STRUCTURAL MECHANISMS OF CYTOPLASMIC INHERITANCE IN WESTERN WHITE PINE (*PINUS MONTICOLA* DOUGL.)

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ABSTRACT

The structural mechanisms governing cytoplasmic inheritance are described for *Pinus monticola* Dougl. and are consistent with patterns of cytoplasmic inheritance determined by molecular studies for some members of the Pinaceae. Ultrastructural observations support paternal plastid inheritance and predominantly-maternal mitochondrial inheritance with the possibility of occasional, low-level, paternal mitochondrial contribution. Although not demonstrated in this study, the possibility of occasional, low-level, maternal plastid contribution is not excluded.

The mature egg cytoplasm of *P. monticola* is organized into three concentric zones; the perinuclear, mid- and peripheral zones. The egg nucleus is central and is surrounded by a narrow, perinuclear zone dominated by egg-cell mitochondria. Outside this zone is a mid-zone which contains numerous small inclusions, scattered organelles and, occasionally, large inclusions. All egg-cell plastids exist as large inclusions which are mostly limited to the peripheral zone of egg cytoplasm and thus are isolated from the perinuclear zone. Body-cell plastids and mitochondria are transferred into the egg with the two male gametes at fertilization. Although small numbers of these body-cell organelles are sometimes carried to the egg nucleus with the fertilizing male gamete, most body-cell plastids and mitochondria remain clustered together and follow some distance behind the fertilizing male gamete. Body-cell organelles which accompany the fertilizing male gamete may escape lysis through their incorporation into the perinuclear

zone. The proembryo neocytoplasm which originates the embryo cytoplasm is formed by the melding of the perinuclear zone with the nucleoplasm released during coenocytic divisions of the zygote nucleus. Paternal mitochondria outside the perinuclear zone appear to degenerate, but clustered body-cell plastids remaining in the egg cytoplasm are not affected and are incorporated into the neocytoplasm probably during migration of proembryo free-nuclei to the chalazal pole of the egg. It is possible that some large inclusions may occasionally be introduced into the neocytoplasm prior to tier formation in the proembryo.

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Chapter 1

INTRODUCTION

Recent reviews of cytoplasmic inheritance in the plant kingdom (Sears, 1980; Connett, 1987) focus almost exclusively on lower plant and angiosperm inheritance mechanisms and only briefly report that conifer plastid inheritance differs from these groups. This is despite results demonstrating predominantly-paternal plastid inheritance in the conifer *Cryptomeria japonica* D. Don (Taxodiaceae) using the classic breeding methods which follow the inheritance of a plastid mutant in reciprocal crosses (Obha *et al.*, 1971). Furthermore, numerous ultrastructural studies by Camefort (1968b, 1969) and others (Chesnoy and Thomas, 1971) describe some aspects of conifer plastid and mitochondrial inheritance mechanisms. More recent molecular techniques using restriction fragment length polymorphisms (RFLPs) have demonstrated that conifers may be unique in seed plants by having strictly- or predominantly-paternal plastid (cpDNA) inheritance, whereas mitochondrial (mtDNA) inheritance varies. In members of the Pinaceae studied thus far, mtDNA inheritance is, most often, strictly maternal. However strict-paternal mtDNA inheritance has been reported in approximately 8% of offspring resulting from controlled crosses of *Pinus banksiana* Lamb. and *P. contorta* Dougl. (Wagner *et al.*, 1991). Although these results may be based on small sample sizes, in *Sequoia sempervirens* D.Don Endl. (Taxodiaceae) (Neale *et al.*, 1989) and *Calocedrus decurrens* (Torr.) Florin (Cupressaceae) (Neale *et al.*, 1991) mtDNA inheritance appears paternal. From these studies it has become evident that organelle inheritance varies, not only from angiosperms but even among conifers. Genetic breeding programs on forest

species must consider organelle-encoded traits and thus require a thorough understanding of their inheritance. This has renewed interest into structural mechanisms regulating cytoplasmic inheritance.

Camefort (1959a,b) was the first to describe ultrastructure of plant sexual reproduction. In studies of fertilization in *Pinus nigra* Arn., he observed a process in which plastids were transformed into large inclusions during megagametophyte development and he determined that both plastids and mitochondria of male origin were transferred to the egg at syngamy. Neither the fate of male organelles nor the origin of embryo plastids were determined. During the next thirty years, ultrastructural studies by Camefort and other French researchers reported that plastids are inherited paternally in five gymnosperm families: Pinaceae, Taxaceae, Cephalotaxaceae, Cupressaceae and Taxodiaceae. In the Pinaceae and Taxaceae, mitochondrial inheritance was determined to be biparental whereas, in the Cephalotaxaceae, Cupressaceae and Taxodiaceae, mitochondria were reported to be paternally inherited (Chesnoy, 1987b). Recent studies of mechanisms of organelle inheritance in *Pseudotsuga menziesii* (Mirb.) Franco (Pinaceae) have clearly demonstrated the mechanism of paternal organelle transmission to the proembryo and the origin of egg-cell large inclusions from plastids in the central cell. They also have estimated that about 10% of mitochondria were paternally inherited (Owens and Morris, 1990; 1991). These results are consistent with previous ultrastructural studies of plastid and mitochondrial inheritance mechanisms and agree with paternal inheritance of cpDNA in this species determined by Neale *et al.* (1986).

The present study into the ultrastructure of fertilization in *Pinus monticola* Dougl. was undertaken to describe structural mechanisms of mitochondrial and plastid inheritance in this species. Using RFLPs, White (1990) has determined that cpDNA inheritance in this species is predominantly paternal, but occasionally biparental. Ultrastructural work on other pines supports paternal plastid inheritance but does not explain how biparental cpDNA inheritance may occur (Camefort, 1966b,c). Understanding the process of cytoplasmic inheritance mechanisms operating in conifers is paramount to understanding plant extranuclear genomes and their evolution.

Chapter 2

LITERATURE REVIEW

2.1 Historical Perspective

Much of the life history and reproductive anatomy of *Pinus* and, to a lesser extent, other conifers was documented prior to the middle of the first decade of this century. Mirbel and Spach (1843) pioneered investigations into gymnosperm embryology, including descriptions of proembryo organization in two species of pine. Hofmeister and later Strasburger conducted meticulously detailed and thorough accounts of pollen formation, pollination mechanisms, gametophyte development, fertilization and proembryo development in *Pinus*. Other significant contributions by a number of investigators followed in the late 1800's. This early literature is reviewed in detail by Ferguson (1904) and briefly by Singh (1978).

Scattered works appeared over the next fifty years on aspects of the life cycle of various species of *Pinus*: sporogenesis, gametophyte development, fertilization and proembryo development in *P. longifolia* Salisb. (Sethi, 1928); oogenesis, fertilization and early proembryo development in *P. lambertiana* Dougl. and *P. monophylla* (Torr. and Frém.) Voss (Haupt, 1941); the pollination mechanism in *P. sylvestris* L. (Doyle and O'Leary, 1935); and sporogenesis, gametophyte development, fertilization and proembryo development in *P. virginiana* Mill. (Thomas, 1951). However, with the advent of the electron microscope, literature on ultrastructure of sexual reproduction in *Pinus* and other conifers advanced rapidly. The French researchers, especially Camefort and Chesnoy, initiated research into cytoplasmic inheritance mechanisms. Their studies

throughout the late 1950's and 1960's are reviewed by Chesnoy and Thomas (1971) and Chesnoy (1987a,b). Except for the ultrastructural studies of organelle inheritance mechanisms by Owens and Morris (1990, 1991) in *Pseudotsuga menziesii* (Mirb.) Franco and Bruns and Owens (1989) in *P. monticola*, these works have remained mostly unconfirmed to date. Recently interest in reproductive ultrastructure has undergone a revival largely as a result of technical developments in molecular genetics which allow the determination of patterns of cytoplasmic inheritance using RFLPs, but these techniques do not reveal underlying mechanisms. Mitochondrial inheritance has been studied using RFLPs in several conifer families. In the Pinaceae, *P. taeda* L. (Neale and Sederoff, 1989) and one offspring from the cross of *P. strobus* L. x *P. griffithii* McClelland (Neale and Sederoff, 1988) demonstrated strict-maternal mtDNA inheritance. However, in intraspecific and interspecific matings of *P. contorta* and *P. banksiana*, 92% of offspring demonstrated strict-maternal mtDNA inheritance and mtDNA inheritance in nearly 8% of offspring was strictly paternal (Wagner *et al.*, 1991). In *Sequoia sempervirens* (Taxodiaceae) (Neale *et al.*, 1989) and *Calocedrus decurrens* (Cupressaceae) (Neale *et al.*, 1991) mtDNA appears paternally inherited. CpDNA in angiosperms is most often inherited strictly from the maternal parent but is occasionally biparentally inherited (Connett, 1987). The Pinaceae, Taxodiaceae and Cupressaceae manifest either strict- or predominantly-paternal cpDNA patterns with RFLP techniques. Strict-paternal cpDNA inheritance is found in *Pseudotsuga menziesii* and *S. sempervirens* (Neale *et al.*, 1986; Neal and Sederoff, 1988), whereas in *Pinus monticola* occasional biparental inheritance occurs (White, 1990). Interspecific matings have produced varied

results. Hybrids of reciprocal crosses of *Picea pungens* Engelm. x *P. glauca* (Moench) Voss (Pinaceae) (Stine and Keathley, 1988; Stine *et al.*, 1989) and *Pinus taeda* L. x *P. rigida* Mill hybrids (Neale and Sederoff, 1989) follow a paternal cpDNA inheritance pattern. *Pinus contorta* (female) x *P. banksiana* (male) hybrids inherit cpDNA exclusively from the paternal parent in more than 90% of crosses (Wagner *et al.*, 1987). In reciprocal crosses of *Larix decidua* Mill. x *L. leptolepis* (Sieb. and Zucc.) Endl. (Pinaceae), although paternal cpDNA inheritance is predominant in hybrids, maternal and heteroplasmic patterns also occur (Szmidt *et al.*, 1987).

2.2 Reproductive Phenology and Anatomy

The Pinaceae comprise the largest family within the Coniferales and reproductive phenology and structure vary greatly among genera. Therefore, except for latter stages which directly affect fertilization, this review is largely restricted to the Pinaceae and particularly to *Pinus* and closely related genera. Pines have one of the longest reproductive cycles of the conifers, wherein more than two years may elapse between initiation of reproductive structures and seed shed. Thus initial stages of gametophyte development, pollination and pollen-tube growth are temporally removed by about one year from the events of fertilization. The early stages, which are outside the scope of the present study, are only briefly outlined below. The reader is referred to Singh (1978) and Chesnoy (1987a) for thorough reviews of sexual reproduction in conifers.

2.2.1 Pollen-Cone and Pre-Pollination Microgametophyte Development

Owens and Molder (1977a,b) completed a thorough light microscope investigation of the phenology and anatomy of the development of pollen cones and seed cones and sexual reproduction of *P. monticola* grown on southern Vancouver Island, British Columbia. Pollen-cones of *P. monticola* differentiate from proximal axillary apices within a long-shoot terminal bud (LSTB) in late summer of the season the LSTB develops. Microsporophyll initiation is complete, or nearly so, before November dormancy. Development resumes in April, sporogenous tissue forms and, about one month post-dormancy, meiosis occurs yielding single-cell microspores. Subsequent divisions produce a mature four-cell pollen grain consisting of two sterile prothallial cells, an antheridial (generative) cell and a tube cell. Dehiscence occurs over several weeks in June (Owens and Molder, 1977a,b). This development is typical of north temperate *Pinus* species although pollen may be shed at the four- or five-cell stage (Owens and Blake, 1985).

2.2.2 First-Year Seed-Cone and Megagametophyte Development, Pollination and Early Pollen-Tube Growth.

In *P. monticola*, as in other soft pines studied thus far (Owens and Blake, 1985), seed-cones differentiate from distal axillary apices within a LSTB in April of the season following LSTB development. Bracts and ovuliferous scales are initiated in May just before pollination. Scales support two ovules each and, prior to pollination, deep within each ovule develops a single megaspore-mother cell (MMC).

Pollination in *P. monticola* as in other *Pinus* species occurs via a pollination-drop mechanism. Air currents transfer pollen to the seed cone where the pollination drop may draw several pollen grains to the nucellar surface (McWilliam, 1958). About one month post-pollination, grains may germinate producing pollen tubes which, prior to dormancy, grow partially through the nucellus towards the megagametophyte (Owens and Molder, 1977b).

Pinus nigra pollen tubes grow in a meandering path preceding dormancy (McWilliam and Mergen, 1958). Although branched pollen-tubes have been observed in *P. sylvestris* (Willemse and Linskens, 1969) this was not reported in *P. monticola* (Owens and Molder, 1977b). *In vitro* production of pectinase and cellulase, believed to aid pollen-tube penetration through nucellar tissue, was demonstrated in pollen tubes of *P. austriaca* Hoess (Paton, 1921), *P. sylvestris* (Stanley, 1958; Willemse and Linskens, 1969) and *P. contorta* (Pettitt, 1985). Release of these enzymes from the pollen tube may dissolve adjacent nucellar cell walls, allowing these cells to be pushed aside by the pollen tube, or cause their degeneration. Passage of *Pseudotsuga menziesii* pollen tubes through the nucellus as described by Owens and Morris (1990) is similar to *Pinus*. Pollen tubes growing towards the megagametophyte contact nucellar cells causing them to degenerate. Meiosis of the MMC of *P. monticola* occurs in early July. Only one of the resulting megaspores, the functional megaspore, survives and subsequently undergoes many free-nuclear divisions until late August or September when development ceases (Owens and Molder, 1977b).

2.2.3 Pre-Fertilization Second-Year Megagametophyte and Late Pollen-Tube Development

Free-nuclear divisions within the megagametophyte of *P. monticola* resume in April and continue for two to three weeks. Estimates on the number of nuclei produced are as high as 2000 in *P. strobus* L. (Ferguson, 1904). Free-nuclear divisions cease in early May and cell-wall formation occurs between all nuclei according to the general pattern in conifers proposed by Maheshwari and Singh (1967). Some cells undergo divisions while cell-wall formation continues in others. Over the next month or more the megagametophyte enlarges (Owens and Molder, 1977b).

2.2.3.1 Late Pollen-Tube Development

Following winter dormancy, starch grains and lipids accumulate in the tube cell of *P. sylvestris*. These are subsequently digested as the pollen tube advances through the nucellus. The antheridial cell divides to form a stalk cell with a granular cytoplasm containing few organelles and a body (spermatogenous) cell with abundant osmiophilic plastids, some of which are surrounded by chains of ribosomes, osmiophilic mitochondria and endoplasmic reticulum formations which are likened in section to watch springs by Willemse and Linskens (1969). Although the body cell of *P. sylvestris* lacks a true cell wall, allowing for migration in the pollen tube (Chesnoy, 1987a), some polysaccharide is present between the cell membranes of the body cell and tube cell in *Pseudotsuga menziesii* (Owens and Morris, 1990). The tube nucleus and body cell, commonly accompanied by the stalk cell, enter the pollen tube.

The tube nucleus is believed to regulate tube elongation and, possibly, cellular development (Chesnoy, 1987a). In *P. menziesii*, tube-cell cytoplasm is more vacuolate than body-cell cytoplasm and becomes even more so during pollen-tube growth. Irregular plastids containing starch, mitochondria, smooth endoplasmic reticulum, rough endoplasmic reticulum, dictyosomes and lipid-like bodies are present in tube-cell cytoplasm although many organelles are somewhat degenerate (Owens and Morris, 1990).

In those Pinaceae which have been studied, the body cell enlarges during pollen-tube growth and its nucleus divides, but cytokinesis does not follow, forming two male nuclei (gametes) in a common body-cell cytoplasm. Proximity of the pollen tube to an archegonium at gamete formation varies with species. In *P. monticola* this division occurs one to two weeks before fertilization when the pollen tube is still within nucellar tissue but has almost reached an archegonium (Owens and Molder, 1977b). The body cell in *P. menziesii* is deeply lobed. Cytoplasmic sheets of the body cell project into embayments in the tube cell where cell membranes may attach, providing a mechanism by which the body cell may be pulled through the pollen tube (Owens and Morris, 1990). In *Picea asperata* Mast. cytoplasmic fingers of the cytoplasm formerly belonging to the body cell and containing the two gamete nuclei surround the stalk cell but no membrane fusion between these cells is reported (Camefort, 1978). Male gametes are reported to be of unequal size in *Pinus nigra* and usually the larger nucleus is distal in the pollen tube. The tube nucleus, two gametes contained within the cytoplasm derived from of the

body cell, and the stalk cell are all located at the pollen-tube apex as it approaches the egg (McWilliam and Mergen, 1958).

In the Cupressaceae, organization of male gametes is similar in *Biota orientalis* Endl., *Calocedrus deccurens* and *Chamaecyparis lawsoniana* (A. Murr.) Parl. (Chesnoy and Thomas, 1971). Two gametes are formed within the pollen tube in *B. orientalis* but, in contrast to the Pinaceae, each gamete is cellular and separated from the tube cytoplasm by a plasmalemma. Cytoplasm is organized into three concentric zones. Immediately surrounding the 30-35 μm nucleus is a 0.5-1.0 μm wide 'juxtanuclear' zone which contains sections of endoplasmic reticulum oriented parallel to the nuclear membrane. Outside this zone is a 3-15 μm wide 'deep' zone which is more electron-dense than the other zones due to the presence of many ribosomes. The deep zone contains almost all cell organelles including ribosomes, abundant, elongate mitochondria, each with an osmiophilic matrix and well developed cristae, small amyloplasts with several starch grains, and groups of vesicles. Just inside the plasmalemma is a 2-3 μm wide 'marginal' zone which has infrequent dictyosomes and golgi vesicles along its inner boundary with the deep zone (Chesnoy, 1969a; Chesnoy and Thomas, 1971; Chesnoy, 1973).

2.2.3.2 Archegonial Development

The number of archegonia formed in *Pinus* may vary from one to seven (Lill, 1976) with three to five produced in *P. monticola* (Owens and Molder, 1977b). In the Pinaceae each archegonium is initiated from a superficial, relatively large archegonial initial present at the micropylar end of the megagametophyte. An unequal division of

the archegonial initial produces a smaller, superficial primary neck cell and a larger, internal central cell (Singh, 1978). At central cell formation in *P. monticola*, a single-layered archegonial jacket, differentiated from surrounding megagametophyte cells, is already recognizable. The central cell enlarges over the following one to two weeks. Divisions of the primary neck cell and derivatives are variable in the Pinaceae and, in *P. monticola*, these divisions result in four to six neck cells arranged in one tier (Owens and Molder, 1977b). An archegonial chamber is formed above the neck cells by upward growth of megagametophyte tissue surrounding the neck cells (Singh, 1978).

The cytoplasm of the ventral canal cell, neck cells and the jacket layer does not contribute to the cytoplasm of the embryo and, therefore, does not directly relate to cytoplasmic inheritance. Ultrastructural descriptions of these cells are limited in this review to *Pinus* and closely related genera in the Pinaceae. The central cell and egg cell are intimately involved in cytoplasmic inheritance as they ultimately contribute to the embryo. Development and structure of these cells is expanded to include comparisons with other conifer families.

2.2.3.3 Central Cell

Central-cell ultrastructure has been described in several conifers. Camefort (1960, 1962, 1965a) investigated the growth and maturation of the central cell of *P. nigra* to determine the origin of cytoplasmic inclusions described previously in the egg (Camefort, 1959a). Historical interpretations were that inclusions were vacuolar in

origin, termed 'granules' or 'proteid vacuoles' by early authors (Ferguson, 1904) and 'vitellus' by Mangenot (1938).

The young central cell of *P. nigra* possesses an electron-translucent cytoplasmic matrix. Dictyosomes, few mitochondria with short tubules, endoplasmic vesicles, and plastids containing few lamellae, osmiophilic inclusions and, rarely, a starch grain are clustered in the peripheral cytoplasm or near the apically-situated nucleus. The density of the peripheral and perinuclear cytoplasm, afforded by the presence of these organelles, contrasts with the internal cytoplasm which is largely electron-transparent and traversed only occasionally with traces of fibrillar cytoplasm. Previously this transparent central area was believed to consist of a large vacuole but the presence of true vacuoles is doubted by Camefort who largely attributes the "voids" in the cytoplasm to the absence of ribosomes and large organelles (Camefort, 1965a).

During rapid growth of the cental cell of *P. nigra*, its dimensions increase ten to twelve times and the cell ultimately reaches 600 μm in length by 300 μm in width. The amount of cytoplasm has been estimated to increase approximately 1000-1700 times and becomes highly organized (Camefort, 1965a). Mitochondria increase in number, often appearing elongate with constrictions which may suggest fragmentation. Some mitochondria become branched. However, Willemse (1974) was unable to identify mitochondria in developing central cells of *P. sylvestris*. Camefort (1962, 1965a) reports that in *P. nigra* a system of single membranes increases, especially in the centre of the cell, conferring a false vacuolar appearance. In section some of these membrane contours are interrupted, allowing continuity between the cytoplasmic area partially

encircled and the general cytoplasm and, rarely, mitochondria and dictyosomes are found within them. Cytoplasm within these simple contours remains electron-translucent. This has often been termed the "foam stage" of archegonial development (Singh, 1978). Formation of small inclusions and the subsequent deformation of plastids further defines this period of central-cell development (Camefort, 1962; 1965a).

The central cell or egg cell of all gymnosperms studied thus far develop numerous small inclusions rich in phospholipids (Singh, 1978; Willemse, 1974; Chesnoy, 1987a). In *P. nigra* central cells, small inclusions measuring 4-5 μm arise from layers of endoplasmic reticulum which appear crescent-shaped and are circular to elongate in section. This orientation partially isolates nodules of cytoplasm which remain connected to the general cytoplasm by short 'peduncles'. Initially the two endoplasmic reticulum membranes remain appressed to each other and the included cytoplasm increases in density (Camefort, 1965a; 1968b).

Large inclusions ultimately measuring 30-50 μm originate from the plastids in the young central cell. In early stages of growth, plastids have a clear matrix with few lamellae and osmiophilic inclusions. In section some plastids appear elongate and to partially encircle a segment of cytoplasm. During plastid development, cytoplasm may be included in several places by localized invagination, creating several cytoplasmic compartments which may only communicate with the general cytoplasm through narrow channels. Accompanying cellular growth the included cytoplasm, which may contain organelles, and the transformed plastids increase in size to constitute the large inclusions of the mature central cell. The plastid nature of these complex formations remains

distinguishable by the presence of a double membrane encircling the various compartments and delimiting itself from the general cytoplasm, whereas the osmiophilic inclusions and lamellae characteristic of young plastids disappear (Camefort, 1963; 1965a; 1968b). Egg cell plastids of *Cephalotaxus drupaceae* Sieb. and Zucc. (Cephalotaxaceae) show an identical development of plastids as described in Pinaceae (Chesnoy, 1987b; Gianordoli, 1974b).

Maturation of the central cell of *P. nigra* is marked by several other cytoplasmic changes. At the end of central cell enlargement the simple membrane systems disappear, liberating their cytoplasmic content which becomes indistinguishable from general cytoplasm. Neither the origin nor function of the single membranes was determined. The reticulum membranes of the small inclusions often separate, creating slightly-inflated, electron-transparent vacuoles which stain for acidic phosphates. Thus the small inclusions appear to have an autophagic function which is triggered following fertilization (Chesnoy, 1987a,b). At the conclusion of central-cell development most cytoplasm is compartmentalized either within small or large inclusions. In both instances the electron density of included cytoplasm increases, commonly more so than does the remaining general cytoplasm and, within both, cytochemical tests show increased production of proteins and phospholipids (Camefort, 1965a).

In the central cell of *Larix decidua* mitochondria are not dispersed but rather assemble under the nucleus (Camefort, 1967a). Although earlier studies of *Pseudotsuga menziesii* (Thomas and Chesnoy, 1969; Chesnoy and Thomas, 1969) report a similar

aggregation of mitochondria near the central-cell nucleus, Owens and Morris (1990) found this to occur in the young egg cell during nuclear migration.

In members of the Cupressaceae and Taxodiaceae much of the volume of the central cell is comprised of a large, central vacuole. Plastid-derived large inclusions are not formed. In *Biota orientalis* (Cupressaceae) mitochondria collect below the nucleus, which is positioned next to the micropyle, and in the basal cytoplasm. Some appear crescent-shaped and partially enclose a portion of cytoplasm (Chesnoy, 1969a,b; Chesnoy, 1971). In *Juniperus communis* L. (Cupressaceae) one or more organelle associations are formed consisting of radially-oriented mitochondria and plastids organized around cytoplasm rich in ribosomes and microtubules. Chesnoy (1967) referred to these associations as 'asteroids' and suggested they represent centres of organelle multiplication. Mature central cells contain electron-opaque, elongate plastids with few lamellae and which are associated with parallel layers of endoplasmic reticulum. In *B. orientalis*, plastids are often grouped (Chesnoy, 1967; 1969a,b).

2.2.3.4 Egg Cell

Just prior to fertilization, in early June in *P. monticola*, a transverse, unequal division of the central cell establishes a large, internal egg cell surmounted by a very small ventral canal cell below the archegonial neck cells (Owens and Molder, 1977b). In *Pinus* the egg cell inherits the majority of central-cell cytoplasm which is dominated and compartmentalized by large and small inclusions and contains numerous mitochondria, vesicles originating from the endoplasmic reticulum and occasional

dictyosomes. Groups of ribosomes are abundant in the ubiquitous small inclusions. Large inclusions line the cell periphery. Their stroma lack starch and the included cytoplasm may become more dense than the general cytoplasm, often supporting various organelles along with small inclusions (Camefort, 1959a; 1965a). Mitochondria of *P. nigra* were reported to be scattered throughout the egg and were not organized into a perinuclear zone. Mitochondria appear circular or slightly elliptical in section and 0.5-0.7 μm in diameter with rare or absent cristae (Camefort, 1966a; 1968b). Mitochondria originally clustered against the egg nucleus in *P. sylvestris* disperse throughout the cytoplasm during egg maturation (Willemse, 1974). In *P. monticola*, Bruns and Owens (1989) observed a narrow perinuclear zone dominated by egg-cell mitochondria. The egg nucleus enlarges in its immediate descent to the centre of the egg where it remains until fertilization. The young egg nucleus of *P. sylvestris* is lobed but with maturation becomes more rounded. Nucleoli increase in number and then decrease prior to fertilization (Willemse, 1974). In *P. nigra* nucleoli were believed to number 200-300 (Camefort, 1964; 1968b). The egg nucleus of *P. nigra* measures about 100 μm (Camefort, 1959a) while in *P. pinaster* Soland. it is ovoid, 120-150 μm in length with an irregular outline and is sometimes more invaginated at the superior pole where the male gamete will fuse at fertilization (Mangenot, 1938). Willemse (1974) noted in *P. sylvestris* that mitochondria, small inclusions, lipid droplets and endoplasmic reticulum are located next to the nuclear periphery and that the length of reticulum strands increase. The appearance of a receptive vacuole just below the ventral canal cell prior to pollen-tube arrival is common (McWilliam and Mergen, 1958).

Mitochondria which collect below the central-cell nucleus in *Larix decidua* and below the central- or egg-cell nucleus in *Pseudotsuga menziesii*, immediately precede the egg nucleus in its migration deeper into the egg cytoplasm. During descent these mitochondria surround the egg nucleus (Camefort, 1967a), while others previously scattered throughout the cytoplasm also move to the nuclear periphery (Thomas and Chesnoy, 1969; Owens and Morris, 1990). Together these maternal mitochondria, along with microtubules in *L. decidua* (Camefort, 1967a,b) and small vesicles and ribosomes in *P. menziesii* (Owens and Morris, 1990), constitute a perinuclear zone, which is 6-7 μm thick in *P. menziesii* and 4-6 μm in *L. decidua*, and stains positive for mtDNA using the Feulgen reaction. Thus the perinuclear zone of mitochondria results from migration of organelles rather than their multiplication (Camefort, 1967a,b; Chesnoy and Thomas, 1969). Perinuclear mitochondria in *L. decidua* and *P. menziesii* are described as practically devoid of cristae and largely electron-transparent (Camefort, 1967a; 1969; Thomas and Chesnoy 1969). Owens and Morris (1990) report that perinuclear mitochondria in *P. menziesii* lack distinct cristae but are very electron-dense. In *Cedrus atlantica* (Endl.) Carr. (Pinaceae) mitochondria are distributed throughout the egg cell (Camefort, 1960).

Many egg cells, which are smaller than those of the Abietaceae, are contained within a single archegonial jacket and share a single neck in the Cupressaceae and most Taxodiaceae. The egg nucleus is situated in the upper half of the cell and is subtended by a large, central vacuole which decreases in size or may disappear during maturation. In *Biota orientalis* small inclusions are peripheral whereas numerous spherical

mitochondria are dispersed in the egg cytoplasm. Occasionally mitochondria encircle a small portion of cytoplasm as was observed in the central cell. Small vesicles abound in the micropylar cytoplasm and around the egg nucleus. Large inclusions are not present in the Cupressaceae and Taxodiaceae. Filamentous plastids may reach 15-20 μm in length in *B. orientalis* and are commonly grouped (Chesnoy, 1969a; 1977). Plastids contain large starch grains and in both *B. orientalis* and *Chamaecyparis lawsoniana* (A. Murr.) Parl., plastids are intimately associated with layers of endoplasmic reticulum (Chesnoy and Thomas, 1971; Chesnoy, 1973). In *C. lawsoniana*, the majority of plastids are located in the cytoplasm of the superior portion of the egg and against the egg nucleus. Mitochondria tend to cluster in pockets of cytoplasm free of small inclusions. In *Juniperus communis* small inclusions surround the mitochondria-plastid associations (Chesnoy, 1967). In the mature egg of *Sciadopitys verticillata* (Thunb.) Sieb. and Zucc. (Taxodiaceae), mitochondria appear degenerate and some plastids develop a granular stroma and may become porous (Gianordoli, 1974a).

2.2.3.5 Ventral Canal Cell

Ultrastructure of the ventral canal cell prior to pollen-tube entry has not been described in *Pinus*. In *Pseudotsuga menziesii* the ventral canal cell is lens-shaped and consists of a large, finely-granular nucleus surrounded by a thin, parietal layer of cytoplasm. A narrow cell wall is present adjacent to the neck cells and, possibly, bordering the egg (Owens and Morris, 1990).

2.2.3.6 Neck Cells

Neck cells are believed to function in delivery of the pollen tube to the egg (Singh, 1978). Variation occurs in neck-cell number, arrangement, size and shape. Scant information exists regarding neck-cell ultrastructure in conifers. In *Pinus virginiana* nuclear divisions may not always be followed by cytokenesis (Thomas, 1951). In *Pseudotsuga menziesii* neck cells have secretory characteristics. Plastids with starch grains, mitochondria, lipid bodies, numerous small vacuoles, rough endoplasmic reticulum and smooth endoplasmic reticulum are contained in the cytoplasm bordering the archegonial chamber. The inner layer of a thick, fibrillar cell wall is undulate adjacent to the archegonial chamber and is possibly the site of cytoplasmic secretions (Owens and Morris, 1990).

2.2.3.7 Jacket Cells

Cells forming the jacket layer of the *Pinus* archegonium (follicle cells of some authors) divide frequently and synchronously to match central-cell enlargement (Singh, 1978). The jacket layer is implicated in egg/proembryo nutrition via enzymatic secretions which are believed to solubilize storage products in surrounding gametophyte cells and these products are thought to be translocated into the archegonium via plasmodesmata and simple pits in the inner tangential walls of jacket cells (Singh, 1978). Cell wall deposition is greater on the central/egg cell side of this boundary. As a result of pit membrane breakdown, probably during specimen preparation, elements of jacket cells including mitochondria, plastids, dictyosomes, endoplasmic reticulum and nuclei

have been reported to pass into the egg (Francini Corti and Maugini, 1964; Willemse, 1974; Chesnoy, 1987a).

P. sylvestris jacket-cell ultrastructure is described by Willemse (1974). During the earliest stages of archegonial development, jacket cells contain large nuclei, their membranes supporting occasional blebs, and the cells are highly vacuolate with few organelles. In jacket cells surrounding the developing central cell, vacuoles disappear and the cytoplasm contains many vesicles, rough endoplasmic reticulum, ribosomes, dictyosomes, plastids and few mitochondria. Mitochondria and plastids multiply. Plastids group together and simultaneously divide, often appearing in section elongated and bowed with constricted centres. Mitochondria with short cristae contain electron-transparent regions. At egg-cell maturation, jacket-cell cytoplasm contains the full compliment of organelles observed during central-cell development but the nuclear membrane lacks blebs. The occurrence in jacket cells of restitution nuclei: nuclei which have undergone endoduplication but which have failed to divide, has been reported in *P. sylvestris* (Singh, 1978).

2.2.4 Fertilization and Early Proembryo Development

The number of archegonia fertilized within an ovule is variable. Commonly only one archegonium is fertilized during the first two weeks of June in *P. monticola* and unfertilized archegonia subsequently abort. In the Pinaceae and Cephalotaxaceae and in other genera with separate archegonia such as *Taxus* and *Sciadopitys*, male gametes are released directly into the archegonium. In *P. monticola*, one pollen tube grows between

the neck cells, penetrates the ventral canal cell and ruptures when it enters the egg, releasing its contents into the egg's superior pole where several receptive vacuoles are positioned (Owens and Molder, 1977b). Neck cells then degenerate (Singh, 1978). Presence of the pollen tube causes neck-cell degeneration in *Pseudotsuga menziesii* (Owens and Morris, 1990; 1991).

Although prior to 1900 it was reported that the *Pinus* stalk cell, tube nucleus and tube cytoplasm are released from the pollen tube along with the body-cell cytoplasm and two gamete nuclei (Ferguson, 1904), it was not until 1938 that Mangenot identified body-cell-derived mitochondria in the egg cytoplasm near the site of tube entry. Egg cytoplasm becomes finely vesicular in the presence of pollen-tube contents. Mangenot (1938) reported that in *P. pinaster* the functional male nucleus, devoid of cytoplasm, immediately moves to the egg nucleus to fuse with it while the male mitochondria, non-functional male nuclei, stalk cell and male cytoplasm containing starch grains remain near the site of entry. There they are reabsorbed during proembryo development, but the non-functional male nuclei and stalk cell may undergo division prior to reabsorption. Camefort (1966a, 1968b) confirmed these observations in *P. nigra*.

Structural differences are noted between maternal organelles and paternal organelles discharged from the pollen tube in *P. nigra*. Male plastids are either grouped around the male gametes or scattered in the male cytoplasm. Scattered plastids are elongate with a largely-transparent matrix and substantial lamellar development. Equidistant between these plastids is a compact grouping of ribosomes which sometimes forms a helix. Male plastids grouped around the gametes are smaller and have a dense

matrix with reduced thylakoid development. In contrast to both types of male plastids, egg-cell plastids exist only as large inclusions. Male mitochondria are also easily distinguished from those of the egg as they are larger, electron-dense and contain many, long cristae while those of the egg are electron-translucent with occasional, short cristae. Neither plastids nor mitochondria of male origin were believed to contribute to the cytoplasm of the embryo (Camefort, 1966a).

Upon approach of the fertilizing male gamete, a depression is formed in the egg nucleus where it will fuse with the male. The male gamete becomes lenticellular and settles into this depression. In several places within the region of contact between nuclei, the membranes of male and female fuse, bridging the nuclei. 'Islets' of included egg cytoplasm are reported inside the zygote nucleus (Camefort, 1965b), but the fate of this cytoplasm was not determined. Mangenot (1938) observed that only maternal mitochondria surround the fusing nuclei and this was confirmed by Camefort (1966a).

Immediately following fusion, the *Pinus* zygote nucleus enters the first of two successive divisions which have been described in detail (Haupt, 1941; McWilliam and Mergen, 1958; Camefort, 1965b). Up to 75% of the zygote nucleoplasm is released at the end of anaphase of the first nuclear mitosis in *P. nigra* (Camefort, 1965c, 1968b). This nucleoplasm, termed neocytoplasm by Camefort (1958), becomes organized throughout subsequent development to constitute the proembryo cytoplasm and functions to shield the proembryo from lysis (Camefort, 1969).

Upon release, the neocytoplasm of *P. nigra* remains distinct from the egg cytoplasm and, following telophase, encompasses both nuclei of the coenocytic

proembryo affording a degree of autonomy for the proembryo. Although as yet largely unstructured, two zones become distinguishable in the neocytoplasm of binucleate proembryos. A deep zone immediately surrounds the nuclei and contains microtubules probably originating from the spindle (Camefort, 1965c,d), endoplasmic vesicles and saccules, ribosomes which are often helically-arranged and are considered a product of nucleolar disorganization and rare dictyosomes and mitochondria (Camefort, 1965c). The marginal zone of neocytoplasm borders the egg cytoplasm and is characterized by extensive endoplasmic reticulum development, derived partially at least from the zygote nuclear membrane (Camefort, 1965c,d). Nucleolar remnants, mitochondria and scattered dictyosomes also occur. Camefort originally presented arguments for neo-/*de novo* formation of both dictyosomes and mitochondria from vesicles in the neocytoplasm but, although he found no evidence supporting the influx of these organelles from the surrounding egg cytoplasm, he did not negate this possibility. Organelles of undetermined identity were also reported in the neocytoplasm (Camefort, 1962; 1965c; 1968b). Nuclei of *P. nigra* grow to 50 μm diameter prior to the second division (Camefort, 1965c).

Simultaneous division of both nuclei produces a four-nucleate coenocytic proembryo. All nuclei become engulfed by neocytoplasm. Smooth endoplasmic reticulum is abundant throughout all neocytoplasm of *P. nigra,* thus obliterating the distinction between deep and marginal zones, and microtubules surrounded by many ribosomes possibly correspond to the 'fibrils' of early authors (see Ferguson, 1904). Disorganization of nucleolar remnants is apparent (Camefort, 1965c). Many osmiophilic vesicles of various sizes (Camefort, 1961a,b) and few dictyosomes (Camefort, 1965c) are

observed throughout the cytoplasm, however numbers of mitochondria may vary. Whereas numerous mitochondria were initially present at this stage (Camefort, 1961b), only a few were identified in subsequent studies (Camefort, 1965c; 1969).

All four nuclei encased in neocytoplasm migrate towards the chalazal pole of the egg. During nuclear migration in *P. nigra*, portions of egg cytoplasm may become incorporated into the neocytoplasm. In all cases, incorporated cytoplasm is comparable in extent of degeneration to cytoplasm in the rest of the egg. At this developmental stage, it is unclear whether the neocytoplasm of the proembryo contains undifferentiated plastids (Camefort, 1966b).

Nuclei settle into a single layer at the base of the archegonium. Neocytoplasm accumulates, now dominated by mitochondria which in size and appearance closely resemble those of the egg. They are 0.5-0.7 μm in diameter in *P. nigra* with a transparent matrix containing poorly-developed cristae and, frequently, an irregular osmiophilic inclusion. Free ribosomes are abundant throughout all neocytoplasm whereas nucleolar remnants have mostly disorganized. Microtubules are rare or absent. Proplastids appear in the neocytoplasm close to the nuclei and begin differentiation. They are 1.0-1.5 μm in diameter, circular or elongated in profile, with well-developed lamellae. Origin of plastids is uncertain. Camefort does not support neoformation of plastids from nuclei as proposed by Bell *et al.* (1966) and questions if some of the rudimentary egg organelles, previously incorporated into the neocytoplasm and identified as mitochondria, might not be undifferentiated plastids which can only be correctly identified at this later stage due to their differentiation (Camefort, 1966b).

Ultrastructure of fertilization in *Larix decidua* was studied by Camefort (1967a,b; 1968a; 1969) and found to parallel *Pinus* in most respects. One of the principle differences relates to the existence and behaviour of egg-cell mitochondria in the perinuclear zone. The perinuclear zone is traversed by the male gamete in its' course to the egg nucleus. Once nuclear fusion has occurred, the egg-cell mitochondria quickly re-surround the zygote nucleus and then penetrate into the neocytoplasm at its formation during the first zygotic division. Neocytoplasm in *Larix* is formed then from the nuclear sap of the zygote and from the perinuclear zone of egg-cell mitochondria. In *Larix* as in *Pinus*, body-cell organelles released from the pollen tube are different in size and appearance from egg plastids and mitochondria and, prior to nuclear fusion, are only observed at the superior pole of the egg. In contrast to the plastid-derived large inclusions of the egg, body-cell plastids measure only 1.0 μm and are electron-opaque with few lamellae. Body-cell mitochondria are spherical with a dense matrix and many cristae whereas egg-cell mitochondria of the perinuclear zone have no cristae and are largely electron-translucent.

In *Larix* binucleate proembryos, a compact group of body-cell plastids and mitochondria is located just outside the boundary of the neocytoplasm in the maternal cytoplasm above the proembryo nuclei. Following the second nuclear division of the zygote, these male organelles are located alongside the neocytoplasm containing the four nuclei or, alternatively, below it. In either of these positions paternal organelles accompany the four nuclei to the archegonial base. At the start of nuclear migration, microtubules which appear in the neocytoplasm after the second mitotic division become

organized in clusters and are oriented in the direction in which the nuclei will proceed (Camefort 1969).

In *Larix*, when all four nuclei have reached the chalazal pole of the archegonium, the cluster of male organelles is within the neocytoplasm, possibly becoming incorporated during nuclear descent. Following the first mitosis which creates two tiers of four cells each, male-derived organelles are dispersed in the neocytoplasm. Some of the plastids contain starch grains and the paternal mitochondria increase in length and thickness and have developed cristae. Maternal mitochondria are also present. The fate of the two morphologically distinct types of mitochondria was not followed (Camefort, 1969).

The pollen tube of *Pseudotsuga menziesii* gains entry into the egg cell by a narrow extension of the pollen tube which grows between the neck cells and through the ventral canal cell in advance of male gamete release. Behaviour of male and female nuclei and organelles during fertilization are similar to descriptions of other Pinaceae with the following exceptions. Prior to nuclear fusion, the egg nucleus develops deep embayments containing perinuclear cytoplasm and, frequently along the nuclear membrane of the fertilizing male nucleus, the two membrane layers separate and the inner layer forms channels into the male nucleus. From fusion until the first free-nuclear division of the zygote, a small cluster of paternal organelles is distinguishable at the depression where the male nucleus fused with the female. As in *Larix*, clustered male organelles follow the zygotic free-nuclei to the chalazal pole but in *Pseudotsuga*, male organelles remain discrete from female cytoplasm during nuclear descent and disperse only once nuclei settle at the archegonial base. While small inclusions are commonly

incorporated into the neocytoplasm at this stage, only occasionally are large inclusions observed (Owens and Morris, 1991).

In the Cupressaceae and in members of Taxodiaceae in which archegonial complexes are formed, the two male gametes are large cells with abundant cytoplasm. These are released above the neck cells which dissociate. Upon entrance of the male gamete into an egg in *Biota orientalis* (Cupressaceae), the marginal zone of cytoplasm is shed while the male nucleus surrounded by the juxtanuclear and deep cytoplasmic zones proceeds to the egg nucleus. As in the Pinaceae, the male nucleus is reported to settle into a depression in the egg nucleus. During membrane fusion the two nuclei turn 180 degrees. The cytoplasm of the juxtanuclear and deep zones comes to lie above the fusing nuclei and gradually surrounds the fusing nuclei, separating them from the egg cytoplasm. The zygote nucleus immediately divides, liberating nucleoplasm which is delimited by nuclear membrane fragments. The male cytoplasm around the zygote nuclei and released nucleoplasm remains cohesive and it is extremely rare that female organelles enter this cytoplasm (Chesnoy, 1977; 1969a,b).

The two zygote nuclei increase in size and the nucleoplasm which encompasses them is invaded by the male organelles so that the discrete halo of nucleoplasm is obliterated or, at least, greatly reduced. Male amyloplasts remain peripheral whereas mitochondria and ribosomes immediately surround the nuclei. These nuclei migrate, accompanied by the envelope of paternal cytoplasm, to the base of the archegonium and, concurrently, egg cytoplasm rapidly lyses beginning in the vicinity of the developing proembryo. During descent, a few maternal mitochondria may enter the proembryonic

cytoplasm where maternal plastids were never observed (Chesnoy, 1969a,b; Chesnoy and Thomas, 1971).

Fertilization in *Chamaecyparis lawsoniana* is essentially the same as in *B. orientalis* (Chesnoy, 1973). In *C. lawsoniana*, division of the zygote nucleus occurs during migration to the base of the archegonium. Proembryo cytoplasm contains both amyloplasts which are not associated with endoplasmic reticulum formations and mitochondria which, in size and appearance, resemble those of the male. Neither plastids nor mitochondria characteristic of egg cytoplasm were found in the proembryo, leading Chesnoy to propose that all plastids and mitochondria are inherited paternally, but the possibility of a reduced female contribution was not excluded (Chesnoy, 1973).

Small inclusions are a universal feature in the central cell and egg cell cytoplasm in all gymnosperms studied. First formed in the young central cell, they accumulate and eventually become so numerous they constitute the greatest proportion of female cytoplasm. Triggered by the arrival of the male gamete(s), acid phosphatase is released from the vacuole which caps the cytoplasmic nodule and this initiates a rapid lysis of female cytoplasm (Camefort, 1966c).

Ultrastructure and cytochemical analysis of egg cytoplasm lysis in *P. nigra* was studied by Camefort. Coinciding with the appearance of neocytoplasm during the first coenocytic division, small inclusions in the egg cytoplasm bordering the neocytoplasm acquire 'dense granulations' and become flocculent, indicating disorganization. In the four-nucleate proembryo prior to nuclear migration, this process is also observed in many small inclusions throughout the egg and in the cytoplasmic fraction of large inclusions.

Initially enzymatic staining for acid phosphatase is intense in many but not all of the small inclusions, often affecting several inclusions in close proximity while others appear unaffected. Following arrival of the four free nuclei at the chalazal pole of the egg cell, both in the included and general cytoplasm, degeneration is advanced. Included cytoplasm is translucent and greatly altered from that of the non-fertilized egg whereas the general cytoplasm appears flocculent. Any maternal cytoplasmic fractions incorporated into the neocytoplasm during nuclear migration are likewise disorganized. Degeneration of egg cytoplasm continues during tier formation in the proembryo (Camefort, 1966c).

Chapter 3

MATERIALS AND METHODS

Second-year seed cones were collected in the spring of 1987 and 1988 from four open-pollinated trees growing in Victoria, British Columbia, Canada. Generally only one or two cones were removed per tree on each collection date. In 1987 collections were made weekly from late April through May, daily until mid-June and weekly from mid-June until early July. In 1988 collections were made biweekly from late April until mid-May, daily from mid-May until the end of May and biweekly to mid-June. Only data from 1988 were used to determine timing and duration of reproductive stages because 1987 results were incomplete and the phenology was abnormally advanced due to mild climatic conditions.

Ovuliferous scales were removed from the central two-thirds along the length of the cone axis and from these approximately fifteen ovules were dissected. Longitudinal cuts were made with a double-edge razor blade on two opposing sides of the micropyle. Peripheral megagametophyte tissue and the chalazal half to two-thirds of the ovule were removed. The small ($<$ 1mm thick) slice of the ovule thus obtained was a distal, median section of megagametophyte tissue containing the archegonia and a portion of the nucellus.

In 1987, samples from each cone were embedded in either Spurr's low-viscosity resin (Spurr, 1969) or Luft's Epon 812 (Luft, 1961). In 1988 all samples were embedded in Spurr's resin because of superior tissue infiltration. For embedding in Epon, specimens were fixed under vacuum for two hours in 4% glutaraldehyde in 0.1

M sodium cacodylate buffer (Ph 7.2) at room temperature, buffer-washed with four changes over one hour, post-fixed in 1% osmium tetroxide for one hour, dehydrated in a graded acetone series for two hours, and infiltrated with propylene oxide overnight or longer on a rotator before embedding in Epon at 60°C for eighteen hours. For Spurr's embedding, specimens were fixed under vacuum for two hours to overnight in cold 2.5% glutaraldehyde and 2% formaldehyde in 50 mM potassium phosphate buffer (Ph 7.2), buffer-washed with four changes over one hour, post-fixed in 1% buffered osmium tetroxide for one hour, buffer-washed overnight and dehydrated in a graded acetone series prior to embedding in Spurr's at 60°C for eighteen hours.

Thick sections (900nm - 1μm) for light microscopy and thin sections (60-70nm) for electron microscopy were cut on a Reichert Om2 or Sorvall MT 5000 ultramicrotome using glass and diamond knives. Thick sections were stained with Richardson's stain (Richardson *et al.*, 1960) and examined and photographed on a Leitz Orthoplan large-field microscope with a Vario-Orthomat camera. Compression of the thin sections from the cutting process was corrected by passing over the sections with a swab dipped in acetone. Sections which were silver-grey became silver by this process. Silver sections were collected on uncoated mesh or notch-dot grids, cleaned with chromic acid and coated with either 0.6% Formvar in 1,2-dichloroethane or 1.0% Parlodion in amyl acetate. Sections were stained with 5% uranyl acetate for ten to fifteen minutes followed by 0.2% lead citrate (Hayat, 1981) for two minutes and then examined and photographed on a Phillips EM300 or JOEL JEM-1200EX transmission electron microscope at 60 kV.

Chapter 4

OBSERVATIONS

4.1 Late Pollen-Tube Development

Most pollen tubes reached the megagametophyte during the last two weeks of May. All pollen tubes were unbranched. In pollen tubes which had fully penetrated the nucellus and reached the megaspore cell wall the body cell, or the cytoplasm derived from the body cell containing the two male gametes, was surrounded by tube-cell cytoplasm which filled the pollen tube, and was located well behind the tube nucleus positioned at the pollen tube tip (Fig. 1). In only one instance was the stalk cell observed and it lay distal to the body cell and was partially surrounded by it (Fig. 2).

4.1.1 Tube Cell

Tube-cell cytoplasm was highly vacuolate. Vacuole contents were mostly electron-transparent or, rarely, flocculant. Tube cytoplasm near the body cell or male gametes contained, in addition to vacuoles, abundant osmiophilic mitochondria, dictyosomes, rough and smooth endoplasmic reticulum and spherical lipid-like bodies. No starch was present and plastids were rare or absent (Fig. 3). In the tube-cell cytoplasm proximal and distal to the body cell, scattered large starch grains were present either as free grains or as one to several grains within a plastid. All plastids were electron-translucent and irregular. Lipid-like bodies and organelles that were abundant in the tube-cell cytoplasm near the body cell or male gametes were less abundant in peripheral regions (Fig. 4). The tube nucleus with a single, large nucleolus was

positioned near the tip of the pollen tube. The nuclear margin was irregular and cytoplasm at the tip was more electron-dense and flocculent than in more proximal areas of the pollen tube. Many of the vacuoles had broken tonoplasts, probably as a result of damage during specimen fixation.

4.1.2 Stalk Cell

The stalk cell was observed only once in an advanced pollen tube where it was partially surrounded by projections of distal body-cell cytoplasm. The stalk-cell nucleus was roughly spherical and small compared with the nucleus of the body cell. The plasmalemma had lost its integrity, the cytoplasm was degenerate and organelles were difficult to identify, probably due to fixation (Fig. 2).

4.1.3 Body Cell and Male Gametes

Before gamete formation the large, roughly-spherical, body-cell nucleus was proximal in the cell, measuring about 44 μm across. Occasionally along the nuclear margin, short and sometimes branched projections of the nucleus extended into surrounding cytoplasm. The nuclear matrix was slightly more electron-dense than the cytoplasm and many small chromatin particles were scattered within it (Fig. 2).

The two gamete nuclei, resulting from division of the body-cell nucleus, remained proximal in the cytoplasm, one preceding the other (Figs. 1, 5). Both nuclei were irregular in shape, measuring about 34 μm by 20 μm, and had nuclear projections similar to those observed in the body-cell nucleus (Figs. 3, 5). Each nucleus contained many

small chromatin particles and, just inside its periphery, varying amounts of electron-opaque structures which appeared to be double-membrane segments associated with ribosomes (Fig. 6).

In both the body cell and in the body-cell cytoplasm around the male gametes, organelles tended to cluster. Mitochondria were more numerous than plastids. Most mitochondria were spherical to slightly ovoid, measuring about 0.47 μm in diameter to about 0.77 μm by 0.33 μm, and were more osmiophilic than plastids (Figs. 7, 8). Rarely, mitochondria were electron-opaque and disc-shaped, appearing in section elongated with constricted centres. Plastids were elongate and lacked starch, measuring about 2.3 μm by 0.51 μm, and were of approximately the same electron-density as the cytoplasm (Figs. 7, 8). Thylakoid development was rudimentary. Cytoplasm immediately surrounding both types of organelles was rich with polyribosomes either associated in small clusters or in helicoid files which tended to encircle organelles (Fig. 7). Throughout the general cytoplasm smooth endoplasmic reticulum was abundant, occasionally with dilated membranes. Sheets of smooth endoplasmic reticulum were oriented roughly parallel to the plasmalemma and lined much of the cell periphery. Vacuoles, mostly with electron-translucent contents, were scattered in a cytoplasm studded with groups of small vesicles. Vesicles were especially common against the plasmalemma. Rough endoplasmic reticulum, dictyosomes and lipid-like bodies were rare.

The body cell and the cytoplasm containing the male gametes were elongate. Long projections of the distal, body-cell cytoplasm which contained the male gametes

extended into embayments in the tube cell, increasing surface area contact and intertwining them. Parallel sheets of smooth endoplasmic reticulum and clusters of mitochondria and plastids were common in these projections (Fig. 5).

4.2 Second-Year Megagametophyte Development

4.2.1 Free-Nuclear Megagametophyte

Mid-April was characterized by free-nuclear divisions in the megagametophyte. Free nuclei (Fig. 9) were peripheral, bordering the megaspore-cell wall, while the centre of the megagametophyte consisted of a large, electron-transparent vacuole. Fixation prior to cellularization was variable, even within a single megagametophyte, but was generally best next to the megaspore-cell wall.

Cytoplasm was dominated by plastids which varied in shape. Plastids surrounding some nuclei contained a single starch granule and thylakoid membranes and commonly measured 2.9 μm by 0.95 μm (Fig. 10), while in other areas plastids rarely contained starch, thylakoid membranes were reduced and plastids were more electron-dense and commonly disc-shaped. In section these latter plastids appeared severely constricted about mid-way along their length and measured about 2.8 μm in length (Fig. 9). Occasionally disc-shaped plastids became concave and more or less encircled a portion of surrounding cytoplasm (Fig. 9). Both rough endoplasmic reticulum and smooth endoplasmic reticulum development was extensive. Reticulum membranes were distended. Many small vesicles were present throughout the cytoplasm and numerous polyribosomes were associated in small clumps or files. Scattered dictyosomes and lipid-

like bodies were present. Mitochondria were impossible to distinguish from plastids. At the onset of cell-wall formation in late April, all plastids were more electron-dense and plastids which had at least partially engulfed a portion of cytoplasm were more common (Fig. 11).

4.2.2 Central Cell

Three to five central cells, each capped by a primary neck cell, were formed at the micropylar end of the megagametophyte by unequal divisions of archegonial initials. Immediately following their formation in late April or early May, central cells began to enlarge. This continued for about two weeks with central cells ultimately measuring about 620 μm by 390 μm. Shape varied with position in the megagametophyte. Internal central cells were oblong whereas peripheral central cells were commonly kidney-shaped.

Most of the early central cell was comprised of a large vacuole. Numerous small vacuoles were contained within the narrow peripheral band of cytoplasm appressed to the plasmalemma and surrounding the nucleus (Fig. 12). Plastids were numerous and similar to those observed in the free-nuclear megagametophyte. Most plastids were disc-shaped with constricted centres and were more electron-dense than the general cytoplasm. Thylakoids were often distended (Fig. 13). Occasionally plastids which had engulfed a portion of cytoplasm were observed. Smooth endoplasmic reticulum was extensive and, in many places, membranes were distended. Small electron-transparent vesicles derived from the smooth endoplasmic reticulum and groups of polyribosomes were numerous. Spherical lipid-like bodies were scattered throughout the cytoplasm (Fig. 14).

As the central-cell enlarged, plastids enlarged many-fold and the stroma became very narrow and increasingly electron-dense. Often along portions of large inclusions, stromal regions were absent so that, in section, all that was visible of the plastid was two double membranes appressed together. Occasionally starch was present. Plastids had commonly engulfed several pockets of cytoplasm (Fig 15). Smooth endoplasmic reticulum, scattered dictyosomes and polyribosome groups were common in the thin cytoplasmic sheets between vacuoles and lipid-like bodies were rare. Mitochondria were oblong to disc-shaped, fairly electron-translucent and lacked cristae. The central vacuole was gradually replaced by many smaller vacuoles. The largest vacuoles remained innermost in the cell while most smaller vacuoles and the majority of cytoplasm were peripheral. This corresponds to the foam stage described by other authors (Fig. 16).

Prior to division of the central cell most vacuoles had disappeared. The general cytoplasm and organelles became more electron-dense (Fig. 17). All central-cell plastids had developed into large inclusions which had engulfed a varying number of cytoplasmic pockets. Starch was absent. Large inclusions varied in size and shape, the largest lining the cell periphery and often measuring 34 μm or more in diameter (Fig. 18). Scattered, concave, disc-shaped mitochondria were common near the nucleus and were more electron-dense than in earlier stages. Cytoplasm partially isolated by disc-shaped mitochondria became more electron-transparent (Fig. 19).

Small inclusions were double-membrane bound sections of cytoplasm which appeared suddenly and dominated the cytoplasm of the dividing central cell (Fig. 20). Origin of small inclusions was interpreted to be from cup-shaped sections of smooth

endoplasmic reticulum which partially isolated nodules of cytoplasm. Short sections of dilated smooth endoplasmic reticulum, polyribosome groups, dictyosomes and mitochondria were common within included cytoplasm.

4.2.3 Primary Neck Cell and Neck Cells

Ultrastructure of the primary neck cell was similar to the early central cell. The nucleus bordered the central cell and the centre of the cell was composed of several large, electron-transparent vacuoles. Most cytoplasm lay against the plasmalemma or surrounded the nucleus and contained elongate plastids with thylakoids, distended smooth endoplasmic reticulum, electron-transparent endoplasmic vesicles, mitochondria which lacked cristae, occasional dictyosomes, polyribosomal groups and lipid-like bodies.

Generally four or more neck cells resulted from divisions of the primary neck cell and its derivatives and they were arranged in a single tier. Neck cells became more electron-dense than other megagametophyte cells and had thick, fibrillar cell walls. Nuclei were proximal and spherical (Fig. 21). The nuclear margin was undulate and nuclei contained many patches of chromatin. Plastids, mitochondria, distended smooth endoplasmic reticulum, endoplasmic vesicles, electron-transparent vacuoles and dictyosomes were numerous. Occasional, small sections of rough endoplasmic reticulum were visible and spherical lipid-like bodies were scattered throughout the cytoplasm. Plastids typically lacked starch and plastid morphology varied. Some plastids were of medium electron-density, ovoid to disc-shaped with little thylakoid development and contained scattered, small plastoglobuli (Fig. 22). Plastids which had become concave

and engulfed a single portion of cytoplasm were most common (Fig. 23). Mitochondria were spherical to flat or concave discs. Cytoplasm which lay inside the mitochondrial disc was less electron-dense than surrounding cytoplasm and commonly became electron-transparent in instances where the mouth of the cavity was severely reduced or, possibly, had disappeared. Many plasmodesmata were present in the cell walls among neck cells and between neck cells and the central cell or ventral canal cell. Neck cells appeared to be secretory with the periphery of the cells dotted with pockets of cytoplasmic secretions. Secretions were located between the plasmalemma and the cell wall and contained both fibrillar material and electron-translucent globules (Fig. 25).

4.2.4 Jacket Layer

Jacket cells differentiated from megagametophyte cells surrounding the early central cell (Figs. 16, 17) and were mostly arranged in a single layer. Some jacket cells were isodiametric, while others were larger and elongate. Nuclear size and shape corresponded to cell size and shape and nuclei were central in the cell and contained one to three large, spherical nucleoli (Fig. 24). One to several cytoplasmic compartments were engulfed by the plastids, forming inclusions which measured 3.7 μm or more across (Fig 26). Commonly two or more plastids created an inclusion (Fig. 27). Mitochondria in the form of concave discs were prevalent. Mitochondria were generally less electron-dense than plastids and had distended cristae (Fig. 26). Rough endoplasmic reticulum, polyribosome groups, distended smooth endoplasmic reticulum and endoplasmic vesicles were numerous. Occasional dictyosomes and small membranous figures were visible.

A source of these membraneous figures appeared to be collapsed plastid inclusions or portions thereof (Figs. 26, 27).

4.2.5 Ventral Canal Cell

During the second or third week of May, central cells divided unequally, each producing a small, lens-shaped ventral canal cell bordering the archegonial neck and a larger, internal egg cell (Fig. 28). A large, osmiophilic and finely-granular nucleus (Fig. 29) comprised most of the cell while a narrow band of osmiophilic cytoplasm was appressed to the plasmalemma (Fig. 30). Organelle identification was difficult in the osmiophilic cytoplasm. Scattered vacuoles of various sizes contained flocculent material (Fig. 30). A cell wall formed between the ventral canal cell and egg cell (Fig. 29).

4.2.6 Egg Cell

The egg nucleus enlarged as it descended to the centre of the cell where it remained until fertilization (Fig. 28). The mature egg cell was comparable in size and shape to the mature central cell. The egg nucleus was finely-granular and ovoid, measuring about 180 μm by 130 μm with an undulate and commonly embayed margin (Fig. 31). During nuclear descent egg cytoplasm became organized into essentially three concentric zones: the perinuclear, mid- and peripheral zones.

4.2.6.1 Perinuclear-Zone Cytoplasm

A narrow, continuous band of perinuclear cytoplasm about 4.2 μm wide was established during nuclear migration and was located immediately outside the egg nucleus (Fig. 31). Ubiquitous, darkly-staining mitochondria allowed for its visibility under the light microscope (Fig. 28). In appearance, mitochondria closely resembled those observed in the mature central cell. Mitochondria were electron-dense, disc-shaped and typically concave, measuring about 0.86 μm across with distended cristae (Figs. 31, 32). Smooth endoplasmic reticulum, polyribosome groups and occasional dictyosomes were the only other organelles present in the perinuclear zone. Plastid-derived large inclusions and small inclusions were absent.

4.2.6.2 Mid-Zone Cytoplasm

A substantial volume of egg-cell cytoplasm encircled the perinuclear zone in a mid-zone dominated by small inclusions (Figs. 28, 31, 33). From egg cell formation until fertilization about one week later, in some small inclusions layers of the double membrane delimiting the small inclusions gradually separated slightly (Figs. 33, 34), creating electron-transparent, cup-shaped vacuoles which, in section, appeared as crescents. As the volume of the vacuoles increased, the electron-density of the included cytoplasmic nodules increased (Fig. 34). In unfertilized archegonia, this process tended to originate around the periphery of the egg. Scattered in the cytoplasm amongst the small inclusions were mitochondria, dictyosomes and lipid-like bodies. Only occasionally

were plastid-derived large inclusions present (Fig. 28). Dilated smooth endoplasmic reticulum and endoplasmic vesicles were abundant.

4.2.6.3 Peripheral-Zone Cytoplasm

Most large inclusions were confined to a peripheral zone adjacent to the plasmalemma. This zone varied in width but commonly was about 74 μm across (Fig. 28). Cytoplasm compartmentalized within large inclusions often became either more or less electron-dense than the general cytoplasm, but otherwise was comparable to mid-zone cytoplasm (Fig. 35). Large inclusions had engulfed many pockets of cytoplasm and usually many of the double membranes which became layered against each other and which delimited the large inclusions and separated stromal regions from included cytoplasm, had lost their integrity (Fig. 36).

4.3 Fertilization

Penetration of the pollen tube between the neck cells and through the ventral canal cell was not observed. Superior egg cytoplasm became finely vesicular with insemination (Fig. 37) and large, electron-translucent receptive vacuoles were present near the site of pollen tube entry (Fig. 38). Remnants of the ventral canal cell and neck cells were common amongst the pollen-tube contents released into the egg. Fertilization occurred about the last week of May.

4.3.1 Movement of Male Gametes and Organelles Inside the Egg

The fertilizing male gamete shed most of the body-cell-derived cytoplasm and enlarged, becoming elongate as it advanced to the egg nucleus. A substantial volume of body-cell-derived cytoplasm, essentially composed of tightly-packed plastids and mitochondria encircled with polyribosomes, followed well behind the fertilizing male gamete but in advance of the non-functional male gamete (Fig. 39). Both the plastids and mitochondria (Fig. 41) were identical in appearance to the organelles which surrounded the male gametes in the pollen tube (Fig. 7). The cluster of paternal organelles was not membrane-bound. Microtubules were associated with the fertilizing male gamete and paternal organelles as they advanced through the egg. Microtubules flanked and trailed behind the fertilizing male gamete, mostly oriented parallel to the direction of travel of the gamete (Fig. 42). Before the first zygotic nuclear division, the paternal organelles had travelled about half-way to the zygote nucleus (Fig. 43). The non-functional male gamete did not proceed much further than the receptive vacuoles.

4.3.2 Nuclear Fusion

The fertilizing male gamete formed a depression in the egg nucleus which created a large area of contact between the two nuclei (Fig. 40). A fusion nucleus was established when, in many places within the zone of contact, the nuclear membranes fused forming channels through which the nucleoplasms became continuous (Fig. 44). A sheet of perinuclear-zone cytoplasm (Figs. 44, 45), commonly containing maternal mitochondria, was contained between the fusing nuclei. The nuclear connections

widened and, as a result, the cytoplasmic sheet was pushed out from between the fusing nuclei. The fusion nucleus persisted for a short time. Commonly small clusters of several paternal organelles were present in the perinuclear-zone cytoplasm where the male gamete had fused (Figs. 39, 46) but the perinuclear cytoplasm surrounding the remainder of the zygote nucleus contained only maternal organelles (Fig. 47). Paternal mitochondria appeared degenerate, both in the cluster migrating towards the fusing nuclei and in the egg cytoplasm bordering the perinuclear zone. These mitochondria enlarged, gradually becoming less electron-dense until they were electron-transparent except for remnants of cristae. The external membrane of some of these mitochondria was affected and may have been interrupted. Paternal plastids remained unchanged. Chains of polyribosomes were associated around these plastids and the affected mitochondria (Fig. 50).

4.4 Proembryo

4.4.1 Free-Nuclear Proembryo

Immediately following fusion of the male and female nuclei, the zygote nucleus divided (Fig. 48) to form two free-nuclei, each of which divided again to form four free-nuclei. All nuclei were positioned in approximately the centre of the archegonium and each nucleus was separated from the degenerating egg cytoplasm by a discrete layer of neocytoplasm (Fig. 49). Neocytoplasm was derived from the perinuclear zone of the zygote and nucleoplasm released during divisions of the zygote nucleus (Fig. 51).

Initially neocytoplasm was organized into two zones. The outer zone adjacent to the egg cytoplasm was derived from the perinuclear zone of the zygote and the inner zone adjacent to the nuclei was derived from the nucleoplasm of the zygote (Fig. 51). Most outer-zone organelles were concave, disc-shaped, maternal mitochondria (Fig. 53), but small numbers of paternal plastids were occasionally visible (Fig. 52). Polyribosome groups, dilated smooth endoplasmic reticulum and occasional lipid-like bodies and dictyosomes were present among the mitochondria. Paternal plastids surrounded by chains of polyribosomes were clustered in the maternal cytoplasm bordering the neocytoplasm of free-nuclei (Fig. 51). Initiated during the first coenocytic division and continuing in the second coenocytic division, nucleoplasm was released from the free-nuclei. This nucleoplasm was initially located immediately outside the nuclear membrane which was highly porous and often deeply invaginated. Both internal and external nucleoplasms were identical in appearance and finely-granular. Where the exuded nucleoplasm contacted perinuclear-zone-derived neocytoplasm, numerous polyribosomes and microtubules were common (Fig. 51). The distinction between the two neocytoplasmic zones was obliterated by the melding of perinuclear-derived organelles and the released nucleoplasm.

The four free-nuclei, each encased in neocytoplasm, migrated to the chalazal end of the egg cell (Fig. 54). During migration the amount of neocytoplasm increased and ribosomes became extremely numerous. Maternal mitochondria and microtubules were present in the neocytoplasm but organelle identification was hindered by the extreme

electron-density of neocytoplasm afforded by the ribosomes. Nuclei formed a single tier and, collectively, were enclosed by a common neocytoplasm (Fig. 55).

4.4.2 Early Cellular Proembryo

Cellularization of the proembryo occurred at the end of May or the beginning of June. Nuclei divided producing eight nuclei arranged in two tiers of four cells each and cell walls formed between nuclei (Fig. 56). Transverse cell walls formed between tiers, followed by perpendicular walls. The upper tier remained open to the degenerating egg cytoplasm. In the lower, primary embryonal tier, which originates the embryo, large inclusions were never observed. Peripheral-zone egg cytoplasm was almost completely excluded from the chalazal pole and any small regions which became incorporated into the neocytoplasm were highly degenerate (Fig. 57). Neocytoplasm of both tiers was very osmiophilic, mostly because of ubiquitous helicoid-files and clusters of polyribosomes. Lipid-like bodies, concave, disc-shaped, maternal mitochondria, smooth endoplasmic reticulum and rough endoplasmic reticulum were numerous (Fig. 58). Irregularly-shaped, osmiophilic plastids measuring 0.80 μm or more across usually contained one starch grain. Plastids appeared to be more numerous in the primary embryonal tier (Fig. 57).

4.5 Degeneration of Maternal Cytoplasm

Coinciding with fertilization, most cup-shaped vacuoles delimiting the small inclusions rapidly increased in volume and the cytoplasmic nodules, partially isolated

within these inclusions, increased in electron-density and became flocculent. This caused the small inclusions to become more spherical. Commonly a very electron-dense patch of cytoplasm developed in the centre of the inclusion and, accompanying this, the electron-density of more peripheral cytoplasm in the inclusion decreased (Fig. 59). This process was initiated in mid-zone cytoplasm bordering on the perinuclear zone and rapidly radiated outward to the plasmalemma. Degeneration of maternal cytoplasm was visible under the light microscope (Fig. 43). Small inclusions within the cytoplasmic compartments of large inclusions changed in a similar manner (Fig. 60). Prior to tier formation in the proembryo, membrane integrity was lost in the small inclusions and in organelles throughout all the maternal cytoplasm outside of the neocytoplasm. Similar degeneration of jacket-cell cytoplasm was common.

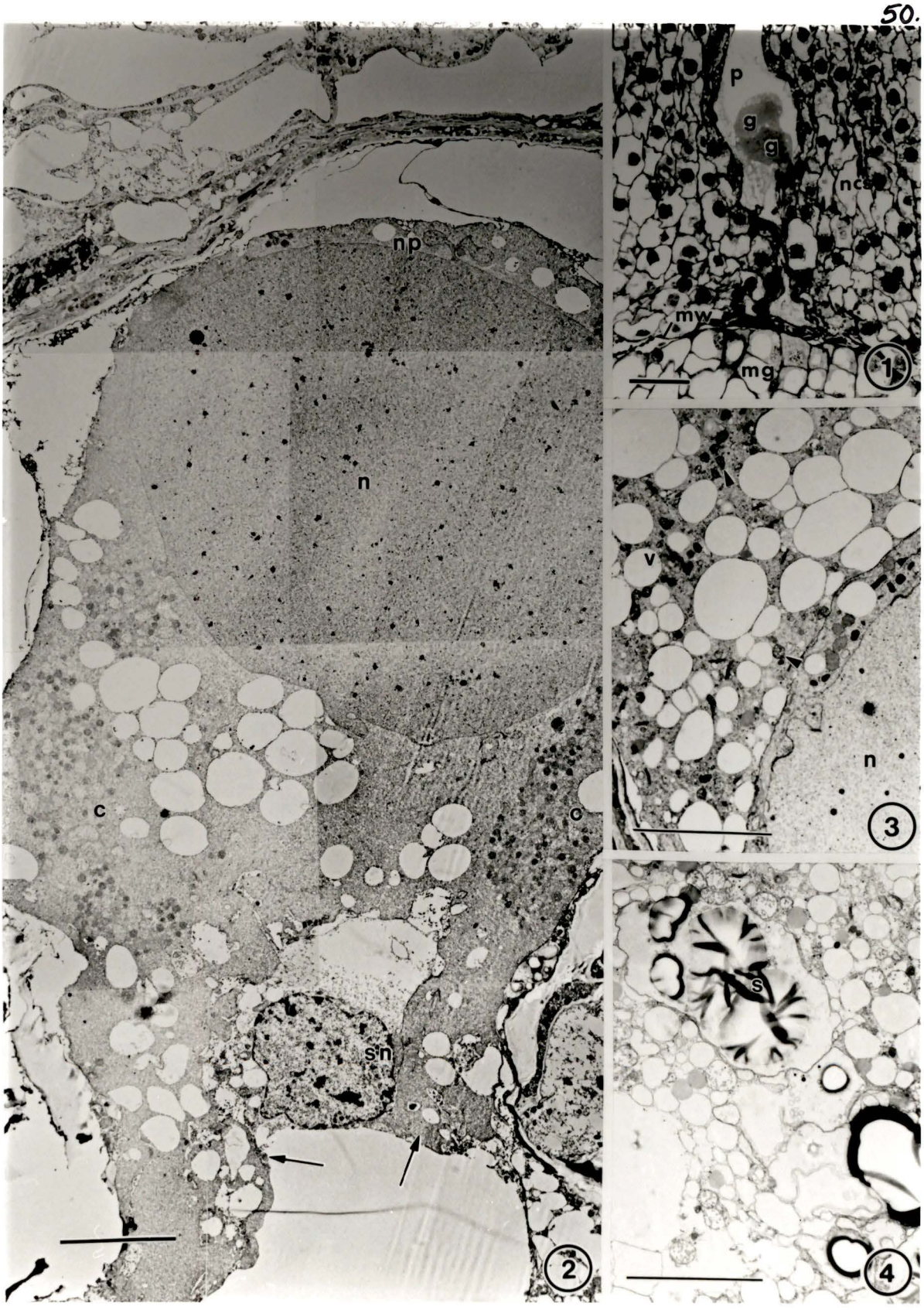
Figs. 1-4. Light microscope and TEM features of the microgametophyte in the nucellus.

Fig. 1. Pollen tube (pt) within the nucellus (ncs). It contains two male gametes (g) and has reached the megaspore cell wall (mw) which bounds the megagametophyte (mg). Scale bar = 50 μm.

Fig. 2. Body cell has a large nucleus (n) with nuclear projections (np) and a cytoplasm rich with clusters (c) of plastids and mitochondria. Distal cytoplasmic projections (arrows) surround the stalk-cell nucleus (sn). Scale bar = 10 μm.

Fig. 3. Highly vacuolate (v) tube-cell cytoplasm which surrounds the male gametes contains many mitochondria (arrowheads). A portion of a gamete nucleus shows nuclear projections.
Scale bar = 5 μm.

Fig. 4. Distal tube-cell cytoplasm is flocculent and contains remnants of plastids with starch grains (s). Scale bar = 5 μm.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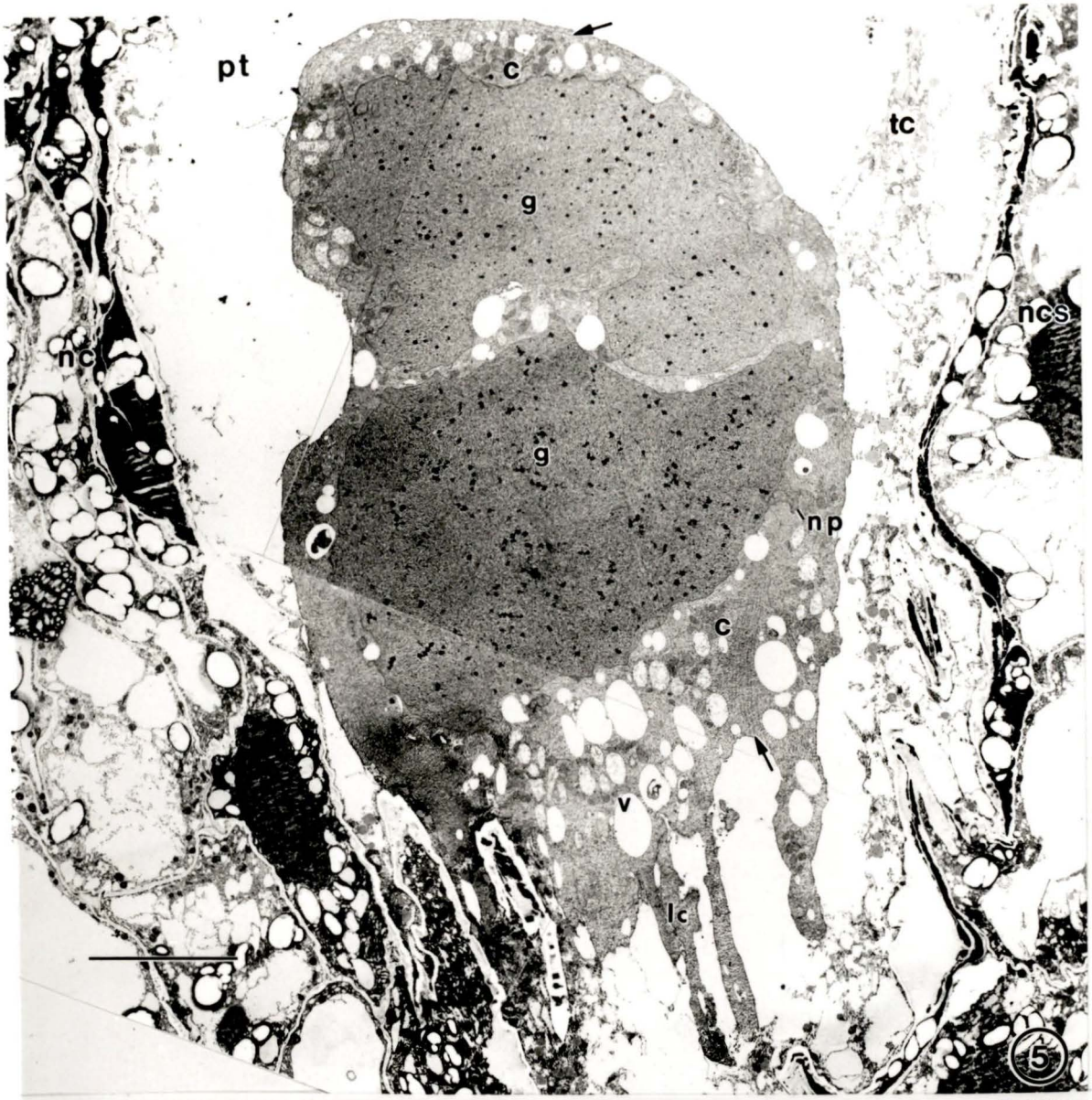
Figs. 5-8. TEM features of the male gametes and body cell cytoplasm in the pollen tube.

Fig. 5. Two male gametes (g) within the pollen tube (pt) have nuclear projections (np). Distal body-cell cytoplasm is deeply lobed (lc) and clusters (c) of mitochondria and plastids are numerous. Sheets of smooth endoplasmic reticulum (arrows) are abundant. ncs, nucellus; v, vacuole; tc, tube cell cytoplasm.
Scale bar = 10 μm.

Fig. 6. Membraneous structures (arrows) inside the nucleus of a male gamete. Small vesicles (sv) and smooth endoplasmic reticulum (ser) are common against the plasmalemma. nm, nuclear membrane. Scale bar = 1 μm.

Fig. 7. Plastids (p), mitochondria (m) and polyribosomes (arrows) in the body-cell cytoplasm prior to gamete formation.
Scale bar = 0.5 μm.

Fig. 8. Plastids and mitochondria in body-cell cytoplasm surrounding a male gamete. Scale bar = 1 μm.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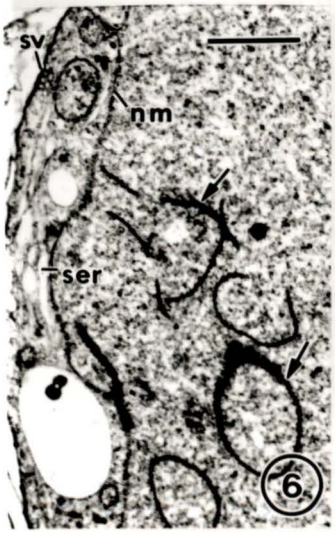
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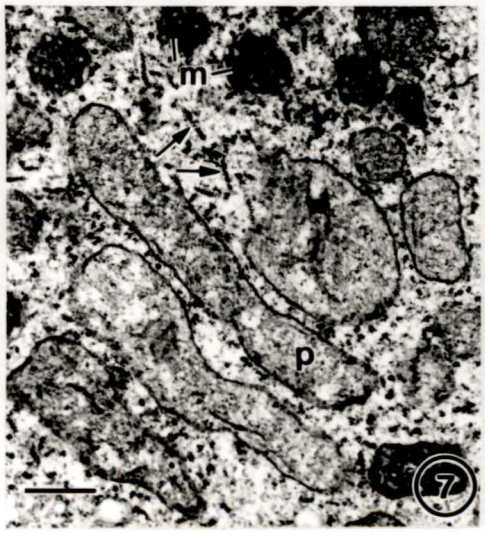
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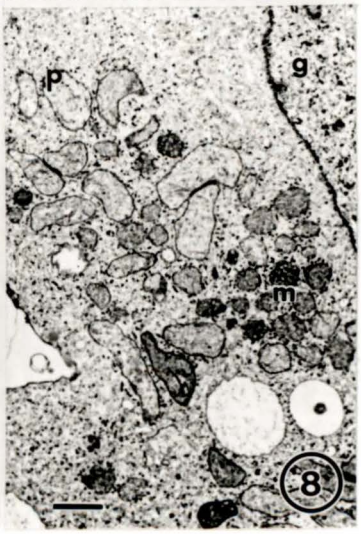
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Figs. 9-15. Light microscope and TEM features of the free-nuclear megagametophyte and central cell.

Fig. 9. Free-nucleus (mfn) surrounded by cytoplasm containing elongate plastids (p) which commonly are constricted (arrows). d, dictyosome. Scale bar = 1 μm.

Fig. 10. Free-nucleus surrounded by irregularly-shaped plastids, some with starch (s) granules. arrows, polyribosomes.
Scale bar = 1 μm.

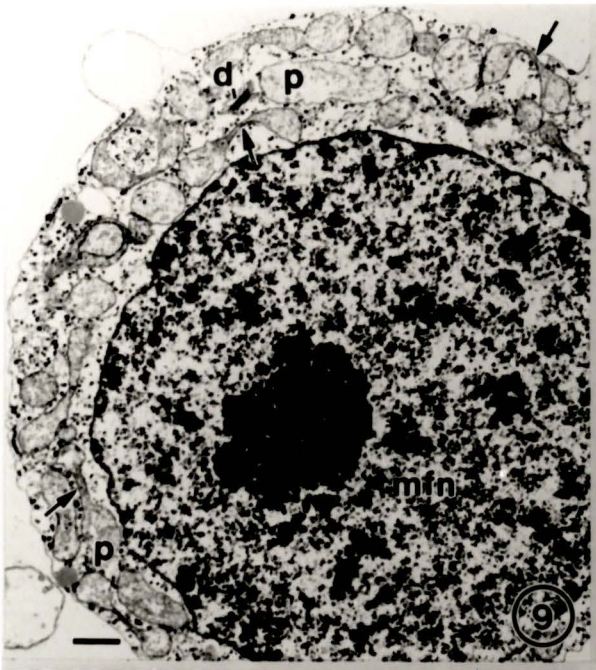
Fig. 11. Free-nucleus during cell-wall formation in cytoplasm with extensive smooth endoplasmic reticulum (arrows) and concave, disc-shaped plastids. Scale bar = 5 μm.

Fig. 12. The early central cell (cc) is highly vacuolate (v) and its nucleus (n) lies next to the primary neck cell (pc). mw, megaspore cell wall; mg, megagametophyte. Scale bar = 50 μm.

Fig. 13. Plastids in the early central cell are elongate to disc-shaped and thylakoid membranes (arrows) are often distended. ser, smooth endoplasmic reticulum. Scale bar = 0.5 μm.

Fig. 14. A disc-shaped plastid in the early central cell has surrounded a portion of cytoplasm in which distended smooth endoplasmic reticulum and polyribosomes (arrow) are visible. lb, lipid-like body. Scale bar = 0.5 μm.

Fig. 15. Portion of a central-cell plastid during the foam stage of development. Plastid is undergoing transformation into a large inclusion. Several cytoplasmic compartments (ci) are bound by the plastid membranes. Stromal region contains starch.
Scale bar = 1 μm.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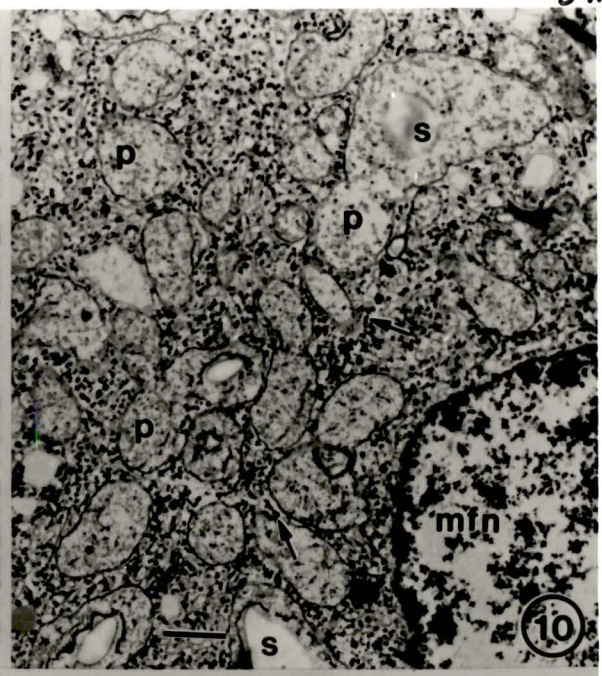
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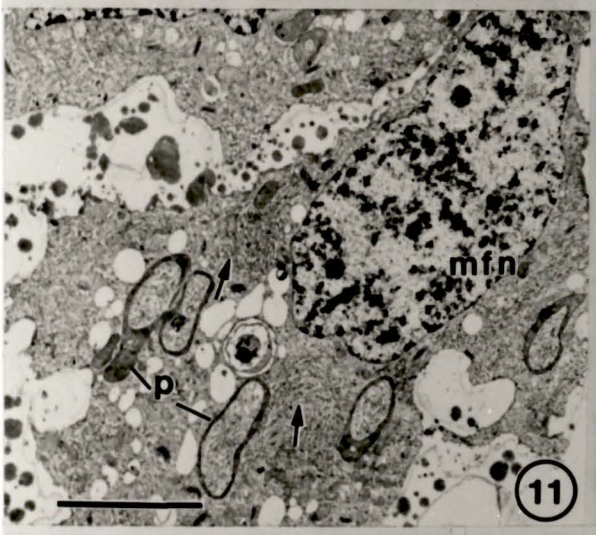
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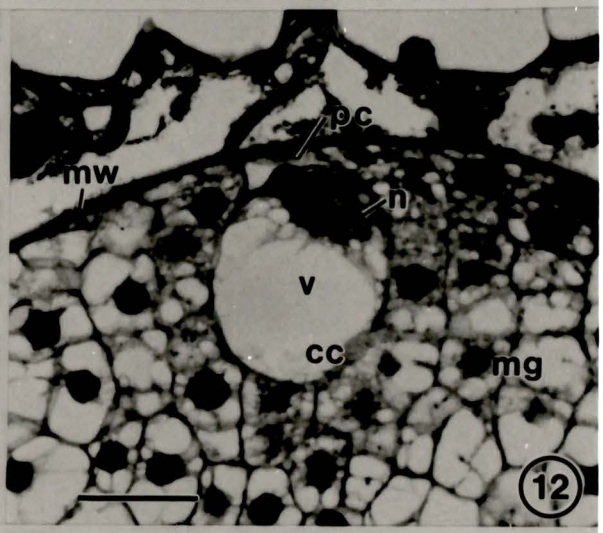
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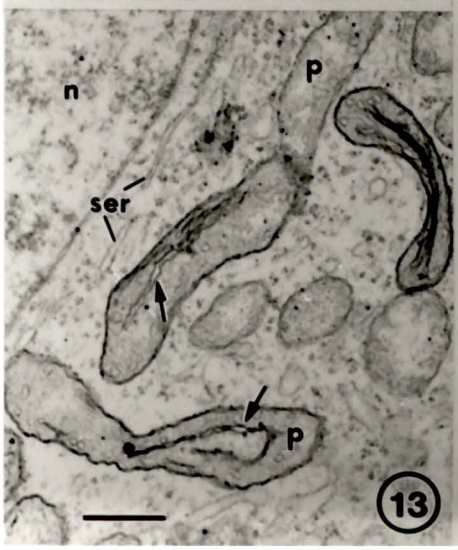
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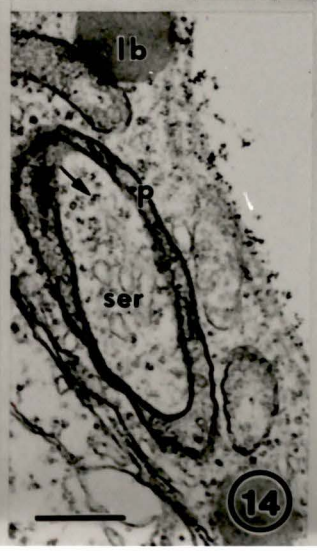
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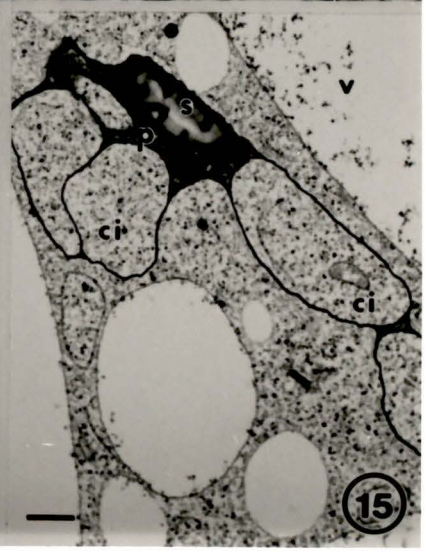
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Figs. 16-21. Light microscope and TEM features of the advanced central cell and neck cells.

Fig. 16. Foam stage of central-cell development. Largest vacuoles (v) are innermost in the cell while the nucleus (n) lies against the archegonial neck and large inclusions (li) are peripheral. Scale bar = 50 μm.

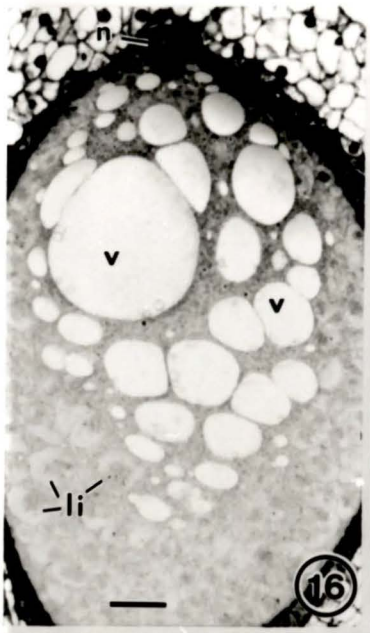
Fig. 17. Vacuoles are less common in the central cell prior to division. aj, archegonial jacket. Scale bar = 50 μm.

Fig. 18. Portion of a large inclusion with many cytoplasmic compartments (ci) and electron-dense stroma (arrows). si, small inclusions. Scale bar = 5 μm.

Fig. 19. Concave, disc-shaped mitochondria (m), distended smooth endoplasmic reticulum (ser) and small inclusions below the central-cell nucleus which is undergoing division. Scale bar = 1 μm.

Fig. 20. Small inclusions are ubiquitous in the cytoplasm of the dividing central cell. d, dictyosome. Scale bar = 1 μm.

Fig. 21. Neck cells (nc) contain large, spherical nuclei and electron-dense cytoplasm. Thick, fibrillar cell walls (cw) have many plasmodesmata and pockets of cytoplasmic secretions (se) collect against the walls. cc, central cell; mg, megagametophyte. Scale bar = 10 μm.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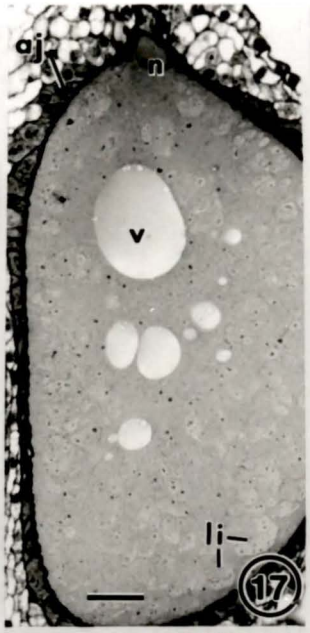
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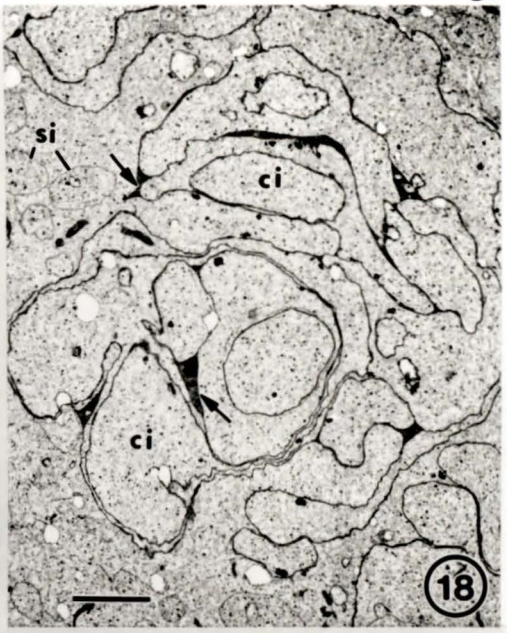
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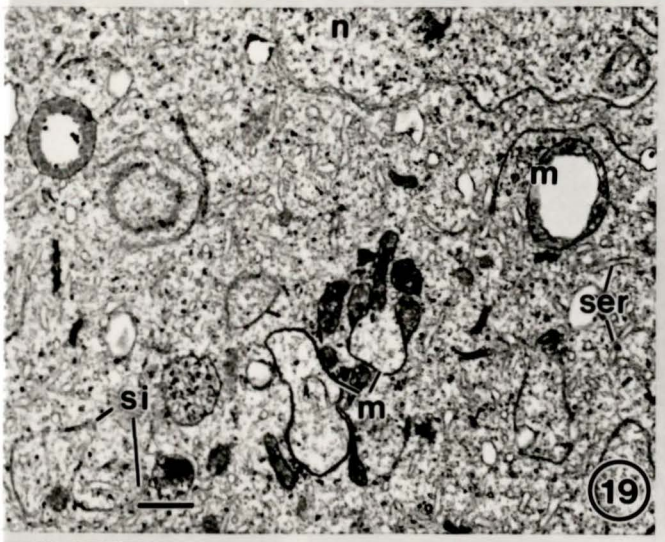
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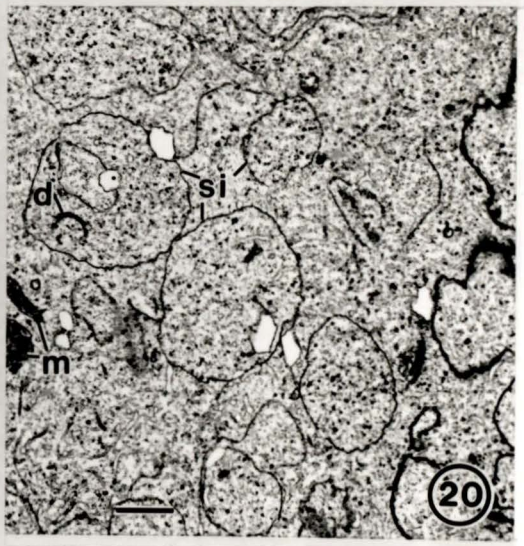
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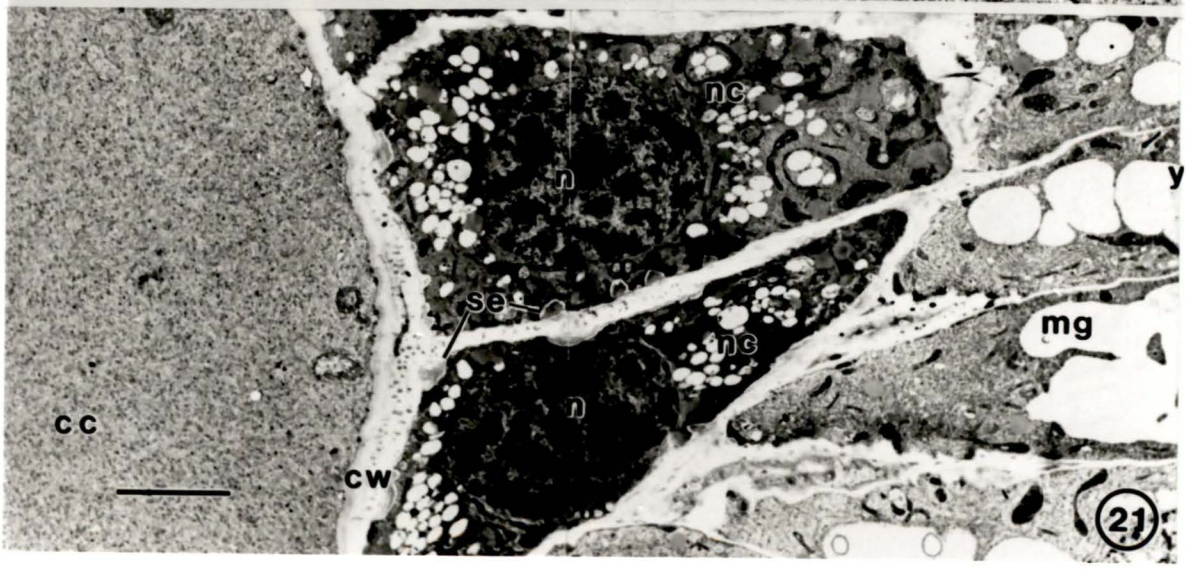
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Figs. 22-27. TEM features of neck cells and jacket cells.

Fig. 22. Ovoid neck cell plastids (p) often contain plastoglobuli (arrows). n, nucleus; cw, cell wall; m, mitochondria; v, vacuole; se, cytoplasmic secretions. Scale bar = 1 μm.

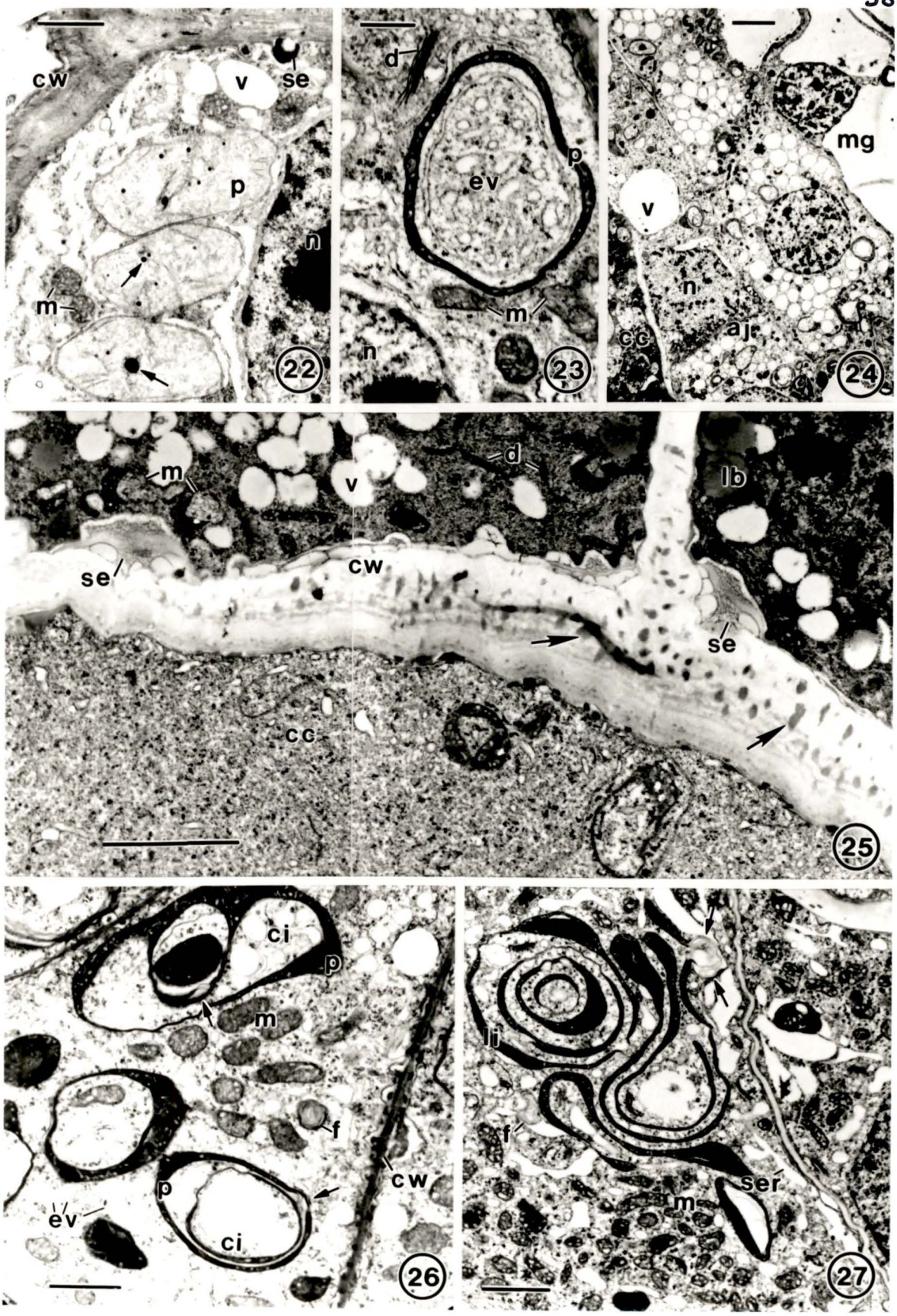
Fig. 23. Concave, disc-shaped, neck-cell plastid is electron-dense. d, dictyosome; ev, endoplasmic vesicles. Scale bar = 0.5 μm.

Fig. 24. Highly vacuolate jacket cells (aj) surround the central cell (cc). mg, megagametophyte. Scale bar = 1 μm.

Fig. 25. Cytoplasmic secretions line the cell walls of the electron-dense neck cells. Cytoplasm is rich with disc-shaped mitochondria, dictyosomes, vacuoles and lipid-like bodies (lb). Cell walls contain many plasmodesmata (arrows). cc, central cell. Scale bar = 5 μm.

Fig. 26. Jacket-cell plastids are very electron-dense and commonly have engulfed several cytoplasmic compartments (ci). Mitochondria are often elongate with distended cristae. Small membraneous figures (f) appear to arise from collapsed plastid membranes (arrows). ev, endoplasmic vesicles. Scale bar = 1 μm.

Fig. 27. Some large inclusions (li) in the jacket cells are formed from several plastids. Collapsed plastid membranes (arrows) form membraneous figures. ser, smooth endoplasmic reticulum. Scale bar = 1 μm.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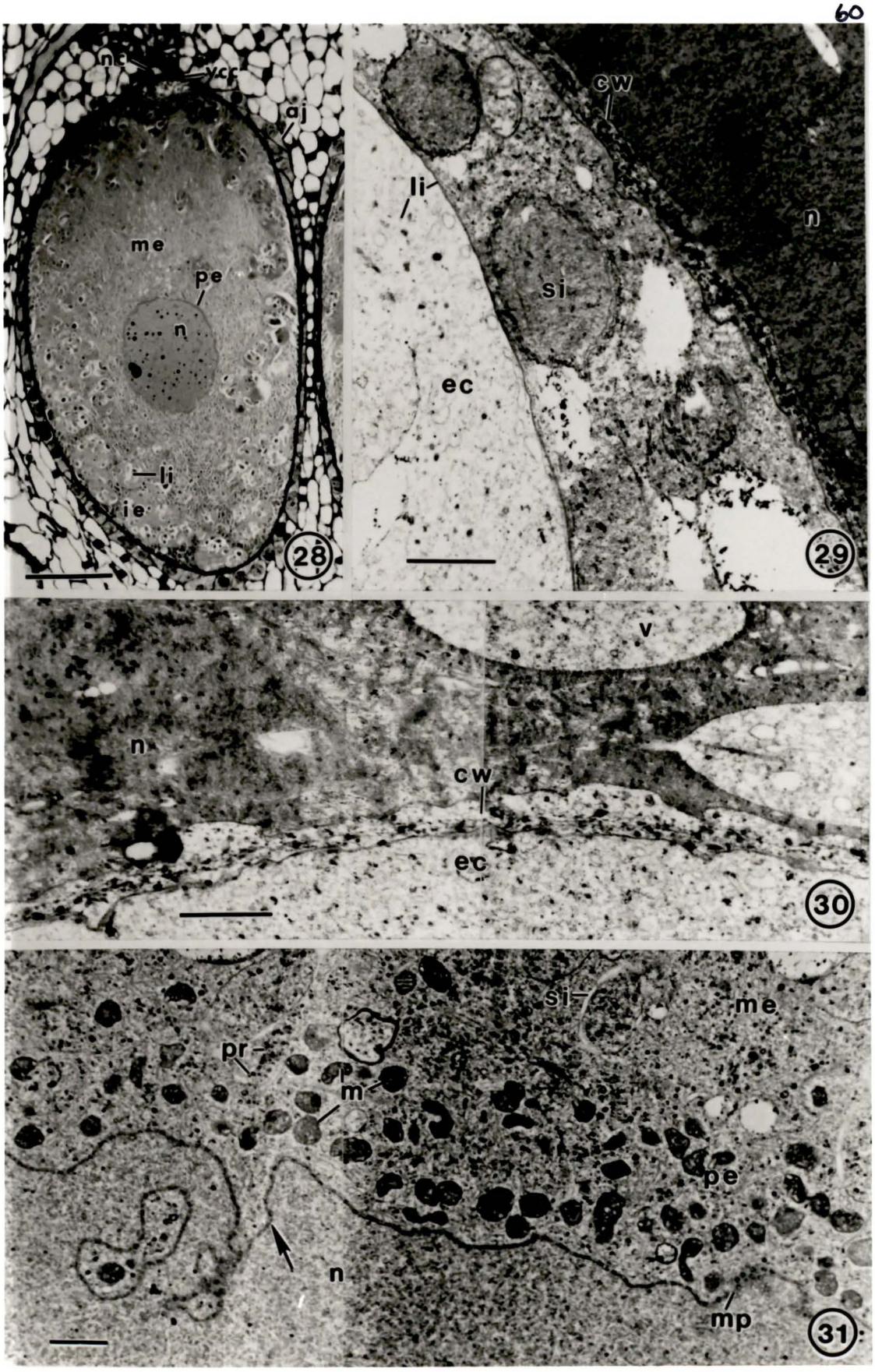
Figs. 28-31. Light microscope and TEM features of the ventral canal cell and egg cell.

Fig. 28. The large egg nucleus (n) is central and cytoplasm is organized into a perinuclear zone (pe), mid-zone (me) and peripheral zone (ie). vcc, ventral canal cell; nc, neck cells; aj, archegonial jacket; li, large inclusion. Scale bar = 100 μm.

Fig. 29. A cell wall (cw) forms between the ventral canal cell and the egg (ec). The ventral canal-cell nucleus comprises most of the cell. si, small inclusion. Scale bar = 1 μm.

Fig. 30. A thin parietal layer of electron-dense ventral canal-cell cytoplasm surrounds the nucleus. Vacuoles (v) contain flocculent material. Scale bar = 1 μm.

Fig. 31. Egg nucleus with embayed margin (arrow) and nuclear-membrane pores (mp). Perinuclear zone contains many electron-dense, disc-shaped mitochondria (m) and polyribosomes (pr). Mid-zone egg cytoplasm with small inclusions surround the perinuclear zone. Scale bar = 1 μm.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Figs. 32-37. Light microscope and TEM features of the egg cell and pollen-tube entry.

Fig. 32. Concave, disc-shaped, perinuclear-zone mitochondria (m) and endoplasmic vesicles (ev) in the egg. Scale bar = 0.5 μm.

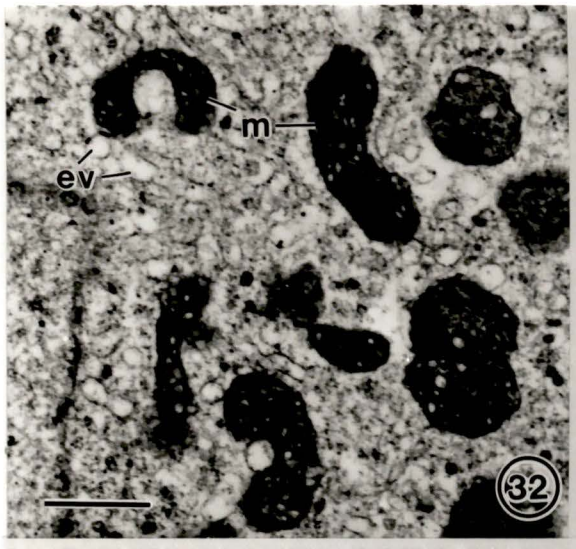
Fig. 33. Small inclusions (si) in the mid-zone of the egg. Endoplasmic reticulum membranes begin to separate (arrows). Scale bar = 1 μm.

Fig. 34. Unfertilized, egg, mid-zone cytoplasm. Cup-shaped vacuoles (v) delimit the small inclusions. Electron-density of cytoplasmic nodules (arrows) in the small inclusions increase. li, large inclusion. Scale bar = 10 μm.

Fig. 35. Peripheral-zone egg-cell cytoplasm with small inclusions, some within large inclusions. Scale bar = 5 μm.

Fig. 36. Integrity of plastid-derived large inclusion membranes is commonly destroyed (arrows). lb, lipid-like body. Scale bar = 1 μm.

Fig. 37. Vesicular, superior egg cytoplasm after pollen-tube (pt) entry. Arrows show male organelles and microtubules. nc, neck cell; aj, jacket cells. Scale bar = 50 μm.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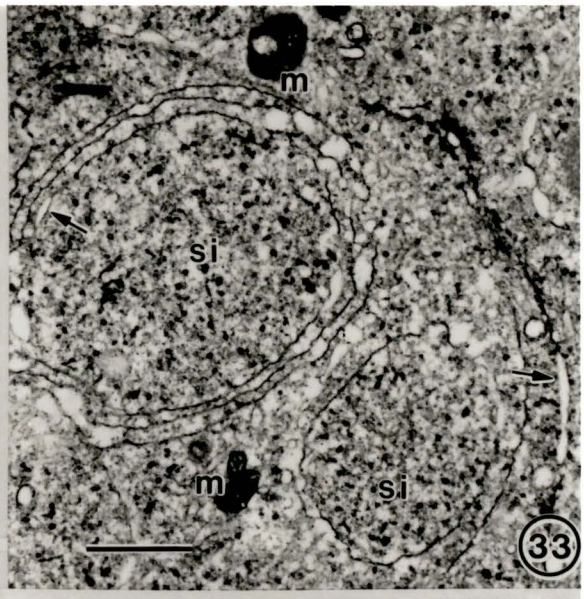
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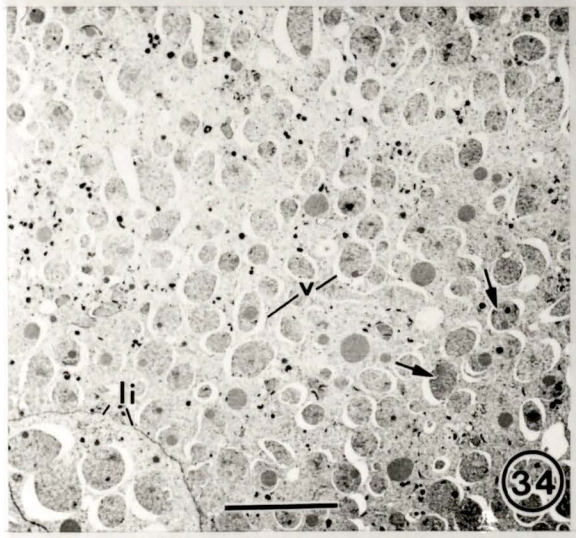
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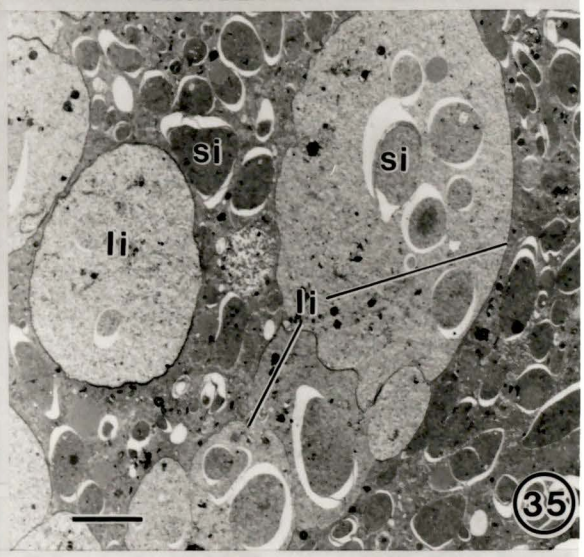
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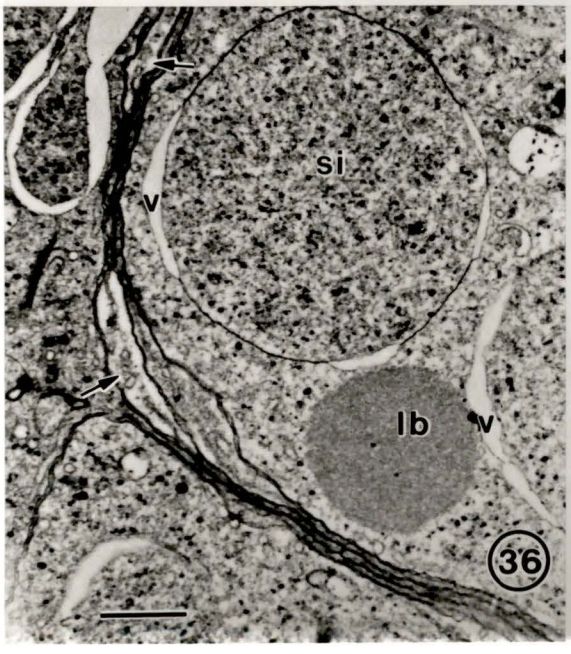
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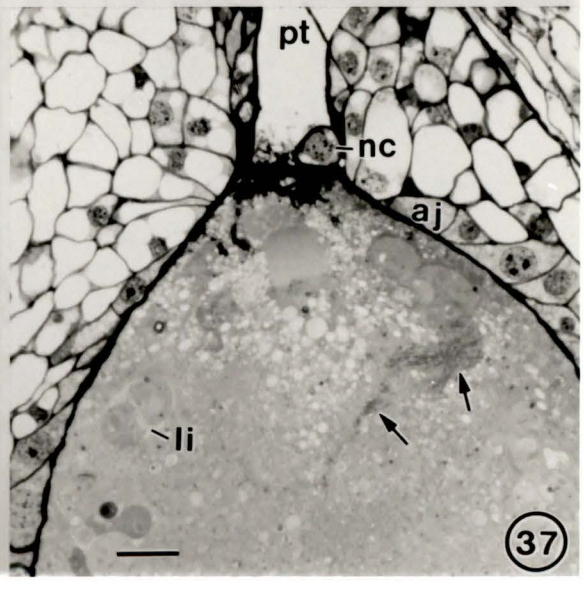
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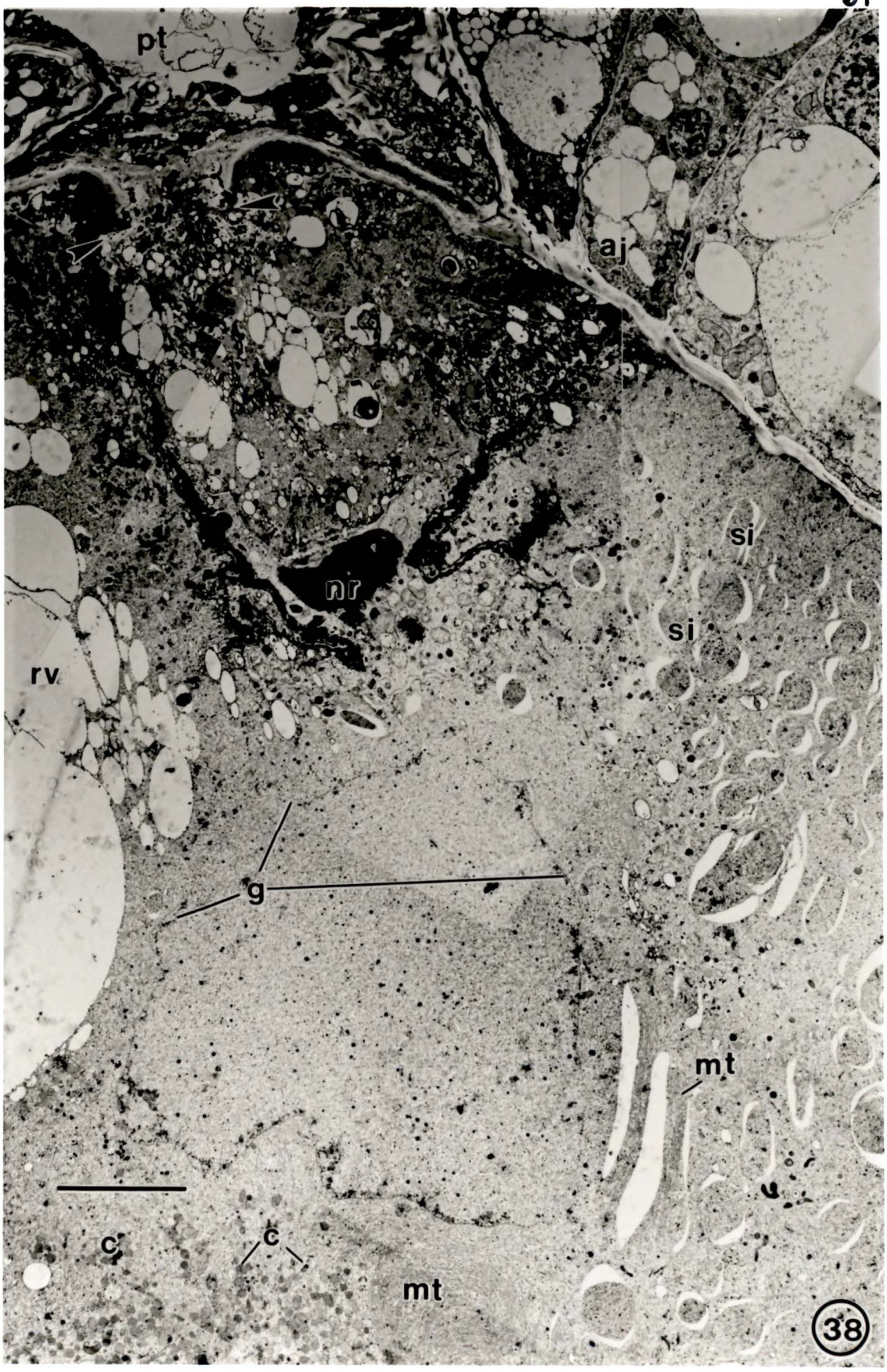
Fig. 38 Superior egg cytoplasm following pollen-tube (pt) entry through neck-cell walls (arrows). Second male gamete (g) alongside receptive vacuoles (rv) and preceded by clustered (c) body-cell-derived plastids and mitochondria. Microtubules (mt) associated with both the second male gamete and paternal organelles. si, small inclusions; aj, jacket cells; nr, nuclear remnants. Scale bar = 10 μm.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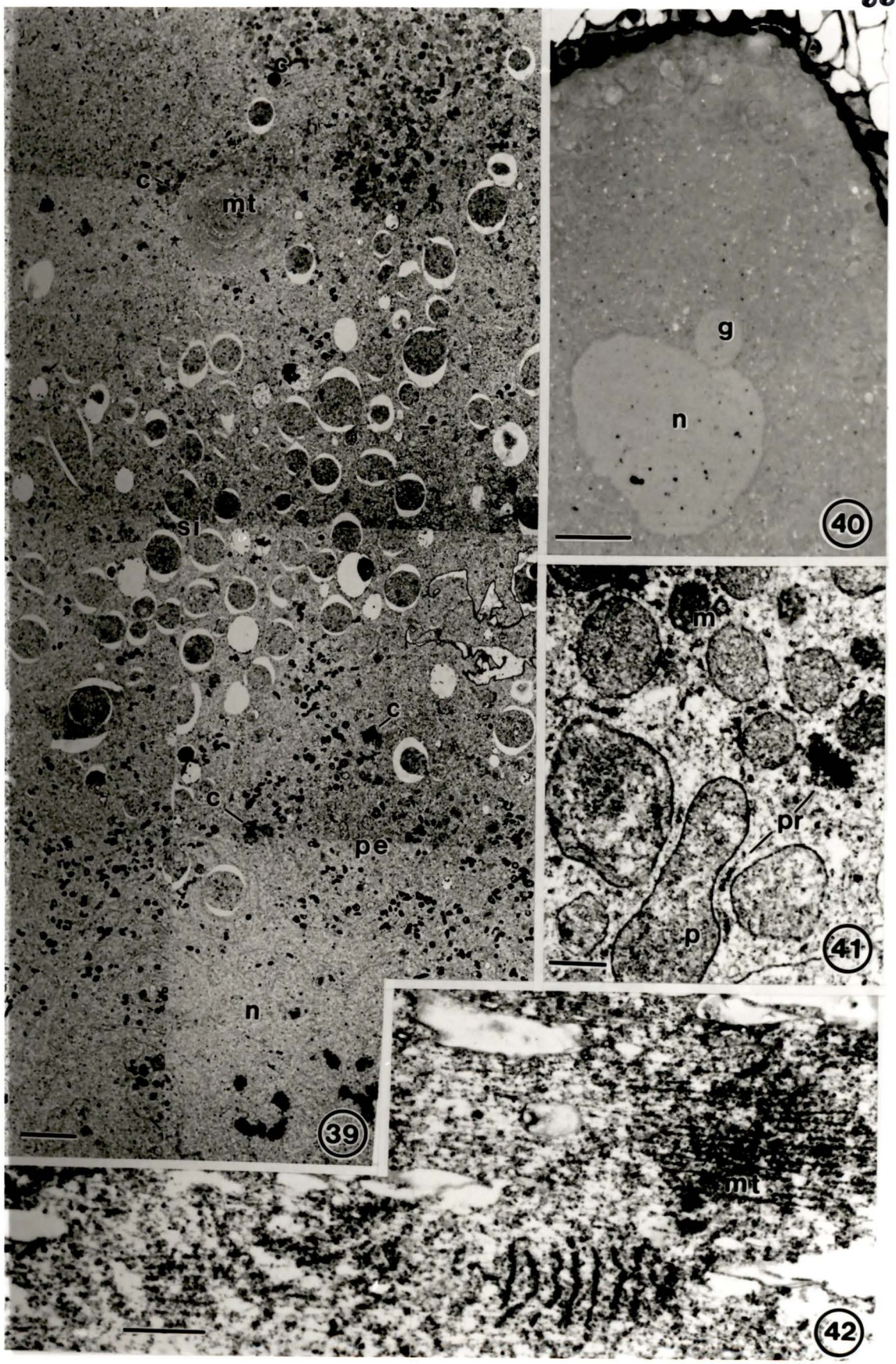
Figs. 39-42. TEM features of fertilization.

Fig. 39. Clusters (c) of paternal plastids and mitochondria in superior egg cytoplasm and in the perinuclear zone (pe) surrounding the fertilized egg nucleus (n). mt, microtubules; si, small inclusions. Scale bar = 5 μm.

Fig. 40. Fertilizing male gamete (g) forms a depression in the egg nucleus at nuclear fusion. Scale bar = 50 μm.

Fig. 41. Clustered, paternal plastids (p) and mitochondria (m) are associated with the polyribosomes (pr). Scale bar = 0.5 μm.

Fig. 42. Microtubules trail behind the fertilizing male gamete. Scale bar = 1 μm.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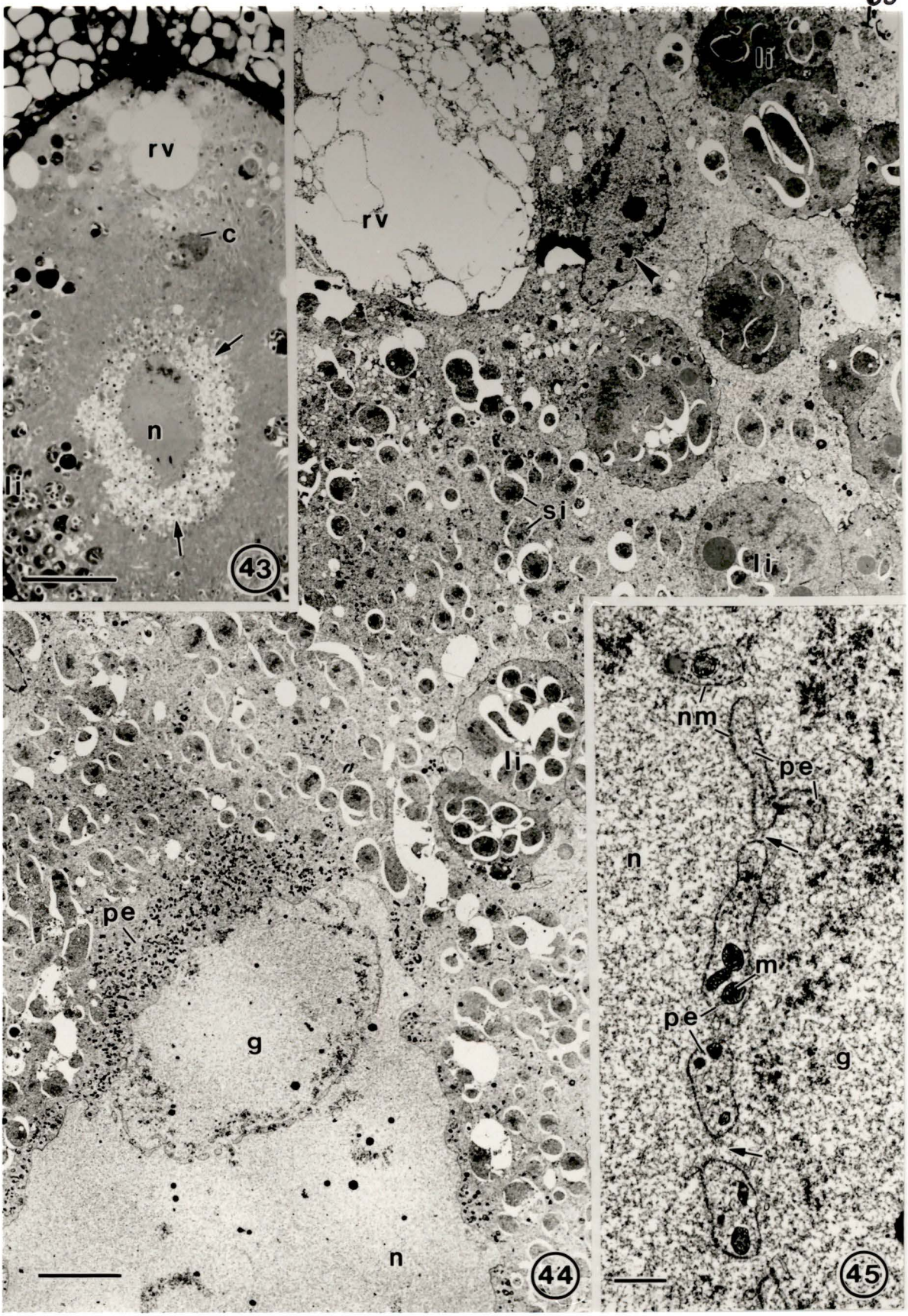
Figs. 43-45. Light microscope and TEM features of nuclear fusion.

Fig. 43. At nuclear fusion, most paternal organelles are in clusters (c) and have advanced about half way to the fusion nucleus (n). Degeneration of the maternal cytoplasm (arrows) is apparent near the nucleus and radiates outward. rv, receptive vacuole; li, large inclusions. Scale bar = 100 μm.

Fig. 44. A fusion nucleus is established between the fertilizing male gamete (g) and the egg nucleus. A male nucleus (arrowhead) remains in superior egg cytoplasm in the vicinity of the receptive vacuoles. pe, perinuclear zone; si, small inclusion. Scale bar = 10 μm.

Fig. 45. In many places between the fertilizing male gamete and egg nucleus, nuclear membranes fuse (arrows). A sheet of perinuclear-zone cytoplasm is contained between the nuclei and often includes maternal mitochondria (m). nm, nuclear membrane. Scale bar = 1 μm.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Figs. 46-51. Light microscope and TEM features of fertilization and the free-nuclear proembryo.

Fig. 46. Paternal plastids (p) and maternal mitochondria (m) in the zygote perinuclear zone at the site of gamete fusion. arrow, polyribosomes. Scale bar = 1 μm.

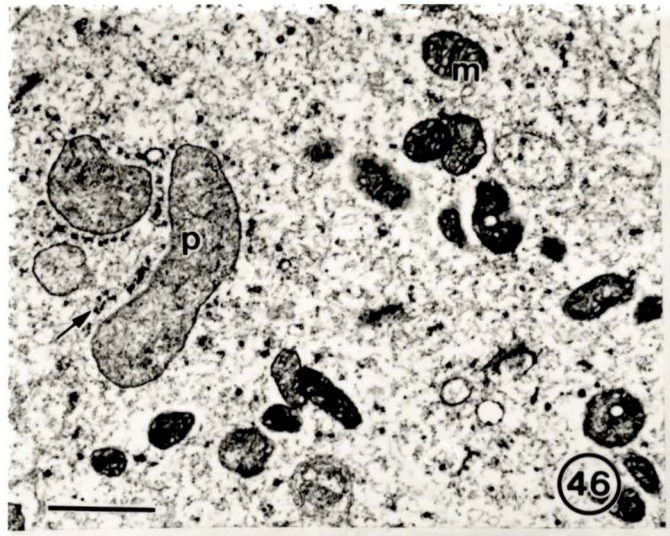
Fig. 47 Perinuclear-zone, maternal mitochondria opposite the site of gamete fusion. Scale bar = 1 μm.

Fig. 48. Zygote nucleus (n) undergoing the first free-nuclear division. arrow, degenerating egg cytoplasm. Scale bar = 50 μm.

Fig. 49. Two of four free-nuclei surrounded by neocytoplasm (neo). Scale bar = 50 μm.

Fig. 50. Paternal plastids, degenerating paternal mitochondria and polyribosomes (arrow) migrating towards the free-nuclei. Scale bar = 1 μm.

Fig. 51. A portion of one of four free-nuclei surrounded by neocytoplasm originating from the nucleoplasm of the zygote (ne) and the perinuclear zone (pe) of the zygote. Clustered (c) paternal organelles and small inclusions (si) are located in degenerating egg cytoplasm. Scale bar = 10 μm.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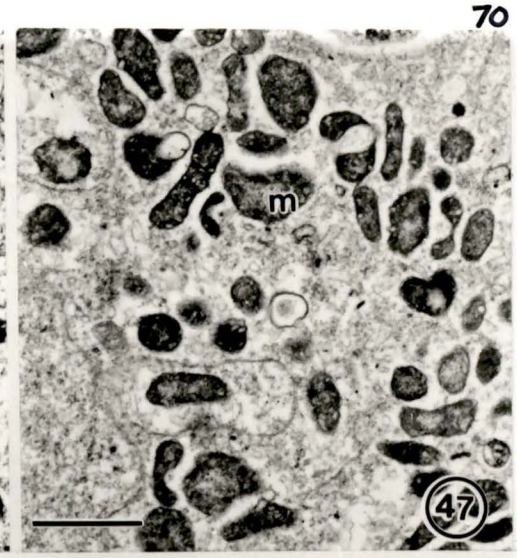
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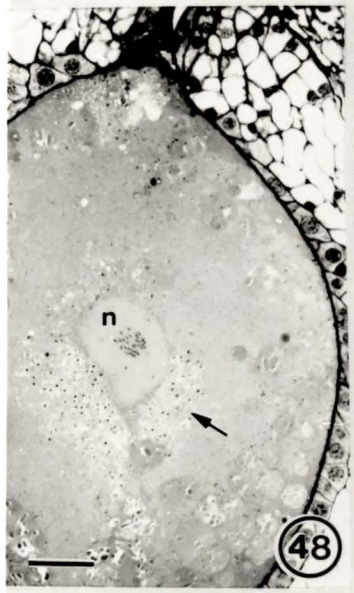
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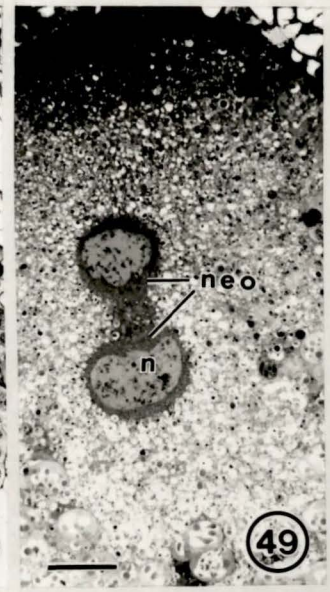
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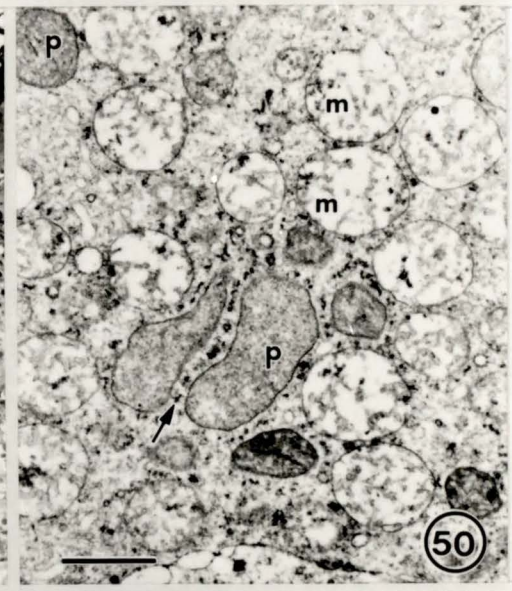
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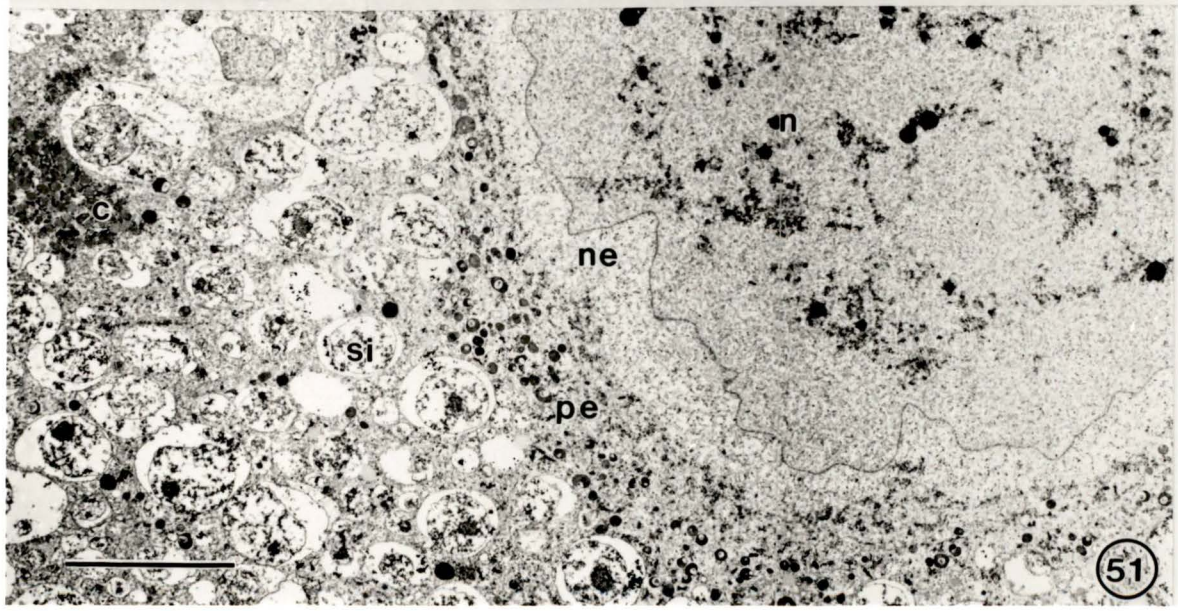
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Figs. 52-60. Light microscope and TEM features of the proembryo and degeneration of maternal cytoplasm.

Fig. 52. Paternal plastids (p) and maternal mitochondria (m) in the outer neocytoplasmic zone of the four-nucleate proembryo. arrowhead, polyribosomes. Scale bar = 1 μm.

Fig. 53. Maternal mitochondria in the outer neocytoplasmic zone. lb, lipid-like body; si, small inclusion. Scale bar = 1 μm.

Fig. 54. Two of four free-nuclei (n), each surrounded by neocytoplasm (neo), descend to the egg chalazal pole. Scale bar = 100 μm.

Fig. 55. Three of four free-nuclei form a single tier.
Scale bar = 50 μm.

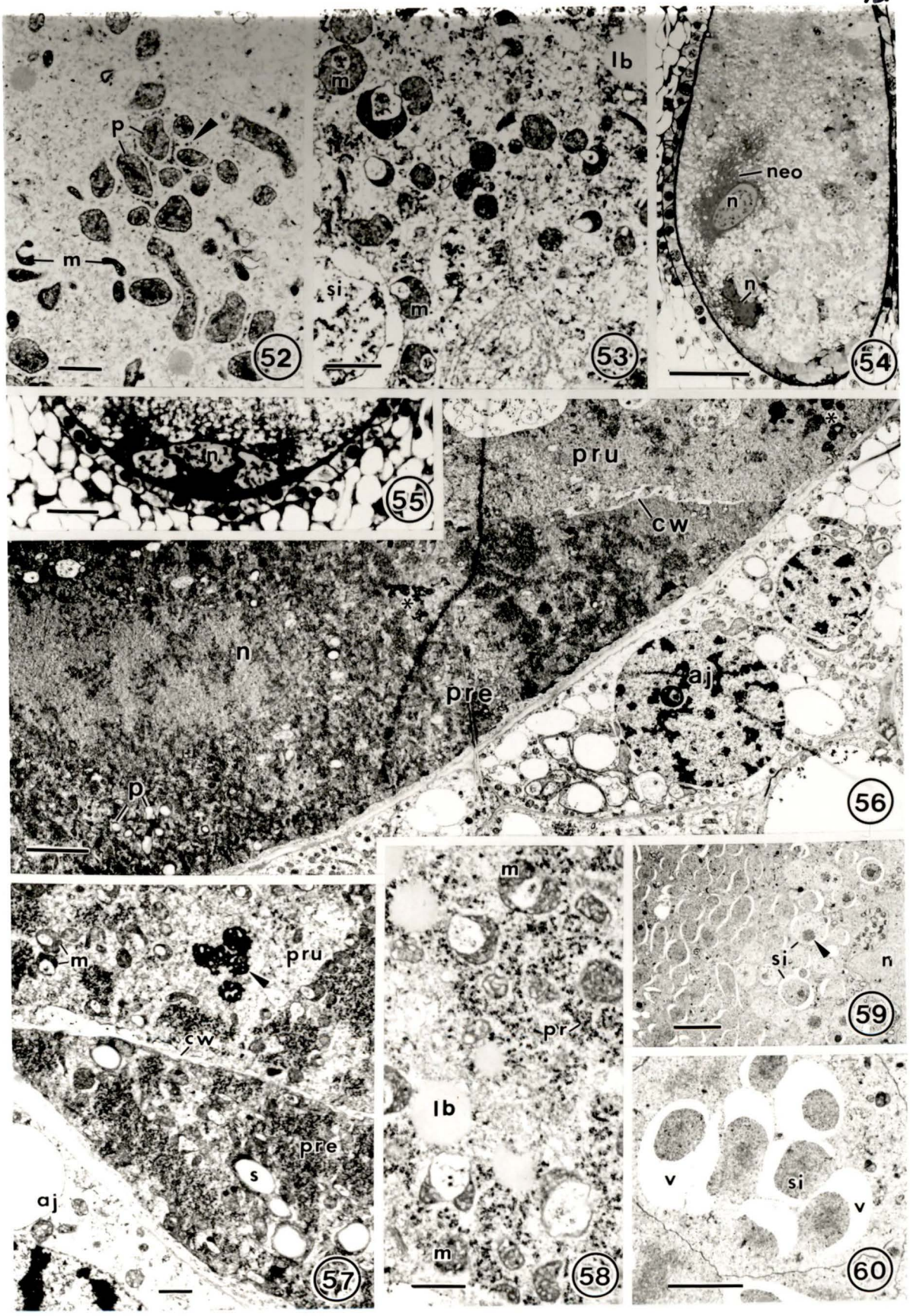
Fig. 56. Primary embryonal (pre) and primary upper (pru) proembryo tiers during cell wall (cw) formation. Plastids contain starch. aj, jacket cells; *, nucleolar remnants. Scale bar = 5 μm.

Fig. 57. Plastids in the primary embryonal proembryo tier contain large starch grains. Maternal mitochondria are numerous. arrowhead, nucleolar remnants. Scale bar = 1 μm.

Fig. 58. Primary-embryonal-tier cytoplasm with lipid-like bodies, maternal mitochondria and polyribosomes (pr).
Scale bar = 0.5 μm.

Fig. 59. Cytoplasmic degeneration at fertilization is initiated next to the zygote nucleus. Small inclusions round up and electron-dense patches (arrow) develop in the centre of the cytoplasmic nodule. Scale bar = 5 μm.

Fig. 60. Small inclusions within a large inclusion become isolated within vacuoles (v) and appear flocculant. Scale bar = 5 μm.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Chapter 5

DISCUSSION

5.1 Pre-Fertilization Microgametophyte Development

5.1.1 Pollen Tube

Although branched pollen tubes have been reported in *Pinus* (Willemse, 1968; Willemse and Linskens, 1969), this was not observed *in vivo* in *P. monticola*. Similar studies of *Pseudotsuga menziesii* revealed the presence of occasional, short, lateral projections of pollen tubes, but only with the careful dissection of the pollen tubes from nucellar tissue (Owens and Morris, 1991) and not during *in situ* observation (Owens and Morris 1990). Whether similar projections of *P. monticola* pollen tubes would be revealed through dissection was not determined.

5.1.2 Body Cell and Male Gametes

The numerous and sometimes branched projections of the body-cell nucleus in *P. monticola* have not been reported in other pines. Prior to division of the body-cell nucleus, distal body-cell cytoplasm formed several large lobes which surround the stalk cell. A similar configuration was observed in *Picea* (Camefort, 1978), but between the stalk cell and the body-cell cytoplasm following gamete formation.

Male gametes in *P. monticola* are roughly equal in size. The complex lobing of distal body-cell cytoplasm observed at this stage was similar in *Pseudotsuga* where Owens and Morris (1990) suggest that the interlocking of the tube cell and body cell serves as a mechanism by which the body cell is pulled through the narrow pollen tube.

Such interlocking of cells may indeed aid in gamete delivery to the egg. Although parallel sheets of smooth endoplasmic reticulum line the body-cell periphery and extend into the cytoplasmic projections, no microtubules were observed in the body cell of *P. monticola*.

The chains of ribosomes which surrounded the body-cell organelles, reported by Willemse and Linskens (1969) in *P. sylvestris*, Camefort (1966a) in *P. nigra* and Camefort (1978) in *Picea*, were also observed in *P. monticola*, where they commonly were helically-arranged and surrounded the tightly-clustered plastids and mitochondria. Because helical polyribosome chains were not observed in the mature egg cell of *P. monticola*, they serve as one feature whereby body-cell organelles can be distinguished from egg-cell mitochondria once the body-cell cytoplasm has entered the egg.

5.2 Pre-Fertilization Megagametophyte Development

5.2.1 Neck Cells

Neck cells in *P. monticola*, as in *Pseudotsuga* (Owens and Morris, 1990), appeared secretory and may be involved in pollen-tube attraction. Pollen tubes grew directly towards the neck cells. Neck-cell cytoplasm was very electron-dense with numerous organelles. Dictyosomes, endoplasmic vesicles and lipid-like bodies were abundant and pockets of electron-dense cytoplasmic secretions were located between the plasmalemma and cell wall all along the periphery of the cells. Plasmodesmata in the cell walls were also electron-dense, possibly because of the presence of these secretions.

5.2.2 Central Cell

Central-cell development and ultrastructure in *P. monticola* was similar to *P. nigra* (Camefort, 1960; 1962; 1965a) with the following exceptions. The young central cell contained electron-transparent vacuoles, delimited from the cytoplasm by a simple membrane, rather than cytoplasmic voids. The simple-membrane contours described by Camefort (1962, 1965a) in the developing central cell and which disappeared with maturation, were not evident in *P. monticola* central cells. Small inclusions appeared suddenly and were numerous in the dividing central cell and were absent in earlier stages.

5.2.3 Egg Cell

The mature egg cytoplasm in *P. monticola* was organized into three concentric zones: the perinuclear, mid- and peripheral zones. This has not been reported in other conifers. The egg-cell perinuclear zone was the maternal origin of proembryo neocytoplasm and, until recently (Bruns and Owens, 1989), was reported to be absent in *Pinus* (Camefort, 1968b) although present in *Larix* (Camefort, 1967a,b; 1968a) and *Pseudotsuga* (Owens and Morris, 1991). The perinuclear zone in *P. monticola* was comparable in size to *Larix*, but smaller than in *Pseudotsuga*. In all three species studied, it consisted largely of mitochondria and polyribosomes. Outside the perinuclear zone in *P. monticola* was a mid-zone dominated by small inclusions with some scattered organelles and, occasionally, large inclusions. Most large inclusions were limited to the peripheral zone lining the plasmalemma.

5.2.3.1 Large Inclusions

Although large inclusions in the *P. monticola* egg cell were bound by double membranes, both at the periphery of large inclusions and separating stromal regions from cytoplasmic compartments, in many places these membranes were broken or absent. This probably resulted from their extreme enlargement. Membraneous figures were often formed from collapsed inclusions or portions thereof. In section, portions of large inclusions in *P. monticola*, unlike *P. nigra* (Camefort, 1965a; 1968b), often lacked some of the double-membrane layers which would be expected between a cytoplasmic compartment and the general cytoplasm surrounding the inclusion. The plastid origin of large inclusions can not be questioned however because of the presence of starch in earlier stages of their development.

The present study differs from earlier ultrastructural reports regarding several aspects of large-inclusion formation in the Pinaceae. Although large-inclusion formation in *P. monticola* was apparent in young central cells, the process of plastid transformation was not initiated at this stage, but much earlier. Even in the earliest megagametophyte stage examined, the free-nuclear megagametophyte, most plastids were concave and had encircled a single pocket of cytoplasm, indicating large-inclusion development. Also, large inclusions were not limited to the central cell and egg cell, but were found in all megagametophyte cells examined except the ventral canal cell. There, organelle identification was impossible because of the extreme electron-density of the cytoplasm. Most plastids of the neck, jacket and the megagametophyte cells which surrounded the central and egg cells had become concave and had engulfed one to several cytoplasmic

pockets. Although the size of the plastids in these smaller megagametophyte cells was limited by cell size and plastids did not ultimately attain the dimensions of central- and egg-cell large inclusions, their structure and development were identical to early developmental stages of central-cell large inclusions and, therefore, they should also be designated as large inclusions. Jacket-cell large inclusions were often formed by several plastids which became entwined early in their development. This also may occur in the other megagametophyte cells. The formation of large inclusions from plastids in *P. monticola* then, appears to occur very early on in development and is characteristic of the entire megagametophyte and not just the central cell and egg cell.

5.3 Fertilization

5.3.1 Fertilizing Male Gamete and Body-Cell Organelles

Previous light microscope and ultrastructural studies on *Pinus* and other Pinaceae (Mangenot, 1938; Camefort 1966a; 1968b; 1969) report that the fertilizing male gamete is devoid of cytoplasm as it descends to the egg nucleus and that only egg-cell mitochondria surround the fusing nuclei. In *P. monticola* small numbers of body-cell plastids and mitochondria surrounded by polyribosomes were sometimes carried with the fertilizing male gamete to the perinuclear zone, in advance of the majority of clustered body-cell organelles which remained well behind the fertilizing male gamete and became incorporated into the neocytoplasm at a much later stage of proembryo development. During and following nuclear fusion, the paternal organelles which had accompanied the male gamete could be found at the edge of the perinuclear zone or inside it.

In *P. monticola*, body-cell plastids in the perinuclear zone were easily identified because no egg-cell plastids were present in or near this zone, but body-cell mitochondria were difficult to identify because male mitochondria were only slightly smaller than egg-cell mitochondria, both were electron-dense and polyribosomes were abundant in the perinuclear zone. Approximately coinciding with nuclear fusion, body-cell mitochondria at the edge of the perinuclear zone and in the cluster migrating towards the egg/zygote nucleus appeared to degenerate. Body-cell plastids appeared unaffected. This has not been reported in other conifers. No degenerate organelles were observed in the perinuclear zone. Paternal mitochondria enlarged, became mostly electron-transparent and contained flocculent structures which may have been cristae remnants. The outer membrane of some mitochondria may have been interrupted. It was impossible to determine whether all body-cell mitochondria outside the perinuclear zone were affected. In *Larix* (Camefort, 1968b; Chesnoy and Thomas, 1971) and *Pseudotsuga* (Owens and Morris, 1991), body-cell organelles followed behind the fertilizing male gamete and arrived near the perinuclear zone prior to division of the zygote nucleus. In *Larix,* body-cell organelles became incorporated into the neocytoplasm at the four-nucleate stage or during nuclear descent (Camefort, 1968a,b). In *Pseudotsuga*, paternal organelles remained clustered until free-nuclei had reached the chalazal end of the egg cell.

5.3.2 Nuclear Fusion

Although earlier studies of fertilization in *Pinus* (Camefort, 1965b; 1969) state that with the approach of the fertilizing male nucleus, the egg nucleus forms an

impression into which the male settles, this was not observed in *P. monticola*. Rather, fusion of the two nuclei was similar to *Pseudotsuga* (Owens and Morris, 1991) in that the male nucleus formed a depression in the egg nucleus. However, these studies (Camefort, 1965b; Owens and Morris, 1991) reported that 'islets' or 'islands' of perinuclear-zone cytoplasm were contained between the fusing nuclei and this terminology is unfortunate. Although, in section, this cytoplasm may appear as distinct portions bound by nuclear membrane, it is a continuous sheet of cytoplasm perforated by the nucleoplasmic channels established between the fusing nuclei. As these channels enlarge, the sheet of perinuclear-zone cytoplasm is gradually pushed out from between the fusing nuclei and into the surrounding perinuclear zone.

5.4 Zygote and Proembryo Development

5.4.1 Neocytoplasm

In studies of *Pinus nigra*, the term neocytoplasm was introduced by Camefort (1958; 1965a,c; 1966b) as a new cytoplasmic territory originating from the nucleoplasm of the zygote released during the first coenocytic division and, through which, the zygote escapes lysis. Through proembryo development the neocytoplasm became progressively organized and eventually constituted the embryo cytoplasm. Camefort (1966b) was unable to determine the origin of plastids and mitochondria which appeared in the neocytoplasm once the free-nuclei had migrated to the chalazal end of the egg, although these mitochondria were believed to be of maternal origin. The concept of neocytoplasm

was subsequently applied to other Pinaceae including *Larix* (Camefort, 1967b; 1968b), *Cedrus* and *Picea* (Camefort, 1969) and *Pseudotsuga* (Thomas and Chesnoy, 1969).

In contrast to *Pinus nigra*, *Larix* possessed a distinct perinuclear zone, and the origin of mitochondria in neocytoplasm was maternal. Egg-cell mitochondria quickly penetrated into the neocytoplasm at its formation. Clustered body-cell plastids and mitochondria were observed to follow the fertilizing male gamete and eventually became incorporated into the neocytoplasm during the four-nucleate proembryo prior to or during free-nuclear migration.

Origin of neocytoplasm in *P. monticola* was similar to *Larix* in that both the nucleoplasm of the zygote and the perinuclear zone contribute to its formation. Initially the nucleoplasm was distinct from the perinuclear zone but, prior to free-nuclear migration, the two zones melded together forming a substantial amount of neocytoplasm. Neocytoplasm origin in *P. monticola* is unique in the Pinaceae studied thus far however, because body-cell mitochondria and plastids are sometimes present in the perinuclear zone along with the numerous egg-cell mitochondria. These body-cell organelles may also contribute to the neocytoplasm early in its development. Although in this study, body-cell plastids and mitochondria were not identified in the neocytoplasm around migrating free-nuclei, body-cell plastids were observed among egg-cell mitochondria in the perinuclear zone and both this zone and the released nucleoplasm escape lysis.

5.4.2 Origin of Proembryo Plastids

Chloroplast DNA (cpDNA) inheritance in *P. monticola* has been determined using restriction fragment length polymorphisms (RFLP) techniques to be predominantly paternal but occasionally biparental (White, 1990). These results agree with similar studies on other conifers in which inheritance of cpDNA is either strictly paternal (Neale *et al.*, 1986; Neale and Sederoff, 1988; 1989; Stine and Keathley, 1988; Stine *et al.*, 1989) or predominantly paternal (Wagner *et al.*, 1987; Szmidt *et al.*, 1987). Ultrastructural evidence presented in this study of *P. monticola* supports strict-paternal plastid inheritance. It seems highly unlikely that maternal plastids remained as proplastids in the perinuclear zone and were incorrectly identified. The occasional occurrence of biparental cpDNA inheritance would more likely be explained through the chance incorporation of one or more large inclusions into the proembryo neocytoplasm. This has been observed in *Pseudotsuga* (Owens and Morris, 1991).

5.4.2.1 Mechanisms of Paternal Plastid Inheritance

Small numbers of body-cell plastids were only sometimes carried with the fertilizing male gamete to the perinuclear zone. Because substantial numbers of plastids were observed in the neocytoplasm of all proembryos in which the four nuclei had reached the archegonial base, these body-cell plastids could not alone be the origin of proembryo plastids. Clustered male organelles were observed near the neocytoplasm of free-nuclei just before migration and, although the moment of incorporation of the clustered organelles into the neocytoplasm was not observed in this study, it is probable

that this occurs during nuclear migration to the chalazal end of the egg. Neocytoplasm in *P. monticola* quickly became very electron-dense as the nuclei began migration, largely because of the appearance of tremendous numbers of ribosomes. As a result, organelles, which themselves are electron-dense, were difficult to identify within the neocytoplasm of migrating nuclei. Once the free-nuclei settled into a single tier and began cellular divisions, starch appeared in the plastids allowing for their identification.

5.4.2.2 Mechanisms of Maternal Plastid Exclusion

The structural mechanisms whereby maternal plastids are completely or largely excluded from proembryo cytoplasm was fully demonstrated in this study and agrees with earlier reports on other species of *Pinus* (Camefort, 1962; 1965a; 1968b), *Larix* (Camefort, 1967a,b) and *Pseudotsuga* (Owens and Morris, 1990; 1991). All egg-cell plastids were transformed into large inclusions. This process was first evidenced when plastids became concave and encircled a portion of cytoplasm. As plastids enlarged, more cytoplasmic pockets were engulfed and starch was completely dissolved from plastid stroma. In the unfertilized mature egg cell of *P. monticola*, large inclusions were confined to the mid- and peripheral zones. In no instances were large inclusions present either near or inside the perinuclear zone which is the sole maternal origin of the proembryo neocytoplasm, or in the primary embryonal tier of the proembryo.

5.4.3 Origin of Proembryo Mitochondria

Results of previous ultrastructural studies of the Cupressaceae and Taxodiaceae (Chesnoy, 1987b) are consistent with RFLP studies undertaken thus far demonstrating paternal mtDNA inheritance in members of these families (Neale *et al.*, 1989; 1991). A layer of male cytoplasm containing plastids and mitochondria surrounds the zygote nucleus in the Cupressaceae, Taxodiaceae and Cephalotaxaceae (Chesnoy, 1987b). This layer alone was determined in ultrastructural studies to originate the proembryo cytoplasm, supporting the concept of paternal mitochondrial inheritance. In *Taxus* (Taxaceae) maternal mitochondria are included in this layer, supporting biparental mitochondrial inheritance (Chesnoy, 1987b). In the Pinaceae molecular studies report strictly- or predominantly-maternal mitochondrial inheritance (Neale and Sederoff, 1988; 1989; Wagner *et al.*, 1991). A perinuclear zone of maternal mitochondria surrounds the zygote nucleus in *Larix* (Camefort, 1967b), *Pseudotsuga* (Owens and Morris, 1991) and *Pinus monticola* (Bruns and Owens, 1989). In *Larix* and *Pseudotsuga*, body-cell plastids and mitochondria ultimately combine with perinuclear zone organelles to form the proembryo neocytoplasm, supporting biparental mitochondrial inheritance. Owens and Morris (1991) estimate that about 10% of mitochondria in *Pseudotsuga* were of paternal origin. Ultrastructural evidence presented in this study of *P. monticola* supports maternal mitochondrial inheritance with the possibility of an occasional, low-level, paternal contribution.

5.4.3.1 Mechanism of Maternal Mitochondrial Inheritance

The mechanism whereby mitochondria were maternally inherited in *P. monticola* was by their aggregation around the egg nucleus in the perinuclear zone. In the mature central cell, mitochondria were common near the nucleus and, during migration of the egg nucleus to the centre of the egg cell, the perinuclear zone was fully established and included most egg-cell mitochondria. The author suspects that this zone may have resulted both from migration of some mitochondria in the mid-zone of the egg and from multiplication of mitochondria previously in the vicinity of the central/egg nucleus. The nucleoplasm released during the zygotic coenocytic divisions and the perinuclear zone of the zygote melded to form the proembryo neocytoplasm which escaped lysis and became the embryo cytoplasm.

5.4.3.2 Mechanism of Paternal Mitochondrial Exclusion

Paternal mitochondria appeared to be completely or largely excluded from the proembryo by degeneration after their release into the egg cytoplasm. Both body-cell mitochondria brought with the fertilizing male gamete to the egg nucleus, but which remained outside the perinuclear zone, and clustered body-cell mitochondria which followed behind the fertilizing male gamete, enlarged and became electron-transparent except for flocculent structures which were interpreted as remnants of cristae. Occasionally the outer membrane of these mitochondria appeared degenerate and was possibly interrupted. Although most or all of the body-cell mitochondria outside of the perinuclear zone were affected, small numbers of body-cell mitochondria which were

sometimes carried with the fertilizing male gamete may have escaped lysis by becoming incorporated early into the perinuclear zone which contributed to the neocytoplasm. The possibility of occasional biparental mitochondrial inheritance is, therefore, not excluded, although the paternal contribution would be small.

Chapter 6

CONCLUSIONS

This thesis confirms that the ultrastructure of sexual reproduction in *Pinus monticola* is similar to other members of the Pinaceae and clarifies several aspects of cytoplasmic inheritance which were hereto incomplete in this genus.

At fertilization, body-cell plastids and mitochondria were transferred to the egg cell with the two male gametes. Two modes for incorporation of body-cell organelles into the neocytoplasm were revealed. Clustered body-cell plastids and mitochondria followed behind the fertilizing male gamete. Occasionally small numbers of these body-cell organelles were carried with the fertilizing male gamete to the egg nucleus where body-cell plastids were sometimes identified inside the perinuclear zone. Although body-cell mitochondria may also have been introduced into the perinuclear zone, they were impossible to identify. Body-cell mitochondria outside the perinuclear zone appeared to degenerate, but no degenerate organelles were present within this zone. Both the perinuclear zone of the zygote and nucleoplasm released during coenocytic divisions of the zygote nucleus contributed to the neocytoplasm of the proembryo. Therefore, small numbers of body-cell plastids and mitochondria could occasionally be introduced along with the egg-cell perinuclear-zone mitochondria, into the neocytoplasm. The larger, paternal-plastid contribution to the proembryo was constituted by the clustered body-cell plastids which were introduced into the neocytoplasm, probably during free-nuclear migration to the chalazal end of the egg.

Maternal plastids were largely or entirely excluded from proembryo neocytoplasm by a process of plastid transformation into large inclusions, which ultimately were isolated from the perinuclear zone as they were located around the periphery of the egg. All central-cell and egg-cell plastids, and possibly all megagametophyte plastids, became large inclusions. Although the dimensions achieved by large inclusions varied among the cell types, the process of transformation was identical in all cells and was initiated in the free-nuclear stage of megagametophyte development, or even earlier.

These structural mechanisms of cytoplasmic inheritance in *P. monticola* are consistent with molecular studies of cytoplasmic inheritance in the Pinaceae. Ultrastructural observations support strict-paternal plastid inheritance, but an occasional low-level, maternal contribution could result if, in some archegonia, one or more large inclusions were incorporated into the neocytoplasm during or following free-nuclear migration. Mitochondrial inheritance appears to be, most often, strictly maternal. Occasional biparental mitochondrial inheritance may result from the introduction, in some archegonia, of body-cell mitochondria carried with the fertilizing male gamete, into the perinuclear zone. Occasional, strict-paternal mtDNA inheritance, determined by Wagner *et al.* (1991) in other *Pinus* species, was not demonstrated in *P. monticola*.

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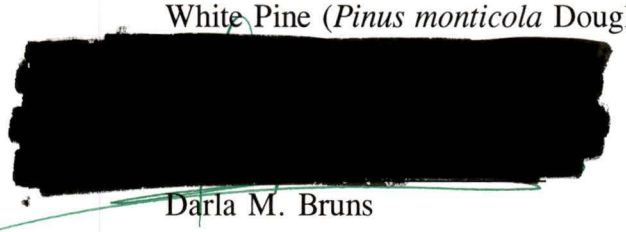
Author: 

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