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Western white pine (*Pinus monticola* Dougl.) reproduction: I. Gametophyte development

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Abstract Male and female gametophyte development are described from light and transmission electron microscope preparations of ovules from first and second year *Pinus monticola* Dougl. seed cones. In the first year of development, pollen tubes penetrate about one-third the distance through the nucellus. The generative cell and tube nucleus move into the pollen tube. The megagametophyte undergoes early free nuclear division. First-year seed cones and pollen tubes become dormant in mid-July. In the second year, seed cones and pollen tubes resume development in April and the pollen tubes grow to the megagametophyte by mid-June. Early in June the generative cell undergoes mitosis, forming two equal-size sperm nuclei that remain within the generative cell cytoplasm. The generative cell has many extensions and abundant mitochondria and plastids. The megagametophyte resumes free nuclear division, then cell wall formation begins in early July. Cell wall formation and megagametophyte development follow the pattern found in other Pinaceae. Three to five archegonial initials form. The primary neck cell divides, forming one tier of neck cells. Jacket cells differentiate around each central cell. The central cell enlarges and becomes vacuolate; then vacuoles decrease in size and the cell divides, forming a small ventral canal cell and a large egg. Plastids in the central cell engulf large amounts of cytoplasm and enlarge. This process continues in the egg, and the peripheral cytoplasm of the egg becomes filled with transformed plastids. Mitochondria migrate around the nucleus, forming a perinuclear zone. The wide area of egg cytoplasm between these two zones has few organelles. A modified terminology for cells involved in microgametophyte development is recommended.

Key words White pine · *Pinus* · Megagametophyte · Egg · Microgametophyte · Sperm

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Introduction

Western white pine (*Pinus monticola* Dougl.) is one of about 31 species of white pines. White pines are divided into two sections: Strobus with 19 species and Parrya with 12 species. The first section has two subsections and the second has three. Western white pine belongs in the subsection Strobi of section Strobus (Critchfield and Little 1966). Western white pine is distributed from coastal and interior British Columbia (B.C.) south to California and Montana in the Cascade and Rocky Mountains. Two other white pines, *P. albicaulis* and *P. flexilis*, occur in B.C. at higher elevations (Fowells 1965). Several other white pines are native to the U.S. and Mexico and others occur in Eurasia from the European Alps to Japan (Bingham 1983).

Early in the 20th century, white pines *P. monticola* and *P. lambertiana* were commercially very important and widely harvested in western North America. Today, they are still potentially some of our most valuable species but their distribution and harvest is limited because of the white pine blister rust (*Cronartium* sp.). White pine blister rust was not reported in North America until a shipment of pine seedlings from France in 1910 infected with the disease was sold in Vancouver. The disease rapidly spread through forests of B.C. and the Pacific Northwest. In the 1950s the U.S. and in the 1970s B.C. began rust-resistant white pine selection and breeding programs. Early attempts to hybridise rust-resistant and rust-susceptible white pine species were largely abandoned because of high incompatibility levels and phenological differences among species (Bingham 1983). Rust-resistant families have been identified and genetic tree improvement programs and seed orchards for the production of rust-resistant seed have been established in coastal and interior regions of the Pacific Northwest and B.C. Classical tree breeding techniques are being used to improve cone and seed production and pollination, as are molecular and tissue culture technologies. These approaches require a better understanding of the reproductive biology of white pine species than is now available in the literature.

The primary purpose of this research was to determine ultrastructurally the male and female gametophyte development and gamete structure of western white pine. A second purpose for this research was to revise and clarify the terminology used for male and to a lesser extent female gametophyte development and reinterpret these structures.

The terminology used for the sequence of mitoses and cell divisions in conifer male gametophyte development goes back over 100 years and has changed over time. Terms for the same structures have varied, often depending only on the species being studied. Some terms are based on misinterpretation of light microscopy. For a complete review see Singh (1978). This leads to considerable confusion even by specialists in the field and, in particular, by students of conifer reproduction. In addition, few of the terms used for conifers are consistent with those used for angiosperms (Johri 1984) although for some structures development may be similar. Terminology used for the archegoniate female gametophyte development of conifers is less variable and quite different than that used with angiosperms, but certain structures unique to conifers, i.e. large inclusions, need to be clarified in order to understand the diversity in conifer cytoplasmic inheritance (Mogensen 1996; Owens and Morris 1990, 1991). A second report will investigate the methods of fertilisation and cytoplasmic inheritance based on a clear understanding of the structures involved.

Materials and methods

Seed cones of western white pine have a 2-year cycle. In B.C., pollination occurs in late May or early June of the first year and pollen germinates within a few days. Pollen tubes develop and penetrate about one-third the length of the nucellus before July when the seed cones, in which the ovules have reached the free nuclear megagametophyte stage, become dormant and pollen tubes stop growing. Second-year seed cones resume development in April when megagametophytes continue free nuclear division and pollen tubes resume elongation (Owens and Molder 1977).

Second-year seed cones were collected, starting in April, from four open-pollinated trees growing in Victoria, B.C. In 1987, collections were made weekly from April through May, daily for the first two weeks of June, then weekly from mid-June until early embryo development began in early July. In 1988, collections were made biweekly from late-April until mid-May, daily from mid-May until the end of May, then biweekly until mid-June. Specimens collected in 1988 were the primary specimens used because they contained the stages of development that were of most interest. Phenology was abnormally advanced in 1997 because of a mild, early spring, so several stages were missed. Several seed cones from each tree were placed in a cooler on ice and taken to the University of Victoria where they were dissected. Ovuliferous scales were removed from the central two-thirds of the length of the cone axis, and from these approximately 15 ovules were dissected per cone. Longitudinal cuts were made with a double-edge razor blade on two sides of the ovules. Peripheral megagametophyte tissue and the chalazal half of the ovule were discarded, and only the 1-mm thick portion containing the nucellus and micropylar portion of the megagametophyte were fixed.

In 1987, samples from each cone were embedded in Spurr's low-viscosity resin (Spurr 1969) and Luft's Epon (Luft 1961). In 1988, all specimens were embedded in Spurr's resin because of superior tissue infiltration. For embedding in Epon, specimens were fixed under vacuum for 2 h in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature, washed 1 h in four changes of buffer, post-fixed in 1% osmium tetroxide for 1 h, dehydrated 2 h in a graded acetone series and infiltrated with propylene oxide overnight on a rotator before embedding in Epon at 60°C for 18 h. For embedding in Spurr's resin, specimens were fixed under vacuum for 2 h to overnight at 4°C in 2% gluteraldhyde and 2% formaldhyde in 50 mM potassium phosphate buffer (pH 7.2), washed in four changes of buffer over 1 h, post-fixed in 1% buffered osmium tetroxide for 1 h, washed in buffer overnight, dehydrated in a graded acetone series and embedded in Spurr's resin at 60°C for 18 h.

Thick sections (900 nm–1 µm) for light microscopy and thin sections (60–70 nm) for electron microscopy were cut on a Reichert Om2 or Sorvall MT 5000 ultramicrotome using glass or diamond knives. Thick sections were stained with Richardson's stain (Richardson et al. 1960) and examined and photographed using a Leitz Orthoplan large-field microscope with a Vario-Orthomat camera. Compression of thin sections was relaxed using acetone fumes passed over the sections. Sections that 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% Parodoni in amyl acetate. Sections were stained with 5% uranyl acetate for 10–15 min followed by 0.2% lead citrate (Hayat 1981) for 2 min, then examined and photographed on a Philips EM300 or Joel Jem-1200EX transmission electron microscope at 60 kV.

Some photographic plates contain light micrographs and low magnification electron micrographs to show the orientation of tissues or cells for which the electron micrographs provide ultrastructural details. The terminology used for male and female gametophyte development is illustrated in Fig. 1A,B.

Observations

When seed cones cease to be receptive, usually in early to mid-June, a pollination drop is exuded from the micropyle. Pollen adhering to the micropylar arms enters the drop, floats up into the micropylar canal and usually settles in the pollen chamber in the tip of the nucellus. Within a few days, pollen germinates and pollen tubes grow into the nucellar tip. Pollen tubes grow between nucellar cells, usually destroying cells they contact. By late July, pollen tubes have grown about one-third of the distance through the nucellus. The seed cone then becomes dormant and pollen tubes stop growing (Owens and Molder 1977). In April of the second year seed cones and pollen tubes resume development.

Pollen tube development

Most pollen tubes reached the megagametophyte during the last 2 weeks of May of the second year. All pollen tubes observed in the nucellus were unbranched. The tube tip was very narrow but broadened where the generative cell was located. The tube nucleus was near the tip of the pollen tube followed by the generative cell. Occasionally the sterile cell was also seen in the pollen tube, but prothallial cells remained in the body of the pollen grain outside the nucellus. In pollen tubes that had penetrated the nucellus and reached the megaspore cell wall, the generative cell cytoplasm contained the two sperm nuclei (Figs. 2, 3, 6) and was surrounded by vacuolated tube-cell cytoplasm.

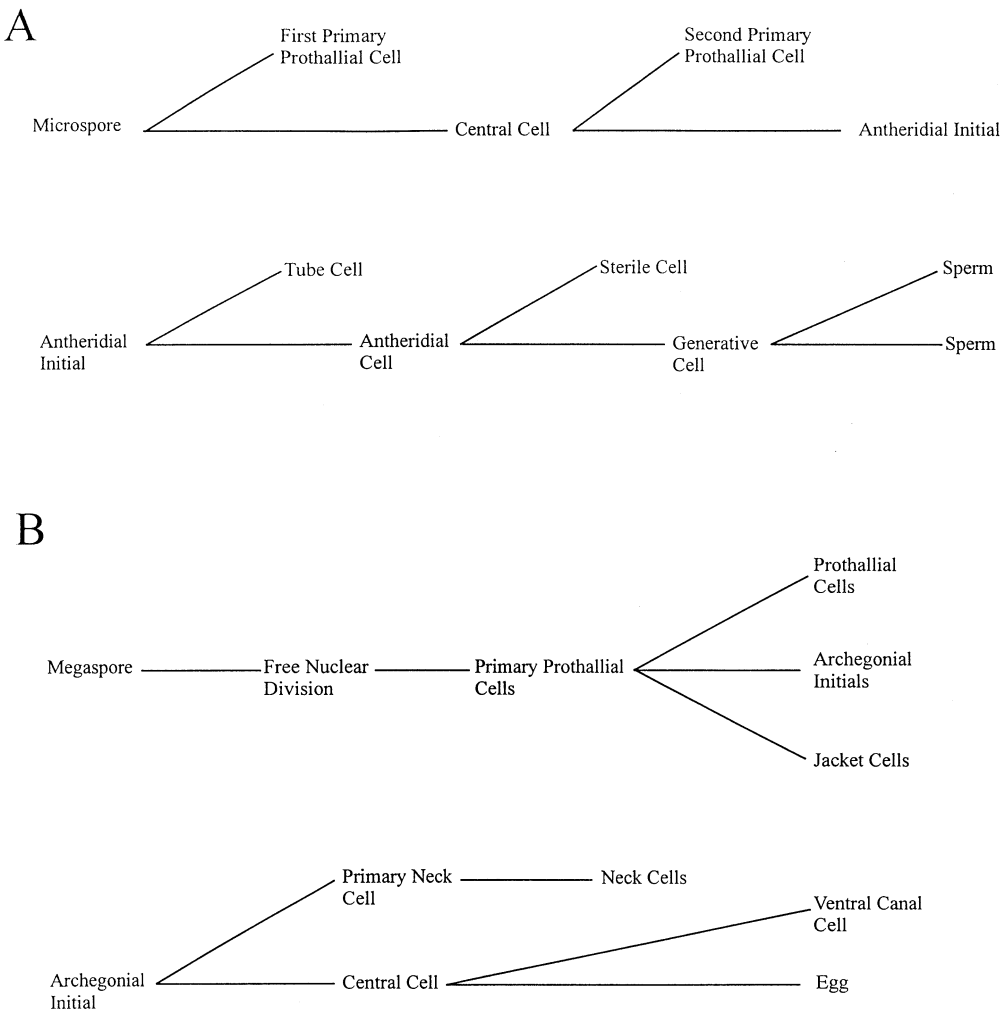


Fig. 1 **A** Male gametophyte development in the Pinaceae and the new terminology used. **B** Female gametophyte development in the Pinaceae and the new terminology used

Tube cell

Tube-cell cytoplasm was highly vacuolated, containing electron-transparent or, rarely, flocculant precipitation within the vacuoles. Tube-cell cytoplasm near the generative cell (Fig. 2) also contained abundant osmiophilic mitochondria, dictyosomes, rough (*rer*) and smooth endoplasmic reticulum (*ser*) and spherical osmiophilic lipid-like bodies. No starch was present and plastids were rare or absent (Fig. 4). In the tube-cell cytoplasm proximal and distal to the generative cell, scattered large starch grains were present (Fig. 5) either as free grains or as one to several grains within a plastid. Lipid-like bodies and organelles that were abundant in the tube-cell cytoplasm near the generative cell were less abundant in peripheral regions. The tube nucleus was positioned near the tip of the pollen tube. The cytoplasm at the tip was more electron-dense than in more proximal areas of the pollen tube.

Sterile cell

The sterile cell usually remained in the corpus of the pollen grain and degenerated. The sterile cell nucleus was spherical and small compared with the generative cell nucleus. The plasmalemma lost its integrity, and the cytoplasm degenerated and organelles became difficult to identify, possibly due to drying of the cell exacerbated by fixation (Fig. 2).

Generative cell and sperm nuclei

Before sperm nuclei formation, the large spherical generative-cell nucleus was proximal in the cell and about 44 µm across. Occasionally along the nuclear margin, short and sometimes branched nuclear projections extended into the surrounding cytoplasm. The nucleus was slightly more electron-dense than the cytoplasm and it contained many small chromatin particles (Fig. 2). Mitosis of the generative cell was not observed. The two sperm nuclei remained proximal in the cytoplasm, one preceding the other (Figs. 3, 6). Both nuclei were irregular in shape, measuring about 34 µm by 20 µm, and had nuclear projections (Fig. 6) similar to those observed in the generative-cell nucleus (Figs. 2, 4). Sperm nuclei contained many small chromatin particles and, just inside the periphery, membranous structures associated with ribosomes (Fig. 7).

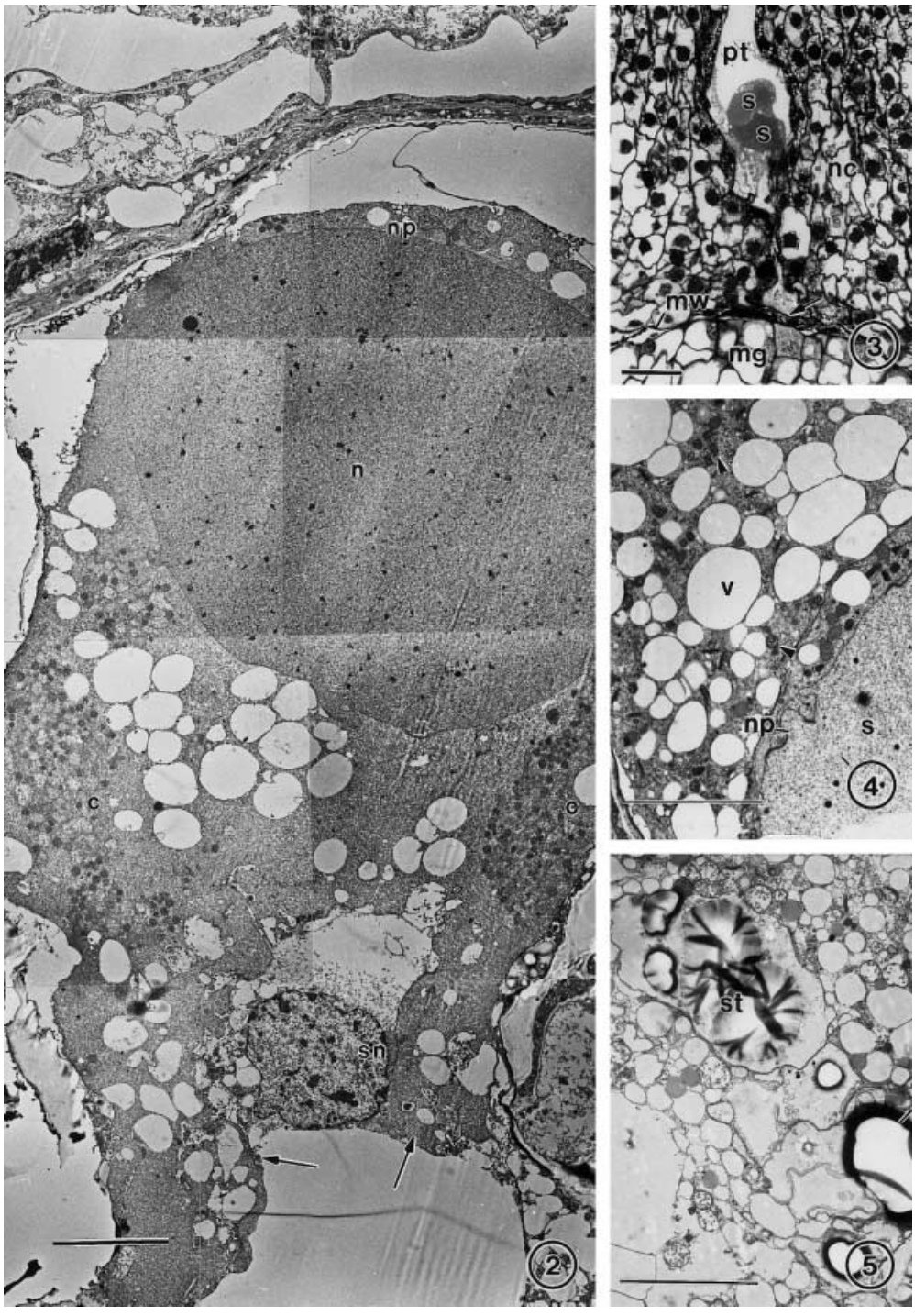
Figs. 2–5 Light (LM) and transmission electron (TEM) microscope features of the male gametophyte in the nucellus during the second year of seed cone development.

Fig. 2 TEM of the generative cell showing a large nucleus (*n*) with nuclear projections (*np*) and cytoplasm with many clusters (*c*) of plastids and mitochondria. Distal cytoplasmic projections (*arrows*) surround the sterile cell nucleus (*sn*). *Bar* = 10 µm.

Fig. 3 LM of the pollen tube (*pt*) within the nucellus (*nc*) showing two sperm (*s*). The tube tip (*arrow*) has reached the megaspore cell wall (*mw*) that bounds the megagametophyte (*mg*). *Bar* = 50 µm.

Fig. 4 TEM of highly vacuolate (*v*) tube-cell cytoplasm with many mitochondria (*arrowheads*). A portion of a sperm nucleus (*s*) shows nuclear projections (*np*). *Bar* = 5 µm.

Fig. 5 Distal tube-cell cytoplasm contains remnants of plastids with starch grains (*st*). *Bar* = 5 µm



Organelles tended to cluster in the generative-cell cytoplasm that remained around the sperm nuclei. Mitochondria were spherical to slightly ovoid and were smaller, more numerous and osmiophilic than plastids. Plastids were elongated, lacked starch and were slightly more electron-dense than the cytoplasm (Figs. 8, 9). Thylakoid development was rudimentary. Cytoplasm was rich with polysomes either associated in small clusters or in helicoid files that often encircled organelles (Fig. 8). Throughout the cytoplasm *ser* was abundant, occasionally with dilated membranes (Fig. 7). Sheets of *ser* were oriented roughly parallel to the plasmalemma and lined much of the cell periphery. Vacuoles, most with electron-translucent contents, were scattered in the cytoplasm. Vesicles were especially common next to the plasmalemma (Fig. 7). Dictyosomes, *rer* and lipid-like bodies were rare. The generative cell, containing the sperm nuclei, was elongate with long distil projections containing parallel sheets of *ser* and clusters of mitochondria (Fig. 6).

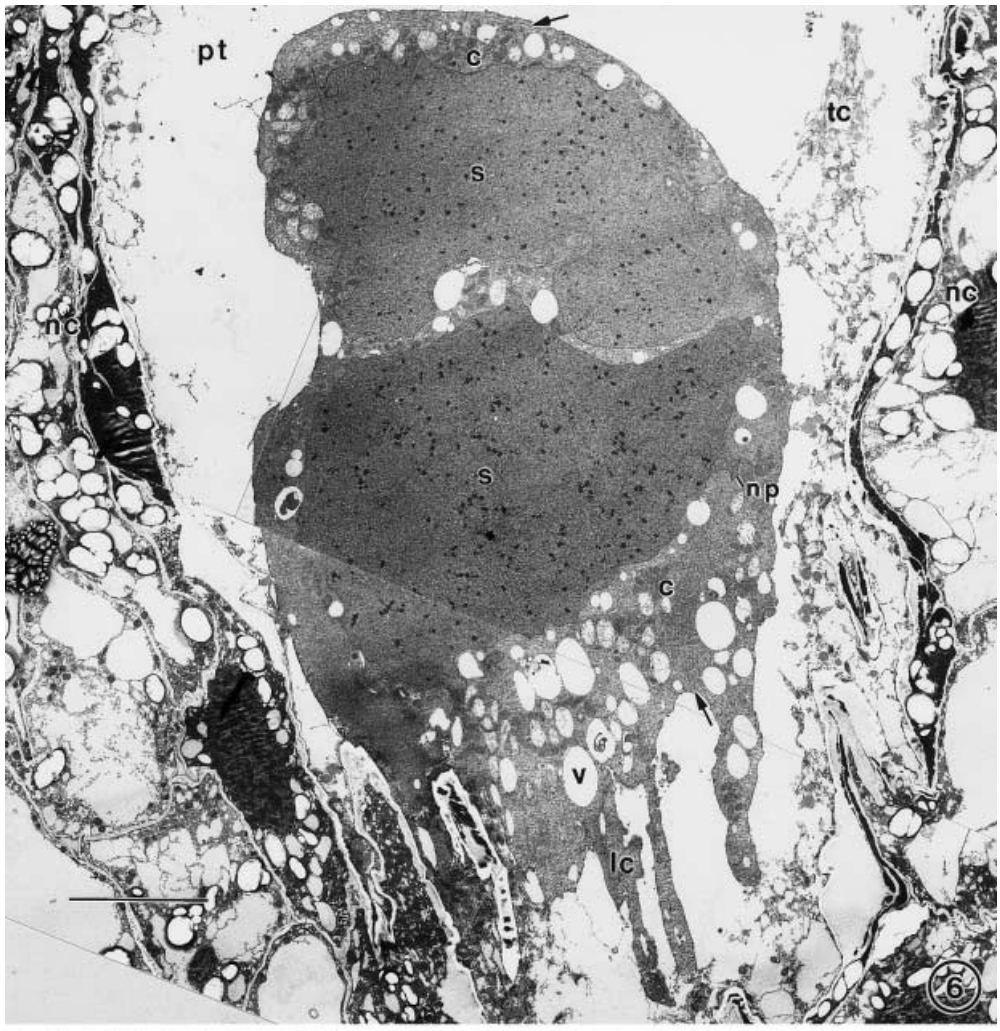
Figs. 6–9 Electron micrographs of sperm and generative-cell cytoplasm in the pollen tube.

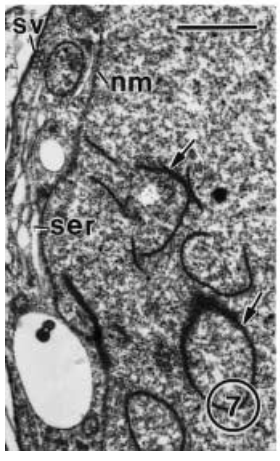
Fig. 6 Two sperm (*s*) within the pollen tube (*pt*) have nuclear projections (*np*). Distal generative-cell cytoplasm has long distal projections (*lc*) and clusters (*c*) of mitochondria and plastids. Sheets of smooth endoplasmic reticulum (*arrows*) are abundant. *nc* Nucellus; *v* vacuole; *tc* tube-cell cytoplasm. *Bar* = 10 µm.

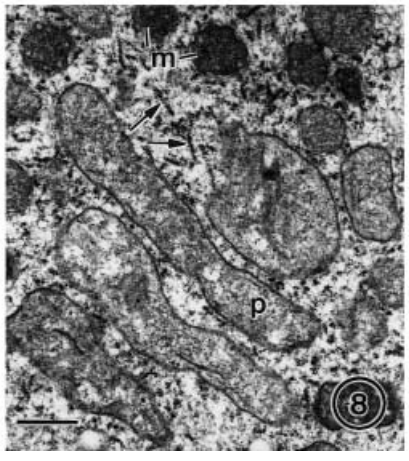
Fig. 7 Membraneous structures (*arrows*) inside the sperm nucleus. Small vesicles (*sv*) and smooth endoplasmic reticulum (*ser*) are common outside the nuclear membrane (*nm*). *Bar* = 1 µm.

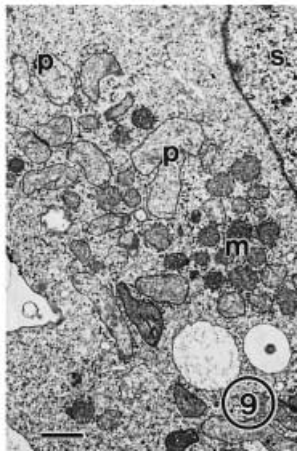
Fig. 8 Plastids (*p*), mitochondria (*m*) and polyribosomes (*arrows*) in the generative cell before sperm formation. *Bar* = 0.5 µm.

Fig. 9 Plastids (*p*) and mitochondria (*m*) in the generative-cell cytoplasm surrounding a sperm. *Bar* =1 µm









Free-nuclear megagametophyte development

Free-nuclear divisions in the megagametophyte occurred during most of April in second-year cones. Free nuclei (Fig. 10) were located near the megaspore-cell wall, while the center of the megagametophyte consisted of a large, electron-transparent vacuole. The cytoplasm had many plastids that varied in shape and some contained a single starch grain and thylakoid membranes (Fig. 11). Occasionally, disc-shaped plastids became concave and encircled a portion of cytoplasm (Fig. 10). At this stage mitochondria were difficult to distinguish from plastids. Both *rer* and *ser* were abundant and reticulum membranes were distended. Many small vesicles were present throughout the cytoplasm and numerous polysomes were associated in small clumps or files. Scattered dictyo-

Fig. 10–16 Light (LM) and electron (TEM) micrographs of the free nuclear megagametophyte and central cell.

Fig. 10 TEM showing a free-nucleus (*mfn*) surrounded by cytoplasm containing elongate plastids (*p*) that often are constricted (*arrows*). *d* Dictyosome. *Bar* = 1um.

Fig. 11 TEM of a free nucleus surrounded by irregularly shaped plastids, some with starch (*st*). *Arrows* Polyribosomes. *Bar* = 1 µm.

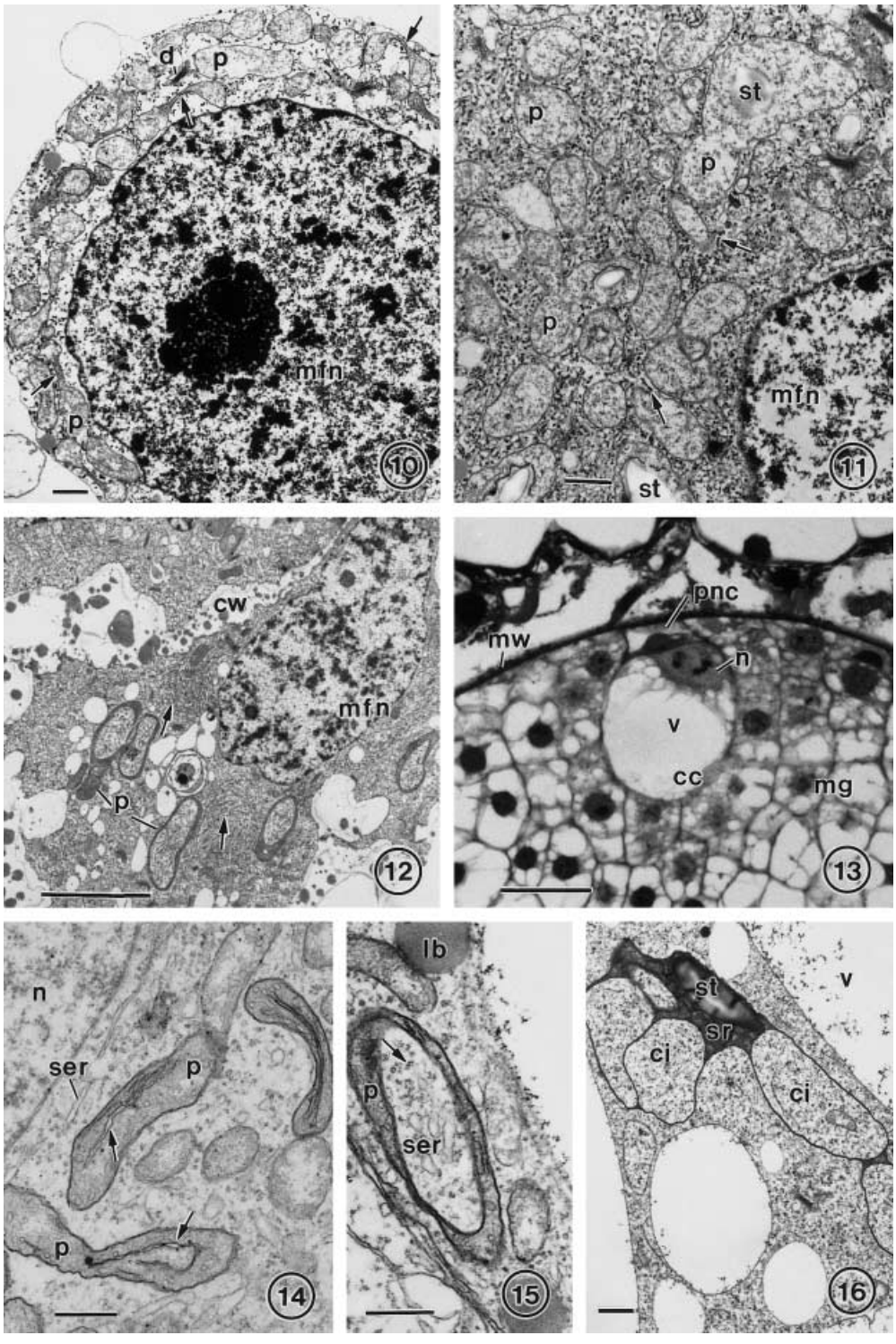
Fig. 12 TEM of a free nucleus during cell-wall (*cw*) formation. The cytoplasm has extensive smooth endoplasmic reticulum (*arrows*) and many plastids have engulfed some cytoplasm. *Bar* = 5 µm.

Fig. 13 LM of an early vacuolated central cell (*cc*). Its nucleus (*n*) lies next to the primary neck cell (*pnc*). *mw* Megaspore cell wall; *mg* megagametophyte; *v* vacuole. *Bar* = 50 µm.

Fig. 14 TEM of the early central cell showing plastids that are elongate and disc-shaped in cross section with thylakoid membranes (*arrows*) that are often distended. *ser* Smooth endoplasmic reticulum. *Bar* = 0.5 µm.

Fig. 15 TEM of an early central cell showing a disc-shaped plastid that surrounded a portion of cytoplasm in which distended *ser* and polyribosomes (*arrow*) are visible. *lb* Lipid body. *Bar* = 0.5 µm.

Fig. 16 TEM of a portion of a central-cell plastid during the frothy stage of central cell development. The plastid is undergoing transformation. Several cytoplasmic inclusions (*ci*) are bound by the plastid membranes. The stromal region (*sr*) contains starch (*st*). *Bar* = 1 µm



somes and lipid-like bodies were present. At the onset of cell-wall formation, plastids became more electron-dense and many had apparently engulfed a portion of cytoplasm (Fig. 12).

Cell-wall formation

Cell-wall formation began in late April or early May and occurred over about 2 weeks. The process was similar to that described by Singh (1978). Each free nucleus became connected to the adjacent free nuclei by spindle fibers. Anticlinal walls were laid down centripetally, separating the nuclei and giving the megagametophyte a hon-

eycomb appearance when viewed in tangential section. The anticlinal walls extended from the periphery toward the central vacuole and cytoplasm filled the vacuole. The anticlinal walls of many adjacent cells joined, forming short pyramidal-shaped cells. Other cells extended to the center of the megagametophyte. Most of the cells underwent mitosis followed by transverse cell-wall formation to form rows of isodiametric cells. Three to five cells at the micropylar end did not divide at that time and remained as large archegonial initials. After the central vacuole had filled with cells, each archegonial initial divided unequally to form a small outer isodiametric primary neck cell and a large pyramidal central cell (Fig. 1B).

Central cell

Immediately following their formation in late April or early May, central cells began to enlarge. This continued for about 2 weeks with central cells ultimately measuring about 620 µm by 390 µm. The central-cell nucleus remained adjacent to the primary neck cell. Most of the early central cell was comprised of a large vacuole with numerous small vacuoles near the nucleus (Fig. 13). Plastids were numerous and similar to those observed in the free-nuclear megagametophyte. Most plastids were disc-shaped with constricted centers and were more electron-dense than the general cytoplasm. Thylakoids were often distended (Fig. 14). Some plastids appeared to engulf a portion of cytoplasm (Fig. 15), based on serial sections. Smooth endoplasmic reticulum was extensive and, in many places, membranes were distended. Small electron-transparent vesicles derived from the *ser* and groups of polyribosomes were numerous. Spherical lipid-like bodies were scattered throughout the cytoplasm (Fig. 15).

As the central-cell enlarged, all plastids engulfed cytoplasm, enlarged many-fold and the stromata became narrow and increasingly electron-dense (Fig. 16). Often in portions of these enlarging plastids stromatal regions were essentially absent or reduced to two appressed double membranes, sometimes with a thin layer of stroma between. Plastids engulfed several pockets of cytoplasm and occasionally starch was present (Fig 16). These transformed plastids are commonly referred to in the literature as large inclusions (Singh 1978). The *ser*, scattered dictyosomes and polyribosomes were common in the central cell, but vacuoles and lipid-like bodies were rare. Mitochondria were oblong to disc-shaped, fairly electron-translucent and they lacked distinct cristae. The large central cell vacuole was gradually replaced by many smaller vacuoles (Fig. 17). This corresponds to the foamy or frothy stage described in the older literature (Singh 1978).

Before division of the central cell, most vacuoles disappeared (Fig. 18) and the cytoplasm became electron dense. All central-cell plastids had been transformed by engulfing varying numbers and sizes of cytoplasmic pockets. The original stroma areas were reduced in size and became very electron dense. The transformed plastids lacked starch, varied in size and shape (Fig. 19) and migrated to the periphery of the cell. Scattered, concave, disc-shaped mitochondria were common near the nucleus and were more electron-dense than in earlier stages (Fig. 20).

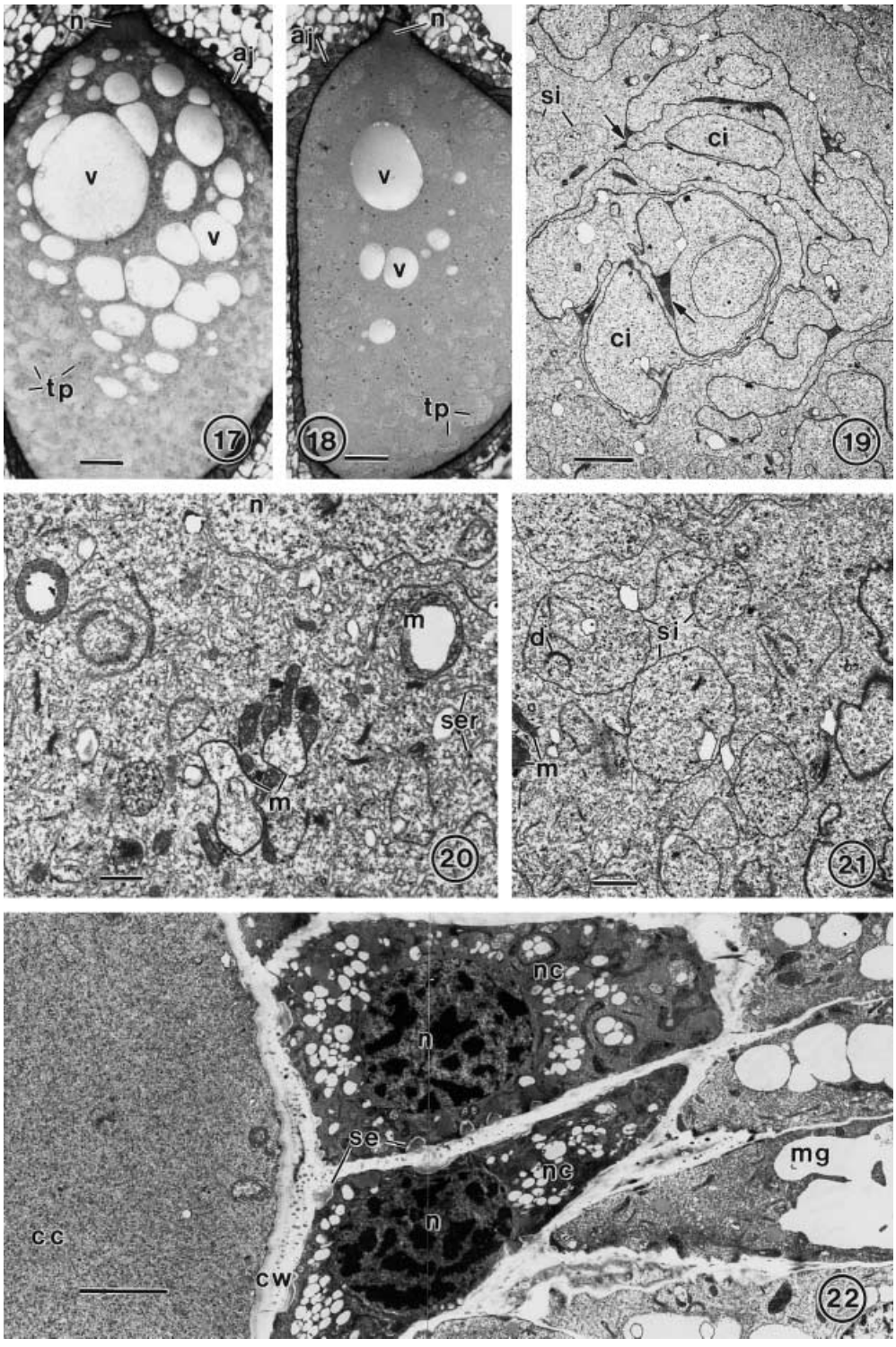
Double-membrane bound sections of cytoplasm appeared throughout the central cell cytoplasm (Fig. 21). These are the small inclusions of older literature (Singh 1978). They were clearly visible in electron micrographs but not in light micrographs where their presence gave the cytoplasm a granular appearance. Their origin was from cup-shaped sections of *ser* that partially isolated pockets of cytoplasm (Singh 1978). Short sections of dilated *ser*, polyribosome groups, dictyosomes and mitochondria were commonly included within the small inclusions.

Primary neck cell and neck cells

The ultastructure of the primary neck cell was similar to that of the early central cell. Generally, four or more neck cells were arranged in a single tier resulting from anticlinal divisions of the primary neck cell and its derivatives. Neck cells became more electron-dense than other megagametophyte cells and had thick, fibrillar cell walls. Nuclei were proximal and spherical and contained many patches of heterochromatin (Fig. 22). Plastids, mitochondria, distended *ser*, endoplasmic vesicles, electron-transparent vacuoles and dictyosomes were numerous. Plastids typically lacked starch and their morphology varied. Some plastids were of medium electron density, ovoid to disc-shaped with little thylakoid development and contained scattered, small plastoglobuli (Fig. 23). Plastids that had become concave and engulfed a single portion of cytoplasm were common (Fig. 24). Mitochondria were spherical, flat or concave and disc-shaped. 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. The periphery of the cells was dotted with pockets of cytoplasmic secretions located between the plasmalemma and the cell wall. These contained both fibrillar material and electron-translucent globules (Fig. 26).

Jacket cells

Jacket cells differentiated from the megagametophyte cells surrounding the archegonial initials and early central cells (Figs. 17, 18). Most were small and isodiametric and arranged in a single layer. Nuclei were central (Fig. 25). Plastids became transformed by engulfing one to several cytoplasmic compartments (Fig. 27), as in the central and egg cells. Commonly two or more transformed plastids created a single inclusion (Fig. 28). Mitochondria were smaller and less electron-dense than plastids and had distended cristae (Fig. 27). Rough endoplasmic reticulum, polyribosome groups, distended *ser* and endoplasmic vesicles were numerous (Fig. 28).



Figs. 17–22 Light (LM) and electron (TEM) micrographs of central and neck cell development.

Fig. 17 LM of the frothy stage of central-cell development. Largest vacuoles (*v*) are innermost in the cell and the nucleus (*n*) lies against the archegonial neck. Transformed plastids (*tp*) are perpheral. *aj* Archegonial jacket. *Bar* = 50 µm.

Fig. 18 LM of later stage of the central cell when vacuoles become less abundant, just before mitosis. *Bar* = 50 µm.

Fig. 19 TEM of a portion of a transformed plastid with many cytoplasmic inclusions (*ci*) and electron-dense stroma (*arrows*). *si* Small inclusions.

Fig. 20 TEM showing the concave mitochondria (*m*) and distended smooth endoplasmic reticulum (*ser*) below the central-cell nucleus that is undergoing mitosis. *Bar* = 1 µm.

Fig. 21 TEM showing abundant small inclusions in the cytoplasm of the dividing central cell. *d* Dictyosome. *Bar* = 1 µm.

Fig. 22 TEM of the neck cells (*nc*) that 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. *Bar* = 10 µm

Ventral canal cell

During the second or third week of May, central cells divided unequally, each producing a small, lens-shaped ventral canal cell and a large egg cell (Fig. 29). The ventral canal cell consisted mostly of a large, osmiophilic and finely granular nucleus (Fig. 30). 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. 31). A thin cell wall formed between the ventral canal cell and egg cell (Figs. 30, 31).

Figs. 23–28 Electron micrographs of neck and jacket cells.

Fig. 23 Three ovoid neck cell plastids (*p*) that often contain plastoglobuli (*arrows*). *n* Nucleus; *cw* cell wall; *m* mitochondria; *v* vacuole; *se* cytoplasmic secretions. *Bar* = 1 µm.

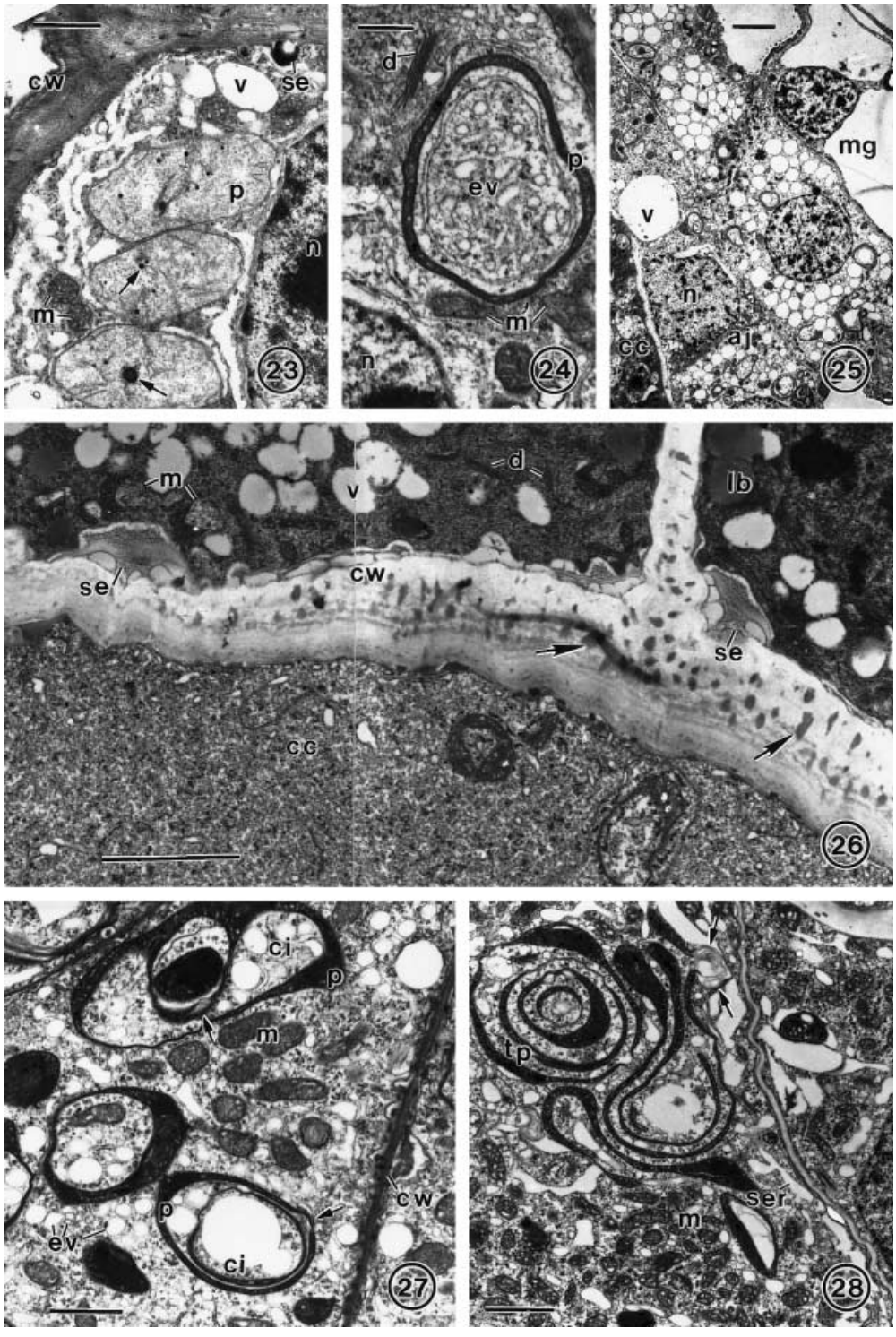
Fig. 24 Concave, disc-shaped neck-cell plastid with electron-dense stromo. *d* Dictyosome; *ev* endoplasmic vesicles. *Bar* = 0.5 µm.

Fig. 25 Vacuolate archegonial jacket cells (*aj*) enclose the central cell (*cc*). *mg* Megagametophyte. *Bar* = 1 µm.

Fig. 26 Cytoplasmic secretions adjacent to the cell walls of the electron-dense neck cells. Cytoplasm is rich with disc-shaped mitochondria, dictyosomes, vacuoles and lipid bodies (*lb*). Cell walls contain many plasmodesmata (*arrows*). *cc* Central cell. *Bar* = 5 µm.

Fig. 27 Jacket-cell plastids are very electron-dense and commonly have several cytoplasmic inclusions (*ci*). Mitochondria are often elongate with distended cristae. *ev* Endoplasmic vesicles. *Bar* = 1 µm.

Fig. 28 Jacket cells sometimes have transformed plastids (*tp*) joined, forming swirls of electron-dense stroma. *ser* Smooth endoplasmic reticulum. *Bar* = 1 µm



Egg cell

Following mitosis of the central cell nucleus, the egg nucleus enlarged as it moved to the center of the egg where it remained until fertilisation (Fig. 29). 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. The margin was undulated and commonly had many embayments (Fig. 32). The egg cytoplasm became organised into three concentric zones: the perinuclear, mid- and peripheral zones.

A narrow, continuous band of perinuclear cytoplasm, about 4.2 µm wide, formed during egg nuclear migration and was located immediately outside the egg nucleus (Fig. 32). Darkly staining mitochondria enhanced the visibility of the edge of the nucleus in light microscopy

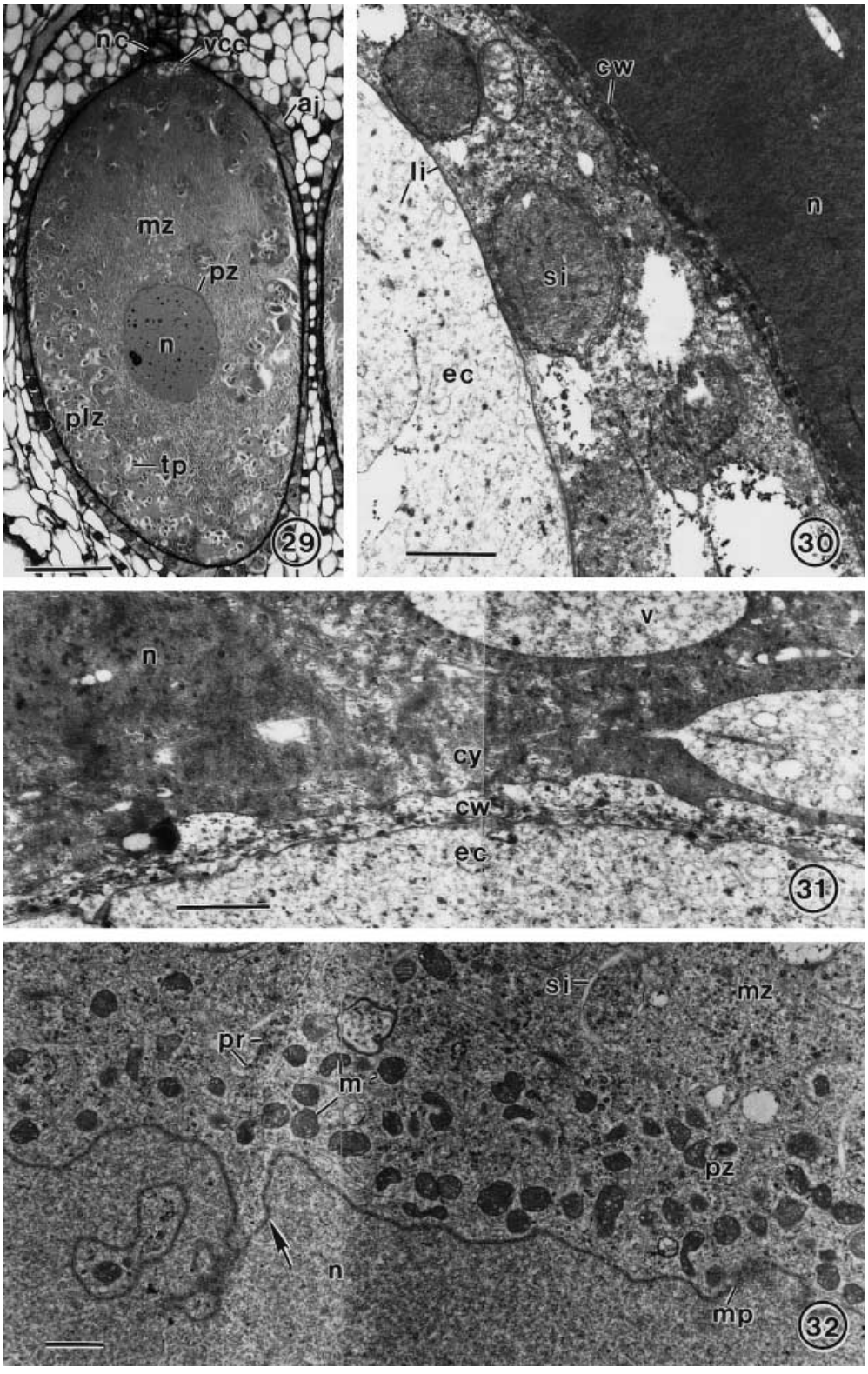
Figs. 29–32 Light (LM) and electron (TEM) micrographs of the ventral canal and egg cells.

Fig. 29 LM of the large egg nucleus (*n*) around which the cytoplasm is organised into a pernicular zone (*pz*), mid-zone (*mz*) and perpheral zone (*plz*). *vcc* ventral canal cell; *nc* neck cells; *aj* archegonial jacket; *tp* transformed plastid. *Bar* = 100 µm.

Fig. 30 TEM of the cell wall (*cw*) between the ventral canal cell and the egg (*ec*). The ventral canal-cell nucleus (*n*) occupies most of the cell. *si* small inclusion. *Bar* = 1 µm.

Fig. 31 TEM of the thin parietal layer of electron-dense ventral canal-cell cytoplasm (*cy*) surrounding the nucleus. Vacuoles (*v*) contain flocculent material. *Bar* = 1 µm.

Fig. 32 TEM of the egg nucleus showing the margin with many embayments (*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 surrounds the perinuclear zone. *Bar* = 1 µm



(Fig. 29). Mitochondria were electron-dense, disc-shaped and typically concave, measuring about 0.9 µm across with distended cristae (Figs. 32, 33). Smooth endoplasmic reticulum, polyribosome groups and occasional dictyosomes were the only other organelles present in the perinuclear zone.

The mid-zone cytoplasm was a thick zone dominated by small inclusions (Singh 1978) (Figs. 29, 32). From the time of central cell division until fertilisation about 1 week later, some small inclusions formed layers of double membranes, separating and delimiting them (Fig. 34). This created electron-transparent, cup-shaped

Figs. 33–38 Electron (TEM) and light (LM) micrographs of the egg.

Fig. 33 TEM showing concave, disc-shaped, perinuclear zone mitochondria (*m*) and endoplasmic vesicles (*ev*). *Bar* = 0.5 µm.

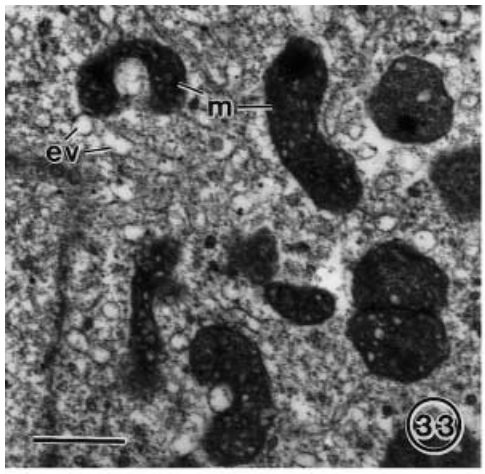
Fig. 34 TEM showing small inclusions (*si*) in the mid-zone of the egg. Endoplasmic reticulum membranes have begun to separate (*arrows*) and vesicles form. *Bar* = 1 µm.

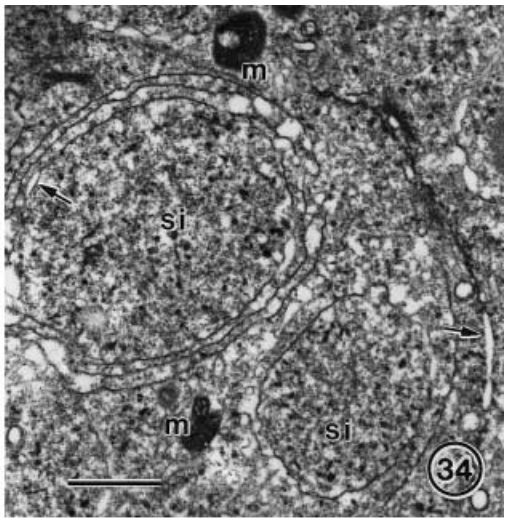
Fig. 35 TEM of unfertilised egg mid-zone cytoplasm. Cup-shaped vacuoles (*v*) delimit the small inclusions. Electron-density of the cytoplasmic nodules (*arrows*) in the small inclusions increase. *tp* Transformed plastid. *Bar* = 10 µm.

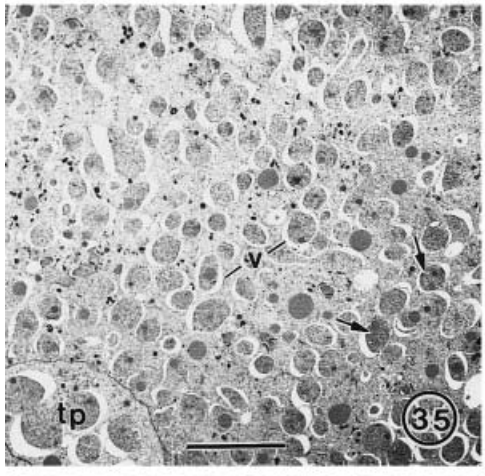
Fig. 36 TEM showing perpheral-zone egg cytoplasm with small inclusions, some within transformed plastids. *Bar* = 5 µm.

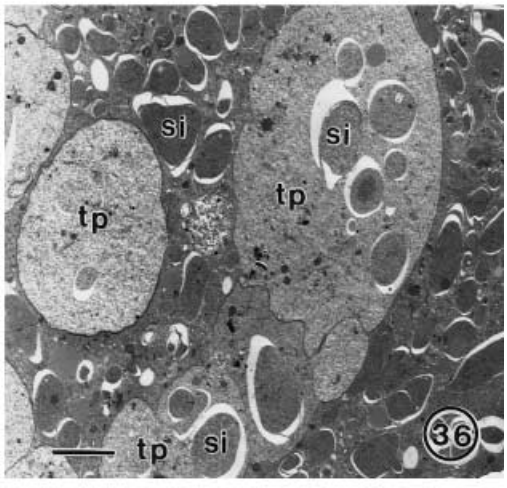
Fig. 37 Integrity of transformed plastid membranes is commonly destroyed (*arrows*). *lb* lipid body. *Bar* = 1 µm.

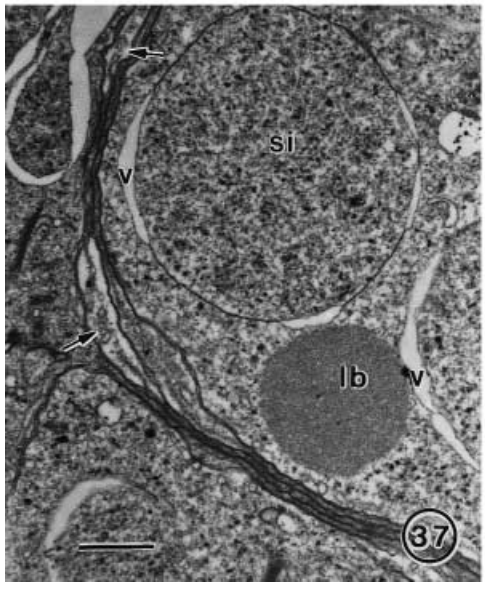
Fig. 38 LM of the micropylar half of the egg at the time of pollen-tube (*pt*) entry. *Arrows* show male organelles and microtubules. *nc* Neck cell; *aj* archegonial jacket cell; *mg* megagametophyte. *Bar* = 50 µm

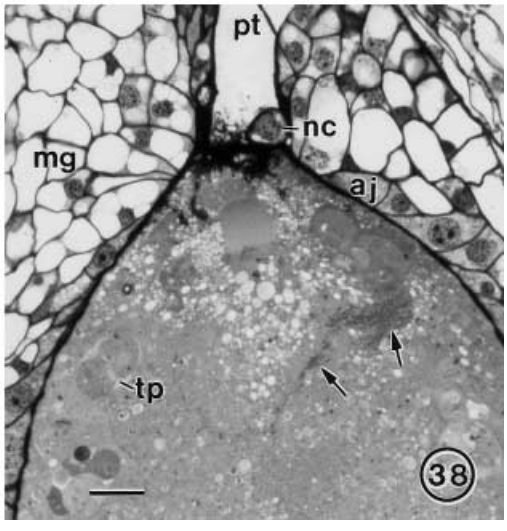












vacuoles that, in section, appeared as crescents with a nodule extending into the crescent. As the volume of the vacuoles increased, the electron-density of the included cytoplasmic nodules increased (Fig. 35). Scattered in the cytoplasm amongst the small inclusions were mitochondria, dictyosomes and lipid-like bodies. Only occasionally were transformed plastids present (Fig. 29). Dilated *ser* and endoplasmic vesicles were abundant (Fig. 34).

Most transformed plastids were confined to the peripheral zone adjacent to the plasmalemma. This zone varied in width but commonly was about 74 µm across (Fig. 29). Cytoplasm within the transformed plastids often became less electron-dense than the general cyto-

plasm, but otherwise was comparable to mid-zone cytoplasm (Fig. 36). Transformed plastids that had engulfed many pockets of cytoplasm had many double membranes layered against each other, causing stromatal regions to no longer be separated from the included cytoplasm. Thus, the perimeter of the transformed plastids began to lose integrity (Fig. 37). The egg retained this appearance until the pollen tube penetrated the neck cells and released its contents into the egg (Fig. 38).

Discussion

Western white pine has a reproductive cycle typical of pines without serotinous cones. Pollination occurs in the spring of the first year. Pollen is taken into the ovules by a pollination drop, then it floats up to the nucellar surface. Pollen germinates within a few days and the pollen tubes grow into about one-third the length of the nucellar tip by mid-July of the first-year; seed cones and pollen tubes then stop growth and become dormant. Ovules that lack pollen abort, and if too few ovules are pollinated in a cone the cone aborts, causing cone drop (Sarvas 1962). Cone drop occurs in the late summer of the first year and can account for significant cone loss. It has been suggested that in pines a hormone, possibly an auxin derived from the pollen, stimulates ovule development (Sweet and Thulin 1969; Sweet 1973), and ovule development promotes cone development. All of the possible causes of cone drop are not well understood. If seed cones survive late summer cone drop and winter losses, development begins again in April of the second year. Ovules resume free-nuclear megagametophyte development and pollen tubes resume their growth through the nucellus. In western white pine, pollen tubes did not branch during growth through the nucellus. Branching is common in some families (Singh 1978) but in the Pinaceae it is rare and considered to be an abnormality. Experimentally, branching has been shown to occur when cones are self-pollinated (Runions and Owens 1999). By mid-June, megagametophytes are mature and pollen tubes have grown to the megaspore cell wall. Here two sperm nuclei are formed and then fertilisation occurs. Seeds are mature and shed usually in September of the second year (Owens and Molder 1977). Phenology may vary by 2–3 weeks among years due to weather.

The early stages of meiosis and pollen development were described in an earlier study (Owens and Molder 1977) and were not repeated in this study. The sequence of cell divisions is the same in all members of the Pinaceae studied thus far (Singh 1978; Owens and Blake 1985) and pollen may be shed at the four- or five-cell stage. Unlike some angiosperms (Johri 1984), no conifers form sperm before pollen is shed. Also, in angiosperms pollen development is simpler with fewer cell divisions than in most conifers and there is a simpler, more standardised terminology for the cells within the pollen. Standardising the terminology for conifer microgametophyte development would reduce the confusion that often exists between conifer families and between angiosperms and conifers. Much of the existing terminology arose from light microscope studies in which cell structure and often function were uncertain. Terminology was often based on cell position which may vary, especially after germination. In the paragraphs following, an alternative terminology is proposed as was used in the Introduction and Results. Each recommended term is shown in italics and is followed, in parentheses, by the previously used term.

Meiosis results in four haploid microspores that separate and develop a thick sculptured outer exine and an inner intine. For several weeks reserves of starch in the Pinaceae or lipids in some other families are stored in the microspores. In the Pinaceae, each microspore divides unequally forming a small *first primary prothallial cell* (prothallial) and a large *central cell* (embryonal). Central cell is recommended because it is consistent with the terminology used for megagametophytes. The central cell then divides unequally forming a *second primary prothallial cell* (prothallial) and an *antheridial initial* (antheridium mother cell). Prothallial cells have no known function, are usually lens-shaped with the second stacked on the first, and both are enclosed in intine. The antheridial initial divides unequally forming a large tube cell and a smaller, thin- walled *antheridial cell* (spermatogenous or generative); these previously used terms are not acceptable because this cell does not directly form sperm. The antheridial cell divides about equally forming a *sterile cell* (stalk or secondary prothallial) and a *generative cell* (body or spermatogenous). Sterling (1963) preferred the term “sterile cell” because it undergoes no further divisions and Singh (1978) preferred “secondary prothallial cell”. However, the latter term is used in the Araucariaceae and Podocarpaceae for cells derived from division of the primary prothallial cells. The sterile cell is usually stacked upon the second prothallial cell and commonly rests in a thick cup-shaped intine but the outer wall remains thin. “Body cell” has traditionally been used but “generative cell” is functionally more accurate and a more widely accepted term used for angiosperms. Pollen is usually shed at this five-cell stage but may be shed at the preceding four-cell stage.

After pollen germination the tube nucleus usually occupies a position near the pollen-tube tip and is followed by the generative cell. The sterile cell may become dislodged and be found in the pollen body or pollen tube, but usually remains firmly in place over the prothallial cells. The generative cell has a thin wall with many extensions and dense cytoplasm with many plastids and mitochondria. It usually follows closely behind the tube nucleus into the pollen tube. The generative cell undergoes mitosis but the two *sperm nuclei* (male gametes) remain within the generative cell cytoplasm until they are released into the egg. Both “sperm” and “male gametes” are widely accepted terms but “sperm” is consistent with angiosperm terminology. Because both sperm nuclei remain within the generative cell cytoplasm for a time, many Pinaceae have been said to have bi-nucleate sperm

(Singh 1978). However, in some species a partial cell wall forms between the sperm nuclei (Runions and Owens 1999). In western white pine the two sperm nuclei are equal- size with no partial cell wall between and they form when the pollen tube reaches the megagametophyte. Sperm nuclei are irregular in shape and tightly packed within the generative cell cytoplasm. They are similar in appearance to sperm nuclei of *Pseudotsuga menziesii* (Owens and Morris 1991) and *Picea glauca* (Dawkins and Owens 1993; Runions and Owens 1999).

The generative cell cytoplasm of western white pine is ultrastructurally similar to that of *Pseudotsuga* (Owens and Morris 1990), *Picea* (Runions and Owens 1999) and *Podocarpus* (Wilson and Owens 1999). Mitochondria and plastids are abundant and tend to cluster. Cristae and thylakoid development are rudimentary. The cytoplasm is rich in polyribosomes and *ser* that is often layered parallel to the plasmamembrane. But *rer*, dictyosomes and lipid bodies are rare. Outside the plasma membrane in *Pseudotsuga* (Owens and Morris 1990) there is a thin polysaccharide layer presumed to be the cell wall. The generative cell boundary in the above genera has many extensions into the tube cell cytoplasm. It was suggested in *Pseudotsuga* (Owens and Morris 1990) that this extensive surface area between the generative and tube cells provides a mechanism by which the large generative cell is pulled through the narrow pollen tube cytoplasm. An analagous structure was observed in the angiosperm *Plumbago zeylanica* (Russell and Cass 1981) in which the vegetative nucleus had many embayments through which the sperm intertwine, allowing the sperm and vegetative nucleus to travel as a unit. In western white pine and the conifers mentioned, microtubules were not involved in generative cell movement. The generative cell appears to be the mechanism of sperm nuclei transport through the pollen tube. However, the time of sperm nuclei formation varies among species. Sperm nuclei form as the pollen tube is growing through the nucellus but before the generative cell reaches the archegonial chamber in western white pine and *Picea* (Dawkins and Owens 1993; Runions and Owens 1999). Division occurs after the generative cell reaches the archegonial chamber in *Pseudotsuga* (Owens and Morris 1991).

Megagametophyte development in western white pine follows the sequence found in other Pinaceae and most other conifers. Terminology is less variable (Singh 1978) than for microgametophytes but some clarification is needed. As in other temperate pines, the free nuclear stage is interrupted by a dormant period. This may not be true in some tropical pines. At the end of the free nuclear stage, secondary spindles connect every nucleus to six adjacent nuclei in a honeycomb fashion. Between these nuclei, cell walls simultaneously form and extend to the central vacuole. These first cells were originally called alveioli (Sokolowa 1890) and later primary prothallial cells. Both terms are still used but the *primary prothallial cells* are more consistent with subsequent terms and microgametophyte development. Primary prothallial cells undergo mitosis and cytokinesis forming the prothallial cells and archegonial jacket cells Prothallial cells frequently become bi- or multi-nucleate in many species. Nuclei may also become polyploid.

Usually several (three to five in western white pine) primary prothallial cells at the micropylar end enlarge forming the archegonial initials. These divide unequally, each forming a small primary neck cell and a large central cell. The former divides to form the neck cells. These are generally four in number and in one tier in western white pine. Two tiers are common in some Pinaceae (Singh 1978) and in the Podocarpaceae (Wilson and Owens 1999). As reported in *Pseudotsuga* (Owens and Morris 1990), *Picea* (Runions and Owens 1999) and in *Cunninghamia* (Singh et al. 1976), the ultrastructure of neck cells in western white pine indicates a secretory function. At maturity, neck cells are polar and cytoplasmic secretions appear along the interface of the protoplast and distal cell wall. Deposits also may appear in the archegonial chamber. These features, plus the generally directional growth of pollen tubes to the neck cells, suggest that archegonial secretions are directing pollen tube growth. Takaso et al. (1996) reported several secretions from the ovule of *Pseudotsuga* and their effect on pollen within the micropylar canal. Ovular secretions began during egg development, about 1 week before fertilisation, as pollen tubes were approaching the megagametophyte. Our recent unpublished results (D. Fernando and J.N. Owens) show high levels of calcium in the megagametophyte of *Pseudotsuga* as the eggs mature.

Several features of central and egg cell development were further clarified in western white pine. The central cells underwent 2 weeks of enlargement during which they became very vacuolate. This was often called the “frothy stage” in older literature (Singh 1978). They then became very electron dense as vacuoles became smaller and disappeared. At that time, plastids within the central cells began to transform into what have been called “large inclusions” in older and recent literature. This term should be replaced by *transformed plastid* because that is how they originate. Small central cell proplastids engulf several pockets of cytoplasm and enlarge many times as the stromata becomes condensed into narrow regions between the cytoplasmic pockets. These large, lobbed or irregular transformed plastids stain readily and are prominent using light microscopy; thus they have been given various names over the years (large inclusions, proteid bodies, paranuclei, vitellus, granules and Hofmeistersche Korperchen) (Singh 1978). As transformed plastids they contain DNA and figure promenantly in cytoplasmic inheritance (Mogensen 1996).

The structure of the mature egg varies in conifers. In the Cupressaceae and Taxodiaceae they are the least modified, having a large central vacuole, evenly distributed organelles and with the plastids remaining untransformed (Chesnoy 1971). At the other extreme are the highly specialised eggs of the Pinaceae. As shown in western white pine, they have no central vacuole but have transformed plastids, a perinuclear zone and the cytoplasm may have concentric zones containing different numbers and types

of organelles (Camefort 1967; Owens and Morris 1990; Runions and Owens 1999). Intermediate in specialisation are the eggs of the Araucaraceae (Owens et al. 1995) that have a large central vacuole and a perinuclear zone but no transformed plastids; Podocarpaceae (Wilson and Owens 1999) that have vacuolate cytoplasm, no perinuclear zone and transformed plastids; and Taxaceae (Anderson and Owens 1999) that have a central vacuole, transformed plastids and a perinuclear zone.

Cytoplasmic compartmentalisation of conifer eggs reaches the maximum in *Pinus*. In western white pine there are three distinct zones: a narrow perinuclear zone that is darkly staining in light microscopy and filled with electron-dense, disc-shaped mitochondria but few other organelles; a wide mid-zone dominated by small inclusions, giving it a lightly staining less electron-dense appearance; and, a darkly staining, electron-dense peripheral zone dominated by transformed plastids. A similar appearance is seen in some of the electron micrographs of *Pinus laricio* (Camefort 1962). The significance of this compartmentalisation of egg cytoplasm is uncertain, although Camefort (1965) suggested that it may prevent lysis of organelles. Transformed plastids and egg cytoplasm compartmentalisation may be most important at fertilisation in allocation of organelles in cytoplasmic inheritance and formation of the neocytoplasm of the proembryo. These topics will be considered in the second paper on western white pine (Bruns and Owens 2000).

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