and Results

Pollen monitoring

Pollen monitors recorded a peak of totara pollen release in the period from October 13-17, 1995 (Monitor A = 21.16 pollen grains per mm² ; Monitor B = 8.66 pollen grains per mm²) (Fig. 116). The two monitors differed in the quantity of pollen captured, and this was most likely due to their relative positions with respect to the prevailing wind direction and proximity to male trees used in the study. However, both monitors recorded a single peak in pollen capture in the same time period. The pollen receptivity period of female trees A, C, and D coincided with the peak of pollen release. The receptive period of female tree B lagged behind by ca. two weeks, however low levels of totara pollen (<1 grain per mm²) were still detected by the monitors at this time.

Due to the isolated position of the field site, the pollen captured by the pollen monitors was predominantly from totara. The field site consisted of a totara stand surrounded by farmland. Two other pollen types were observed. Three-winged pollen identified as that of *Dacrycarpus dacrydioides* was captured in the 24 October collection, and was likely to have originated from a stand ca. 1.5 km to the west of the field site. Triangular fern spores were observed in mid-November collections.

Pollination success

The average number of pollen grains found in the micropyle of totara ovules collected after micropyle closure was 4.52 (Tree A, 4.7 ± 2.51 ; Tree B, 3.25 ± 1.96 ; Tree C, $5.75 \pm$

3.03; Tree D, 4.35 ± 2.83). An analysis of variance (ANOVA) was performed on the data from the four female trees, using the equation:

$$Y_{IJ} = \mu + T_I + e_{(IJ)}$$

where Y_{IJ} = number of pollen grains in the micropyle of ovule J on tree I

and $I = 1 \dots 4$

T = female tree

$J = 20$

The results of the ANOVA are presented in Table 3. The effect of the female tree was found to be statistically significant ($F = 3.102$; $F_{\text{crit}}(3, 120) = 2.68$, $p < 0.05$). Subsequent analysis of the variance components showed that the tree contributed 9.5% of the variance within the sample population (Table 3).

Fertilization success

Ovules from the four female trees used in this study were categorised as to whether they contained no embryos, one, two or three embryos. No ovules were observed to contain more than three embryos. A χ^2 analysis was performed on the data set, with a null hypothesis that the proportions of ovules in each embryo category were the same in all four female trees (Table 4). The χ^2 value was 4.42 ($\chi^2_{\text{crit}}(df = 9, p < 0.05) = 16.919$) and as there was no evidence for an alternative hypothesis, the null hypothesis was accepted. Therefore each female tree was representative of the population, and the proportions of each embryo category could be pooled across all four trees. The majority

of ovules (48.6%) contained a single embryo (Table 4). No embryos were found in 27.1% of ovules, and 24.3% of ovules contained two or three embryos (Table 4).

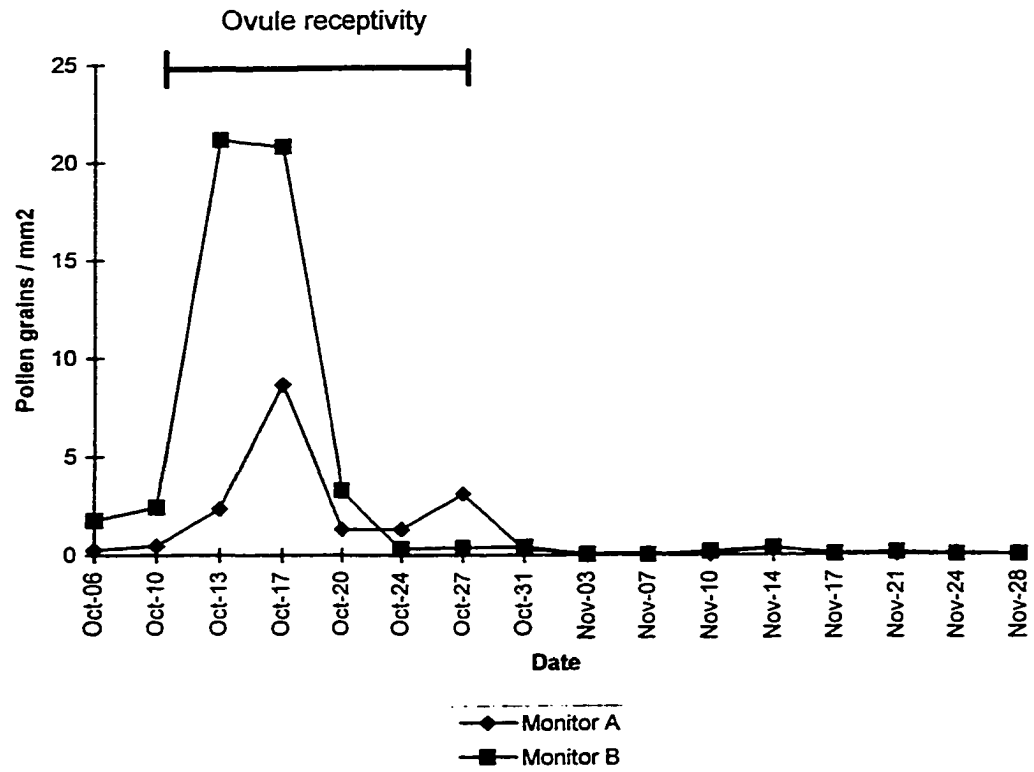


Figure 116. Totara pollen grain release with respect to ovule receptivity, as measured by two pollen monitors at the Auckland field site in 1995.

Table 3. ANOVA results for analysis of number of pollen grains captured in the micropyle of four female totara trees.

Source of Variation	df	Mean Square	F-ratio	Variance Component	Percentage Variance Component
Tree (T)	3	21.25	3.102 *	0.72	9.5 %
Error	76	6.85		6.85	90.5 %
	Σ 79			Σ 7.57	Σ 100 %

* significant at $p < 0.05$

Table 4. Proportion of ovules in different embryo categories in fertilization success sampling of four female totara trees.

Embryo Category	Tree				Total	Percentage of Ovules ²
	A	B	C	D		
No Embryos	6 (9.5) ¹	12 (9.5)	11 (9.5)	9 (9.5)	38	27.1 %
1 Embryo	18 (17)	16 (17)	17 (17)	17 (17)	68	48.6 %
2 Embryos	9 (7)	5 (7)	6 (7)	8 (7)	28	20 %
3 Embryos	2 (1.5)	2 (1.5)	1 (1.5)	1 (1.5)	6	4.3 %
Total	35	35	35	35	140	100 %

¹ Expected values for each category in χ^2 analysis.

² Percentages were calculated from pooled samples of all female trees.

³ H_0 : The proportion of ovules in each embryo category is the same in all female trees.

Alternative hypothesis : The proportion of ovules in each embryo category is not the same in all female trees.

$$\chi^2 = \sum \frac{\text{Observed}^2}{\text{Expected}} - N = 4.42$$

Expected

$$\chi^2 \text{ crit (df = 9, p < 0.05) = 16.919}$$

No evidence for the alternative hypothesis.

Discussion

Analysis of the relative timing, pollination and fertilization success in totara shows that male and female trees are homogynous as pollen release is simultaneous with ovule receptivity. Totara does not appear to exhibit the degree of dichogamy typical of some other conifer species. Dichogamy results in a temporal separation of male and female maturation and/or receptivity periods as a mechanism to prevent self-fertilization and possibly coordinate allocation of assimilates in monoecious species (Owens, 1995; Ramirez and Berry, 1997). Large differences between male and female maturity periods can adversely affect pollination success in dioecious species such as totara. The male strobilus of totara reaches maturity before the female strobilus is receptive (i.e. the pollen grains have completed all mitoses and are considered mature) but pollen release appears to be delayed to coincide with ovule receptivity.

There was an average of 4.52 grains in the micropyle of totara ovules, and 9.5 % of the variation in the sample population was attributable to the particular female tree from which the ovules were collected. In *Dacrydium cupressinum* ovules were reported to contain a single pollen grain, and the presence of more than one pollen grain was uncommon (Norton et al., 1988). The average number of pollen grains supports observations that the ovule receptivity period matched the peak of pollen release. The variation between female trees can be partially explained by differences in the relative timing of early stages of ovule development. Ovules did not become receptive until division of the megaspore mother cell (see Chapter 4), and the precise timing of this varied between female trees. Female B was especially late in reaching the megaspore

tetrad stage, and did not become receptive until two weeks after the other female trees. Female B had the lowest average number of pollen grains in the micropyle, which reflects the fact that the peak of pollen release was over before the ovules were receptive. The limited observations of totara recorded in other studies suggest that the seasonal timing of pollen release and ovule receptivity differs between populations. McEwen (1988) recorded totara pollen release as not occurring until mid-November at a field site ca. 300 km south of the one used in this study. Production of pollen cones in other New Zealand podocarp species has been described as irregular from year to year, and this has been suggested to adversely affect seed set in some species (Norton et al., 1988; Ogden, 1985). Bergin and Kimberley (1992) identified considerable variation between provenances of totara with respect to seedling height and growth form, and Hawkins et al. (1991) identified significant variation in frost hardiness. Although no work has been specific to phenological traits in adult totara, it is reasonable to expect that the variation seen in other traits might be observed in timing of shoot development and therefore the timing of the reproductive cycle.

The majority (48.6 %) of ovules contained a single embryo in samples taken in January (the early embryo stages). Chapters 5 and 7 described the splayed out shape of the pollen tube as it contacted the megagametophyte, and it was observed that the male gametes formed in this pollen tube fertilized an archegonium long before competing pollen tubes broke through to fertilize surrounding archegonia. Observations of early and late embryo stages in totara and other podocarp species have shown that the oldest and most well-developed embryo displays dominance over other embryos, and outgrowth of embryonal suspensor cells often obstructs the growth of smaller embryos (Buchholz, 1941; Looby

and Doyle, 1944b; Boyle and Doyle, 1954; Doyle, 1954). It appears that the product of the first fertilization event has a developmental advantage and is therefore favoured in the simple polyembryony mechanism of totara. Quinn (1965; 1966a; 1966b) found that a single embryo gained dominance over other smaller embryos even in the cleavage polyembryony mechanism of *Dacrydium*. It would be extremely interesting to track the genetic contribution of the first pollen tube to penetrate the nucellus, to see if the dominant embryo is always its genetic offspring.

Many pre- and post-zygotic factors have been documented as having an effect on seed yield in conifers (Owens and Blake, 1985). Pre-zygotic factors include a paucity of male trees, adverse weather and wind conditions, dichogamy and poor pollen quality. There did not appear to be a problem with the relative numbers of male and female totara trees at the field site in this study, however the effect of the irregular pollen cone crops noted in other podocarp species would require investigation over a number of seasons. The difference in pollen monitor estimates of pollen release of totara is likely to be due to their position with respect to wind direction. Although this suggests a non-uniform pollen flow within the field site, Sorensen and Webber (1997) found that regardless of how much pollen is available to ovules (whether wind-dispersed or artificially applied) 10 - 40% of seeds were empty at maturity in a number of conifer species. Totara does not have the same problem as *Pinus*, where if more than 20% of the ovules in a cone are not pollinated, the cone will abscise (Sarvas, 1962). Abscission of one of the two ovules in a totara strobilus does not appear to adversely affect the development of the other. The delay in development of one of the two ovules in totara would appear to extend the possible receptivity period of the strobilus, but make abscission of one ovule more likely

due to lack of pollination. The majority of mature totara strobili have only one seed attached to the receptacle. Preliminary observations of totara pollen quality suggest that there is a ca. 85% germination rate, and this is therefore unlikely to be a significant pre-zygotic factor.

Post-zygotic factors include abortion of the zygote, embryo or megagametophyte at various stages in development (Owens and Blake, 1985; Owens, 1995). In totara, 27.1% of mature ovules which appeared healthy and viable externally did not contain an embryo. Further analysis would be required to determine at which stage(s) embryo abortion occurred. Complete investigation of such factors on the seed yield in totara would require observations over many seasons, preferably in a controlled environment where the effect of pollination and receptivity periods could be quantified more precisely than in a field site. Although the duration of this study precluded a multi-seasonal assessment, preliminary observations suggest that ca. 20% of ovules abort after pollination, and a further 10% abort before seed maturity. If this information is combined with the 27% of seeds found not to contain an embryo in the fertilization success study, and assume that it is likely that one of the two ovules on a strobilus will abscise, the reproductive success can be estimated by :

$$RS = \text{no. of viable seeds/no. of ovules} \times \text{surviving ovules/strobilus}$$

$$RS = 43/100 \times \frac{1}{2} = 21.5\%$$

This gives an estimate of pre-germination reproductive success, and a study of germination rates would be required to see how many of these seeds were viable.

Preliminary results of seeds from each of the female totara trees in this study suggest that

ca. 15% of seeds germinated and produced a healthy seedling. Studies of *Dacrydium cupressinum* have shown 1-7% of sound seed is viable (Norton et al., 1988).

Confirmation of preliminary results in totara will require experimentation over a number of seasons. Podocarp seeds are recognised as having a limited viability period on the forest floor and in storage, due to their high lipid content and high metabolic and respiration rates after seed fall (Dodd and Van Staden, 1981; Dodd et al., 1989a; 1989b). Although seeds have been maintained deep in forest litter or in artificial storage for up to two years (Norton et al., 1988; Dodd et al., 1989b), seed viability declines quickly. The implications for the production and storage of totara seed for silvicultural purposes are that: (a) seed production in totara is subject to some pre- and post-zygotic factors but approximately 20% of the strobilus crop will produce a viable seed; and, (b) that the main problem for totara growers will be the maintenance of a seed bank of desirable parent trees due to the difficulty of maintaining seed viability.

Chapter 10

General discussion and directions for future research

This study has described many aspects of the reproductive biology of *Podocarpus totara*, and has documented ultrastructural detail of male and female gametophyte morphology and the mechanism of fertilization for the first time in a member of the Podocarpaceae. Significant findings of this study include :

1. Totara has a two-year reproductive cycle from cone initiation through to seed maturity. There is a nine-month period of dormancy occurring between cone initiation and emergence, but the period from pollination through to seed maturity is continuous and complete in eight months.
2. Pollen is shed at a six-nucleate stage. The mature pollen grain consists of a tube cell containing a tube nucleus, a sterile nucleus, three prothallial nuclei and the body cell. The prothallial nuclei remain in close association with the body cell until gamete formation.
3. Pollen release and receptivity of female strobili are synchronous. The average number of pollen grains in the micropyle of ovules is 4.52.
4. More than two megagametophytes are formed in ca. 5% of ovules. This is likely to result from development of more than one megaspore. Both megagametophytes are capable of forming archegonia, although pollen tube growth is directed towards the more micropylar of the two.

5. The body cell nucleus divides to form two unequal male nuclei once the pollen tube has contacted the megagametophyte. The larger nucleus remains central in the body cell cytoplasm, surrounded by dense accumulations of mitochondria, plastids containing small starch granules and small vesicles which are released into the pollen tube. The smaller gamete is excluded to the periphery of the body cell cytoplasm but remains in association with the large gamete until pollen tube entry into the archegonium.
6. The egg cell consists of a large ovoid egg nucleus situated in the micropylar end of the egg cell, and a small lenticular ventral canal nucleus appressed to the egg cell wall near to the neck cells. There are three main cytoplasmic areas within the egg cell. The perinuclear zone is dense in mitochondria and surrounds the egg nucleus. The micropylar zone of cytoplasm near the ventral canal nucleus is devoid of organelles except for numerous smooth ER membranes. The chalazal zone of cytoplasm surrounding the perinuclear zone and stretching around the large chalazal vacuole, contains cup-shaped maternal plastids and small inclusions interspersed with small vacuoles and thin cytoplasmic strands.
7. Male and female cytoplasmic organelles are different in morphology. Mitochondria in the male gamete have a darkly-staining matrix and numerous cristae, and have an elongated vermiform shape. Plastids in the male gamete contain small starch grains and a darkly-staining stroma. Mitochondria in the egg cell are ovoid, with a lightly-staining granular matrix and few cristae. Egg cell plastids are transformed into a cup-shaped structure as the egg cell matures, and do not appear to retain the normal function of plastids.

8. Both male nuclei enter the egg cell at fertilization, but only the larger nucleus migrates to the egg nucleus and fuses with it. The bulk of the body cell cytoplasm accompanies the large male nucleus, mingles with the perinuclear zone around the egg nucleus, and remains surrounding the fusion nucleus as it undergoes mitosis. The neocytoplasm of the zygote is therefore composed of maternal and paternal mitochondria, and paternal plastids. Modified maternal plastids in the form of large inclusions degrade soon after fertilization, and are actively excluded from the proembryo cytoplasm.
9. The proembryo consists of 16 free nuclei prior to cell wall formation. A varying number of relict nuclei are excluded from the proembryo cytoplasm. Cell walls form around two tiers of nuclei, and after one further division, the proembryo is made up of an open tier of 7-9 nuclei, a suspensor tier of 7-9 cells and a single binucleate embryonal tier cell.
10. The binucleate embryonal tier cell develops a thickened cell wall at the chalazal tip. Active secretion of vesicles with electron-transparent contents forms a network of vesiculate material next to the chalazal cell wall area. This area is likely to be equivalent to the cap cell region of the embryos of some other conifer species, and secretes enzymatic substances to aid in embryo penetration through the megagametophyte.
11. A system of simple embryony operates within totara ovules. The first embryo to penetrate into the megagametophyte appears to have a developmental advantage. Nearly 50% of ovules contained a single embryo at the end of early embryo stages. The splayed growth of the first pollen tube to reach the megagametophyte, appears to

restrict the penetration of archegonia by competing pollen tubes until well after fertilization. Cleavage polyembryony was not observed.

The lack of study of podocarp species has always left a puzzling gap in our knowledge of how derived the family Podocarpaceae is within the Coniferales. The modes of cytoplasmic inheritance in all seven conifer families are presented in Table 5.

Angiosperm species are united by predominantly maternal inheritance of mitochondria and plastids (Mogensen, 1996). In contrast, conifer species combine paternal inheritance of plastids with paternal or biparental inheritance of mitochondria (Table 5). As RFLP studies increase the range of species analysed, it appears that the situation is not quite as simple as it appears and that so-called "leakiness" of organelle inheritance (e.g. some maternal inheritance of plastids in species such as *Larix* (Szmidt et al., 1987) and some paternal inheritance of mitochondria in *Pinus* (Wagner et al., 1991)) may blur the generalizations about inheritance patterns across entire families. However, the addition of information for Podocarpaceae supplied by this study does allow confirmation of a pattern of predominantly paternal plastid inheritance in all conifer families, and variations in the maternal and paternal contributions of mitochondria dependent on the mechanism of fertilization and the structure of the male and female gametes (as discussed in Chapter 7).

If we compare the pattern of similarity of cytoplasmic inheritance pattern among families, the Podocarpaceae shares an inheritance mechanism most similar to the Taxaceae, Pinaceae and possibly the Cephalotaxaceae (Owens and Morris, 1991; Mogensen, 1996). Morphological analyses of the phylogeny of the Coniferales such as that of Hart (1987) show that the Pinaceae diverged early in the lineage, followed by the

Family	Inheritance based on ultrastructure		Male gametes	Perinuclear zone	Transformed maternal plastids	Inheritance based on RFLP results	
	cpDNA	mtDNA				cpDNA	mtDNA
Pinaceae	p ¹	m ²	Nuclei	+	+	p ³	m
Podocarpaceae	p	m/p ⁴	Nuclei	+	+	?	?
Taxaceae	p	m/p	Nuclei	+	+	?	?
Cephalotaxaceae	p	m/p	Nuclei	-	+	?	?
Araucariaceae	p	p	Nuclei	+	-	?	?
Cupressaceae	p	p	Cells	-	-	p ³	p
Taxodiaceae	p	p	Cells	-	-	p ³	p

Table 5. Cytoplasmic inheritance in conifers (adapted from Owens and Morris, 1991)

¹ p, paternal inheritance

² m, maternal inheritance

³ Inheritance of plastids in these families has been shown to be predominantly paternal, but with some “leakiness” of maternal plastids.

⁴ m/p, both maternal and paternal inheritance

Podocarpaceae and then a divergence of the Taxaceae and Cephalotaxaceae from the remainder of the families. The *rbcL* (Rubisco large subunit) molecular phylogenetic study carried out by Chase et al. (1993) agrees with the order of derivation of the Pinaceae, Podocarpaceae, Taxaceae and Cephalotaxaceae suggested by Hart (1987). Other molecular studies vary in the order of derivation of families in the Coniferales, depending on which gene sequence is used in the analysis. However, the Pinaceae and Podocarpaceae remain relatively constant in molecular analyses as diverging first and second respectively (Stefanovic et al., 1998). Miller (1988) goes even further in his morphological analysis of the Coniferales and suggests that that the Podocarpaceae were derived in a distinct clade within the Coniferales, also including the Pinaceae, Cephalotaxaceae and Araucariaceae. Given the constancy of phylogenetic position, the cytoplasmic inheritance mechanism shown in these four families (i.e., predominantly paternal plastid and biparental mitochondria inheritance) could perhaps be interpreted as basal, and the remaining conifer families (Araucariaceae, Cupressaceae and Taxodiaceae) have developed variations of this mechanism.

The next step in the process will be to confirm the pattern of cytoplasmic inheritance in the Podocarpaceae with: (a) studies of other podocarp species; and, (b) RFLP studies to quantify parental contributions. The foundation for molecular work in members of the Podocarpaceae has already been laid. Cato and Richardson (1996) devised PCR primers for microsatellite (simple sequence repeat) areas in the chloroplast genome (cpDNA) of several *Pinus* species, which were also found to amplify cpDNA sequences in totara, *Podocarpus nivalis* and *P. hallii*, and Kelch (1998) used 18sRNA primers to construct a phylogeny of the Podocarpaceae. The basis for testing maternal and paternal cpDNA and

mitochondrial genome (mtDNA) complements in the zygote and proembryo is therefore available.

There have been many analyses of embryogeny in the Podocarpaceae with the intention of highlighting evolutionary trends within the family. The findings of these analyses (Buchholz, 1941; Looby and Doyle, 1944b; Boyle and Doyle, 1954; Doyle, 1954; Chowdhury, 1962; Konar and Oberoi, 1969b) are summarised in the following:

1. Basal genera tend to undergo five free nuclear mitoses (i.e., a total of 32 free nuclei) before wall formation in the proembryo, the first two of which take place before migration to the chalazal end of the archegonium in most species. Genera which are recognised as more derived undergo only four free nuclear mitoses (i.e., 16 free nuclei) and only one mitosis occurs before migration to the chalazal area of the archegonium. *Totara* belongs in the latter category, having 16 free nuclei prior to wall formation in the proembryo.
2. There are two trends in reduction of the embryonal tier of the proembryo. In the most basal podocarp species, the embryonal tier contains up to 15 binucleate cells (Quinn, 1966a) observed some embryonal tier cells to be uninucleate in *Dacrydium*) in a storied arrangement considered to indicate differing cell functions. The stories segregate a cap region, and two different sorts of embryonal cell; elongated cells on the periphery, enclosing an area of small isodiametric embryonal cells located in the centre of the embryonal tier. In more derived genera, there is a trend towards reduction in the number of embryonal tier cells (to the most extreme case e.g. *totara* where there is only a single embryonal cell), and a rearrangement from a storied embryonal tier to all the cells arranged in one tier.

3. Genera with only simple polyembryony are considered to be less derived than “advanced” species which develop cleavage polyembryony. This point has been hotly debated mainly because the proliferation of detached suspensor cells has led to reports of cleavage polyembryony in species which in fact only have simple (e.g. *totara*), and because the precise mechanism of cleavage in species with only a few embryonal tier cells remains unclear. Buchholz (1941) found that each binucleate embryonal tier cell underwent a mitosis at the end of early embryo stages, and simultaneously formed cell walls around each of the four daughter nuclei. Later studies have not been able to confirm this simultaneous wall formation, and have therefore not been able to verify that the resulting embryonal mass can undergo cleavage to form more than one embryo.

Analysis of *totara* with respect to these trends suggest that it occupies a highly derived position because only four free nuclear divisions occur before wall formation in the proembryos, and because the embryonal tier has been reduced to a single binucleate cell. In contradiction, *totara* is placed in a less-derived position because the polyembryony mechanism is simple only. The trends described above segregate genera such as *Prumnopitys*, *Nageia* and *Sundacarpus* into a so-called “primitive” group (Doyle, 1954; Chowdhury, 1962), and *Podocarpus*, *Saxegothaea*, *Phyllocladus*, *Dacrydium* and *Microstrobos* into an “advanced” group. The morphological and molecular phylogeny of the Podocarpaceae carried out by Kelch (1997; 1998) do not agree with the arrangement of species into these two groups. Molecular analysis of 18sRNA sequences suggest that basal (or “primitive”) genera within the family include *Phyllocladus*, *Prumnopitys* and *Dacrydium* (Kelch, 1998) and while the morphological analysis does not completely

agree with the molecular work, the arrangement of genera into groups on the basis of embryogeny does not appear to be a good representation of the level of evolution of each genus. Genera identified as highly derived in Kelch's (1997; 1998) analyses have smaller numbers of embryonal tier cells than those identified as basal, but any trends as far as the derivation or cleavage polyembryony or number of free nuclear divisions in the proembryo are concerned are not supported. Many authors have commented on the enormous range of embryological features observed within the Podocarpaceae, and even within the genus *Podocarpus*. After having reviewed all the literature, it appears that two problems are responsible for current disagreement over the derivation of genera within the family: incomplete or unreliable observations of a number of genera, and the complete absence of any information for some genera, and the tendency to designate certain features as "primitive" or "advanced" with questionable justification. The conclusions reached here are that there is a tendency within the Podocarpaceae for a reduction in embryonal tier cell number in the more highly-derived members, but that there does not appear to be a clear trend in polyembryony mechanism. Observations of key genera such as *Acmopyle* and *Microcachrys* will be required to confirm these patterns.

Direction of future research

There are two main areas in which future research could be directed to augment and improve our current understanding of reproductive biology in totara: the phenology of the reproductive cycle and associated factors, and further anatomical studies.

Phenology of the reproductive cycle

1. We do not currently know much about what governs the relative development of the two ovules on a totara female strobilus. It is likely that the difference in developmental stage aids in extending the receptive period of the strobilus, but we do not know if there is a pattern to which ovule is developmentally older, or at what point the ovules synchronize (if ever). It would be useful to follow the fate of marked ovules to determine precise timing of abortion, and factors which influence the health of both ovules such as pollination period, resource allocation etc.
2. There is evidence that the timing of pollen release, and presumably ovule receptivity differs among geographical locations in New Zealand. A provenance test to quantify these differences, and to see if they are an inherited trait or an environmental response would help in selecting parent trees for future plantation tree stocks.
3. Insect damage to male trees especially resulted in the entire loss of a pollen cone crop on one tree used in this study. There was also a case of insect infestation of pollen cone primordia resulting in early outgrowth (by 9 months) of abnormally-shaped and sized pollen cones. Investigation of how widespread this problem is will be necessary for successful seed orchard location and operation.
4. Larger sample sizes for pollination, fertilization and abortion counts would aid in producing more reliable statistical results. It will be important also to pinpoint specific times of ovule loss. Ovule loss due to lack of pollination is relatively easy to ascertain, but it is harder to predict the timing and effect of post-zygotic factors causing embryo loss.

5. Some totara trees carry consistently heavier seed crops than others (Hugh Hulse, pers. comm. 1995). We do not know a great deal about the site preferences of totara, so it would be interesting to find out if seed crop follows a genetically-inherited masting pattern, or if site factors such as water availability favour some trees.

Further anatomical study

1. Egg cell and proembryo chemical fixation was problematic in this study, and resulted in poor sectioning quality of ovules at stages when the cytoplasm was especially dilute and lipidic (e.g. around the times of fertilization and free nuclear division in the proembryo). Although it is likely to be the high lipid content which obstructs adequate fixation and resin infiltration, dewaxing or defatting methods are extremely damaging to delicate egg cell and megagametophyte tissue. Alternative fixation methods such as freeze substitution may help to preserve the cytoplasm integrity of delicate stages. The use of confocal microscopy in the location of microtubules and other cytoskeletal elements during fertilization and proembryo development would help to visualize processes of cytoplasm movement within the egg cell.
2. As noted earlier in this discussion, RFLP analysis of cytoplasmic inheritance in totara would help to confirm the mechanism postulated in this study. Ultrastructural observations are limited to times before proembryo development in which maternal and paternal mitochondria are still distinguishable. RFLP analysis would allow quantification of the relative contributions of either parent to the proembryo cytoplasm.

3. Histochemical investigation of a number of anatomical features would help to confirm their function e.g. possible secretions by the egg cell or megagametophyte tissue directing pollen tube growth; the mechanism by which maternal and paternal cytoplasm contents mingle and migrate with the fusion nucleus; the contents of the vesicular network developed within the binucleate embryonal cell.
4. As two megagametophytes are found in 5% of totara ovules, it would be interesting to confirm their origin (i.e. as the product of two megaspores as opposed to the abnormal growth of one megaspore), and to track the survival rate of ovules containing two megagametophytes.

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