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POLLEN BIOLOGY OF *PICEA GLAUCA* (MOENCH) VOSS.

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ABSTRACT

Pollen development, pollen forcing, pollen germination and pollen tube growth were examined in *Picea glauca* (Moench) Voss.

Pollen development was studied from breaking of dormancy in mid-February to dehiscence. The effects of forcing were studied by comparing development and quality of pollen from potted grafts grown in a heated greenhouse or at ambient temperature. It was found that although forcing increased the rate of pollen development, it also decreased sucrose levels in cones containing 5-cell pollen and decreased the ability of the pollen to produce seed. Abnormal pollen was found in both force and control treatments.

Pollen hydration, germination and tube growth were studied *in vivo* and *in vitro*. Events were similar during hydration and germination, although pollen germinated faster in liquid medium than in the ovule. Differences in starch accumulation of body cell plastids and in pollen tube zonation became apparent in early stages of pollen tube growth. A penetration peg was formed *in vivo* when the pollen tube contacted nucellar cells. Projections formed by the tube tips appeared to penetrate between nucellar cells, resulting in a branched pollen tube. Plasmatubules were found at and near the pollen tube tip, and are thought to be involved in nutrient absorption. The stalk cell separated from the corpus of the pollen and moved in advance of the body cell into the pollen tube. Stalk and body cells remained within the corpus of the pollen *in vitro*.

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Chapter 1

INTRODUCTION

The life cycle of higher vascular plants consists of a prominent diploid sporophytic generation and a reduced haploid gametophytic generation. Through meiosis, the sporophyte produces male and female spores which develop into gametophytes. The pollen grain and the tube it produces upon germination represent the male gametophyte. The mature male gametophyte contains two male gametes. The function of the mature pollen grain is dispersal. The function of the pollen tube is delivery of the male gametes to the ovule.

In tree breeding programs, pollen forcing is a technique used to enhance the rate of pollen development so pollen is available for specific genetic crosses. In pollen forcing treatments, the rate of pollen-cone development is artificially accelerated by allowing the cones to develop in a warmer than ambient temperature. *Picea glauca* (Moench) Voss (white spruce) is an economically important conifer in Canada with a transcontinental distribution (Fowells 1965). Of a total of 1,223,705 hectares of forested land planted in Canada between 1980 and 1985, 430,236 hectares were planted with *P. glauca* seedlings (Teske 1989). Tree improvement and breeding programs are in place. In B.C. there are 4,050 parent *P. glauca* trees selected and registered with a tree breeding and/or seed orchard program (Anonymous 1989). The effect of forcing on *P. glauca* pollen development has not been studied in detail, although the treatment does have detrimental effects on pollen cone development and pollen yield per cone in *P. engelmannii* (Ross 1988).

A study of pollen biology is not complete without examining the events which occur during pollen germination and pollen tube growth. Pollen germination in a liquid medium has often been used to test pollen quality and viability (Owens and

Blake 1985). It has not been determined how closely the events which occur during germination *in vitro* approximate those *in vivo*.

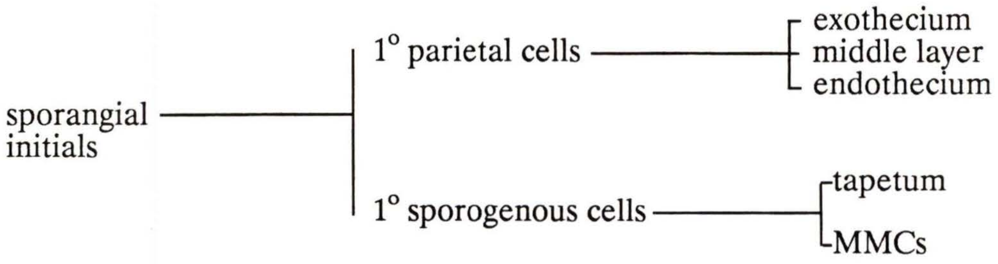
In this thesis, several aspects of pollen biology in *P. glauca* are examined: 1. The development of pollen under natural conditions is described in detail, and adverse effects of elevated temperatures on pollen development are determined. 2. The effects of forcing on: a) the rate of pollen development; b) the sucrose, glucose and starch levels in the pollen; and, c) the ability of the pollen to produce seed are determined. 3. *In vitro* pollen germination and pollen-tube growth are examined histochemically and ultrastructurally, and comparisons are made to *in vivo* events. 4. Development of the pollen tube *in vivo* is studied ultrastructurally with particular emphasis on movement of the body cell in the pollen tube, role of the stalk cell and events at the pollen tube tip.

Chapter 2

LITERATURE REVIEW

2.1. Pollen Development

Sporogenous cells of gymnosperms are derived from sporangial initials, meristematic cells in the microsporophyll. In many gymnosperms these initials are hypodermal in origin, giving rise to primary parietal cells and primary sporogenous cells. The primary parietal cells undergo further cell division to form a multilayered sporangial wall underlying the epidermis. In most conifers the sporangial initials comprise the outermost layer of the sporangium. They divide periclinally to form the inner primary sporogenous cells and the outer primary parietal cells. The latter produce the epidermis (exothecium) as well as the middle layer and inner wall (endothecium). The primary sporogenous cells form the archesporium. They undergo mitosis forming microspore mother cells (MMCs) within the microsporangium (Fagerlind 1961; Singh 1978; Moitra and Bhatnagar 1982). The developmental sequence is:



2.1.1. Tapetal Development

The tapetum is a layer or two of uni- and bi-nucleate cells which encompass the mass of MMCs and function as nutritive tissue. The origin of the tapetum appears to be variable between species. It is generally accepted that the tapetum is derived from the innermost layers of the microsporangial wall (Singh 1978; Moitra and Bhatnagar 1982). Fagerlind (1961) observed this in *Picea, Pinus* and *Larix*. Walles and Rowley (1982) found that tapetal cells differentiated from parietal cell layers in *P. sylvestris*. In *P. banksiana* (Dickinson and Bell 1976a) and *Taxus baccata* (Pennell and Bell 1985) the tapetum is derived from the outermost layer(s) of sporogenous cells.

The earliest sign of tapetal differentiation in *T. baccata* is the flattening of the peripheral cells of the archesporium. The radial walls between these cells become sinuous. There are fewer plasmadesmata in the walls between the differentiating tapetal cells and the more central sporogenous cells than in the radial walls separating the future tapetal cells. Cell walls of future tapetal cells are thinner than the walls of the neighboring future MMCs. Differentiating tapetal cells have larger vacuoles, and the cristae of their mitochondria are more inflated than in other archesporial cells (Pennell and Bell 1985).

In *P. banksiana*, differentiation of MMCs and tapetal cells begins when the protoplasts of the central sporogenous cells contract, rupturing plasmadesmatal connections. The central cells contract up to 60% of their original volume, while the peripheral 1-2 cell layers do not. The central cells form the MMCs. The outer cell layers become radially compressed as they differentiate into tapetal cells. Layers of rough endoplasmic reticulum (RER) form around the periphery of the tapetal cells. In contrast to *T. baccata*, tapetal cell walls increase in thickness, by dispersion of the cellulose microfibrils. Accompanying this stage of differentiation is a 30% increase in microsporangium volume (Dickinson and Bell 1976a).

There are two distinct patterns of tapetal development, plasmodial (or amoeboid) and secretory (or glandular). In the plasmodial tapetum the cell walls are lysed and the intact protoplast invades the loculus surrounding the developing pollen (Maheshwari 1950). Before anthesis, the plasmodium dehydrates and the remnants are deposited on the pollen (Esau 1977). In the secretory tapetum, cells remain *in situ* but go through various secretory phases interspersed with periods of dedifferentiation (Rowley and Walles 1985a,b, 1987, 1988). Tapetal cells form a peritapetal membrane which envelopes the microsporangium, and Ubisch bodies which migrate into the loculus and modify the pollen surface. Ultimately, the tapetal cells degenerate. The events occurring during development of the tapetum differ between species. In gymnosperms, the tapetum is generally of the secretory type (Moitra and Bhatnagar 1982). However, several exceptions have been reported. The secretory tapetum of *Taxus* becomes partly amoeboid when the plasmalemma ruptures and the contents intrude into the loculus at the end of its developmental cycle (Pennell and Bell 1986). In *P. banksiana* portions of the cytoplasm separate from the tapetal cells and migrate into the loculus (Dickinson and Bell 1972, 1976b). Similar development occurs in the periplasmodial tapetum of *Larix* (Mikulska *et al.* 1969; Said and Dumas 1988).

Just before meiosis in *L. decidua* (Mikulska *et al.* 1969), *P. sylvestris* (Walles and Rowley 1982), *P. banksiana* (Dickinson and Bell 1976a) and *Pseudotsuga menziesii* (Singh *et al.* 1983), tapetal cell walls are digested and these cells become intensely basophilic due to an increase in the number of free ribosomes. This basophilia decreases somewhat when meiosis begins. At that time tapetal cytoplasm also contains a well developed RER system, many Golgi bodies with Golgi vesicles containing fibrillar material, multivesicular bodies and autophagic vesicles (Walles and Rowley 1982). In *Pinus sylvestris*, a cell wall is reformed on the parietal cell side of the middle lamella adjacent to the tapetal cells. A lamellation is present in the position of the former middle lamella, similar to the peritapetal membrane of *P. banksiana* (Dickinson 1970, 1971). Echlin (1971) suggested the lamella is required either as a synthetic site for polymerization of sporopollenin precursors or as a passive framework on which the sporopollenin is deposited.

When MMCs have completed meiosis, protoplasts of tapetal cells enlarge. Plastids increase in number and aggregate around the nucleus. Large intercellular cytoplasmic channels form. Tapetal cells become vesicular. Coated vesicles fuse with the plasmalemma and uncoated vesicles containing fibro-granular material are released intact into the loculus then rupture (Dickinson and Bell 1976b). Dickinson and Bell propose that these contain precursors for sporopollenin.

In *P. banksiana*, the peritapetal membrane is formed as tapetal cells deposit lipid globules on the remains of the outer middle lamella of the tapetum followed by a coating of sporopollenin. A similar, but much thinner layer is deposited on the remnants of the tapetal middle lamella towards the MMCs. The deposition of lipid and sporopollenin stops when the tetrads have formed (Dickinson and Bell 1970, 1976b). The peritapetal membrane is acetolysis resistant, and is thought to function as a culture sac for the young microspores (Heslop-Harrison 1969). The lipid layer is removed in later stages of microsporogenesis. This suggested to Dickinson (1971) that the peritapetal membrane may be involved in controlling pollen dessication.

The formation of Ubisch bodies, or orbicules, by tapetal cells occurs concurrently with the formation of the peritapetal membrane, and by a similar mechanism. A mature Ubisch body is made up of a core of lipid coated by sporopollenin. The lipid globule originates within the tapetal cell in association with RER cisternae (Risueno et al. 1969; Willemse 1971e; El-Ghazaly and Jensen 1986; Rowley and Walles 1987). In *P. sylvestris*, as the immature Ubisch bodies leave the tapetal cell, they are coated with part of the plasmalemma glycocalyx (Rowley and Skvarla 1975; Rowley and Walles 1987). This glycocalyx is a surface coating on the plasmalemma composed of polysaccharides, proteins and lipids. It is proposed to contain receptors for the sporopollenin which is deposited once the Ubisch bodies are in the loculus. In *P. sylvestris*, Ubisch bodies are produced repeatedly, in waves, from the beginning of meiosis until the young microspore stage (Rowley and Walles 1987).

There is a very different mode of development of Ubisch bodies in *P. banksiana*. Once the microspores are released from the tetrad, drastic changes occur within the tapetal cytoplasm. Fragments of cytoplasm are isolated from the cell by fusion of vesicles with each other, then with the plasmalemma, then they are released into the loculus. Any lipid material is coated with sporopollenin, forming Ubisch bodies. Finally, the nuclear membrane breaks down, the plasmalemma is lost, and remaining cytoplasm disintegrates. During this time, the peritapetal membrane disappears, the microsporangium dries and dehiscence occurs (Dickinson and Bell 1972, 1976b).

2.1.2. MMC Development

Premeiotic development of sporogenous cells and MMCs in gymnosperms has been described for *Podocarpus* (Vasil and Aldrich 1970), *Ceratozamia* (Audran 1979, 1981), *Pseudotsuga menziesii* (Singh *et al.* 1983) and *Taxus baccata* (Pennell and Bell 1985). After tapetal differentiation, sporogenous cells enlarge. In *Podocarpus*, plastids contain small starch grains. In *T. baccata*, plastids loose their starch and become dedifferentiated. The dedifferentiation of plastids is common during sporogenesis (Pennell and Bell 1985). Plastid membranes become less osmiophilic, perhaps indicating increasing saturation of lipids which would result in increased membrane permeability (Bell 1983). In *Podocarpus* and *Taxus* ribosomes are aggregated into polysomes. In *T. baccata* there is a decrease in basophilia, sporogenous cell nuclei are large and contain membranous tubular inclusions thought to be microtubules, measuring 35 nm diameter and up to 0.5 μm in length.

Subsequently, nuclear vacuoles arise by invagination of the inner nuclear membrane. These persist until leptotene (Pennell and Bell 1985).

The beginning of the MMC stage is marked by the deposition of the "special wall" beneath the sporocyte primary cellulose wall. In angiosperms and probably most gymnosperms this wall consists of callose, a β-1,3-polyglucan. In *Ceratozamia*, this is composed of hemicellulose (Audran 1979, 1981). In *Podocarpus*, the plasmalemma withdraws from the cellulose wall, vesicular contents are deposited into this space and electron-dense material, proposed to be sporopollenin is deposited on the outer surface of the plasmalemma (Vasil and Aldrich 1970). Similarly, in *Pseudotsuga menziesii* the plasmalemma is withdrawn from the original cell wall and a layer of loose fibrillar material is laid down. A non-uniform layer of electron-dense material, thought to be newly formed callose, forms on the outside of the plasmalemma (Singh *et al.* 1983) . Subsequent vesicle deposition of loose fibrillar material increases the thickness of the new cell wall. In *Pinus sylvestris* a new thin cell wall is deposited consisting of two electron-dense layers of thin fibrillar material separated by fibrils (Walles and Rowley 1982).

2.1.3. MMC Meiosis

Chromosome behaviour during MMC meiosis has been described for several conifers: *Picea pungens* (Fechner 1973), *P. sitchensis* (Moir and Fox 1975), *Pinus contorta* (Ho and Owens 1974b), *Tsuga heterophylla* (Owens and Molder 1971; Ho and Owens 1974a), *Pseudotsuga menziesii* (Owens and Molder 1971; Allen and Owens 1972), *Larix* (Ekberg *et al.* 1968; Owens and Molder 1971; Hall 1982) and *Thuja plicata* (Owens and Molder 1971). The phenology varies between these species. MMCs of *Tsuga*, *Pseudotsuga*, *Thuja* and most species of *Larix* overwinter at pachytene or diffuse diplotene (Owens and Molder 1971; Luomajoki 1982; Owens

1982). *L. gmelinii* in Finland completes meiosis before winter dormancy (Luomajoki 1982). MMC meiosis in *Taxus* lasts from late October until early December (Pennell and Bell 1987). *Chamaecyparis* and *Juniperus* complete MMC meiosis and pollen is mature before winter dormancy (Owens and Molder 1974). Other species, such as *Picea* and *Abies* (Mergen and Lester 1961; Winton 1964; Fechner 1973; Moir and Fox 1975; Owens and Molder 1980; Luomajoki 1982; Owens 1982; Harrison and Owens 1983), do not start MMC meiosis until dormancy has ended in the spring. *Pinus* overwinters at the sporogenous cell stage (Owens 1982).

Meiosis is not synchronous in most conifers. For example, in *P. engelmannii* a microsporangium may contain meiocytes in metaphase I to telophase II (Singh and Owens 1981a). The stage of development may vary between microsporangia within a cone. In *P. sitchensis*, differentiation of microsporangia occurs basipetally (Moir and Fox 1975), whereas in *Pinus contorta* (Ho and Owens 1974) and *Larix laricina* (Hall 1982), differentiation occurs acropetally. In *T. baccata*, meiosis is more or less synchronous within a microsporangium (Pennell and Bell 1985). In other species, such as *Tsuga heterophylla*, the stage of microsporangial development is uniform throughout the cone, but the stage of pollen development within a microsporangium may vary (Colangeli and Owens 1988).

Reports of cytoplasmic changes during meiosis are scattered and generally do not distinguish between the particular stages of meiosis. Those that do include *Taxus baccata* (Pennell and Bell 1985), *Ceratozamia* (Audran 1981) and *Pinus sylvestris* (Willemse 1971a, 1971b, 1971c). Of particular interest are development of nuclear vacuoles and invaginations, and changes in plastids, mitochondria, ribosomes and other cytoplasmic components.

2.1.3.1. Nuclear Vacuoles and Invaginations

A phenonemon, which appears to be widespread, is the presence of nuclear vacuoles and nuclear invaginations at different stages of meiosis, and in the tetrad stage. The early occurance of tubular, membranous inclusions in sporogenous cell nuclei of *T. baccata* (Pennell and Bell 1985) may be related to the later development of nuclear vacuoles. The tubular inclusions disappear and nuclear vacuoles, containing fibrillar substances which are present until zygotene, arise by invagination of the inner nuclear membrane. Similar inclusions have been found in *P. sylvestris* zygotene nuclei (Willemse 1971a). In *T. baccata* (Pennell and Bell 1985), the vacuoles become electron-lucent and by pachytene they are no longer present. At this time, both membranes of the nuclear envelope invaginate into the nucleus, forming channels which Pennell and Bell suggest may be the result of traction exerted on the membrane by the condensing chromosomes. The synaptonemal complexes are attached at one end to the inner membrane at the tip of the invaginations or end within 200 nm of the tip. The outer membrane of the nuclear envelope is often continuous with ER.

In *Podocarpus*, nuclear invaginations are present during the late tetrad stage while the ectexine (sexine) is being formed (Aldrich and Vasil 1970; Vasil and Aldrich 1970). The nucleoplasm bordering these invaginations contain chromatin-like material. The number of invaginations decreases at the end of sexine formation to only a few notches or folds by the time nexine 2 is formed (Vasil and Aldrich 1970). The authors propose the invaginations are channels for the transport of exine precursor substances and provide greater surface area for synthesis of ribosomal subunits and rRNA which takes place after meiosis. In *Pinus sylvestris* such nuclear invaginations have not been reported but nuclear vacuoles present during interphase II may be related to pollen wall formation which starts after the early tetrad stage (Willemse 1971b). During the late tetrad stage in *Podocarpus* (Aldrich and Vasil 1970; Vasil and Aldrich 1970), the outer nuclear membrane becomes distended in areas, forming electron-lucent vesicles. These bleb off into the cytoplasm. Later during nexine formation, the outer membrane is often contiguous with the ER.

Nuclear invaginations in *Pinus banksiana* arise and undergo structural changes throughout tetrad development (Li and Dickinson 1987). In young tetrads they are cylindrical, but develop into sac-like structures by the late tetrad stage, similar to those seen in *Podocarpus*. The sacs of *Pinus banksiana* contain RNA. It was proposed that these function as specially adapted structures for the transfer of mRNA, which apparently passes directly across the nuclear envelope rather than through a pore (Li and Dickinson 1987).

2.1.3.2. Plastids, Mitchondria and Cytoplasm

Information on changes in cytoplasmic organelles in conifers is scattered. However, well documented changes occur in plastids and mitochondria of angiosperms throughout meiosis, in particular *Lilium* (Dickinson and Heslop-Harrison 1977; Dickinson 1981; Dickinson and Wilson 1983; Bird *et al.* 1983). In early prophase there is a loss of starch in the plastids and division profiles are present. In *Taxus baccata*, this occurs at pachytene (Pennell and Bell 1985). In angiosperms, plastids undergo total dedifferentiation until they resemble double-membraned inclusions containing only osmiophilic droplets and small vesicles or membranous tubes. After prophase membrane particle associations (MPAs) arise in the plastids and become fully developed by the tetrad stage. The MPAs are composed of RNA in a protein matrix, and probably also contain DNA (Dickinson 1981). In the late tetrad stage, MPA division precedes plastid division so that each daughter plastid contains one MPA (Bird *et al.* 1983). MPAs synthesize carbohydrate which is replaced by starch when the young microspores are released from the tetrad.

Lilium mitochondria change from the orthodox to the condensed form in early prophase, and division profiles are present. In late prophase some mitochondria contain relatively large amounts of acid phosphatase in their cristae (Bird *et al.* 1983), an enzyme commonly associated with autophagy (Matile 1975). During the tetrad stage, the mitochondria accumulate electron-opaque material in their matrices, and after division in the late tetrad stage each daughter mitochondrion contains an electron-opaque aggregate. Mitochondria return to the orthodox form when young microspores are released from the tetrad (Bird *et al.* 1983). Dedifferentiation of the mitochondria is not as extreme as that of the plastids (Dickinson and Heslop-Harrison 1977).

Cytoplasmic events are also well documented during meiosis in angiosperms. In early prophase double-membraned inclusions (DMIs) and multi-membraned inclusions (MMIs) develop as ER cisternae form cup-shaped profiles, then fuse, encapsulating ribosomes (Dickinson and Heslop-Harrison 1971). DMIs are commonly seen in male meiosis, while MMIs are characteristic of female meiotic cells, probably because their larger size will accomodate them. A large number of cytoplasmic ribosomes outside the DMIs disappear during prophase, while many of those inside persist. By late prophase it is difficult to discern DMIs from dedifferentiated plastids. The DMIs persist until the young microspore stage, at which time the envelope changes. Parts of the membrane become electron-opaque and coated with ribosomes, the rest of the membrane is lost. The edges of the persistant membrane fuse, forming plates of membrane coated with ribosomes, which move to the surface of the cell and play a major role in synthesis of the nexine wall layer. In this way sporophytic information could be retained in the gametophyte (Dickinson and Heslop-Harrison 1971).

Information on plastid, mitochondrial and cytoplasmic developments in conifers is scattered. Plastids of *Pinus sylvestris* MMCs in zygotene have a granular content and rarely contain membranes; some division profiles are present. There is no change through to prometaphase II, at which time many plastids contain a large starch grain. From interphase II to early tetrad there is a decrease in the size of the starch grains and some fragments of membrane are visible. The starch is completely gone by late tetrad, however amylogenesis recommences once the young microspores are released form the tetrad (Willemse 1971a,b,c). No MPAs were noted in *P. sylvestris*. However, osmiphication is required for MPAs to show up (Dickinson 1981), and Willemse's material was fixed in a potassium permanganate fixative.

Other reports on starch accumulation in plastids of conifers are somewhat contradictory. In *Taxus baccata*, premeiotic plastids are undifferentiated, but by early leptotene plastids have accumulated starch (Pennell and Bell 1985). This disappears by pachytene. No MPAs are present. In *Ceratozamia*, early prophase plastids are granular and closely associated with ER. By leptotene, starch synthesis has begun, and the rate of synthesis increases during zygotene (Audran 1981). Most prophase plastids of *Pseudotsuga* are undifferentiated, and contain smooth or rough lamellae. Some amyloplasts are present (Singh *et al.* 1983).

There is also a dearth of information on mitochondrial development during male meiosis in conifers. Premeiotic mitochondria of *T. baccata* are undifferentiated. By early leptotene they contain numerous cristae but have dedifferentiated again by pachytene (Pennell and Bell 1985). In *P. sylvestris*, mitochondria contain some osmiophilic material and few cristae and do not change from zygotene through to diakinesis. From prometaphase I to telophase II they contain electron-lucent material and few cristae. Again this material was fixed in potassium permanganate (Willemse 1971a). *Ceratozamia* prophase mitochondria are granular (Audran 1981). *Larix* mitochondria of post-meiotic interphase microspores have a poorly developed inner membrane, and are difficult to distinguish from plastids (Gorska-Brylass *et al.* 1984). Changes in mitochondrial enzyme activities in *Larix* suggest a switch in the metabolic activity in the transition from diplo- to haplophase (Chirwot *et al.* 1987).

There are similarities in ribosome population changes in conifers and angiosperms. Polylammellar concentric structures similar to MMIs of *Lilium* are present in *Ceratozamia* during prophase (Audran 1981). These are destroyed during pachytene. Singh *et al.* (1983) reported the presence of autophagic vesicles in prophase cytoplasm of *Pseudotsuga* which resemble the DMIs of *Lilium*. Further development of these has not been examined. In *Ceratozamia* prophase MMCs there is a decrease in the number of cytoplasmic ribosomes, and a degeneration of cytoplasm along the plasmalemma.

In *Pinus sylvestris*, "lipid complexes" arise in zygotene, consisting of lipid globules connected to large electron-lucent vesicles, the whole complex surrounded by osmiophilic dots. These disperse during pachytene. In diplotene two forms of lipid globules are present: one is electron-dense and contains osmiophilic dots which disappear by diakinesis, the other is less electron-dense and contains no dots. The latter are present throughout diakinesis (Willemse 1971a). The lipid complex reappears in telophase II, and Willemse (1971b) proposed that these and the lipid granules of diakinesis provide products required for callose wall synthesis.

2.1.3.3. Callose Wall Formation

In conifers there are two phases of callose wall formation. The first occurs during the diplotene stage of meiosis when a callose wall forms around the MMCs, completely surrounding the cytoplasm (Singh 1978). The second phase of callose wall formation occurs after meiosis II. In conifer MMCs, meiosis I is generally not followed by cytokinesis. After meiosis II, cell walls form simultaneously between the four free haploid nuclei (Willemse 1971d; Kurmann 1989a,b). Callose is deposited in these walls centripetally (Singh 1978). In *Larix*, callose wall formation occurs in both telophase I and telophase II. Just before the callose wall is synthesized, high peroxidase activity and an accumulation of calcium ions occur in the phragmoplast area. This disappears at the time of callose wall synthesis (Tretyn *et al.* 1987). This may indicate that mitochondria are involved in the process.

The callose wall is a polymer of β-1,3-polyglucan. Experiments with radioactive compounds show that the callose wall is impermeable to nucleosides (Heslop-Harrison and McKenzie 1967) and amino acids (Southworth 1971) but permeable to acetate, glucose and precursors of polysaccharides and lipids (Albertini *et al.* 1987). The callose wall is also involved in exine wall patterning (Waterkeyn and Bienfait 1970; Kurmann 1989a,b).

2.1.4. Pollen Wall Development

Pollen wall formation in gymnosperms has been described for *Abies* (Lepouse 1971), *Ceratozamia* (Audran 1980), *Larix* (Said and Dumas 1988), *Pinus banksiana* (Dickinson 1971), *P. sylvestris* (Willemse 1971c,d; Rowley 1981; Rowley and Walles 1985c), *Podocarpus* (Vasil and Aldrich 1970), *Taxus baccata* (Rohr 1977; Pennell and Bell 1986) and *Tsuga canadensis* (Kurmann 1989b) and thoroughly reviewed (Singh 1978; Moitra and Bhatnagar 1982). The pollen wall consists of two main parts, the sporopollenin-containing exine and the cellulosic intine. The exine consists of a sculptured ectexine (or sexine) and a non-sculptured endexine (or nexine). The ectexine of gymnosperms generally consists of a tectum which forms a layer joining pillar-like bacula. In *Tsuga*, the bacula are absent and the convoluted tectum rests directly on the foot layer (Kurmann 1989b). The foot layer (nexine 1) is the outer layer of the endexine; the inner layer of the endexine is the lamellated nexine (nexine 2). The intine is a cellulosic wall underlying the exine, next to the plasmalemma.

2.1.4.1. Ectexine Formation

The ectexine is formed during the tetrad period, when the microspore is enclosed by a callose wall (Dickinson 1971; Heslop-Harrison 1971; Willemse 1971c,d; Rohr 1977; Singh 1978). In *Pinus banksiana* (Dickinson 1971) small frequent invaginations are formed on the plasmalemma, then Golgi vesicles move to the cell surface, adjacent to the plasmalemma. The vesicles fuse to each other, forming a new cell membrane inside the original plasmalemma. The membranes of the original plasmalemma and the membrane portions of the Golgi vesicles cut off from the cytoplasm become undiscernable, and a primexine is formed of this material. The new plasmalemma is displaced inwards, away from the callose wall by a fibrillar material which is deposited between the primexine and the plasmalemma. In saccate pollen, the inward displacement is greater and the PAS-positive fibrillar contents are more condensed in the area where the sacci form (Dickinson 1971), probably a consequence of the greater number of Golgi vesicles in this region (Willemse 1971d). Between the sacci at the distal pole where the leptolemma forms, small invaginations of the plasmalemma occur (Dickinson 1971), but Golgi vesicles are infrequent, perhaps because the close proximity of the nucleus represents an obstacle (Willemse 1971d).

In other species, a primexine is formed by fusion of Golgi vesicles with the plasmalemma and secretion of their contents between the plasmalemma and the callose wall. Where this occurs, contact is lost between the callose wall and the plasmalemma, and callose is no longer deposited. Where contact persists, callose wall formation continues, so that callose protrusions are formed (Willemse 1971c; Audran 1980; Kurmann 1989a). The callose protrusions are actually sinuous bands of variable thickness which anastomose over the surface of the microspore and are reminiscent of superficial exine organization in the mature pollen grain. Where the sacci develop, connections between plasmalemma and callose remain intact and long protrusions are formed in the callose wall (Willemse 1971d).

In *Ceratozamia* (Audran 1980), tubules thought to be of lipoprotein are formed between the callose bands and are perpendicular to the microspore surface. Similar structures, called tufts, are found in various other species where they are thought to be part of the glycocalyx of the plasmalemma (Rowley 1981). Rowley has proposed that their substructure is composed of helical core subunits within a supercoiled binder subunit. According to this model, the tufts of the glycocalyx are perpendicular to the plasmalemma. Electron-dense osmiophilic material is deposited on the primexine, and along and between the tufts, forming the bacula. The primexine is thought to be composed of a mat of these tufts. Elongated electron-dense elements are seen during primexine formation of *Tsuga* (Kurmann 1989b). The trilaminar tapes of *Podocarpus* primexine (Vasil and Aldrich 1970) may be a result of binder subunits in phase. There was no evidence of a helical glycocalyx sub-structure in acetolyzed pollen of *Juniperus* (Southworth 1986).

In *Podocarpus*, the two sacci of the pollen are formed and become distended during ectexine deposition (Vasil and Aldrich 1970). In *Pinus banksiana*, the ectexine forms first in the area which the sacci will occupy (Dickinson 1971). In winged pollen, sporopollenin is thickly and evenly deposited in the sexine over the whole grain, except at the leptolemma where the pollen tube will emerge (Vasil and Aldrich 1970; Dickinson 1971).

2.1.4.2. Endexine Formation

The foot layer of the endexine (nexine 1) appears to develop as extensions from the bases of the bacula and by lamellae arising from the plasmalemma (Heslop-Harrison 1971). In Rowley's (1981) model, deposition of sporopollenin on the tufts of the glycocalyx form the endexine. In *Tsuga*, tripartite lamellae appear outside the plasmalemma (Kurmann 1989b). In *Larix*, *Podocarpus* and *Pinus sylvestris*, sporopollenin is deposited on tapes, forming the foot layer (Vasil and Aldrich 1970; Willemse 1971d; Said and Dumas 1988). In *P. banksiana*, the plasmalemma thickens and a new plasmalemma forms beneath it. This occurs repeatedly to form the lamellae of the foot layer (Dickinson 1971). In *Taxus baccata*, the plasmalemma withdraws from the bacula and a polysaccharide periplasm is formed. In this periplasm, numerous isolated plates of the same electron density as the ectexine arise. These have a thin electron-lucent coating. These plates grow and meld together, forming a thin continous layer which then thickens (Rohr 1977). All plasmalemma connections to the callose wall are severed at this point (Willemse 1971d). The foot layer lays adjacent to the plasmalemma and thus separates the contents of the sacci from the plasmalemma (Moitra and Bhatnagar 1982). While the foot layer forms, the callose wall encompassing the microspores begins to break down.

The inner layer of the endexine (nexine 2) is formed when the microspore is released from the tetrad. In *Podocarpus*, fine granular and globular particles of sporopollenin coalesce between the plasmalemma and the foot layer. *Podocarpus* has a third nexine layer which is laid down after mitotic division of the microspore, in the same fashion as nexine 2 (Vasil and Aldrich 1970). In the mature pollen wall of *P. sylvestris*, the nexine 2 layer cannot be distinguished from the foot layer (Willemse 1971d).

When the microspores are released from the tetrad into the thecal fluid, their volume increases greatly and the sacci expand. The increase in microspore volume is due to vacuolization of the microspore (Audran 1979; Kurmann 1989ab). To accomodate this increase in growth the previously undulated nexine straightens and the bacula spread further apart (Audran 1979, 1980, 1981). Sporopollenin in the thecal fluid acretes on the pollen wall increasing the thickness of the exine (Singh 1978).

2.1.4.3. Intine Formation

The intine is the innermost pollen wall. Intine synthesis begins once the outer callose wall is dissolved and the microspore is released from the tetrad. There is considerable variation in the intine of gymnosperms. The intine of *Pinus* consists of an incomplete, variously thickened, outer callosic layer and a continous inner pecto-cellulosic layer. The cellulosic intine of *Picea* is a continuous layer of equal thickness. In mature pollen of both *Picea* and *Pinus*, the degenerated prothallial cells are embedded within the intine. In *Pinus*, they are embedded within the callosic layer of the intine (Martens and Waterkeyn 1961). The intine of *Tsuga* is fibrillar, and is composed of a narrow outer layer and a thicker, more electron-dense inner layer. The intine of *T. canadensis* is much thicker at the distal region than elsewhere, and it undulates on the distal face (Kurmann 1989b). The intine of *Podocarpus* is a single homogenous layer which is thinner at the prothallial-cell (proximal) pole than elsewhere (Vasil and Aldrich 1970).

2.1.5. Male Gametophyte Development

The number of mitoses in pollen grains of gymnosperms is variable. Moitra and Bhatnagar (1982) have reviewed the literature on pollen development in the various classes and families of gymnosperms. The terminology used has been reviewed by Singh (1978), who proposed the following scheme, which I will use. The microspore divides unequally to form a small prothallial cell and a central cell. The latter divides to form a second small prothallial cell and an antheridial initial. Division of the antheridial initial results in a large tube cell and an antheridial cell. The antheridial cell divides to form the sterile stalk cell and the body cell. It is the latter which will divide to form the two male gametes after pollination. Prothallial cells are thought to be vestigial remnants of a once prominent male prothallium. The function of the stalk cell is unknown, but it may have originally functioned as a spermatogenous cell (Chamberlain 1935). The tube cell eventually forms the pollen tube.

Within the Coniferales there is considerable variation from the general scheme. No prothallial cells are formed in Taxodiaceae, Cupressaceae, Cephalotaxaceae, or Taxaceae, thus the microspore nucleus functions as the antheridial initial (Singh 1978; Moitra and Bhatnagar 1982). The pollen of these taxa is shed at the 1-cell (microspore) or 2-cell (tube and antheridial cells) stage. In Araucariaceae and Podocarpaceae, pollen development follows the general scheme, but the prothallial cells may proliferate, resulting in 6 to 40 prothallial cells in Araucariaceae, and 1 to 8 in Podocarpaceae (Moitra and Bhatnagar 1982). In Pinaceae, mature pollen has 4 [*Pinus* (Ho and Owens 1974b; Moitra and Bhatnagar 1982)] or 5 cells [*Abies* (Singh and Owens 1981b, 1982), *Picea* (Miyake 1903;

Hutchinson 1915; Rauter and Farrar 1969; Owens and Molder 1979; Singh and Owens 1981a), *Larix* (Goc 1986), *Tsuga* (Colangeli and Owens 1988), *Pseudotsuga* (Allen and Owens 1972)]. In 4-cell pollen, the antheridial cell doesn't divide before pollen is shed. Recently, mature 3-cell pollen has been reported in *Cathaya*, a Pinaceae native to China (Wang and Chen 1986).

Prothallial cells in Pinaceae form during deposition of the intine. In many species these cells degenerate, resulting in darkly staining lens-shaped inclusions within the intine at the proximal pole of the pollen grain [*Tsuga* (Colangeli and Owens 1988), *Abies lasiocarpa* (Singh and Owens 1981b), *Picea glauca* (Owens and Molder 1979)]. An early description of *P. glauca* mistakenly stated prothallial cells were not embedded within the intine (Hutchinson 1915). The prothallial cells of mature *Abies grandis* pollen do not degenerate, and although they are somewhat flattened and contain little cytoplasm, the nucleus is still discernable (Singh and Owens 1982).

Most ultrastructural studies on gymnosperm pollen concentrate on pollen wall development, rather than post-meiotic development of the microspore and its derivitives. The structure of cells within the pollen grain has been examined in *Larix* (Goc 1986; Goc and Gorska-Brylass 1986) and *Abies* (Lepouse 1969). At the antheridial cell stage in *Larix*, the smaller antheridial cell and the larger tube cell are similar ultrastructurally, but the distribution of the organelles differs. The mitochondria and plastids of the antheridial cell are evenly distributed, whereas the tube cell mitochondria and plastids are restricted primarily to perinuclear and parietal zones. This is in part a result of the numerous vacuoles in the tube cell (Goc and Gorska-Brylass 1986). The antheridial cell divides to form the stalk and body cells. In *Larix* the resultant body cell has a greater number of pleiomorphic mitochondria and a more highly developed ER system than the parent antheridial cell (Goc 1986). As in *Abies* (Lepouse 1969), the stalk cell of *Larix* is smaller than the body cell. The tube cell contains many vacuoles and more starch than the other cells. In *Abies*, each cell is limited by a slightly undulating plasmalemma which is separated from the neighboring plasmalemma by electron-lucent spaces. Occasionally there are clear vesicles within the intercellular spaces. These spaces react positively with PAS, but not specifically for cellulose or callose. Lepouse (1969) proposes they are composed of pectin. In *Podocarpus* the walls between the cells of the pollen grain are of pecto-cellulose (Audran 1979).

2.1.6. Dehiscence

There are no detailed studies on the dehiscence mechanism of conifers. Generally the axis of the pollen cone elongates about the time pollen matures (Colangeli and Owens 1988). Spiral thickenings develop in the walls of the exothecial cells of the microsporangium, except in those cells which form the dehiscence line (Owens and Molder 1979; Singh and Owens 1981a, 1982). The position of this dehiscence line on the microsporangium appears to vary. In *Pseudotsuga* (Allen and Owens 1972) and *Tsuga* (Colangeli and Owens 1988) the line is oriented horizontally to the microsporophyll axis; in *Picea glauca* it is oriented longitudinally. Pollen is released when the microsporangia dry and the exothecium breaks at the dehiscence line. Conifers are wind pollinated.

2.2. Pollen Forcing

The rate of pollen development in conifers has been artificially accelerated by allowing the cones to develop in a warmer than ambient temperature. This is commonly known as pollen forcing. Various methods of increasing the temperature include bagging the pollen-cone-bearing shoots with polyethylene bags (Boyer and

Woods 1973; Beers *et al.* 1981), putting branches cut from trees into a warm room or environmental chamber (Winton 1964; Chira 1965; Jonsson 1974; Dorman 1976; Beers *et al.* 1981; Colangeli 1989; Colangeli and Owens 1989), and placing whole trees, potted grafts or rooted cuttings in greenhouses or environmental chambers (Ekberg *et al.* 1968; Ross 1988; Colangeli 1989).

Although such treatments are successful in speeding the time to pollen shed, detrimental effects have been reported. Germination of *Picea excelsa* pollen from cut branches forced at 15 to 17°C was 34% compared to 64% in pollen forced at 10 to 12°C and 87% in that developing under ambient conditions in Czechoslovakia (Chira 1965). For *Tsuga heterophylla* cut branches at room temperature, the time of collection of branches had a marked effect on the quantity and quality of pollen produced. Pollen cones on branches collected prior to meiosis and up to bud burst (free microspore stage of pollen development) produced a significantly lower quantity of pollen, and a significantly higher proportion of abnormal pollen when compared to those collected at later dates (Colangeli 1989; Colangeli and Owens 1989). However forcing rooted cuttings of *T. heterophylla* in an environmental chamber at 18°C and low humidity caused no significant difference in quantity or quality of pollen produced (Colangeli 1989). This suggests that the cut branch method of forcing pollen induces physiological stress, probably related to moisture and nutrient deficiencies (Colangeli 1989).

In contrast to *T. heterophylla*, potted grafts of *Picea engelmannii* forced in a polyhouse showed a high percentage of underdeveloped and rotted cones, and a decrease in pollen yield per cone (Ross 1988). Meiotic irregularities were found in *Larix* grafts forced in a polyhouse (Eriksson *et al.* 1970). Forcing resulted in increased chromosome number in *Pinus* and *Picea* where the spindle mechanism was disrupted and neither diads nor tetrads were formed (Chira 1965, 1974).

Forcing *Picea excelsa* pollen resulted in plasmolysis of MMCs, which then continued development, forming mature pollen with decreased germinative capacity (Chira 1965). Detrimental effects of forcing on anatomical development of pollen have also been reported. In some cycads, placing branches bearing cones in a warm (20°C), humid environment resulted in meiotic abnormalities. These included abnormal plane of cell division, lack of wall formation and abnormal and incomplete wall formation (Audran 1969). None of these reports discuss detrimental effects on tapetal development.

2.3. Pollination

Pollination mechanisms of conifers have been thoroughly reviewed (Doyle 1945; Dogra 1964; Konar and Oberoi 1969; Singh 1978; Owens 1980; Owens and Blake 1985). *Pinus* and *Picea* have a pollination drop mechanism (Doyle and O'Leary 1935; Doyle and Kane 1943; Doyle 1945; McWilliam 1958; Sarvas 1962, 1968; Dogra 1964; Lill and Sweet 1977; Owens and Molder 1979; Owens *et al.* 1981; Singh and Owens 1981a; Owens and Blake 1984, 1985; Owens *et al.* 1987). Pollen sifts down between the bracts of the seed cone and adheres to the secretory droplets formed by the epidermal cells on the micropylar arms. Nucellar cells secrete a pollination drop which, in *P. engelmannii*, contains 4.3% glucose and 3.8% fructose (Owens *et al.* 1987). The large droplet is exuded between the micropylar arms and picks up any adhering pollen. Pollen may also land directly on the pollination drop and be drawn into the micropylar canal. Pollen settles in or near the pollen chamber of the nucellus, which resulted from collapse of the cells lining the chamber. In *P. engelmannii* germination occurs within a few days (Owens *et al.* 1987), whereas one to three weeks elapse before germination in *P. glauca* (Rauter and Farrar 1969; Owens and Molder 1979).

Cupressaceae, Taxodiaceae, Taxaceae and Podocarpaceae also have a pollination drop mechanism. In *Chamaecyparis*, it is similar to that described above but the ovule is flask-shaped, the micropyle cylindrical and the micropylar canal is short (Owens *et al.* 1980; Owens and Blake 1985).

Most other species have an integument to which the pollen adheres and no large pollination drop (Owens and Blake 1985). In *Abies* and *Cedrus*, the integument is funnel-shaped and the edges fold inward, bringing any adhering pollen into the micropylar canal. The nucellus elongates towards the pollen, and a depression or pollen chamber forms in the tip (Doyle 1945; Owens and Molder 1977; Singh and Owens 1981b, 1982).

In *Pseudotsuga* and *Larix*, the integument forms two unequal lobes with stigmatic hairs to which the pollen adheres. The lobes fold inwards, bringing pollen into the micropylar canal where it elongates and sheds its exine. Nucellar cells divide causing the nucellus to grow down the micropyle towards the pollen (Owens and Morris 1989). Tube formation occurs when the elongated pollen grain contacts or comes into close proximity to the nucellus (Allen 1963; Allen and Owens 1972; Owens and Molder 1979b; Ho 1980; Owens *et al.* 1981a; Villar *et al.* 1984; Owens and Morris 1990).

Tsuga has two types of pollination mechanisms. In *T. mertensiana*, pollen adheres to integumentary flaps, germinates and forms a long tube which grows through the micropylar canal to the nucellus (Owens and Blake 1983). In *T. heterophylla*, the pollen adheres to fine cuticular wax hairs on the bract, and germinates, forming a very long tube. The tube grows along the bract to the micropyle of an adjacent ovule, and down the micropyle to the nucellus (Colangeli and Owens 1988).

2.4. Pollen Germination and Tube Growth

Pollen germination and tube growth through the nucellus have been studied in conifers at the light microscope level since the turn of the century (see reviews by Chamberlain 1935; Sterling 1963; Singh 1978; Owens and Blake 1985). These studies generally describe the position of the pollen in relation to the nucellus at the time of germination, the course the pollen tube takes to reach the archegonium, the movement of the tube nucleus, body and stalk cells into the pollen tube, the position of the body cell when it divides to form the male gametes, and the phenology of these events.

There are few detailed ultrastructural studies on pollen germination and tube growth in conifers. Species in which some of these aspects have been studied *in vivo* include *Biota orientalis* (Chesnoy 1969, 1975), *Juniperus communis* (Duhoux and Chesnoy 1978), *Pinus sylvestris* (Willemse and Linskens 1969), *Pseudotsuga menziesii* (Owens and Morris 1990), and *Taxus baccata* (Pennell and Bell 1986). Aspects of the ultrastructure of pollen germination and tube growth have been studied *in vitro* for *Juniperus communis* (Duhoux 1972a, 1972b, 1974, 1980, 1981; Duhoux and Chesnoy 1978), *Taxus baccata* (Pennell and Bell 1986), *Picea, Pinus, Abies* and *Cedrus* (Pettitt 1985).

2.4.1. Pollen Germination

There are three basic mechanisms of pollen germination in conifers (Singh 1978; Owens and Blake 1985). In saccate pollen, which includes most of the Pinaceae except *Pseudotsuga*, *Larix* and *Tsuga heterophylla*, there is a germinal furrow, the leptolemma, at the distal pole between the sacci. In *Pinus, Picea, Abies* and *Cedrus*, the exine at the leptolemma consists only of endexine (Pettitt 1985). Hydration of the pollen causes the lamellations of the nexine at the leptolemma to separate from each other, revealing the microfibrillar nature of the nexine between the lamellae. Hydration also causes the pollen corpus to swell, and the sacci to separate exposing the leptolemma. Enzymes are released primarily from the leptolemma at this time, and later from the tube itself. These enzymes include acid phosphatase, esterase, protease and amylase (Pettitt 1985). Germination of saccate pollen begins by rupture of the endexine at the leptolemma, and emergence of a pollen tube whose wall is an extension of the intine (Ho and Sziklai 1971; Singh 1978; Pettitt 1985; Owens and Blake 1985).

In *Pseudotsuga* and *Larix*, the exine breaks irregularly and is cast off as the pollen elongates (Singh 1978; Owens and Molder 1979b; Owens and Morris 1990). Elongation of the pollen takes about 6 weeks in *Pseudotsuga*. In the elongated pollen, the stalk cell cytoplasm is very dense with abundant ribosomes, mitochondria and lipid globules. The body cell cytoplasm is less dense and contains poorly differentiated plastids with small starch grains. The body cell is irregularly shaped, with long extensions into the tube cell cytoplasm. The boundary between the tube and body cells consists of a pellucid space delimited by the plasmamembrane of each cell, and transversed by what appear to be plasmodesmata. A small tube forms only when the elongated grain reaches the nucellus (Owens and Morris 1990). Pollen elongates but no tubes are formed *in vitro* (Ho and Rouse 1970; Ho and Sziklai 1972; Charpentier and Bonnet-Masimbert 1983).

In Cupressaceae, Taxaceae and Taxodiaceae the intine swells rapidly, and the tube cell bounded by the intine squeezes out through a papilla in the exine, rupturing the latter (Singh 1978). In *Juniperus*, an operculoid plugs a pore in the exine. Upon hydration, the intine swells, pushes the operculoid out of the pore, and the rate of hydration increases. The male gametophyte is forced out through the pore and the exine ruptures (Duhoux 1982). The intine is composed of three layers.

As the male gametophyte grows, a pollen tube wall is formed under the inner intine. The intine is finally ruptured at the distal pole and is cast off during pollen tube growth (Duhoux 1972a, 1972b). In *Taxus baccata*, division of the microspore nucleus to form the antheridial cell and the tube cell occurs concomitant with germination *in vitro* (Pennell and Bell 1986).

2.4.2. Pollen Tube Growth

The duration of pollen tube growth in conifers varies from a few days in *Pseudotsuga* (Owens and Morris 1990) to over a year in *Pinus, Saxegothaea* and *Podocarpus* (Konar and Oberoi 1969). In most conifers, pollen tubes penetrate the nucellus and grow more or less directly toward the archegonium. In some Taxodiaceae (*Athrotaxis* and *Sequoia*), the tube grows laterally between the integument and the nucellus before it penetrates the latter. In Podocarpaceae, when the pollen tube reaches the megagametophyte it splays out forming a thin disk (Konar and Oberoi 1969). In *Podocarpus* (Singh 1978) and in the Araucariaceae (Konar and Oberoi 1969), the pollen tube branches as it grows through the nucellus.

The tube nucleus usually moves into the tube shortly after the tube is formed (Konar and Oberoi 1969). Most pollen tubes, both *in vivo* and *in vitro*, are vacuolate and the tube-cell cytoplasm is restricted to a fairly thick apical zone, and a peripheral area, with a few strands spanning the vacuoles (Willemse and Linskens 1969; Duhoux 1980; Pennell and Bell 1986; Owens and Morris 1990). In pollen tubes of *Picea, Pinus, Abies* and *Cedrus* grown *in vitro*, a pattern of zonation is obvious in the young pollen-tube cytoplasm. Immediately behind the tube tip there are two types of membrane bound vesicles; the smaller contains electron-dense granules and the larger contains dispersed fibrillar material. Mitochondria and ER are located just proximal to this, and further back from the tip are dictyosomes with golgi vesicles, and amyloplasts (Pettitt 1985). *Taxus* pollen tubes growing *in vivo* also show cytoplasmic zonation. Plastids near the wall of the pollen-tube tip are elongated and undifferentiated, whereas those close to the body cell, back from the tip, are spherical and well differentiated. The ER and Golgi bodies are primarily in the central region of the tube tip cytoplasm. Golgi bodies are often associated with p-particles, thought to be involved with wall formation (Pennell and Bell 1986).

When pollen is shed before division of the antheridial cell, timing of this division varies according to species. It may occur before the cell moves into the tube, as in *Pinus* (Konar and Oberoi 1969; Owens *et al.* 1982) and *Taxus* (Pennell and Bell 1986), as the antheridial cell is moving into the tube, as in Taxodiaceae (Konar and Oberoi 1969), or once it is in the tube, as in *Thuja* (Owens and Molder 1980b). There is variability in cell movement in pollen which contains stalk and body cells. The stalk cell often breaks away from the pollen wall and moves with the body cell into the tube in *Pinus* (Ferguson 1901), *Picea* (Miyake 1903; Moshkovich 1988) and *Larix* (Owens and Molder 1979b). In *Abies lasiocarpa*, the stalk and body cells exit the pollen grain when the tube has penetrated the nucellus halfway, but these cells move separately into the tube (Singh and Owens 1981b). In *Pseudotsuga*, the body cell moves into the tube behind the tube nucleus. The body cell remains in the extremely lobed condition generated during pollen elongation in the micropyle (Owens and Morris 1990). Generally the stalk cell remains attached to the pollen wall, but it occasionally breaks away and moves together with the body cell into the tube (Allen and Owens 1972). In *Biota*, the wall of the stalk cell breaks, and the stalk nucleus with some of its cytoplasm moves with the body cell into the tube tip. At the tube tip, the body cell is proximal to the stalk and tube nucleus, and is surrounded by tube cytoplasm. As it is migrating down the tube, the body cell is only slightly irregular in shape (Duhoux 1980).

In conifers, cytokinesis may or may not follow mitosis of the body cell. In Cupressaceae, Taxodiaceae and Araucariaceae, which have an archegonial complex, cytokinesis occurs and two equal-sized male gametes are formed (Singh 1978). In *Biota* (Duhoux 1980), *Juniperus* (Duhoux and Chesnoy 1978) and *Thuja* (Owens and Molder 1980b), division occurs when the body cell is just above the archegonial complex before penetration of the neck cells. In *Biota*, the cytoplasm of the resulting male gametes has three distinct zones around the nucleus: the outer marginal zone has no organelles, the middle zone contains mitochondria, amyloplasts and ribosomes, and the juxtanuclear zone contains no organelles, but ribosomes and some RER are present (Duhoux 1980).

In taxa where archegonia are separated, cytokinesis does not occur and the two male nuclei are contained within the body cell cytoplasm (Singh 1978). In *Taxus*, a cell plate begins to form, but a cell wall is not formed (Pennell and Bell 1986). Division of the body cell to form the two male gametes can occur at various stages of pollen-tube growth. In *Abies* it occurs near the tube apex while still within the nucellus (Singh and Owens 1981). In *Picea*, Miyake (1903) found division occured when the body cell was halfway down the tube, but Singh and Owens (1981) found only one instance of division, at the proximal end of the pollen tube. In its final stages of development in the pollen tube, the boundary of the body cell cytoplasm of *Taxus* becomes discontinous and the whole contents of the body cell are released into the pollen tube (Pennell and Bell 1986).

Chapter 3

METHODS AND MATERIALS

3.1. Pollen Development and Pollen Forcing

In early February, 1986, 3 clones of *Picea glauca* (clones 3, 21 and 52) and 2 clones of *P. engelmannii* (1675 and 1676) at the B.C. Ministry of Forest Research Station, Victoria were chosen for the pollen development/pollen forcing study based on their potential pollen-cone crop. The stage of pollen-cone development was monitored at a gross morphological level (Table 1), and the stage of pollen development within representative cones sampled from each clone was determined microscopically using squash preparations of the microsporophylls stained with 0.5% acetocarmine in 45% acetic acid (Jensen 1962). On March 5 when MMCs of the slowest developing clone (clone 52) reached meiosis, ramets of each clone were separated into two treatment groups, forced and control. Ramets were divided so that each treatment was assigned trees of matching vigor and approximately equal numbers of pollen cone buds from each clone. Control trees were used to study the development of pollen under ambient growing conditions. The effect of forcing on pollen development was studied anatomically and physiologically.

Control trees were placed in an unheated polyhouse with open sides, protected from rain but exposed to ambient temperatures. Trees assigned to the forcing treatment were placed in a heated polyhouse and subjected to 17°C days and 10°C nights. As ambient temperatures increased and daytime temperatures in the heated polyhouses rose above 17°C, fans circulated air through the polyhouse from outside. During the latter part of the experiment fans were on continuously. No attempt was made to control humidity in the polyhouses.

Table 1. Stages used to classify pollen-cone bud development in interior spruce

STAGE	POLLEN CONE MORPHOLOGY
1	dormant pollen-cone bud
2	pollen-cone bud swelling
3	pollen-cone bud bursting
4	pollen cone 25-50% emerged from bud scales
5	pollen cone 50-75% emerged from bud scales
6	pollen cone fully emerged from bud scales
7	elongation of cone axis
8	dehiscence

Collections of pollen-cone buds were made biweekly from March 6 to late April when all pollen cones had shed their pollen. Pollen-cone buds were collected from two ramets of each of three clones (clones 3, 52 and 1675) for each treatment. From each of these ramets three pollen-cone buds, representing the range of stages (early, mid and late) present in the clone for that treatment, were collected and classified according to Table 1. Thus 6 pollen-cone buds/clone/treatment were collected.

3.1.1. Microscopy

Pollen cones were dissected out of their bud scales and their length recorded. Twelve microsporophylls were dissected from each of the cones and fixed in 4% glutaraldehyde in sodium cacodylate buffer for 2 hours, rinsed in buffer, post-fixed in 1% osmium tetroxide in double-distilled H_2O for 1 hour, then dehydrated in a graded series of ethanol and embedded in L.R. White (Biorad Laboratories). The remains of the pollen cones were frozen in liquid nitrogen on the day of collection for subsequent carbohydrate analysis.

3.1.1.1. Electron Microscopy

Embedded specimens were sectioned with a glass or diamond knife on a Reichert Om2 or Sorvall MT 5000 ultramicrotome. One micron (μm) sections were stained with Richardson's stain (Richardson *et al.* 1960) for light microscopy. Thin silver sections were picked up on either uncoated 100-200 mesh copper grids, or on 50-75 mesh grids coated with 1% Parlodion in amyl acetate or 0.6% Formvar in 1,2-dichloroethane. Sections were then stained with 2% uranyl acetate for 30 minutes and counter-stained with 0.2% lead citrate for 5 minutes (Hayat 1970). Sections were examined on a Phillips EM300 or a JEOL-1200EX transmission electron microscope (TEM) at 60kV.

Unfixed mature pollen of each treatment in all clones was dusted on metal stubs coated with double sticky tape, then gold coated with a Technics sputter coater for 4 minutes. These were examined on a JEOL 35 JSM scanning electron microscope (SEM) at 10kV.

3.1.1.2. Histochemistry

Histochemical tests were performed on 1 μm thick sections. The presence of callose was tested with 0.05% water-soluble aniline blue (CI 42755) in 67 mM Sorenson's phosphate buffer pH 8.5 (O'Brien and McCully 1981). Cell wall formation was examined using 0.01% aqueous Cellufluor (Polysciences Inc. lot 44504). The periodic acid-Schiff's (PAS) reaction was used to test polysaccharides, including starch (Jensen 1962). IKI was used specifically for starch (Jensen 1962). Sudan black B in 70% ethanol stained lipids blue-black and starch remained white (Bronner 1975).

3.1.1.3. Light Microscopy

A Leitz Orthoplan largefield microscope equiped with a Vario-Orthomat camera system was used to study anatomical development and non-fluorescent histochemistry. The two fluorescent stains, aniline blue and Cellufluor, were detected using the same microscope equiped with a G filter block system (excitation range 350-460 nm, suppression at 515 nm) and a Pleomopak 2 Fluorescent Vertical Illuminator.

3.1.2. Carbohydrate Analysis

The remnants of pollen cones frozen in liquid nitrogen were later freeze-dried and analyzed for glucose, sucrose and starch. Pollen which had been previously collected from the forced and control ramets, and stored for 1 year at 4°C was also analysed. Carbohydrate analysis was carried out using the technique routinely used by the B.C. Ministry of Forests Research Branch, North Road, Victoria, B.C. on pollen (Webber 1988). Freeze-dried samples were weighed, then homogenized in 10 mls sodium acetate buffer (8.2034g sodium acetate + 5.3 mls acetic acid/l dH_2O) pH 4.5-4.8 for 30 seconds with a Brinkman Homogenizor Polytron PTA 10TS. The homogenates were shaken on a Lab-line multi-wrist shaker at setting 4 for 30 minutes, centrifuged for 10 minutes at 3000 rpm, the supernatant decanted and the pellet resuspended in 10 mls of fresh sodium acetate buffer. The shaking and centrifugation steps were repeated and the second supernatant added to the first supernatant. This was made up to 25 mls with sodium acetate buffer. The pellet was resuspended in 25 mls NaOAc buffer, capped and placed in a 90°C oven overnight. The next day the hot extract was homogenized for 30 seconds. Three 150 μl aliquots were taken from each of the hot and cold extracts. These were paired and assayed for sucrose, glucose and starch.

3.1.2.1. Enzyme Assays

For enzyme assays, sucrose and starch were first broken down into their simple sugar components. Sucrose was digested with invertase (Sigma I-9253) (1 mg/ml sodium acetate buffer). Starch was digested with a solution of 100 mg amyloglucosidase (Sigma A7255) and 2 units amylase (Sigma A-6380) in 100 mls NaOAc buffer. 150 µl of reagent was mixed with one hot and one cold extract aliquot. For glucose, 150 µl of NaOAc buffer was used in place of enzyme reagent. Enzyme digestion was carried out for two hours at 40°C, then 600 µl of glucose oxidase reagent (GOR) added to each digest. GOR consisted of 150 units glucose oxidase (Sigma G-6125) and 4 mg peroxidase (Sigma P-8250) in 100 ml 0.1M phosphate buffer with 1.0 ml o-dianiside-HCl (Sigma D-3252). The samples were placed in a 40°C oven for one hour, then the reaction was stopped by addition of 900 µl 5N HCl. The decrease in pH also changed the color of the solution from brown to magenta. Absorbance values were measured against reagent blanks on a Pye Unicam SP.800 Spectrophotometer at 540 nm. Absorbance values of the glucose assays were subtracted from sucrose and starch assays. Glucose, sucrose and starch standards ranging from 10-100 ppm were prepared at the same time as sample assays. Simple linear regression (Zar 1974) was used to calculate the unknown concentrations in ppm. Values were transformed to mg/mg dry weight x 100 (= mg%). The data were analysed using General Linear Model (GLM) for unbalanced ANOVA (Steel and Torrie 1980; Ray 1982), by clone, treatment and stage of pollen development and by clone, treatment and stage of pollen-cone development. In the latter, stages 2 and 3, stages 4 and 5, and stages 6, 7 and 8 (see Table 1) were bulked into 3 groups.

3.1.3. Seed Efficiency

As pollen cones matured and approached dehiscence, those which were about to shed their pollen were collected from the trees and brought into the lab. Cones were kept separate according to clone and ramet. Pollen collected from these cones was pooled according to clone and treatment. Thus there were 10 pollen lots (2 treatments, 5 clones/treatment). This pollen was used to pollinate field planted white spruce trees at the B.C. Ministry of Forest Skimikin Seed Orchard, Salmon Arm, B.C.

In mid-May, 1986, 3 grafts of white spruce at Skimikin were chosen based on the number of female cones on the grafts which were almost at the receptive stage. These included two grafts of clone 671 and one of clone 682. Branches bearing female cones were enclosed in paper pollination bags before the cones became receptive, to prevent uncontrolled pollination. Two bags per tree with 2-4 cones per bag were brush pollinated on either May 15 or May 16 when secretory droplets were present on micropylar arms, indicating cone receptivity (Owens *et al.* 1987), and again on May 18. Pollination bags were left on the trees until cones closed, then removed and replaced with mesh insect bags. Just before cones opened to shed seed in August, they were collected and individually bagged in small paper bags. Cones were dried and seeds removed and x-rayed at the Canadian Forestry Centre, Victoria, B.C, with a Faxitron x-ray machine (Hewlitt-Packard) at 350 mA, 12.5 kV, onto Industrex 600 paper.

Since each fertile scale was theoretically capable of producing two filled seed, seed efficiency (SEF), the ratio of filled seed to potential seed, was calculated as:

$$\text{SEF} = [\text{filled seed}/\ (\#\ \text{fertile scales x 2})]\ \text{x}\ 100$$

(Colangeli and Owens 1989). Significant differences in SEF between treatments, overall and clonally, were tested using GLM (Steel and Torrie 1980; Ray 1982). Clonal differences within treatment groups were tested using Student Newman Keuls (SNK) (Zar 1974). Percent figures were tested with and without arcsine transformation.

3.2. Pollen Germination and Pollen Tube Growth

3.2.1. *In Vitro*

A polyclonal mix of white spruce pollen[1] which had been stored at -4°C and 4% moisture content was used for *in vitro* studies. When removed from storage it had a respiration rate of 15.82 µl O_2/min/g and a germination rate of 87%.

Pollen was germinated on agar or in a liquid medium. In both cases a modified Brewbaker and Kwack (1963) pollen germination medium (PGM) was used. This contained 300 ppm H_3BO_3, 300 ppm $Ca(NO_3)_2\ 4H_2O$, 200 ppm $MgSO_4\ 7H_2O$ and 100 ppm KNO_3 in dH_2O. The optimal sucrose concentration was determined by germinating pollen in PGM containing 0, 5, 10, 15 or 20% sucrose. PGM containing 4.27% glucose and 3.8% fructose, the sugars found in the pollination drop of Engelmann spruce (Owens *et al.* 1987), was also tested.

After 3 and 6 days, percent germination and length of pollen tubes were recorded. Pollen grains were considered germinated when the length of the pollen tube was equal or greater than the diameter of the pollen grain. Data were analyzed using Duncan's multiple range test and SNK (Zar 1974) at $p = 0.05$. A 5%

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[1] Pollen provided and tested by Dr.J.E.Webber of the B.C. Ministry of Forests.

sucrose liquid PGM was subsequently routinely used, except where noted.

3.2.1.1. Electron Microscopy

Dry, hydrated and germinated grains were fixed as described in section 3.1.1., but subsequent to alcohol dehydration were put through propylene oxide and embedded in Spurr's low viscosity medium (Spurr 1969) at 60°C for 18 hours. *En bloc* staining with 2% aqueous uranyl acetate following fixation in 4% glutaraldehyde in 0.1 M phosphate buffer and post-fixation in 1% OsO_4 in veronal acetate buffer was also used for dry pollen. Specimens were sectioned and stained as described in section 3.1.1.1.

3.2.1.2. Histochemistry

Histochemical tests were performed on pollen during hydration, germination and tube growth (Table 2). For most tests, unfixed pollen tubes grown in liquid medium as described in Section 3.2.1. were used. In some instances, 1 μm sections of L.R.White embedded material were used. To determine whether starch would be an acceptable carbon source, pollen was germinated on a sugar-free PGM containing 0.5% soluble starch and 1% agar, and the preparations stained with IKI (Nolan and Davis 1984; Pettitt 1985). To determine whether proteins were released from the pollen tube, pollen was grown on PGM containing 1% agar, and stained with Coomassie Blue stain-fixing medium (0.25% Coomassie Blue in methanol:H_2O:acetic acid 25:73:2) (Heslop-Harrison *et al.* 1973; Pettitt 1985). To test for proteinase activity, 1% gelatin was added to the agar PGM, and the Coomassie Blue stain-fixing medium used (Pettitt 1985).

Table 2. Summary of histochemical tests

CHEMICAL CONSTITUENT	REAGENT	FIXATION	REFERENCE
Callose	Aniline blue	GA/Os_4	O'Brien and McCully 1981
Starch	IKI	none	Jensen 1962
Lipids	Sudan IV	Lewitsky's	Jensen 1962
	Sudan Black B	"	" "
	Neutral Red	none	Clarke 1981
	Nile Blue	"	Gahan 1984
Nucleic acids	Acridine Orange	"	Clarke 1981
	Acetocarmine	"	Jensen 1962
Proteins and Proteases	Coomassie-Blue	see text	Heslop-Harrisson *et al.* 1973; Pettitt 1985

3.2.2. *In Vivo*

3.2.2.1. Cone Induction

In the spring of 1986, five clones of white spruce potted ramets, with 18-24 ramets per clone were subjected to cone-induction treatments (Ross 1988) to provide sufficient female flowers for 1987 pollinations. The trees were sprayed to wetness, weekly, with a foliar spray of 400 mg/ml gibberrellin $A_{4/7}$ (34 A_4:60 A_7, w/w, ICI Plant Protection Division, England) ($GA_{4/7}$) in 0.05% Aromox C-12/W Surfactant (Armax Chemical Corp. Ltd., Saskatoon, Canada). Spraying commenced on April 7 when 75% of the ramets in a clone had flushed, and continued until the end of rapid shoot elongation (May 20 for clone 28, June 4 for clones 3, 21, 52 and 168). Shoot elongation was monitored weekly from April 7 to June 26. Trees were top-dressed with Osmicote on May 27 and were kept in a heated polyhouse (30°C day/20°C night) from June 11 to June 26, when they were moved outdoors to overwinter.

3.2.2.2. Pollination, Collections and Microscopy

To study pollen germination and pollen development *in vivo*, female cones on potted ramets of white spruce clones 3, 21, 28, 52 and 168 were brush pollinated in the spring of 1987 with a polyclonal mix of white spruce pollen. Pollinations were started when ovuliferous scales began to reflex and were repeated every 2-3 days until ovuliferous scales began to close. Female cones were collected from the trees beginning two days after the last pollination until embryos were present in the ovules. Ovuliferous scales were removed from the cone axis, and dissected. In those cones where the pollen had not yet germinated, the integument was left covering the nucellus, and a thin wafer (< 1 mm), which included the integument surrounding the micropylar canal and the nucellus on which the pollen was situated, was dissected from the ovuliferous scale. In those cones where a pollen tube had formed and penetrated the nucellus, the integument was removed, and a thin wafer dissected from the nucellus.

Some dissected nucelli were fixed in McClintock's, stained with the Feulgan reaction for DNA (Jensen 1962), and squashed to monitor the morphology of the tube during growth and the distance the pollen tube had penetrated the nucellus. The rest of the nucelli were fixed in glutaraldehyde and OsO_4 and after alcohol dehydration were infiltrated and embedded in Spurr's (Section 3.2.1.1) or in Luft's Epon 812 (Luft 1961) at 60°C for 24 hours. Resin embedded specimens were sectioned, sections collected on uncoated 200 mesh copper grids or on coated notch-dot grids and stained for viewing on the TEM (Section 3.1.1.1.).

Chapter 4

OBSERVATIONS AND RESULTS

4.1. Pollen Development

4.1.1. Sporogenous Cells

In mid-February, when pollen cones resumed development, microstrobili within the bud scales were about 4 mm long. The microsporophylls on the microstrobili were less than 1 mm long. Each microsporophyll contained two abaxial microsporangia enclosed by an outer exodermal layer, and 2-3 layers of elongate parietal (inner wall) cells. Each microsporangium consisted of a slightly flattened tapetal layer and a mass of sporogenous cells, and were separated from each other by 2 to 6 parietal cell layers (Fig. 1).

Sporogenous cells were isodiametric, tightly packed and had very thin cell walls. They measured 20-24 μm in diameter and contained a large, generally round nucleus (17 μm diameter). Within the nucleus 1-3 prominent, darkly staining nucleoli often contained light staining areas. The cytoplasm contained a dense ribosome population, irregularly-shaped undifferentiated plastids and round and oval mitochondria (Fig. 2). Vacuoles were small, compared to those of the parietal cells.

Tapetal cells were difficult to distinguish from sporogenous cells at the light microscope level. They had a similar ultrastructure to sporogenous cells (Fig. 3) but ribosomes were often aggregated into polysomes and plastids were more differentiated. Thylakoids of tapetal cell plastids often separated, forming an electron-lucent space not found in sporogenous cell plastids, while the matrix was electron-dense, like plastids of sporogenous cells.

4.1.2. Differentiation of Sporogenous Cells into MMCs

By the end of February, the number of sporogenous cells had increased due to mitotic divisions. The cell shape changed from isodiametric to rounded (Fig. 4) and had enlarged to 30-35 μm with nuclei of 20 μm and nucleoli of 6 μm. A dense fibrous layer was laid down inside the original cell wall, followed by a more loosely packed, electron-dense fibrillar layer, marking the beginning of the special wall (Audran 1981) of the differentiating MMCs. This thickened cell wall stained pink-purple with Richardson's stain. About this time plasmadesmatal connections between sporogenous cells were broken (Fig. 6).

Changes also occurred within the differentiating cell cytoplasm. The density of ribosomes decreased as the cells increased in size, and at the light microscope level the cytoplasm was more lightly staining than in earlier stages. Large pleiomorphic plastids, measuring up to 3 μm, often contained a single starch grain (Figs. 4,6).

In these early stages of MMC differentiation, tapetal cells were quite variable. In those which still had intact cell walls, a complex ER network was interwoven throughout the cell and the cytoplasm had regressed from the cell wall leaving a space containing fibrillar material. In other tapetal cells, the cell walls were digested away to the middle lamella, the cytoplasm had regressed and the whole area thus created contained fibrillar material (Figs. 4,5), which stained positively with PAS for polysaccharides. Such fibrillar material was also found inside vesicles of various sizes within the tapetal cell cytoplasm (Fig. 5).

4.1.3. MMC Development

As differentiation continued, the loculus expanded and the MMCs separated and became round. The thecal fluid contained dense flocculent fibrillar material which stained positive for polysaccharides (Fig. 7). This was very similar to the fibrillar material found in the extra-cytoplasmic tapetal spaces. There were no remnants of the original sporogenous cell wall. The thick, fibrous MMC wall (Fig. 8) tested positive for polysaccharides and negative for callose. The cells contained abundant plastids, some of which were amyloplasts, and lipid globules. The cell diameter was about 40 μm, that of the nuclei about 28 μm. The nucleoplasm stained lightly with Richardson's stain. The MMC nuclei were in the pachytene stage of meiosis, with condensed chromosomes and prominent nucleoli.

As MMC development continued, their shape changed from round to crenate, elongated or angular. MMCs at this stage were smaller than earlier (35 μm diameter), as were their darkly staining nuclei (20 μm diameter). The cytoplasm stained darker blue and contained more starch than before (Fig. 9).

Before the first division, MMCs elongated, reaching 65 μm in length. They generally contained little to no starch during this period, but lipids were present within the cytoplasm. The lipids were often found only in those MMCs located in the basal half of the microsporangium. Flocculent polysaccharide material was still present in the matrix of the locule around the MMCs.

Meiosis was synchronous within a microsporangium, but not within a cone. However, some microsporangia contained cells at various stages of meiosis. After the first meiotic division, the daughter nuclei moved to each pole of the elongate cell and the cells became lobed (Fig. 10). A callose wall was deposited around the periphery of the cell inside the polysaccharide MMC wall. At the end of the second meiotic division, each of the daughter nuclei migrated to one of the four poles. Callose walls formed between the nuclei, resulting in a tetrad of microspores. These intratetrad callose walls were formed from the periphery inwards, and were thinner than the outer callose wall. The outer callose wall was especially thick where the intratetrad callose wall joined (Fig. 11). Polysaccharide material was deposited inside the callose wall shortly after intratetrad walls formed. This probably represented the early stages of primexine development.

After the callose walls formed, light microscopy revealed shiny yellow and white globules within the loculus (Fig. 11), and the density of the flocculent polysaccharide material decreased. These globules stained negative for polysaccharides, callose, starch, and lipids. They did not have the characteristic red stain of phenolics with the PAS reaction. Under the TEM they appeared as an electron-lucent area delimited by a fine fibrous layer (Fig. 12). Some of the structures had a thicker outer fibrous layer than others, and probably represented the shiny yellow globules viewed under the light microscope, while those with the thinner fibrous layer probably were the white globules. These structures were also found in the cytoplasm, vacuoles and extra-cytoplasmic space of the tapetal cells. They were most abundant during the period when the tetrad was enclosed within the callose wall, but tended to be scattered in the loculus during subsequent pollen development.

4.1.4. Male Gametophyte Development

In the early tetrad stage the cytoplasm and nuclei stained lightly, and nucleoli were prominent. The microspores measured 33 μm at their widest point; the nuclei measured 17 μm in diameter. Some starch was present in the cytoplasm of the tetrads, but lipid was only present in tetrads located in the proximal part of the microsporangium.

Sacci differentiated shortly after all callose walls of the tetrad formed. In early stages of saccus development, two thick lens-shaped areas which tested positive for polysaccharides formed on each microspore (Fig. 11). As the sacci expanded, the bacula became obvious on the inner surface of the tectum (Fig. 13) and neither the primexine nor the bacula of the wings tested positive for polysaccharide. The expanding sacci were filled with dense flocculent polysaccharide material.

In the late tetrad stage, when the sacci measured 25 μm across, the microspore cytoplasm was dense, and contained abundant starch. The body of the microspore was funnel-shaped and measured about 28 μm across the widest part. The callose wall separating the microspores still fluoresced strongly when stained with aniline blue, whereas that surrounding the microspores fluoresced much less intensely than it did in earlier stages, indicating a decrease in the callose content of this wall.

The beginning of intine formation was marked by the presence of another layer of polysaccharide material outside the pollen cytoplasm beneath the endexine. During this stage, saccus expansion continued and callose in the outer wall disappeared completely. However, the intratetrad callose wall was still present and apparently held the microspores together. As the sacci increased in size, the microspores began to separate. However, even after total separation of microspores, the remnants of the callose walls which held the microspores together could be seen free in the loculus up to the 2- and 3-cell stage of pollen development. These remnants no longer tested positive for callose.

The microspore expanded as vacuoles formed in the cytoplasm. The vacuoles enlarged until the nucleus appeared suspended in the centre of the microspore by strands of cytoplasm extending to the cell periphery. The vacuoles then appeared to fuse, becoming large and irregular (Figs. 14,15). Starch was generally abundant in the cytoplasm at this time.

Soon after the release of the microspores from the tetrad, the sacci expanded greatly, appearing very convoluted and filling the space within the loculus. The bacula of the sacci lengthened and anastomosed inwards (Fig. 15). The exine over the proximal pole of the microspore contained very closely spaced bacula. As the microspore enlarged, the space between the bacula increased, indicating the expansion of the foot layer to which the bacula are attached. Intine deposition continued.

The first division of the proximal microspore nucleus resulted in the formation of a small prothallial cell and a large central cell (Fig. 16). Aniline blue staining showed the wall separating the prothallial cell from the central cell contained callose. The prothallial cell was disk shaped, then became flattened against the proximal pole and enclosed within the intine. Unequal division of the central cell formed a second small prothallial cell and the large antheridial initial. The second prothallial cell was flattened against the first and also enclosed within the intine. These cells degenerated, forming thin, dense inclusions within the intine of mature pollen (Fig. 18).

The antheridial initial divided to form the large tube cell and the small antheridial cell (Fig. 17). The reniform tube nucleus lay towards the distal pole of the pollen grain. The antheridial cell divided to form the stalk cell towards the proximal pole and the body cell near the centre of the pollen grain (Fig. 18). Intine deposition finished around the time of the final cell division within the pollen grain. In the mature pollen grain the intine extended over the shoulders and sides of the stalk cell but did not completely encapsulate it (Fig. 18).

The corpus of the 5-cell pollen measured approximately 65 μm across and about 50 μm high. The layers of the pollen wall were clearly visible with light microscopy. Plastids were present in the body and tube cells. Many small round vacuoles occurred within the tube cell cytoplasm.

At this stage of pollen development, secondary thickenings had developed in the cell walls of all cells of the exothecium except those constituting the dehiscence line, which were thin walled. The dehiscence line was two cells wide and ran longitudinally along the abaxial surface of each microsporangium (Fig. 19). Separation occurred between these two rows of cells as dehiscence began. As dehiscence progressed the pollen dehydrated. The cytoplasm became more dense and darkly staining, and the vacuoles became flattened and generally followed the contour of the pollen grain. After these initial stages of dehydration, fixation of the pollen was difficult.

4.1.4.1. Dry Pollen

When shed, mature dry pollen of white spruce contained 5 cells. The 2 degenerated prothallial cells were embedded in the intine. A 0.25 μm layer of intine lay between the nexine and the first prothallial cell. The degenerated prothallial cells were flattened to a lens shape 21 μm by 0.39 μm. The stalk cell measured 21μm by 8 μm and contained a central elongate nucleus (10.8 μm by 4 μm). The body cell was rounder and larger (19 μm by 17 μm) than the stalk cell, with a larger nucleus (12μm by 8 μm) and the cell margin towards the tube nucleus was irregularly lobed. The tube cell was the largest cell in the pollen grain (Fig. 18).

Dry pollen was vacuolate, with the tube cell containing the highest proportion of vacuoles per cytoplasmic area (Fig. 18). Lipid globules were distributed around the periphery of the stalk and body cells, and throughout the tube cell. Cytoplasm of all three cells was very similar in dry pollen. The ground cytoplasm was extremely dense with free ribosomes. Mitochondria were in the inactive orthodox form. Plastids were round, elongate, u-shaped and cup-shaped. They contained very little inner membrane structure and only an occasional small starch grain. An extensive ER system, few Golgi bodies, and many small vesicles were present throughout the cells (Figs. 20,21,22). The lamellae of the nexine were very closely spaced and electron-dense. At the leptolemma, these appeared to merge, forming dark spherical areas (Fig. 23).

The walls between the cells of the pollen grain were not cellulosic. They contained some fibrous material, but many small, round vesicles and a middle lamella were also present between the plasmalemmae of neighboring cells.

4.1.5. Tapetal Development

At the MMC stage of pollen development, tapetal cells were either uninucleate or binucleate (Fig. 7) and nuclear division was still occurring in some of the uninucleate cells. Tapetal cells were at various stages of development. The least differentiated tapetal cells, a stage which was no longer seen as MMCs entered meiosis, were more lightly staining than or had similar staining characteristics to the MMCs. Elongated plastids were visible at the light microscope level. Starch and small vacuoles were present in the cytoplasm. The nuclei were at interphase, and had a diameter of approximately 14 μm, about 2/3 the diameter of MMC nuclei. Cell walls stained positive for polysaccharides. As differentiation continued, the cytoplasm of the tapetal cells became more dense and darkly staining (Figs. 7,9,10). Large vacuoles developed. The cytoplasm pulled away from the cell walls and the space created was filled with flocculent polysaccharide material. This flocculent material also filled the space between the separating MMCs, and was present in the vacuoles of the tapetal cells. The walls between the tapetal cells were sometimes difficult to discern, giving the appearance of a syncitium. However, upon staining with PAS, it became apparent that at least remnants of radial walls were present, which were much thicker towards the loculus than towards the parietal cells.

During the tetrad stage flocculent polysaccharides decreased in the vacuoles and extracytoplasmic space of the tapetal cells. Shiny globules (see section 4.1.3.)

were present within the tapetal cell cytoplasm and vacuoles within the loculus (Figs. 12,13). Some plastids contained starch granules or lipid globules, but most also contained numerous electron-lucent and electron-opaque bodies. RER was abundant throughout the cytoplasm. Cell walls on the parietal side of the tapetum were digested to the middle lamella, and radial walls were reduced to a thin fibrillar structure. Walls facing the loculus consisted of loosely woven fibers, through which the shiny round globules appeared to pass (Fig. 12).

As the microspores separated, the beginning of tapetal cell degeneration was marked by the loss of the cell wall towards the loculus and the appearance of Ubisch bodies on the tapetal cell surface. These were small bead-like structures with the same staining characteristics as the exine of the pollen grains (Fig. 14). At this stage, Ubisch bodies consisted of a round electron-lucent core surrounded by a thick coating (Fig. 24). As degeneration continued, Ubisch bodies were abundant near the tapetum (Fig.24), and sometimes were dispersed throughout the loculus (Figs. 15,16). A continuous, thin turquoise layer, apparently the peritapetal membrane, could be distinguished between the degenerated tapetal cells and the inner layer of parietal cells. In places, the small shiny globules, described above, fused with this membrane.

Remnants of tapetal cells could be seen until the 5-cell stage of pollen development (Fig. 24). Throughout the period of tapetal degeneration, Ubisch bodies were continually produced and sometimes seemed to fuse with the exine of the pollen grains. Ubisch bodies became concentrated in a thin layer around the periphery of the loculus seemingly interconnected by membranous remains of the tapetal cells. The electron-lucent core of Ubisch bodies at this stage of development was sometimes horseshoe-shaped as well as round and oval (Fig. 24).

4.1.6. Abnormal Development

Anomalies occurred throughout pollen development which led to the formation of abnormal pollen grains in both control and forced trees. Various anomalies originated at or before the tetrad stage. Degeneration of microspores was first evident at the tetrad stage, when the walls between the microspores had formed or were developing. Degenerating tetrads were generally scattered throughout the loculus, amongst normal tetrads. Often, one microsporangium of a microsporophyll contained more degenerating tetrads than the other. Degenerating tetrads stained darkly, microspores were concave and elongate nuclei were appressed to the concave surface whereas normal microspores had round nuclei with lightly staining nucleoplasm (Fig. 25). In some instances, not all of the microspores of the tetrad degenerated at the same rate (Fig. 26). Degeneration of pollen cytoplasm also occurred but to a lesser extent at later stages of pollen development, resulting in undersized, collapsed pollen grains.

Abnormal wall development also began during the tetrad stage, and had several ramifications. In the most extreme cases, the callose wall between the four microspore nuclei did not form completely, and since this is the template for the pollen wall, the latter did not form in those areas where the callose wall was missing. This resulted in cytoplasmic continuity between the pollen grains, and a lack of complete separation of the grains (Fig. 27). Although this pollen did not necessarily degenerate, it was difficult to determine the number of cells within or their origin.

In the less extreme forms an abnormal exine formed. The tectum over the corpus of spruce pollen normally is microverrucate (Owens and Simpson 1986). In some pollen, the tecta of the microspores of a tetrad were fused, and bacula were poorly developed (Figs. 28,29). These microspores either developed for a short time, then degenerated resulting in collapsed grains, or continued to develop, forming what appeared to be normal pollen except for the fusion of the grains (Figs. 30,31). The degree of fusion of the microverrucae varied within a fused tetrad, and between fused tetrads. In some areas, microverrucae were so completely fused that it was difficult to tell where one pollen grain ended and the other began (Fig. 29). In other areas it appeared that as the pollen grains developed, the connections between the pollen grains had been stretched, and in some cases broken, leaving pointed structures on the pollen surface (Figs. 31-33). In some instances, the connections were less tenuous, and a sheet-like structure resulted from stretching (Fig. 34).

Abnormal saccus development was common (Figs. 35,37). This was found in, but not restricted to pollen which was joined at the exine. In some pollen, sacci were absent (Fig. 35). In other cases, sacci did not expand (Fig. 36), and where they would normally develop the ectexine separated little from the foot layer and bacula often connected the two layers resulting in very convoluted sacci (Figs. 36,37).

In a few cases, the plane of cell division within the pollen was affected by the forcing treatment, so the tube nucleus lay adjacent to the antheridial cell nucleus instead of below it (Fig. 38). This did not affect wall development and was not seen in pollen from control trees.

When a loculus contained a high proportion of abnormal or degenerated pollen, the secretory products of the tapetum also appeared atypical. When pollen degeneration occurred at the tetrad stage, the loculi with high proportions of degenerated pollen contained fibrous material rather than the normal flocculent material and the shiny globules (Fig. 11) were often absent (Fig. 26). When pollen degeneration occurred in the free microspore stage, tapetal cells appeared to have degraded rather than following the normal secretory stages of development. Late in tapetal development, when normally only Ubisch bodies and membranous strands remained, Ubisch bodies in microsporangia with a high proportion of abnormal pollen tended to have a slit-like electron-lucent core (Fig. 39) rather than a round or oval core (Fig. 24).

4.2. Effects of Forcing on Pollen Physiology

4.2.1. Seed Efficiency

Pollen forcing significantly decreased seed efficiency when compared to controls. In individual clones forcing caused significantly lower seed efficiency only in clones 3, 52 and 1675. There were significant differences in seed efficiency between clones in both forced and control treatments.

Table 3. Pollen Forcing Trial Seed Efficiency.
Student-Newman-Keuls test for variable seed efficiency (SEF).
* denotes significant difference.

CLONE	TREATMENT	MEAN SEF	PR>F	
3	control	35.7	0.0002	*
	force	15.772		
21	control	31.674	0.4099	
	force	25.255		
52	control	18.980	0.0001	*
	force	5.718		
1675	control	12.645	0.0001	*
	force	3.858		
1676	control	17.160	0.1365	
	force	24.918		
overall	control	22.816	0.0013	*
	force	14.599		

4.2.2. Carbohydrate Levels

The levels of sucrose, glucose and starch in the pollen cones were determined, and the data analyzed according to stage of pollen-cone development (as described in Table 1, Sec. 3.1.) and stage of pollen development within the cone. Some significant differences were found in each case. Glucose levels were significantly lower in forced than control pollen cones at stage 5 in clone 3 (pr>f = 0.0171), and at stage 6 in clones 52 (pr>f = 0.0153) and 1675 (pr>f = 0.0361). Sucrose levels were lower in forced cones of clone 52 at stage 7 (pr>f = 0.0268). When data were analyzed according to stage of pollen development, there were significantly lower sucrose levels in forced pollen of clone 52 at the 4-cell stage (pr>f = 0.0054) and at the 5-cell stage (pr>f = 0.0042). This corresponds to the low sucrose level found in forced cones of clone 52 at stage 7. When values for all clones were bulked for each stage of pollen development, control 4-cell pollen showed higher levels of glucose (pr>f = 0.0457), sucrose (pr>f=0.0295) and starch (pr>f = 0.0423) than forced pollen at this stage. Also, control 5-cell pollen showed higher levels of sucrose than forced (pr>f = 0.0458).

4.2.3. Rate of Development

Forcing affected the rate of pollen (Fig. 40) and pollen-cone development (Fig. 41). These graphs represent the length of time a particular stage of pollen or pollen cone was found on the ramets of a treatment. Data represent the means of only three clones (3, 52 and 1675), therefore statistically significant differences are obscured, but trends may be seen.

Forcing tended to decrease the amount of time pollen remained in each stage of development (Fig. 40), with the greatest differences found in the tetrad and 1- and 2-cell stages. Control pollen remained at the MMC stage about 4 days longer than forced pollen, 10 days longer at the tetrad stage, 11 days longer at the 1-cell stage, 9 days longer at the 2-cell stage, 4 days longer at the 3-cell stage, 2 days longer at the 4-cell stage, and 5 days longer at the 5-cell stage.

Forcing tended to decrease the period of time pollen cones were found at particular stages of development (Fig. 41). Stage 2 pollen cones were found on control trees for 31 days versus 13 days on forced trees. Stage 2 was the period of pollen cone swelling before bud-burst. In control trees this included MMC separation and loculus expansion (Fig. 7), meiosis (Fig. 10), tetrad formation (Fig. 11), and separation of 1-cell microspores (Fig. 14). During stage 2, pollen cones elongated within the bud scales from 5 mm to 9 mm. Many of the pollen abnormalities originated during stage 2. The remaining stages of pollen cone development, except stage 3, occurred over a longer time on control trees compared to forced trees. The first cones to reach these stages were smaller and contained pollen at an earlier developmental stage than pollen cones which reached these stages later, in both forced and control trees.

Forcing advanced the time of pollen shed in all clones (Fig. 42). Forced trees of clones 3 and 52 shed pollen about two weeks earlier than control trees. In clones 21 and 1676 the difference was about 10 days and in clone 1675 there was 1 week difference. Differences between the clones were also evident. Clones 3, 21, 52 and 1675 had similar trends in pollen shed, although clones 21 and 1675 had greater overlap between forced and control pollen shed. Clone 1675 shed both forced and control pollen earlier than the other clones.

4.3. Pollen Germination and Tube Growth

4.3.1. *In Vitro*

Of the germination media tested, PGM + 5% sucrose, PGM + 10% sucrose and dH_2O + 5% sucrose gave equally high percent pollen germination. After 3 days in these media, pollen tubes were longest in dH_2O + 5% sucrose (360 µm), followed by PGM + 5% sucrose (340 µm), and PGM + 10% sucrose (260 µm). After 6 days, those in dH_2O + 5% sucrose measured 490 µm, those in PGM + 5% sucrose 530 µm, and those in PGM + 10% sucrose 400 µm. PGM + 5% sucrose was used in all subsequent studies.

4.3.1.1. Hydration, Germination and Pollen Tube Growth

Upon hydration, separation of the nexine lamellae revealed a fibrous matrix of similar structure to the intine. Hydration of the nexine also decreased the size of the dark spherical areas found in the nexine at the leptolemma.

In the early stages of hydration (1.5 hours) the U-shaped and cup-shaped plastids found in dry pollen disappeared, leaving only round or oval plastids. These had more complex internal membrane structure than plastids in dry pollen. Some plastids contained one or more double-membraned inclusions, the contents of which were more similar to the ground matrix of the plastids than to the cytoplasm (Fig. 43).

Distribution of the organelles within the tube cell was polar. Plastids were concentrated around the tube nucleus and the areas bordering the stalk and body cells but were absent from the periphery of the pollen grain. In the latter area abundant ER, Golgi bodies, mitochondria, lipids and vesicles were found (Fig. 44). Vacuoles and ribosomes were present throughout although ribosome density was lower than in dry pollen and there was an increase in the number of polysomes (Fig. 43). Banks of smooth and rough ER followed the contours of the tube cells below the sacci. The shape of the vacuoles also reflected the contours of the pollen grain (Fig. 45). The plasmalemma of the tube cell was sinuate next to the intine, with some deep intrusions into the cell (Fig. 44). Walls between the stalk, body and tube cells were very thin and undulate (Figs. 43,46).

Within 3 hours of placing the pollen into the medium, starch began to accumulate in many of the plastids and cristae of the mitochondria began to swell (Fig. 46). Vesicles with electron-dense contents were present within the tube-cell cytoplasm near the leptolemma. Just outside the plasmalemma, electron-dense globules appeared to have been deposited on the intine (Fig. 47). Similar structures were present within some vacuoles of the tube cell (Fig. 46). A thin layer of loosely woven cellulosic fibers was non-uniformly distributed between the intine and the plasmalemma at the leptolemma (Fig.45) and below the sacci (Fig. 47).

Hydration resulted in physical swelling of the pollen grain, causing the sacci to expand outward and expose the leptolemma (Fig. 48). This, coupled with the deposition of new cell wall material caused the foot layer to stretch and the lamellae of the nexine to separate. After 12 hours of hydration, germination of the pollen began as the foot layer and nexine split and the tube began to emerge (Figs. 48,49). The wall of the emerging tube was continuous and identical in appearance to the intine. In some cases the foot layer and nexine did not split, resulting in a lack of germination and a build up of wall material between the intine and the plasmalemma (Fig. 50).

After 24 hours in the medium, pollen tubes had elongated 50-120 μm. Starch was abundant in all 3 living cells. In the tube cell, amyloplasts were distributed within the corpus around the body and stalk cells and throughout the pollen tube except at the tip (Fig. 51). Most other tube cell organelles were concentrated towards the tip of the extending pollen tube. The proximal portion of the pollen tube was vacuolate.

When stained with acridine orange the cytoplasm in the tip of the pollen tube displayed bright orange fluorescence indicating high RNA concentration. This corresponded to an extensive dome of RER, which dominated the cytoplasm of the tube tip (Fig. 52). Behind the RER cap were located microtubules, mitochondria, dictyosomes, polysomes, vesicles, vacuoles, lipids and large starch grains (Figs. 52,53).

As the tube nucleus began to move into the pollen tube the body and stalk cells remained in the corpus, still attached to the intine. The stalk cell was more vacuolate, and the starch grains within its amyloplasts were generally larger than in the body cell. Typical cell walls were lacking between the stalk, body and tube cells; instead these areas contained some fibrillar strands and small round inclusions. Vesicles within the body cell appeared similar to these inclusions (Fig. 54). Cells were connected by plasmadesmata (Fig. 55). Most of the tube-cell cytoplasm moved into the pollen tube. Within the corpus, the tube-cell cytoplasm was restricted to a thin layer surrounding the stalk and body cells (Figs. 54,55,58), a layer around the periphery of the corpus and cytoplasmic strands traversing the large vacuolar areas.

After 2-3 days of pollen-tube growth the tube-cell cytoplasm remained concentrated towards the tip of the pollen tube, maintaining the same zonation pattern. Most of the tube cell was vacuolate, with cytoplasmic strands along its length. Pollen tubes demonstrated active cytoplasmic streaming. The tube nucleus was located behind the area of high starch concentration (Figs. 56,57). Stalk and body cells contained less starch and remained in the corpus (Fig. 58).

When pollen was germinated on 1% agar PGM containing 0.5% soluble starch, pollen tubes contained much less starch than when grown on PGM containing a sugar source. When grown on 1% agar PGM containing 1% gelatin and stained with Coomassie blue, the media around and in front of the elongating pollen tubes lacked the deep blue stain indicating a loss of protein from the media. Pollen tubes also stained positively for protein. Pollen tubes did not stain positive with aniline blue, indicating a lack of callose.

4.3.2. *In Vivo*

Pollen germination and tube growth occurred more slowly within the ovule than in artificial media. Early stages of germination occurred within two days of pollination. The nexine split in several places at the leptolemma, and breaks occurred in the foot layer apparently by separation of overlapping units of the foot layer (Fig. 59).

4.3.2.1. Early Pollen Tube Growth

4.3.2.1.1. Tube Cell

A pollen tube formed and grew towards the nucellar cells. Early stages of *in vivo* growth mimicked that seen *in vitro*. The tube tip was thick and rounded until the pollen tube began penetration of the nucellus. Then it became narrower and irregularly shaped as it pushed its way between degenerating nucellar cells. Cytoplasm of nucellar cells broke down in advance of the pollen tube and cells bordering the tube contained primarily flocculent material (Fig. 60).

As pollen tube growth progressed, most of the tube-cell cytoplasm moved into the pollen tube and the portion of the tube cell within the corpus became very vacuolate (Fig. 61). The oval tube nucleus measured 4 μm by 6 μm, and contained a large nucleolus (2 μm diameter). The nucleus was surrounded by vacuolate cytoplasm (Fig. 62) containing many large starch grains, pleiomorphic plastids, golgi bodies, lipid globules and ribosomes. Tube-cell plastids (Fig. 63) were generally smaller and the stroma of the plastids and amyloplasts were much more electron-dense than those of the body and stalk cells.

The *in vivo* tube-cell cytoplasm displayed a different zonation than *in vitro* grown tubes. The majority of amyloplasts and plastids were located very near the tube nucleus in the pollen grain, while few were distributed in the tube. Other cellular components were distributed throughout the cytoplasm.

4.3.2.1.2. Stalk Cell

The stalk cell contained several large vacuoles in the proximal portion of its cytoplasm. The plasmalemma of the stalk cell separated from the intine at the proximal pole and the resultant space was filled primarily with flocculent material and several round vesicular bodies. The latter were also present where plasmalemmae of stalk and body cells separated from their cell walls. Where the intine surrounded the shoulders of the stalk cell, dense fibrillar substances were present between the intine and the plasmalemma and the intine extension was reduced compared to earlier stages (Fig. 64). Plasmadesmata connected stalk and body cells but were not apparent between stalk and tube cells.

Plastids and amyloplasts of the stalk cell were irregularly shaped, contained few thylakoids and had granular stroma. They often included cytoplasm. This appeared to occur by the plastids elongating and surrounding a portion of the cytoplasm (Figs. 63,64). Mitochondria were small, spherical to oval, and contained well-developed cristae in the inactive, orthodox form (Fig. 64). The nucleus was smaller and more condensed than those of body or tube cells (Fig. 61).

4.3.2.1.3. Body Cell

Plastids of the body cell had similar morphology to those of the stalk cell. Several amyloplasts contained starch grains much smaller than those of stalk and tube cells (Fig. 63). No large starch grains were present. Many lipid globules were distributed throughout the cytoplasm, as in the stalk and tube cells. Lipid globules were generally smaller than in the tube cell. Body-cell cytoplasm was less dense than that of the stalk cell.

4.3.2.2. The Advanced Pollen Tube

Two to three weeks after pollination, pollen tubes had grown about 400 μm and penetrated the nucellus by passing between degenerating nucellar cells. Pollen tubes had small branches along their length (Fig. 65), some of which were very thin, resembling haustoria. The tube tips formed projections which appeared to penetrate between degenerating nucellar cells (Fig. 66). Deeper within the nucellus, degenerated cells contained flocculent material and starch was the last identifiable nucellar component.

The stalk cell separated from the corpus wall apparently by disruption of the connection of the intine extension and the stalk cell (Fig. 67). As the stalk and body cells moved into the pollen tube the stalk cell was found at the distal end of the body cell (Figs. 72,74). It was not determined whether the body/stalk cell complex rotated or whether the stalk cell overtook the body cell within the pollen tube. Each of the cells were limited by their own plasmalemma. Cells were intertwined, with the spaces between the body and tube cells often very large (Fig. 71) while those between the stalk and body cells were minute (Fig. 72). The body-cell nucleus divided forming two male gametes when the body cell was in the proximal part of the pollen tube (Fig. 74).

4.3.2.2.1. Tube Cell

The tube cell contained distinct zones. The portion of the tube cell within the corpus contained only a few strands of cytoplasm and several amyloplasts with large starch grains. The body/stalk-cell complex occupied about 100 μm of the proximal portion of the pollen tube. Distal to this, the next 100 μm of the tube-cell cytoplasm contained many amyloplasts, each with one large starch grain. On either side of the body cell, strands of tube-cell cytoplasm contained amyloplasts, RER, golgi bodies, active condensed mitochondria, small plastids with little internal membrane structure, lipid globules and a large number of vesicles (Fig. 71). The latter were polymorphous: some had very darkly staining contents, others had less darkly staining, fibrous contents. Tube-cell cytoplasm was vacuolate throughout, the vacuoles containing flocculent material and, occasionally, darkly staining globules similar to the vesicles found within the cytoplasm (Fig. 68).

The tube nucleus was located near the pollen-tube tip. As in earlier stages, it was round or oval containing a large prominent nucleolus. The cytoplasm in the distal-most 75 μm of the tube cell was distinct from more proximal cytoplasm. No amyloplasts were present, RER was abundant and lipids were sparse (Figs. 68,69,70). Small tubule-like projections with an outer diameter of about 0.063 μm, continuous with the plasmalemma, extended from the plasmalemma towards the tube wall through the extracytoplasmic space (Figs. 69,70).

4.3.2.2.2. Body Cell

The body cell within the pollen tube was elongate, measuring about 120 x 27 μm. There were long, narrow extensions of the tube cell into the body cell which resulted in a high degree of surface area contact between the cells (Fig. 71). The distal end of the body cell was very irregular, with long fingers extending into the tube cell (Figs.

72,73). Microtubules in the tube cell appeared to be associated with these extensions of the body cell (Fig. 73). The large nucleus with its prominent nucleoli was located at the proximal end of the body cell (Fig. 71).

Mitochondria and plastids were abundant throughout the cytoplasm (Fig. 71). Plastids were oval to elongate and contained electron-dense stroma and few thylakoid membranes. The mitochondria were oval (1.3 x 0.6 μm), appeared to be in the inactive orthodox state and were more numerous than the plastids (Fig. 71). Small oval vacuoles were present. The cytoplasm was dense with ribosomes and SER. No RER and few Golgi bodies were present. Lipid globules were absent in the body cell (Fig. 72).

At mitosis, the body-cell cytoplasm became polarized. Figure 74 shows the body cell at metaphase. The plastids, mitochondria and golgi bodies were restricted to the distal end (Fig. 74). Between the metaphase chromosomes at the proximal pole and the organelles was an area with a high concentration of polysomes and microtubules (Fig. 75).

Division of the body-cell nucleus resulted in two male nuclei (Fig. 76). Plastids and mitochondria surrounded each of the nuclei. The margins of the nuclei were often irregular with finger-like projections extending into the cytoplasm (Figs. 76,77). Within these projections groups of tubular structures with an outer diameter of about 0.045 μm were apparent (Fig. 77). These tubular structures were not restricted to the projections but were around the periphery of each male nucleus just inside the nuclear membrane (Fig. 78). Between the nuclei (Fig. 76) an incomplete cell plate had apparently formed (Fig. 78).

4.3.2.2.3. Stalk Cell

The small stalk cell was distal to the body cell, about 200 μm from point of pollen tube emergence from the corpus. It was extremely intertwined with the tube and body cells, making it difficult to distinguish. However, subtle cytoplasmic differences existed (Fig. 72). The stalk cell lacked the extensive RER system and large vesicular population found in the tube cell. The stalk cell contained abundant ribosomes and several large vacuoles. Plastids generally contained thylakoids which were sometimes swollen. Some plastids contained small starch grains whereas in the body cell no amyloplasts were present. Mitochondria were smaller and less abundant than in the body cell.

Figs. 1-6. Normal pollen development. Sporogenous cell stage.

Fig. 1. Light micrograph of a cross-section of a microsporophyll in mid-February. Microsporangia consist of tapetal (t) and sporogenous (sp) cells enveloped by exothecial (e) and parietal (p) cells. x230.

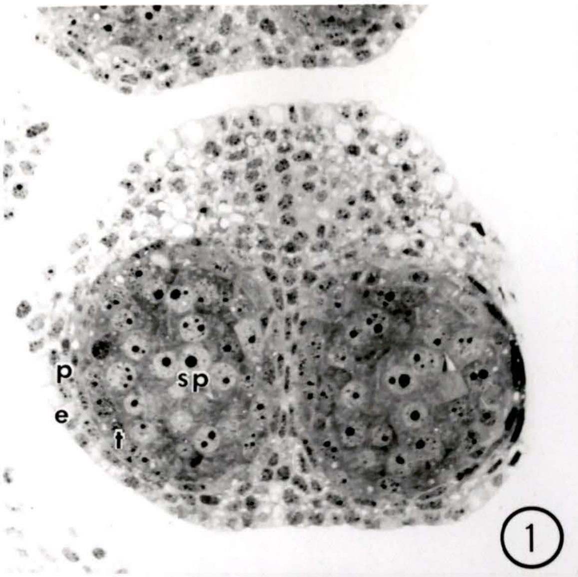
Fig. 2. TEM of sporogenous cells. Cell walls, (w); plastids, (pl); mitochondria, (mt); vacuoles, (v); nucleus (n). x11,070.

Fig. 3. TEM of tapetal cells. Plastids are more differentiated than those of sporogenous cells. x11,070.

Fig. 4. TEM of a sporogenous cell and neighboring tapetal cells late in February. Intact tapetal cells have complex ER network (arrow). Other tapetal cells (*) have begun secreting fibrillar material (fm). x1,937.

Fig. 5. TEM of sporogenous and tapetal cells. Tapetal cell wall digested to middle lamella (ml) and cytoplasm has regressed. Vacuoles and extracytoplasmic space contain fibrillar material. x6,273.

Fig. 6. TEM of special cell wall of two neighboring sporogenous cells. The original cell wall (w) is outside the dense fibrous (f) and fibrillar (fb) layers of the special MMC wall. Broken plasmadesmatal connection (arrow). Some plastids contain a single starch grain (sg). x11,070.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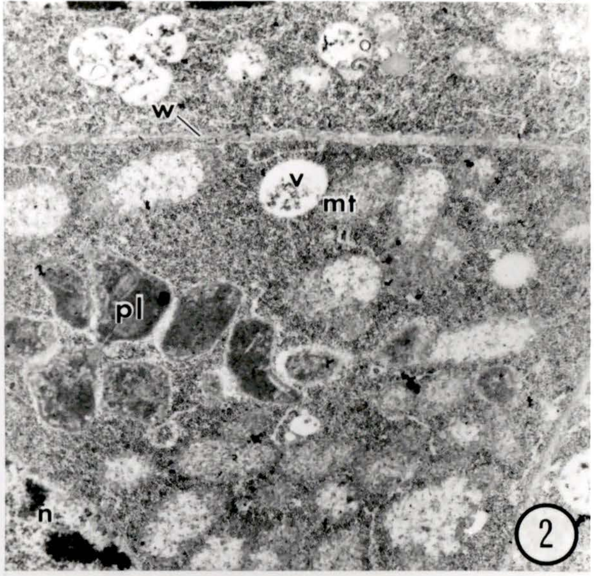
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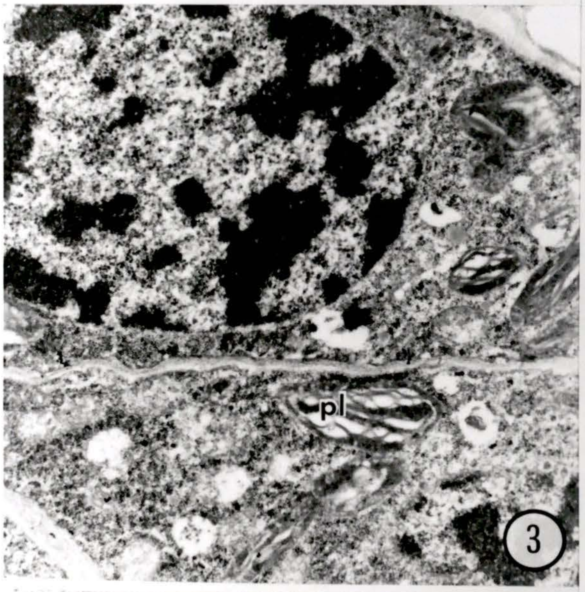
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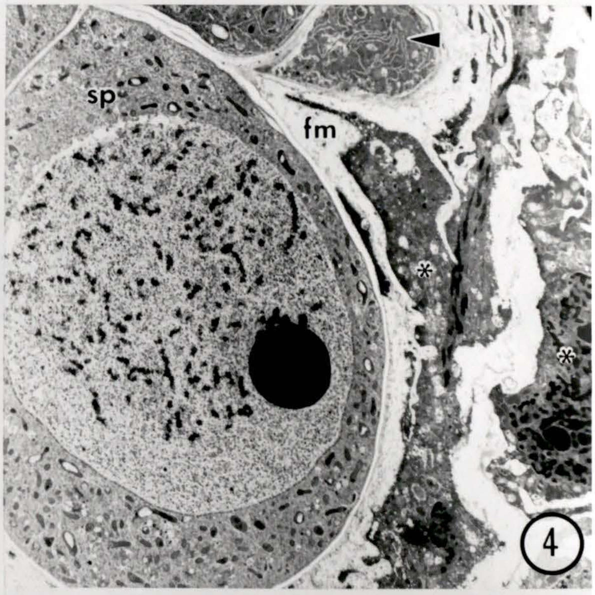
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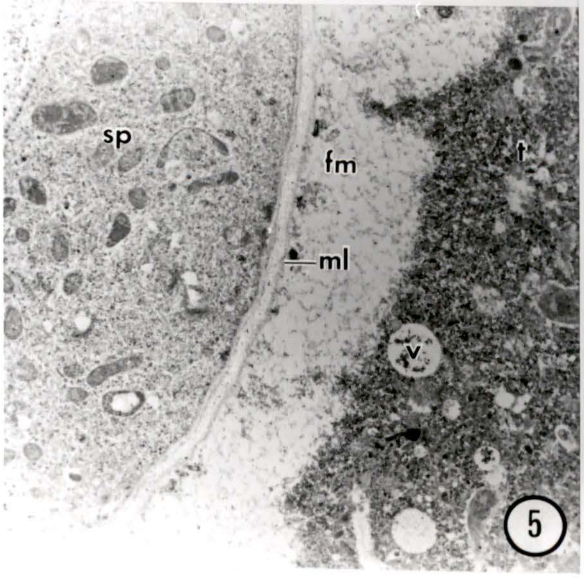
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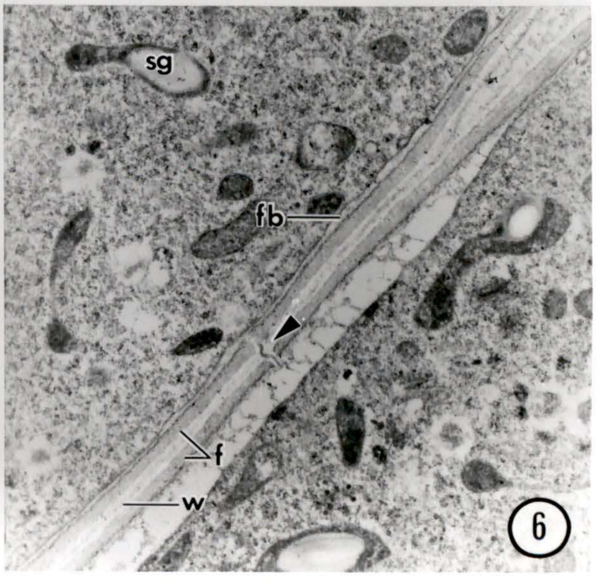
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Figs. 7-12. Normal pollen development. MMC to early tetrad.

Fig. 7. Light micrograph of a longitudinal section of the proximal portion of a microsporangium in early March. MMCs are suspended in a flocculent matrix surrounded by uni- and bi-nucleate tapetal cells (t). Exothecium, (e); parietal cells, (p). x260.

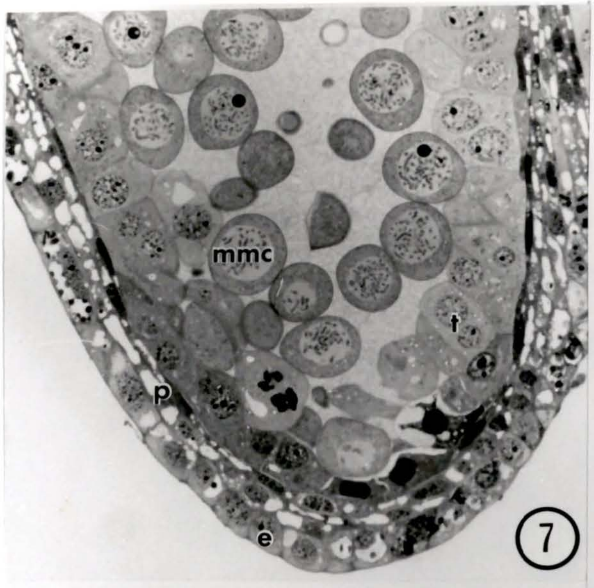
Fig. 8. TEM of portion of the thick MMC wall (w) surrounding MMC cytoplasm (cyt). x33,210.

Fig. 9. Light micrograph of a longitudinal section through a microsporangium before meiosis, irregular shaped MMCs with dense cytoplasm (arrows) appeared in sectioned material, and the vacuoles (v) of tapetal cells increased in size. x370.

Fig. 10. Light micrograph of MMCs at meiosis. Some MMCs have completed meiosis (single arrow) while others have not (double arrow). Tapetal cells are more vacuolate. x270.

Fig. 11. Light micrograph of a section through a microsporangium. Callose walls (c) have formed around and between microspores of the tetrad (td). Early saccus development is indicated by the presence of PAS-positive areas on the microspores (s). Globules present in the loculus (arrow). x620.

Fig. 12. TEM showing portion of a tapetal cell and tetrad. Globules are present within the loculus, and in the vacuole and wall of the tapetal cell (arrows). x5,124.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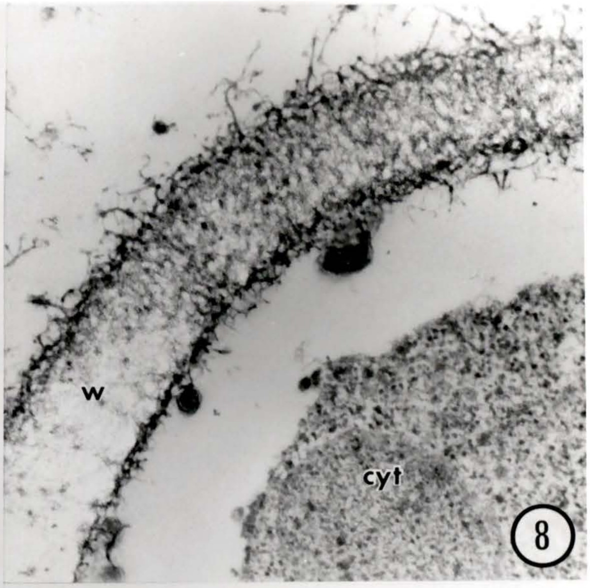
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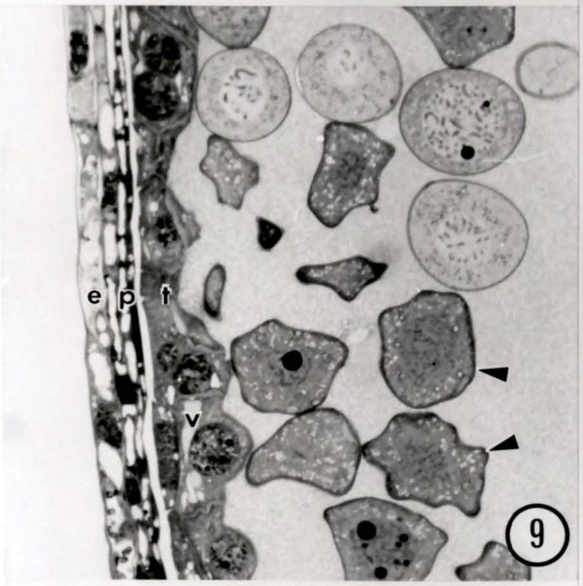
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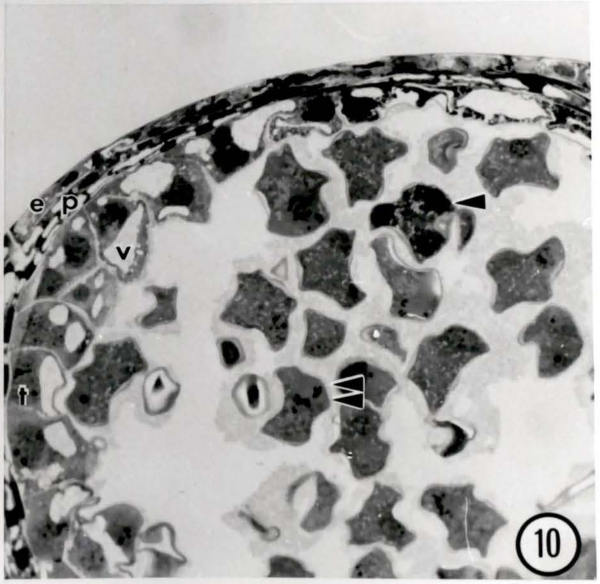
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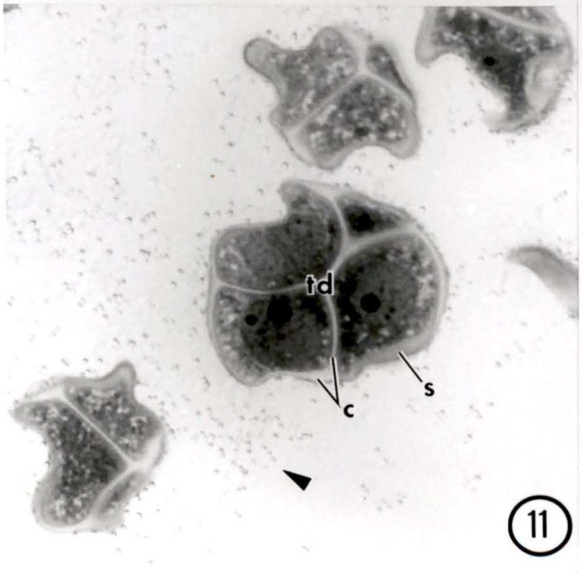
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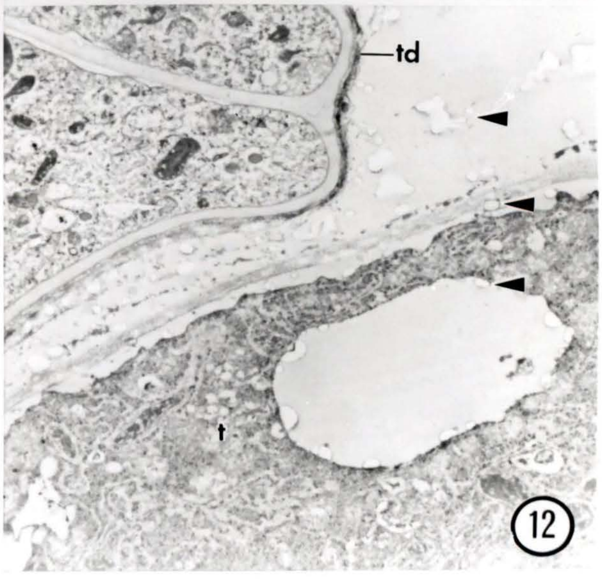
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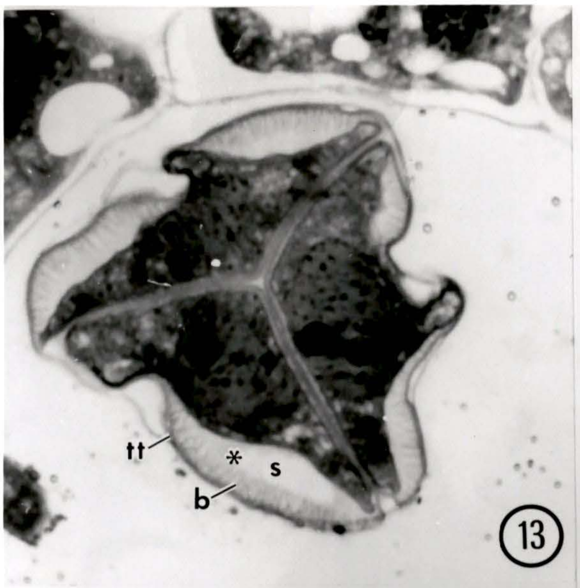
Figs. 13-16. Light micrographs of normal pollen development. Late tetrad to 2-cell pollen stage.

Fig. 13. Three microspores of the tetrad. Sacci (s) with outer tectum (tt), inner bacula (b) and dense PAS-positive material (*). x1,380.

Fig. 14. Microspore separation and saccus expansion. Vacuolar area (v) of microspore cytoplasm has increased. Tapetal cell walls have degenerated. Ubisch bodies (u) are apparent on tapetal cell surface. x155.

Fig. 15. Free microspores. Sacci have expanded greatly, bacula are extensive and vacuoles are large. x550.

Fig. 16. Two-cell pollen with small prothallial cell (pc) and large central cell (cc). Ubisch bodies are abundant near the degenerating tapetum and can be seen in the loculus. x250.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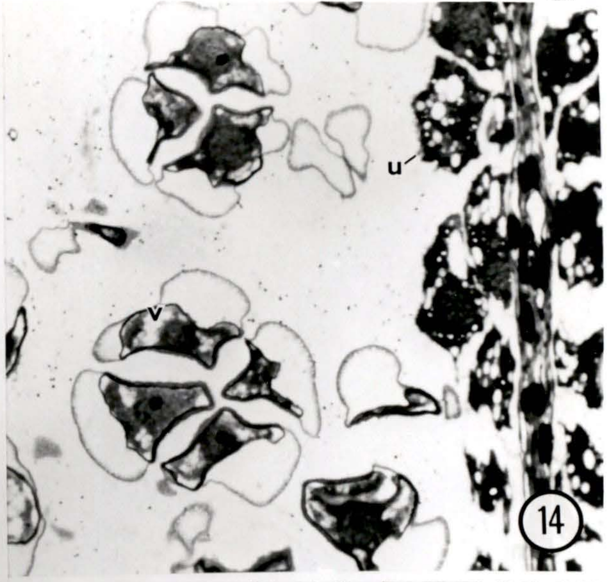
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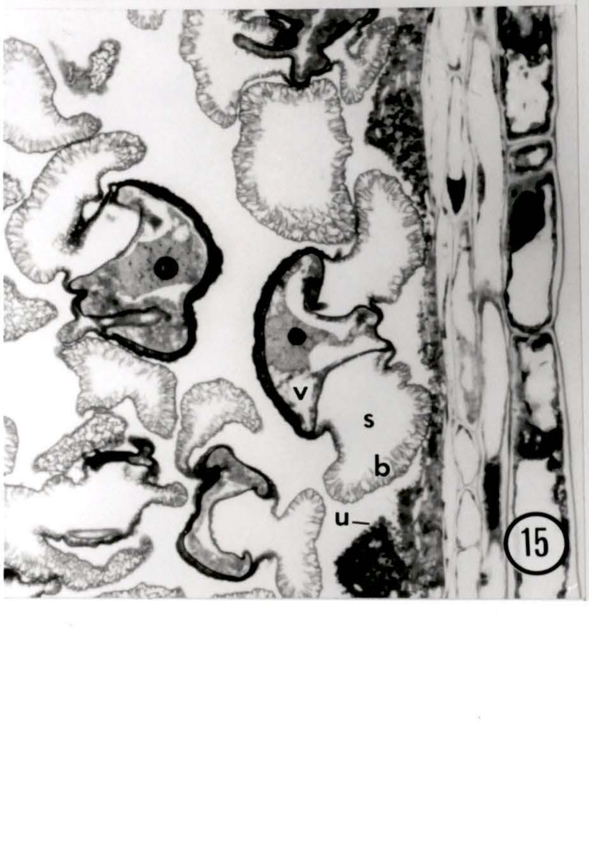
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Figs. 17-21. Normal pollen development. 4- and 5-cell pollen stage.

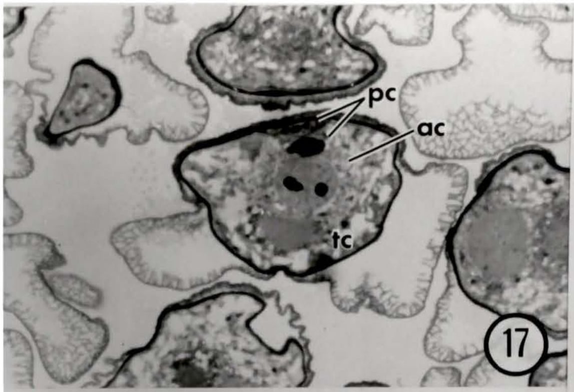
Fig. 17. Light micrograph of four-cell pollen containing 2 prothallial cells (pc), antheridial cell (ac) and large tube cell (tc). x313.

Fig. 18. TEM of dry 5-cell pollen: two flattened prothallial cells embedded in the intine at the proximal pole, stalk cell (sc), body cell (bc) and tube cell (tc). Leptolemma, (l). x 1,107.

Fig. 19. Light micrograph of a cross section through a microsporangium approaching dehiscence. Radial walls of the exothecial cells (e) have secondary thickenings (arrows). Cells comprising the dehiscence line are thin-walled (double arrows). Tapetal cells have degenerated and Ubisch bodies (u) envelope the loculus. x313.

Fig. 20. TEM of body-cell cytoplasm. Golgi bodies, (g); mitochondria in the orthodox form, (mt); variously-shaped plastids (p) with little internal membrane structure and an occaisional starch grain (sg); ER. x16,605.

Fig. 21. TEM of the tube-cell cytoplasm near the leptolemma. Small vesicles (ve) are abundant. x40,180.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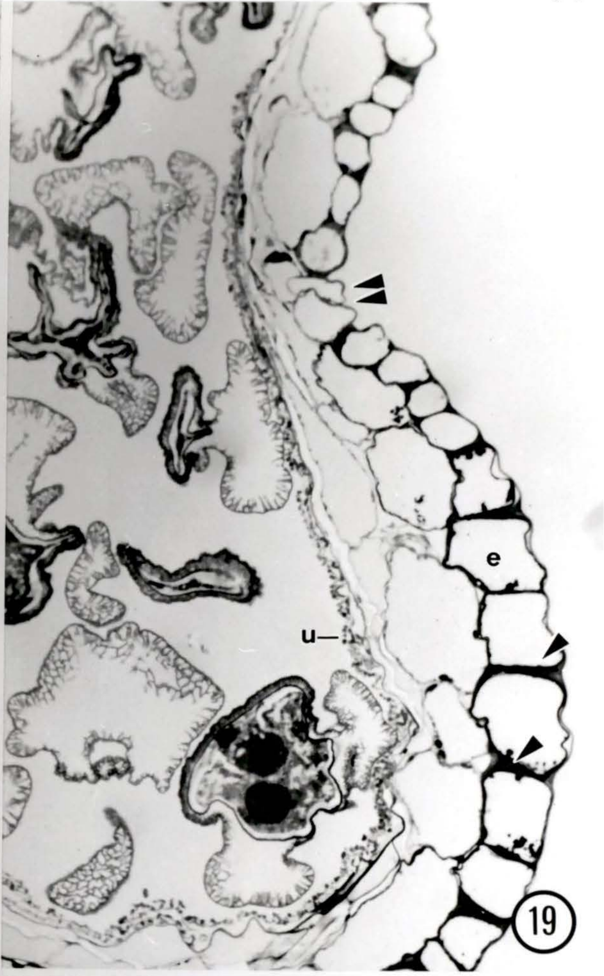
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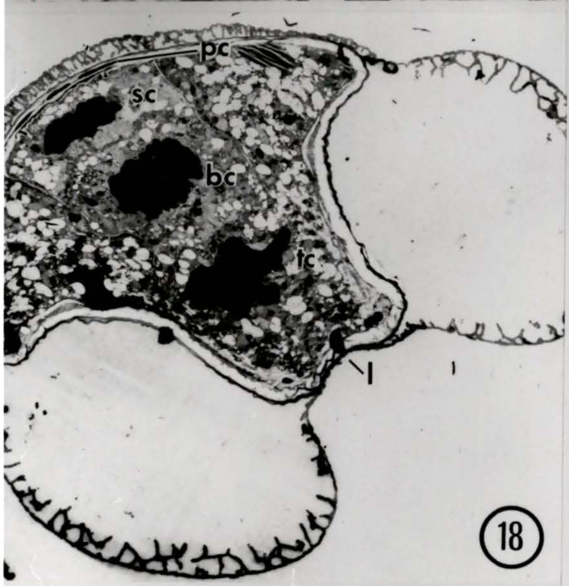
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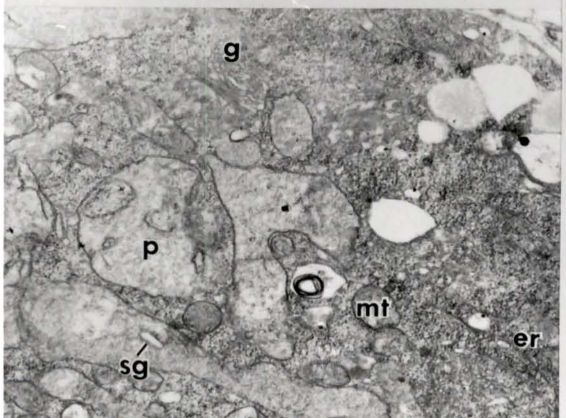
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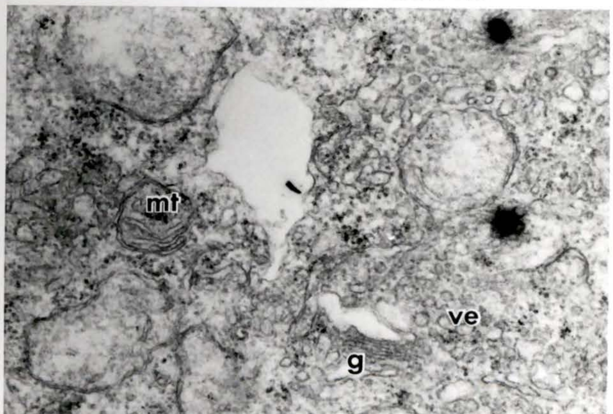
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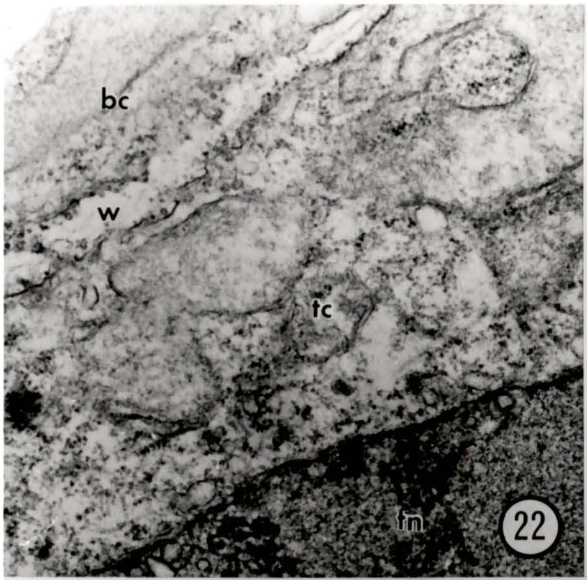
Figs. 22-24.	Normal pollen development. 5-cell pollen stage.
Fig. 22.	TEM of the wall (w) between tube (tc) and body (bc) cells. Tube nucleus (tn). x10,204.
Fig. 23.	TEM of the leptolemma of dry pollen. Lamellae of nexine (n) fuse to form large spherical areas. Tectum (tt) and foot-layer (fl) of ectexine fuse. Intine, (i). x19,632.
Fig. 24.	TEM of degenerated tapetal cell (t) at 5-cell pollen stage. Ubisch bodies, (u). x10,000.
Figs. 25-29.	Abnormal pollen development.
Fig. 25.	Light micrograph of normal and degenerating (*) tetrads within a loculus towards the end of callose wall formation. Note concave nucleus (n) of degenerating tetrad. x555.
Fig. 26.	Light micrograph of a slightly later stage. Cytoplasmic degeneration of some microspores is complete, while others within the same tetrad have not completely degenerated. x445.
Fig. 27.	Light micrograph. Incomplete wall formation (arrow) results in cytoplasmic continuity between microspores of a tetrad. x470.
Fig. 28.	TEM at the microspore stage. Lack of separation due to fusion of tecta (arrows) of adjacent microspores. x1,200.
Fig. 29.	TEM at the five-cell stage. Tecta of adjacent pollen grains are fused (arrows). Pollen cytoplasm has degenerated. x10,000.


bc
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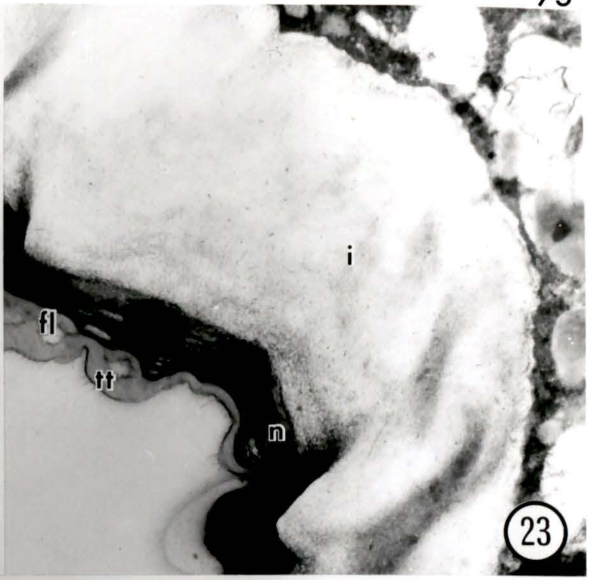
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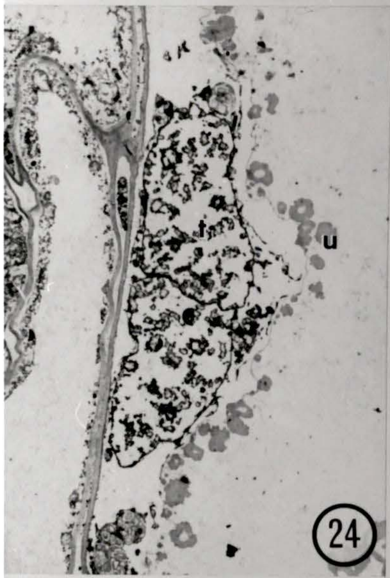
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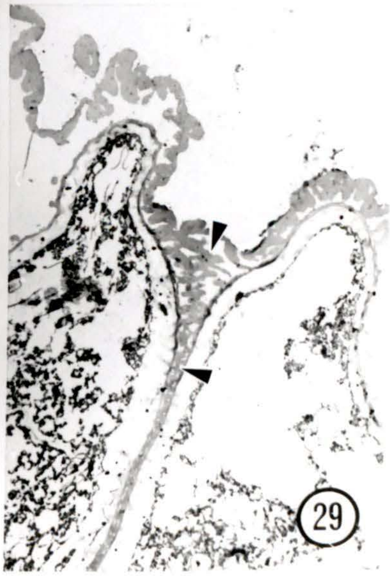
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Figs. 30-34. Abnormal pollen development.

Fig. 30. Light micrograph of section through abnormal pollen at the 2-to 3-cell stage. Tecta of the pollen wall are fused (arrows), but pollen cytoplasm is normal. x440.

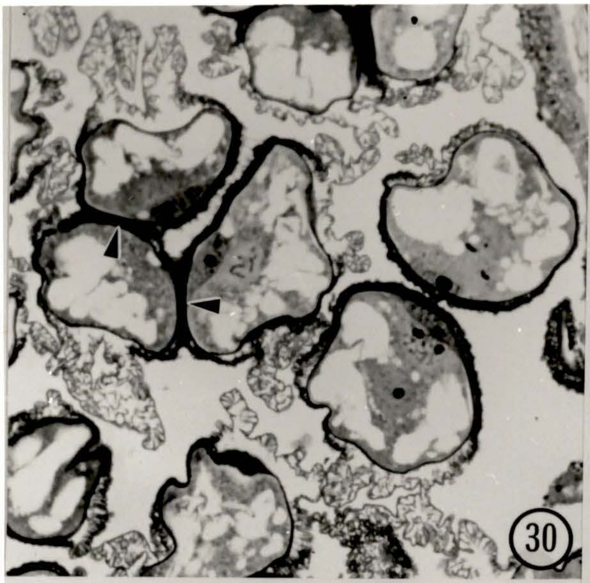
Fig. 31. SEM of shed, dry pollen. Fused pollen whose cytoplasm has degenerated (single arrow) also show collapse of sacci (s). Fused pollen with normal cytoplasm (double arrow) have well-formed sacci. x300.

Figs. 32-34. Details of Fig. 31.

Fig. 32. Extensive tectal fusion (arrow) in pollen with collapsed cytoplasm. x1,380.

Fig. 33. Expansion of pollen grain appears to have caused stretching and breakage of some tectal connections (arrow). x1,380.

Fig. 34. Connection at the base of the sacci of two adjacent pollen grains appears to have stretched into a thin sheet-like layer (arrow). x755.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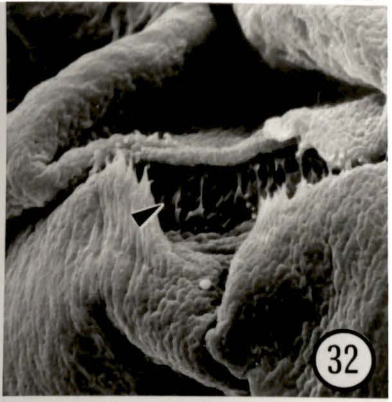
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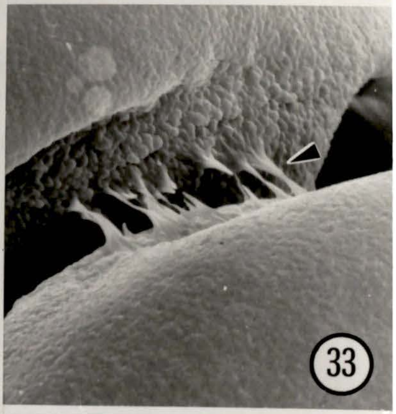
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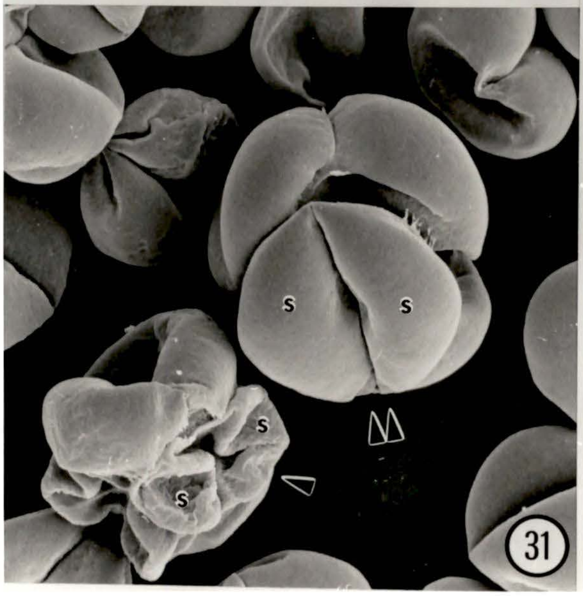
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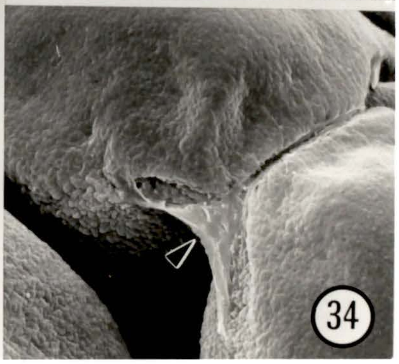
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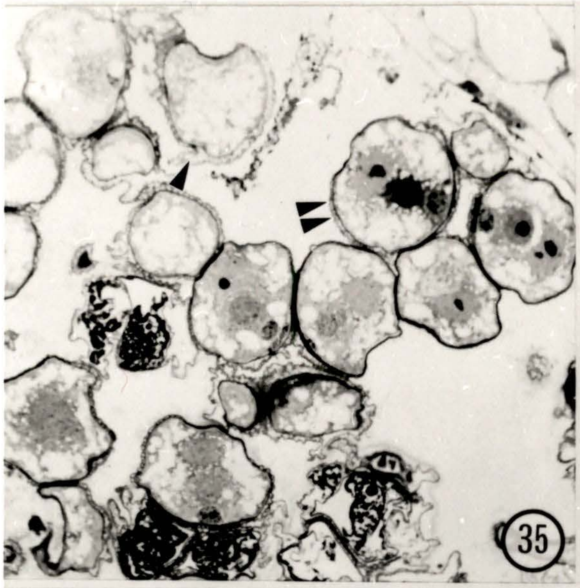
Figs. 35-39.	Abnormal pollen development.
Fig. 35.	Light micrograph of fused pollen with sacci which are poorly developed (arrow) or absent (double arrow). x265.
Fig. 36.	Light micrograph of pollen whose bacula have not separated from the foot-layer at the sacci (arrow). x270.
Fig. 37.	SEM of pollen with normal (*) and abnormal sacci. x255.
Fig. 38.	Light micrograph of section through 4-cell pollen. Tube nucleus (tn) lays adjacent to antheridial cell nucleus (an) instead of below it due to abnormal plane of cell division. x10,000.
Fig. 39.	TEM of abnormal Ubisch bodies (u) illustrating the slit-like electron transparent core. x10,000.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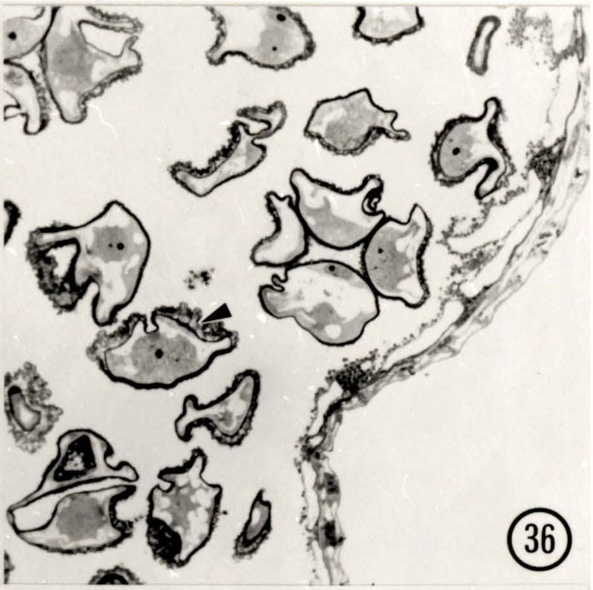
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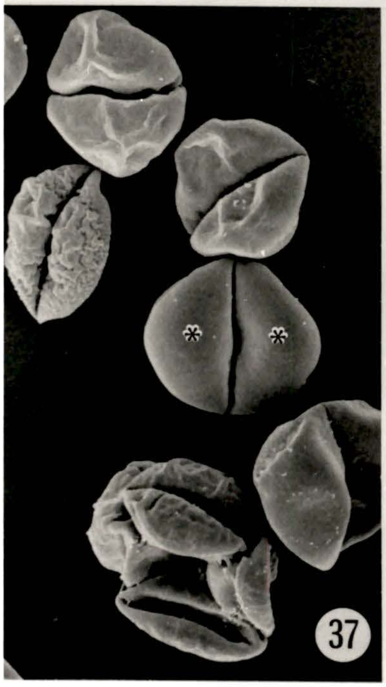

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Fig. 40. Average number of days each stage of pollen development was present on ramets of clones 3, 52 and 1675 in control (▨) and forced (■) treatments.

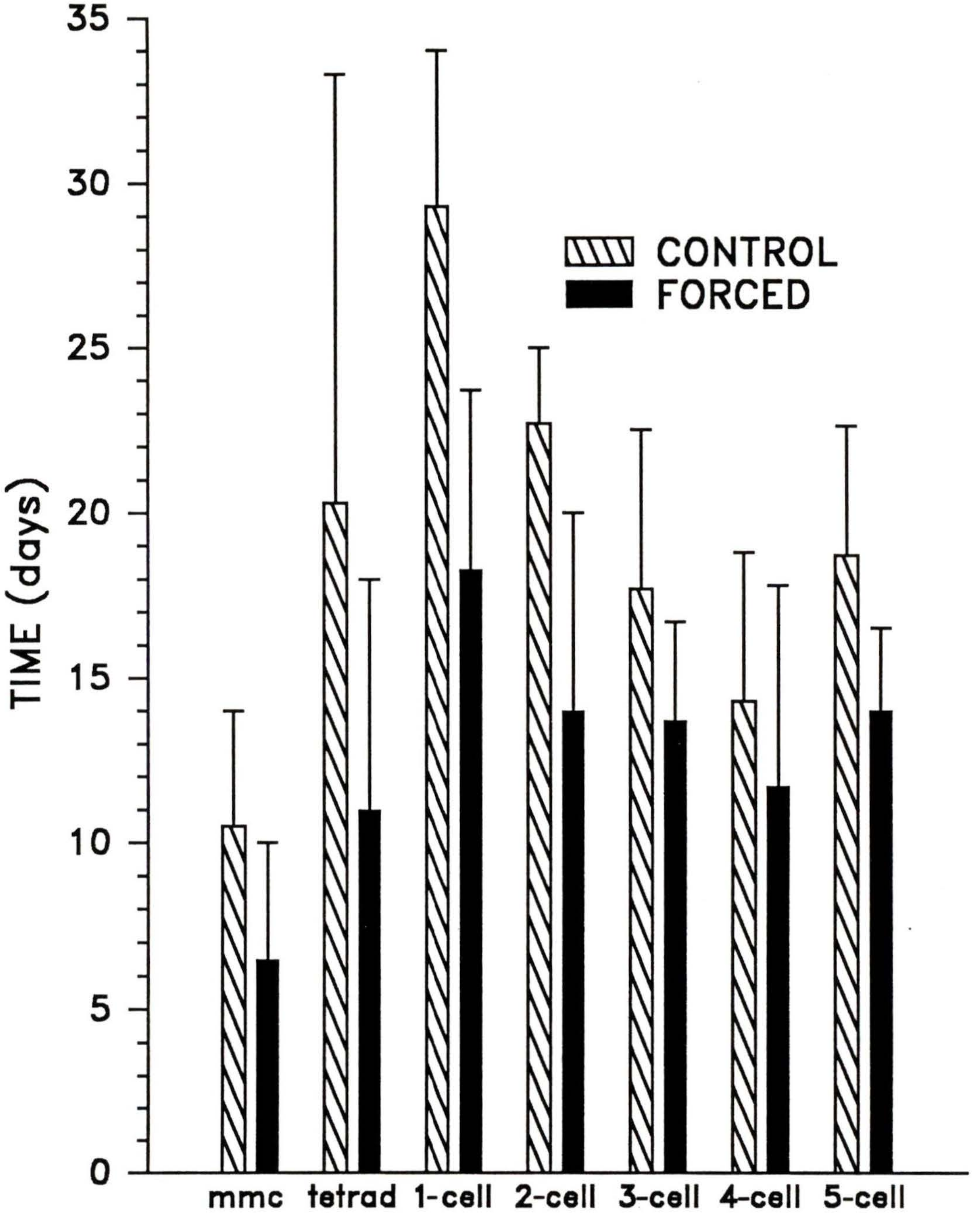

35
30
25
20
15
10
5
0
TIME (days)
CONTROL
FORCED
mmc
tetrad
1-cell
2-cell
3-cell
4-cell
5-cell
STAGE OF POLLEN DEVELOPMENT

Fig. 41. Average number of days each stage of pollen cone was present on ramets of clones 3, 52, and 1675 in control (▨) and forced (■) treatments. Stage 2 = pollen-cone bud swelling; stage 3 = pollen-cone bud bursting; stage 4 = pollen cone 25-50% emerged from bud scales; stage 5 = pollen cone 50-75% emerged from bud scales; stage 6=pollen cone fully emerged from bud scales; stage 7 = elongation of cone axis; stage 8 = dehiscence.

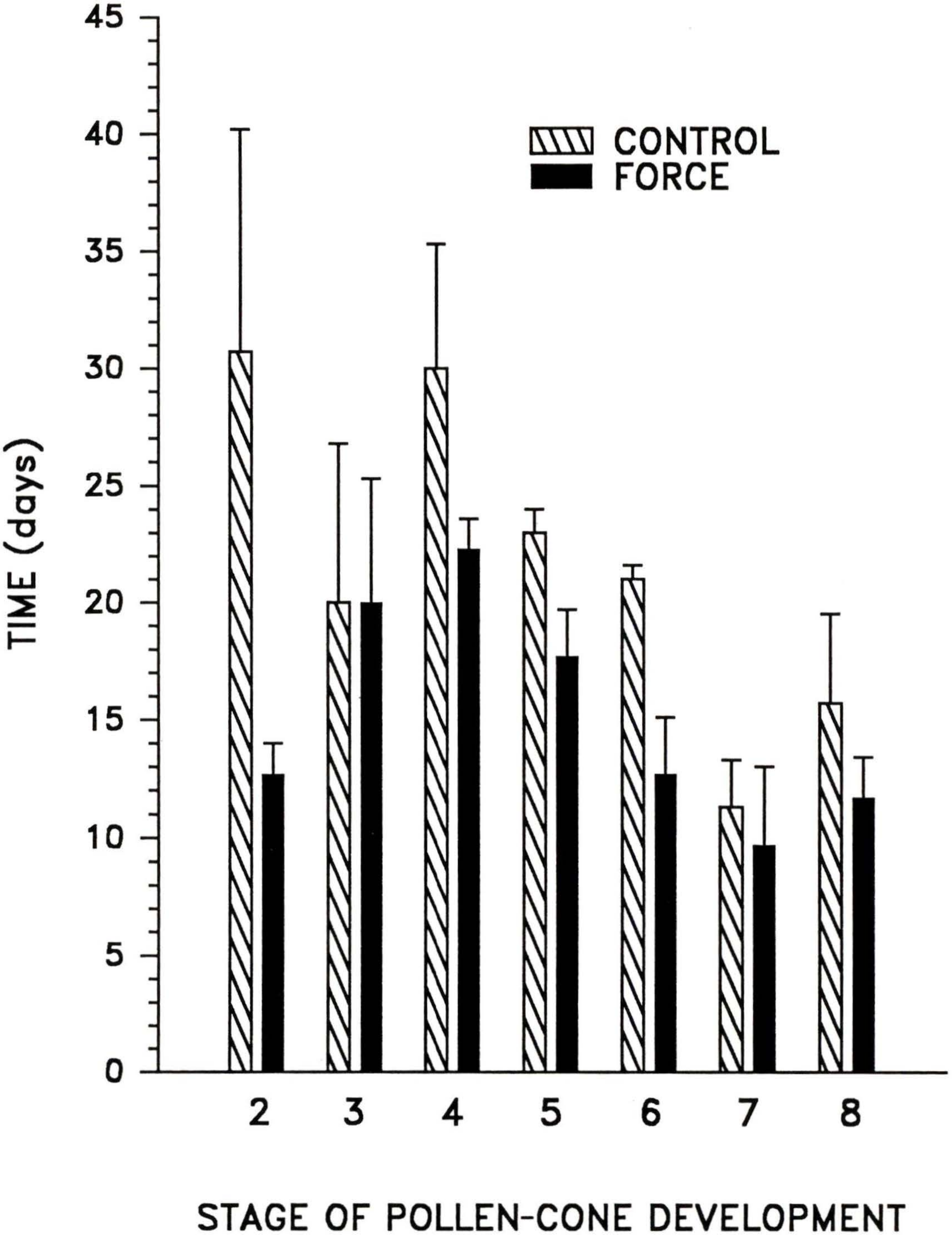
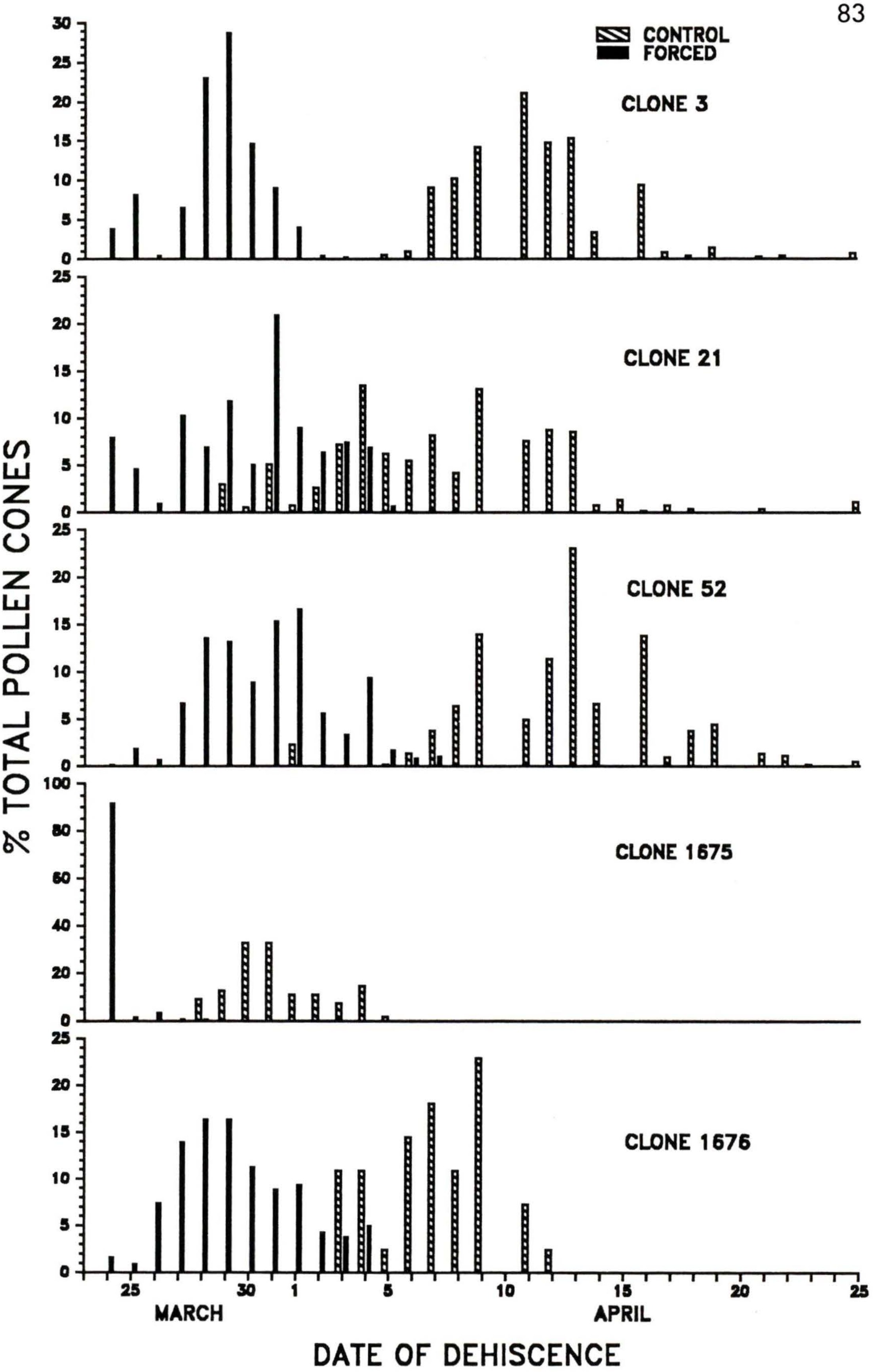

45
40
35
30
25
20
15
10
5
0
TIME (days)
CONTROL
FORCE
2
3
4
5
6
7
8
STAGE OF POLLEN-CONE DEVELOPMENT

Fig. 42. Dates of dehiscence of pollen cones of each clone in control (▨) and forced (■) treatments.


▨ CONTROL
■ FORCED
CLONE 3
CLONE 21
CLONE 52
CLONE 1675
CLONE 1676
% TOTAL POLLEN CONES
0
5
10
15
20
25
30
40
60
80
100
25
30
1
5
10
15
20
25
MARCH
APRIL
DATE OF DEHISCENCE

Figs. 43-47. TEMs of pollen in the early stages of hydration *in vitro*.

Figs. 43-44. 1.5 hours in germination medium.

Fig. 43. Tube (tc) and body (bc) cell cytoplasm. Plastids (pl) often have one or more double-membraned inclusions (dmi). Cell wall (w); lipids (l); mitochondria, (mt); vacuoles, (v); ER, (er). x18,265.

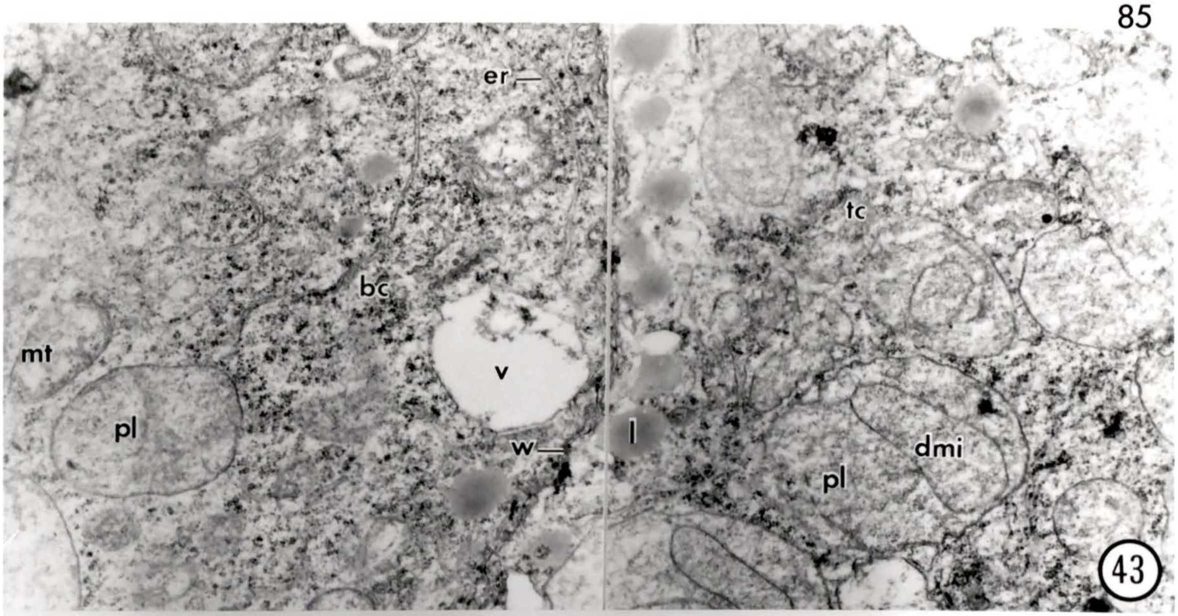
Fig. 44. Leptolemma region of hydrated pollen grain. Plastids are absent. Polysomes (arrows) and small vesicles (ve) are abundant. Intine, (i); nexine, (ne); ectexine, (ec). x14,190.

Figs. 45-47. 3 hours in germination medium.

Fig. 45. Longitudinal section through leptolemmal portion of pollen grain showing loosely woven cellulosic fibres (arrow) between intine and plasmalemma, and directionality of vacuoles (v). x5,213.

Fig. 46. Starch accumulation (sg) in plastids of the tube cell. Note electron-dense globules (gl) in vacuoles of tube cell. Stalk cell, (sc). x6,410.

Fig. 47. Electron dense globules and loosely woven cellulosic fibres are present between intine and plasmalemma (arrow). Lamellae of the nexine have separated. Saccus, (s). x19,219.



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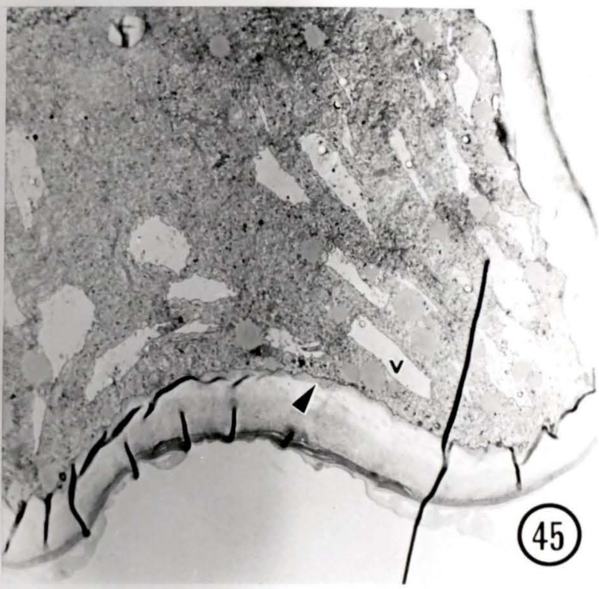

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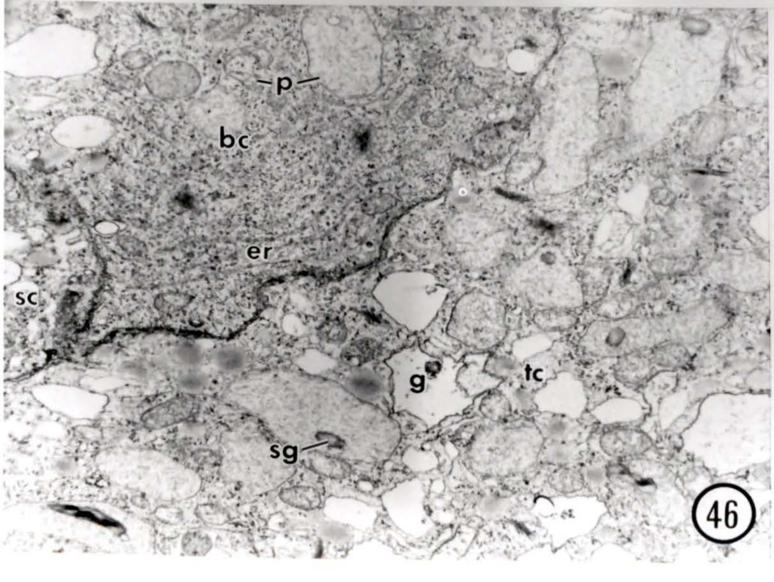

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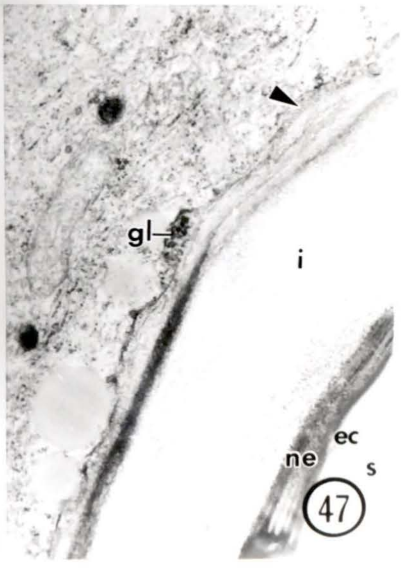

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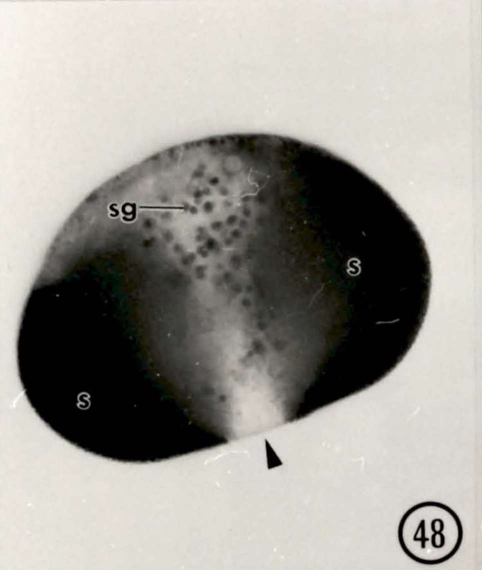
Figs. 48-49.	Pollen in early stages of germination after 12 hours in germination medium.
Fig. 48.	Light micrograph of whole grain stained with IKI. Sacci, (s); leptolemma (arrow); starch, (sg). x587.
Fig. 49.	TEM of longitudinal section through leptolemma region showing broken nexine (ne) and ectexine (ec), and emerging pollen tube (arrow). x12,440.
Figs. 50-53.	24 hours in germination medium.
Fig. 50.	TEM of longitudinal section through leptolemma region of hydrated grain which did not germinate. Note distinct layer (arrow) between intine (i) and plasma-lemma (pl). x12,470.
Fig. 51.	Light micrograph of whole germinated pollen stained with IKI. Starch is absent at the tip of the pollen tube. x275.
Fig. 52.	TEM of a longitudinal section through the apex of the pollen tube. Large starch grains, lipids (l) and mitochondria (mt) are located proximal to an extensive dome of RER (er). Pollen tube wall, (w). x8,150.
Fig. 53.	TEM of portion of pollen tube proximal to apex showing cytoplasmic detail. Microtubules (arrows). x14,628.



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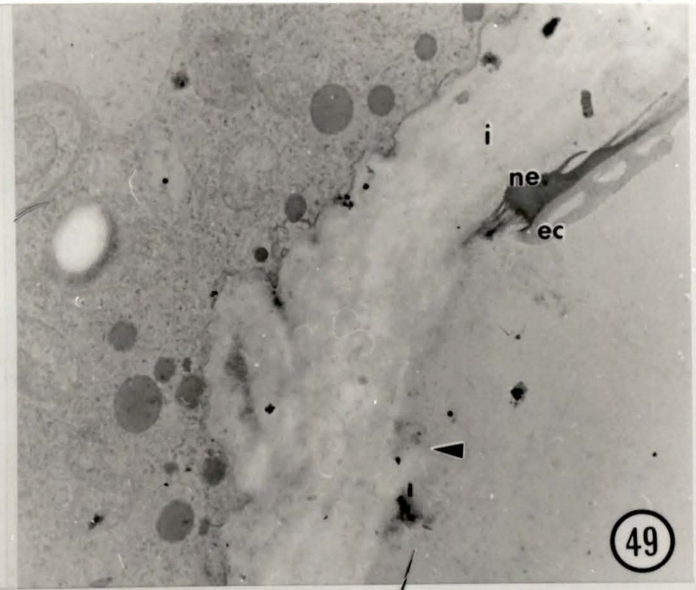

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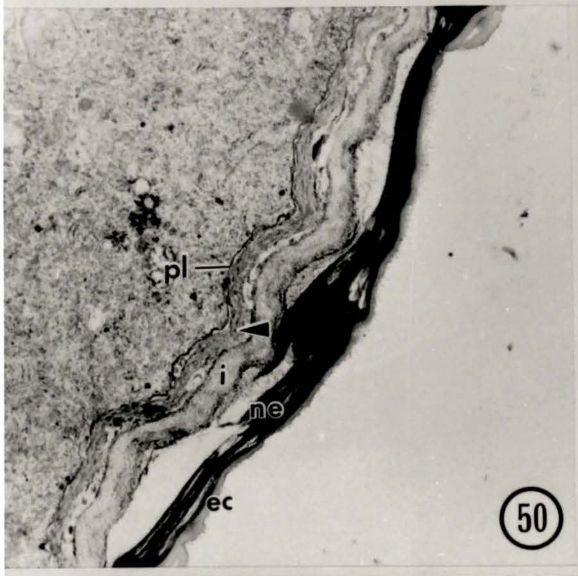

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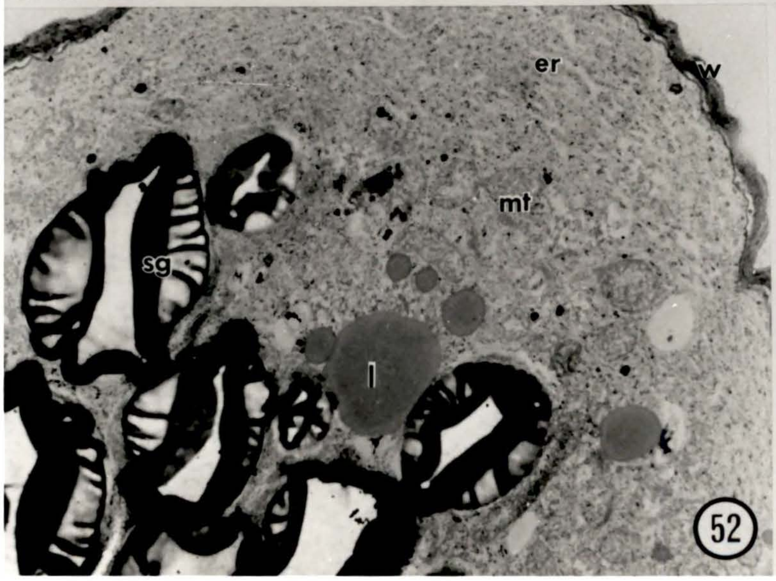

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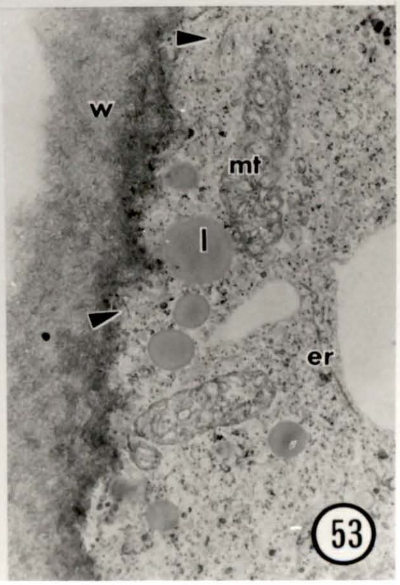

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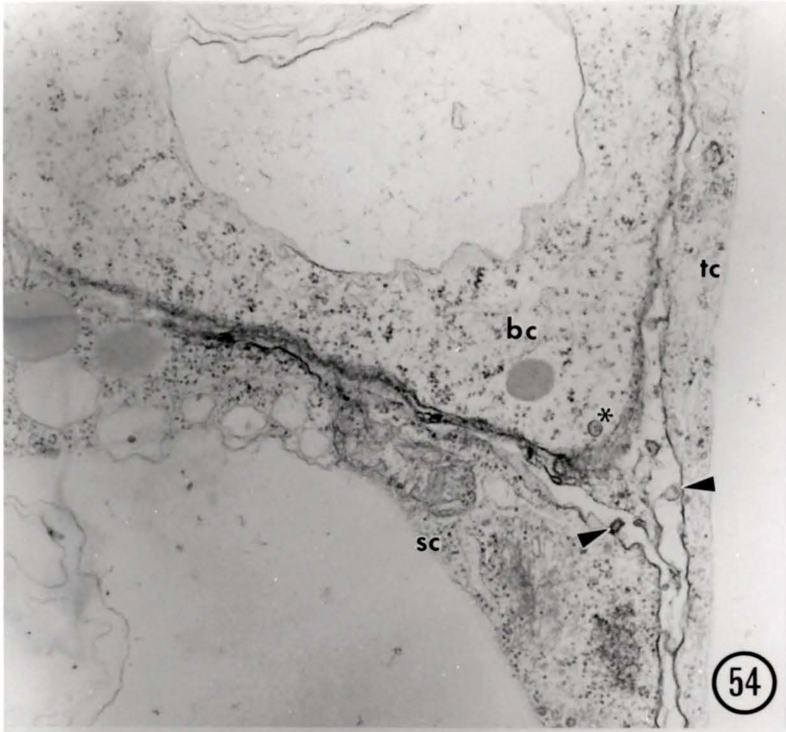
Figs. 54-55.	24 hours in germination medium. TEMs.
Fig. 54.	Portions of stalk (sc), body (bc), and tube (tc) cells. Note lack of typical cell walls and presence of inclusions (arrows) between the cells and within the cytoplasm of the body cell (*). x25,750.
Fig. 55.	Plasmadesmata (arrow) between tube and body cells. x25,750.
Figs. 56-58.	3 days in germination medium. Light micrographs.
Fig. 56.	Whole pollen tube stained with IKI. The highest concentration of starch (arrow) lies proximal to the starch-free apical dome. x238.
Fig. 57.	Whole pollen tubes stained with acetocarmine. Tube nucleus (tn) lies proximal to the area of highest starch concentration seen in Fig. 56. x240.
Fig. 58.	Longitudinal section through pollen tube. Stalk and body cells remain within the corpus of the pollen and contain relatively little starch. x280.



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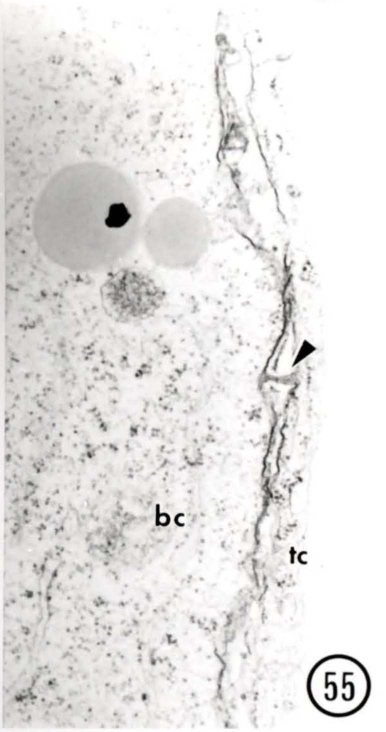

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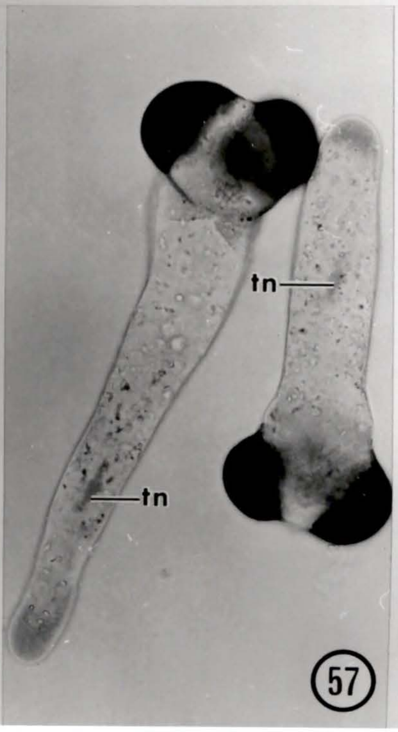

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Figs. 59-64. *In vivo* pollen germination and pollen tube growth.

Fig. 59. TEM of the leptolemma of the pollen grain two days after pollination. Overlapping plates of the foot layer appear to be sliding apart (arrows). Nexine has split and separated. Intine, (i). x17,500.

Figs. 60-64. Pollen tube 1 week after pollination.

Fig. 60. TEM of the tip of the pollen tube (pt) becomes irregularly shaped as it penetrates into the nucellus. Nucellar cells (nc) degenerate in advance of the pollen tube. x8,500.

Fig. 61. Light micrograph of a longitudinal section through a pollen tube as it penetrates the nucellus. Stalk (sc) and body (bc) cells are still attached to the pollen grain. The tube nucleus (tn) is in the corpus. x340.

Fig. 62. TEM of portion of tube (tc) and body cells. Tube cell plastids contain large starch grains (sg) and have electron dense stroma. x10,000.

Fig. 63. TEM of portion of stalk, body and tube cells. Plastids of stalk and body cells often include cytoplasm (*). Body cell contains very few, small starch grains (arrow). x10,000.

Fig. 64. TEM of the proximal portion of stalk cell. Fibrillar material (fm) is present between stalk cell and intine (i), and intine extensions are reduced (arrow). x20,000.


i
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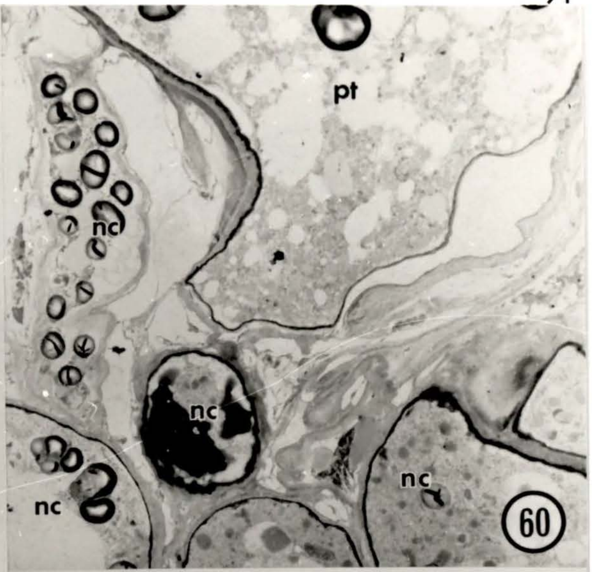
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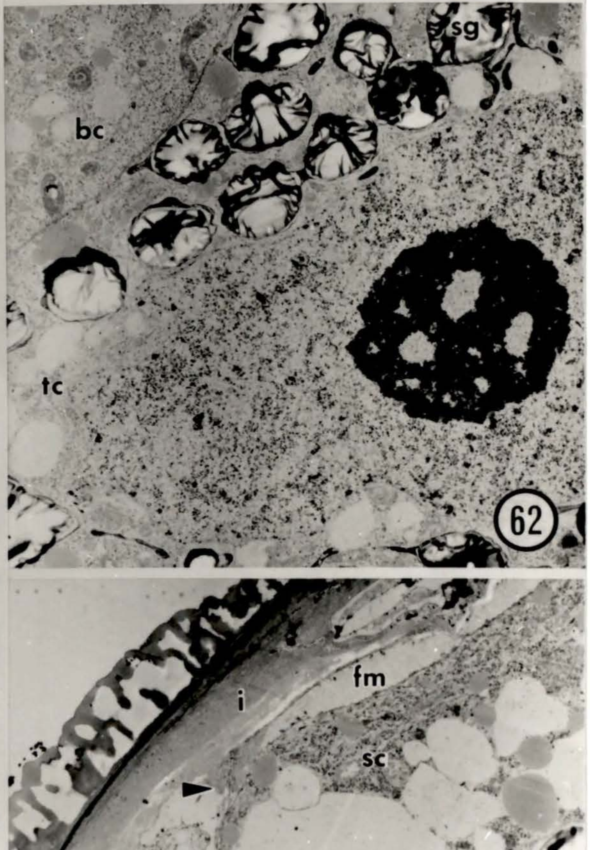
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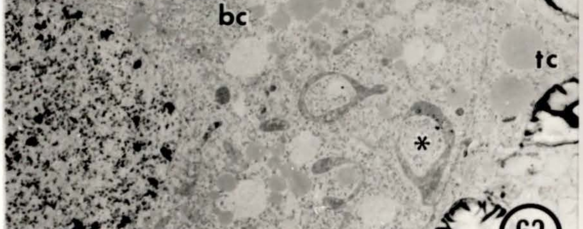
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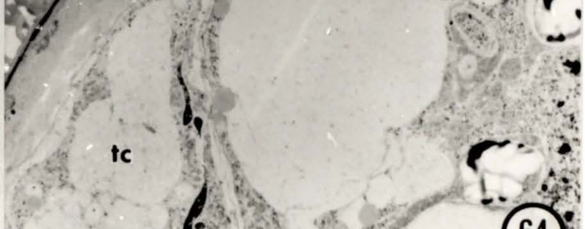
fm
i
sc
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64

Figs. 65-70. Pollen tube 3 weeks after pollination.

Fig. 65. Phase micrograph of a squash preparation of a pollen tube (pt) 3 weeks after pollination, demonstrating the branched nature of the tube tip (arrows). Sacci, (s); nucellar cells, (nc). x115.

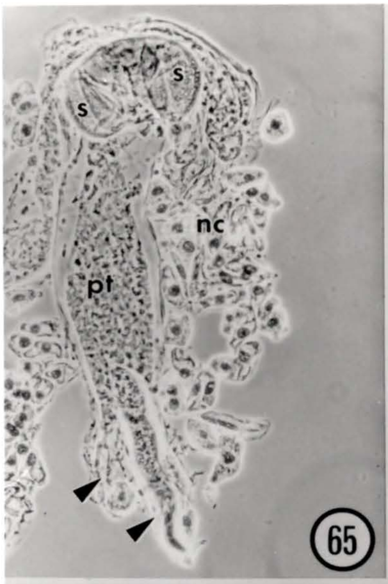
Fig. 66. TEM of pollen-tube tip and surrounding nucellar cells. x1,440.

Fig. 67. TEM of remains of the intine extension where the stalk cell separated (arrow). x20,000.

Fig. 68. TEM of portion of the tube-cell cytoplasm about 70 μm from the tube tip. Pollen tube wall, (w); degenerated nucellar cell, (nc); nucellar cell wall (ncw). x12,315.

Fig. 69. Detail of tube-cell cytoplasm about 35 μm from the tube tip. Small tubule-like projections (tu) are abundant and extend from plasmalemma to the pollen tube wall. x9,140.

Fig. 70. Detail of boxed area in Fig. 66 about 20 μm from tube tip. Tubule-like projections present (arrow) are not as abundant as in Fig. 69. x4,975.



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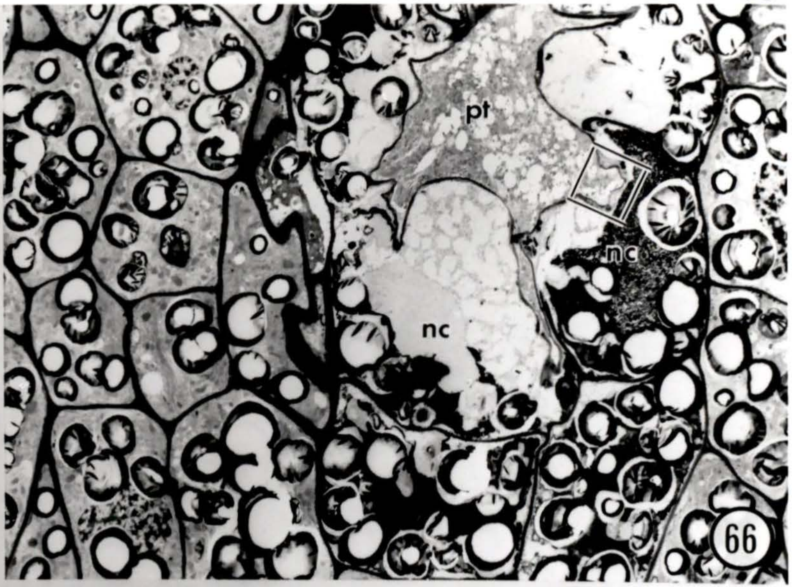

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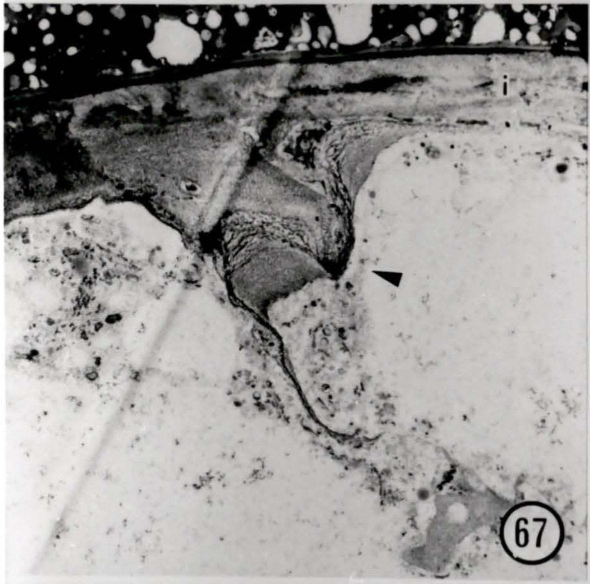

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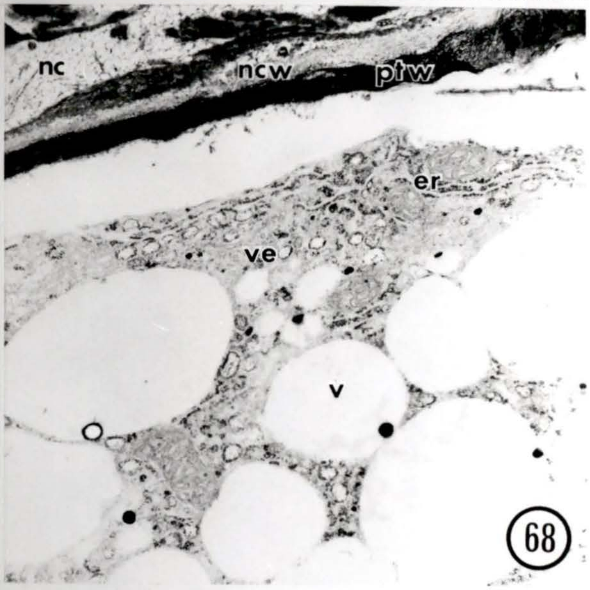

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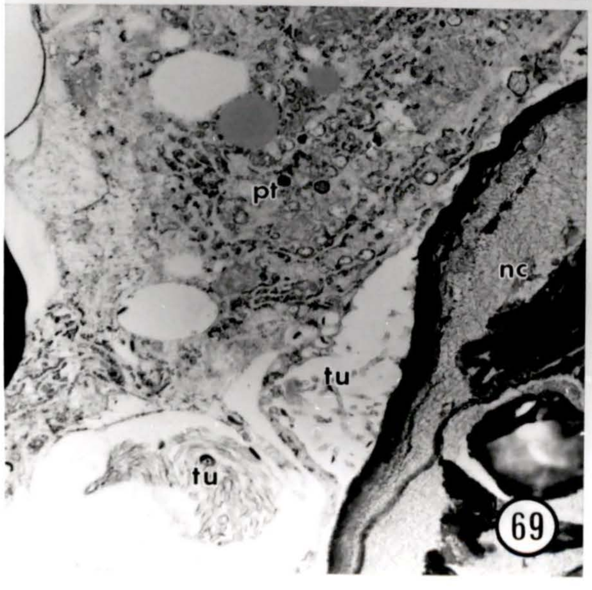

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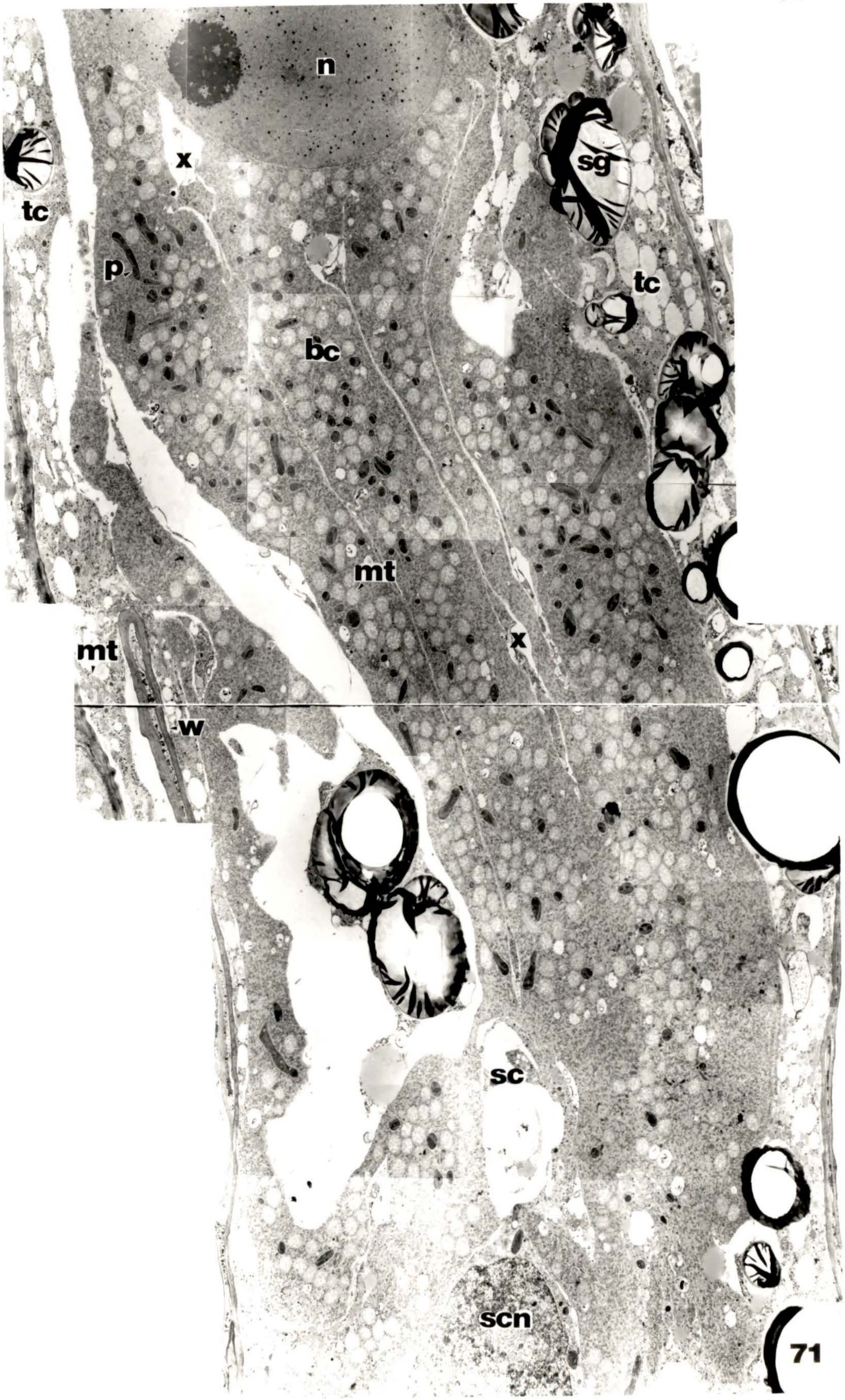
Fig. 71. TEM composite of a portion of the pollen tube within the nucellus 3 weeks after pollination, containing the body cell (bc). Nucleus (n) of body cell has not divided. Tube-cell (tc) cytoplasm on either side of the body cell contains amyloplasts with large starch grains (sg), RER, Golgi, lipids, elongate mitochondria (mt), and small plastids. Long, narrow strips of tube-cell cytoplasm (x) extend into the body cell. Body-cell plastids (p) contain no starch grains and mitochondria are round. Extensions of the body cell encompass the stalk cell (sc). Stalk cell nucleus, (scn); pollen tube wall (w). x2,590.


n
x
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sg
p
tc
bc
mt
mt
x
w
sc
scn
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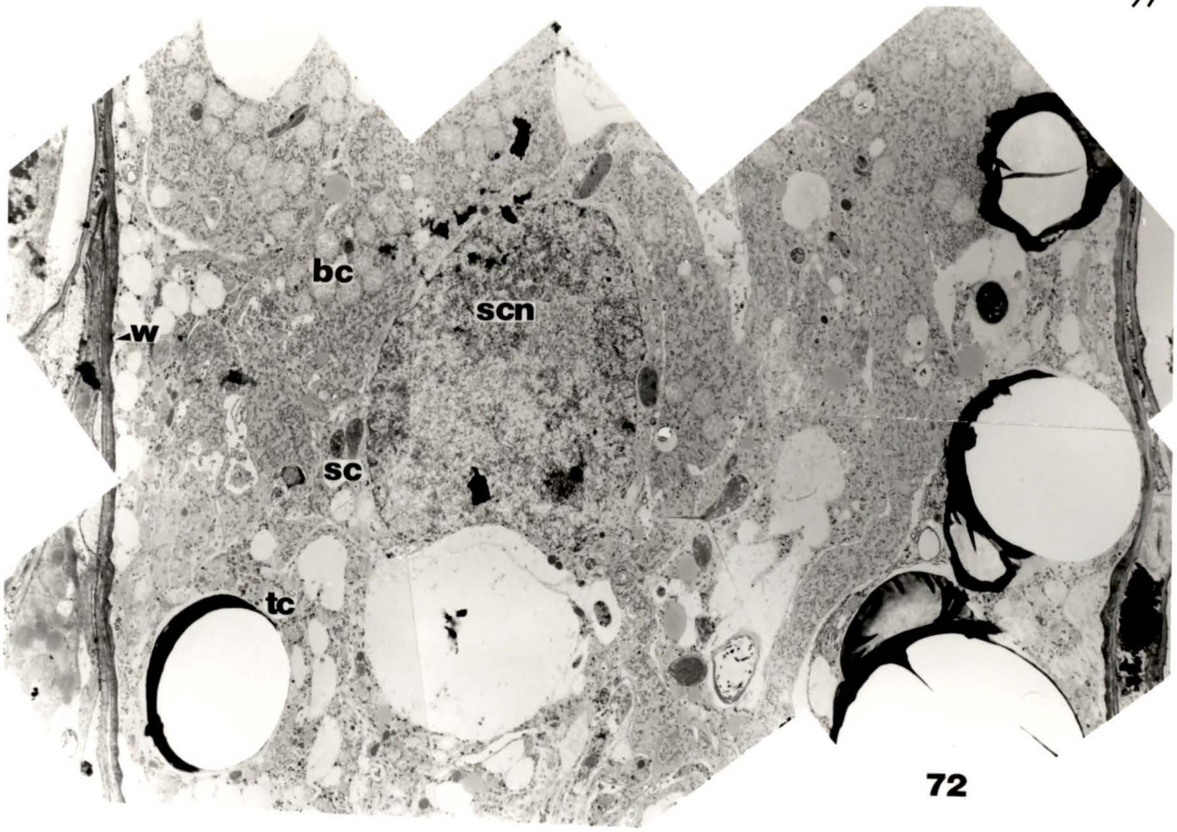
Fig. 72. TEM composite of a longitudinal section of the portion of a pollen tube at distal end of the body cell (bc), 3 weeks after pollination. The stalk cell (sc) is entwined in extensions of the body and tube (tc) cells. Pollen tube wall (w); stalk cell nucleus (scn); lipids (l). x3,700.



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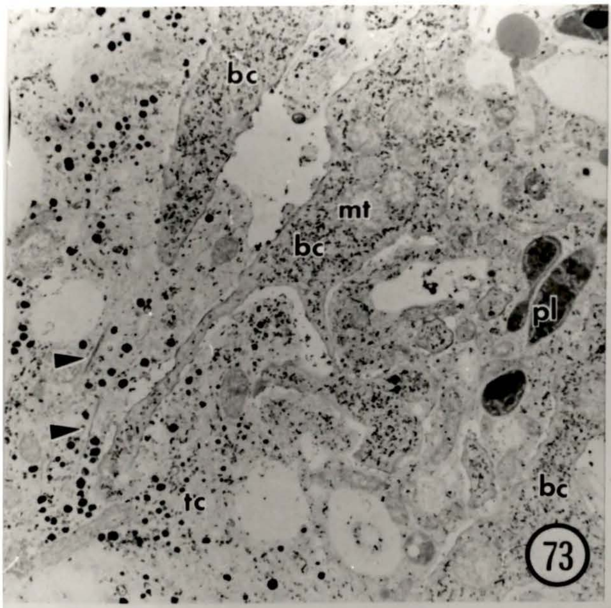
Figs. 73-75. TEMs of a portion of the pollen tube 3 weeks after pollination.

Fig. 73. Distal-most portion of body cell (bc) has finger-like projections in close proximity to microtubules (arrows) within the tube cell (tc). Mitochondria, (mt); plastids, (p). x5,330.

Fig. 74. Somewhat oblique section of pollen tube within the nucellus showing polarized cytoplasm of the body cell at mitosis. Between the chromosomes, which are in an adjacent section at the proximal pole (single arrow), and the more distal group of organelles (double arrows) is an area containing polysomes and microtubules (*). x1,400.

Fig. 75. Detail of * area in Figure 74. Microtubules (arrows) amid polysomes. x37,900.



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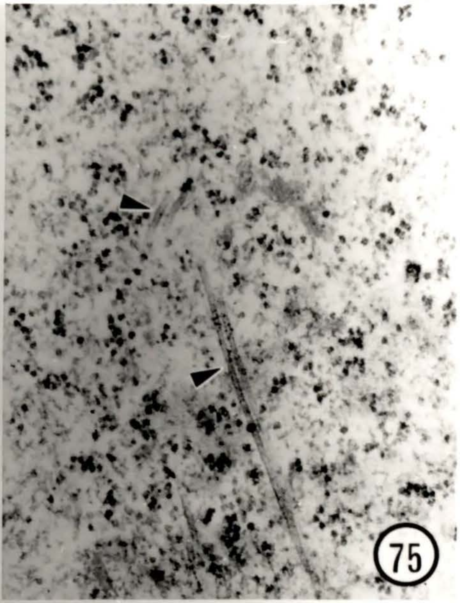

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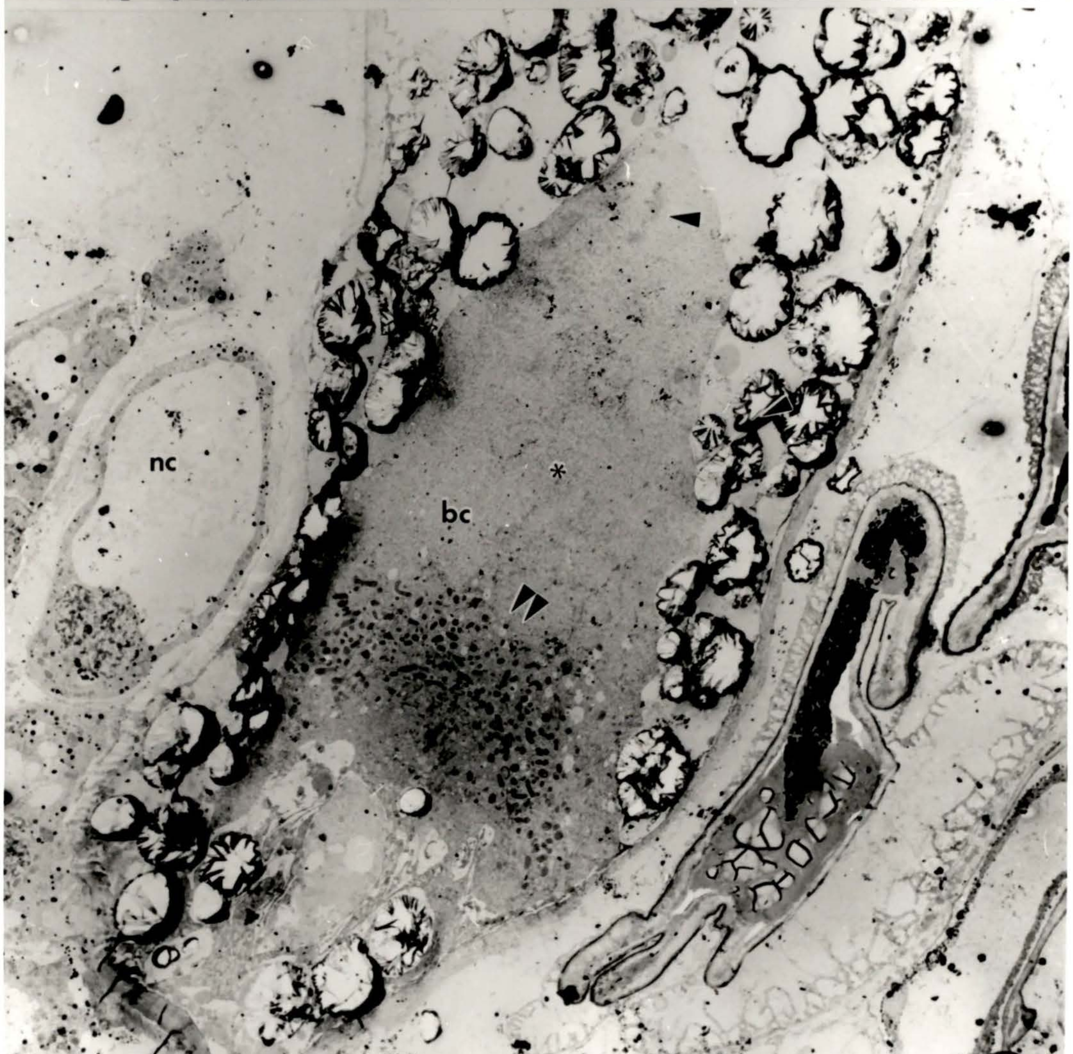

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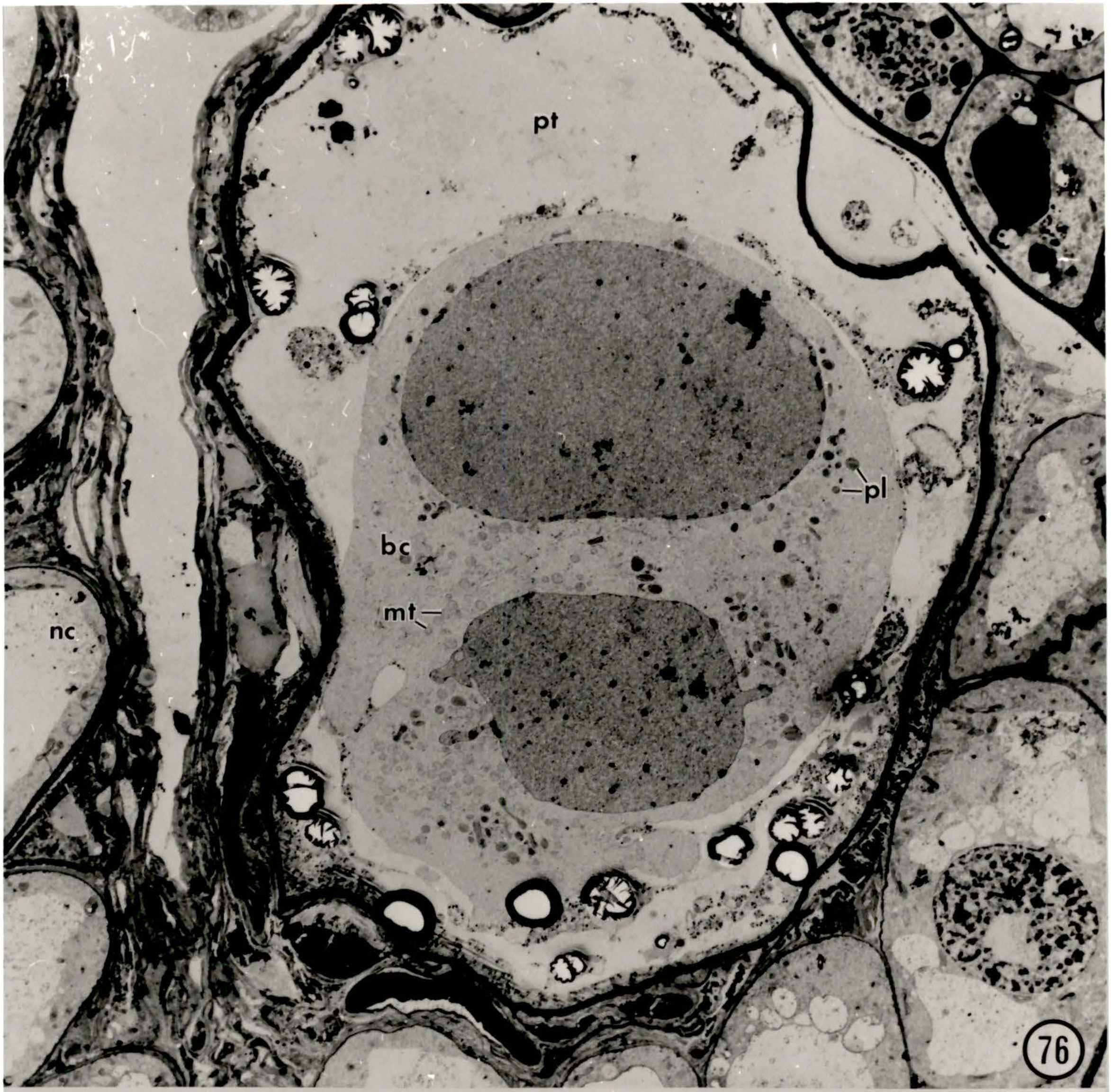
Figs. 76-78. TEMs of the body cell with 2 male nuclei 3 weeks after pollination.

Fig. 76. Oblique section of pollen tube containing body cell with 2 male nuclei, each surrounded by plastids (p) and mitochondria (mt). Nucellar cells, (nc). x1,730.

Fig. 77. Detail of nuclear lobes containing tubular structures (arrows). x10,540.

Fig. 78. Detail of incomplete cell plate (cp) separating the 2 nuclei. Also note tubular structures (arrows) around periphery of nuclei. x7,950.



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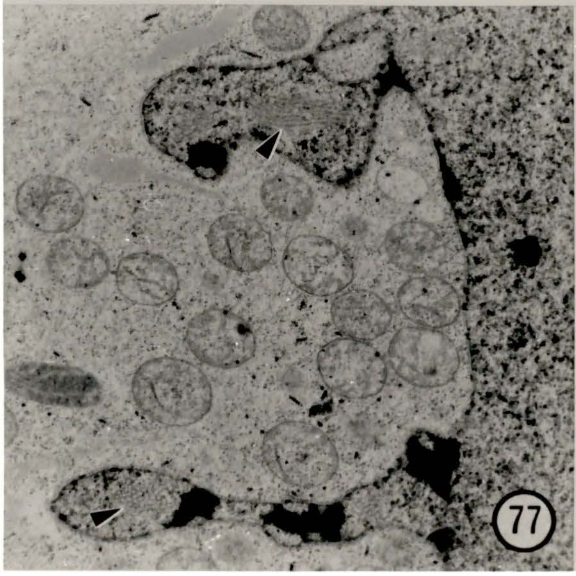

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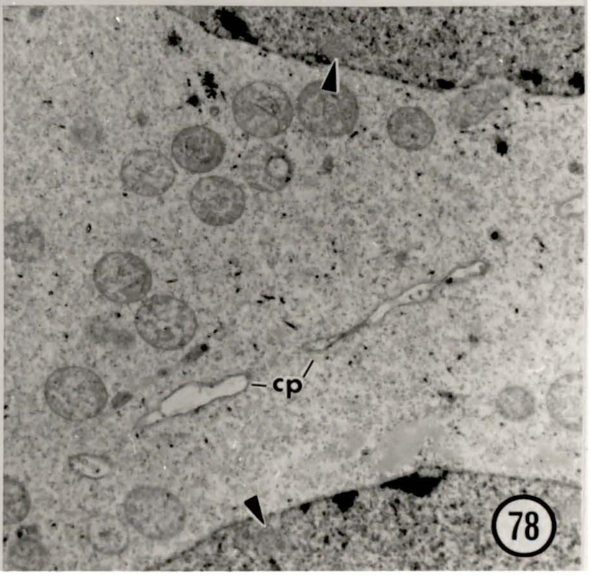

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Chapter 5

DISCUSSION

5.1. Pollen Development

5.1.1. Sporogenous Cells

When pollen cones of *Picea glauca* broke dormancy in the spring, they were at the sporogenous cell stage, undergoing several mitoses before differentiating into MMCs. Dormant pollen cones of *P. engelmannii* reportedly contain MMCs (Singh and Owens 1981a), however the description of these MMCs provided by these authors matches that of sporogenous cells in this study. Differentiation of sporogenous cells into MMCs is marked by the formation of the special wall (Audran 1981).

5.1.2. Tapetal Development

The earliest indication of tapetal differentiation was in mid-February at the end of sporogenous cell development, when plastids of tapetal cells contained more internal membrane structure than those of sporogenous cells and polysomes were present within the tapetal cytoplasm but absent in sporogenous cell cytoplasm. Otherwise tapetal and sporogenous cells were virtually identical. This suggests the tapetum of *P. glauca* is derived from the outermost layer of sporogenous cells as in *Pinus banksiana* (Dickinson and Bell 1976a) and *Taxus baccata* (Pennell and Bell 1985) rather than the innermost layer of parietal cells.

Like most gymnosperms, *Picea glauca* has a secretory tapetum. There is no evidence of cellular migration into the loculus as was reported for *Taxus* (Pennell and Bell 1986), *Pinus banksiana* (Dickinson and Bell 1972,1976b) and *Larix* (Mikulska *et al.* 1969; Said and Dumas 1988). As in *P. sylvestris* (Walles and Rowley

1982), there was an increase in tapetal cell basophilia just before MMC meiosis in *Picea glauca*, probably indicating an increase in the number of free ribosomes and subsequent protein synthesis. However, unlike other species in the Pinaceae [*L. decidua* (Milkulska *et al.* 1969), *Pinus banksiana* (Dickinson and Bell 1976a), *P. sylvestris* (Walles and Rowley 1982), and *Pseudotsuga menziesii* (Singh *et al.* 1983)] where digestion of tapetal cell walls occurred prior to meiosis, only a portion of the radial walls appeared affected at that stage in *Picea glauca*, with most wall digestion occurring in the tetrad stage. The tapetal cell walls towards the loculus were not completely digested until the microspores began to separate.

Stages of tapetal cell development can be related to stages of pollen development. While tapetal cells were secreting flocculent-fibrillar PAS-positive material into the loculus and extra-cytoplasmic cellular areas (Fig.7), thick PAS-positive fibrous MMC cell walls were forming. This secretory stage continued through to callose wall formation in the MMCs. Similar material was described in vesicles and channel-like dilations of ER in tapetal cells of *Pinus sylvestris* at the pachytene stage of MMC development (Rowley and Walles 1985a,b, 1988). In *Allium*, no polysaccharide was found in the MMC stage of development, but was found at the tetrad stage (Gori 1982). This polysaccharide originated in dictyosomes and vesicles of the tapetum, and persisted until after the tetrads were freed. However, a high percent of the free microspores were found to degenerate and Gori suggested that this accumulation of polysaccharides resulted from anomalous tapetal metabolism and that normally free simple sugars should have been present instead of polysaccharides. In *Picea glauca* the presence of polysaccharides within the loculus appears to be normal. However, when there were many degenerating microspores within a loculus the locular contents appeared fibrous rather than flocculent, perhaps a result of anomalous tapetal metabolism.

Once all callose walls had formed after meiosis II, a second form of secretory product was seen in the loculus. These were the shiny globules which did not stain positive for lipid, starch or polysaccharides. These globules appeared to be large vesicles which were released intact from the tapetal cells. They were apparently secreted by the tapetum and migrated towards the tetrads, lodging on the remnants of the MMC wall surrounding the callose wall. These do not resemble pro-orbicules, since the latter are lipidic, and these globules did not stain positive for lipids. These are electron-lucent but for a few fibrous strands (Fig.12).

Mempham and Lane (1969) found that the plasmodial tapetum of *Tradescantia bracteata* degraded the callose of the tetrad at the end of meiosis, and that the tapetum was capable of degrading callose for up to 48 hours after release of the microspores. They found that prior to callose degradation, electron-lucent vesicles were present in the tapetal cytoplasm and proposed that these contained the enzymes responsible for callose degradation. It is possible that the globules seen in the present study were vesicles responsible for the breakdown of the callose wall since they appeared after the tetrads had formed and their appearance was coincident with a decrease in the aniline blue-induced fluorescence of the callose wall surrounding the tetrad.

In *Pinus banksiana* two types of vesicles were found in the tapetum at the tetrad stage: large vesicles containing fibro-granular material, which were dispersed intact into the loculus, and small coated vesicles whose membranes fused with the plasmalemma and whose contents were released from the protoplast (Dickinson and Bell 1976b). The large vesicles resemble those found in the present study, however the ultimate fate of these vesicles was not described. The authors proposed that both types contain precursors for sporopollenin, but did not discuss the possibility that they may be involved in callose degradation.

Co-incident with the appearance of the shiny globules in the loculus was a decrease in the density of the PAS-positive fibrillar-flocculent material. This may be related to the degradation of the callose wall. Callose allows the passage of certain molecules such as glucose and acetate, but limits the passage of amino acids (Southworth 1971) and nucleosides (Albertini *et al.* 1987). Thus if the globules did contain a β-1,3-glucanase, and affected the integrity of the callose wall, this might allow passage of polysaccharides which were present within the loculus, or conversely, release of enzymes from the microspores and subsequent decrease of the polysaccharides. When there was a large proportion of abnormally developing tetrads, the shiny globules were often absent and fibrous material was present within the loculus. If in fact these globules are involved in the breakdown of the callose wall, a lack of them would result in failure of the microspores to separate. Also, the ability of the microspores contained within the tetrad to absorb nutrients from the loculus could be affected, resulting in degeneration of the microspores and a build-up of nutrients within the loculus.

Rowley and Walles (1988) reported that MMCs in *P. sylvestris* at diplotene take up globules "that appear to be lipoidal", and fibrillar material. However this is the stage when the callose wall is formed around the MMC, and it is unlikely that such large molecules would pass through this wall.

The final stage of tapetal development includes the degeneration of tapetal cells and the formation of Ubisch bodies. Ubisch-body formation began at the time when microspores were released from the tetrad, and continued throughout pollen development. A proposed function of Ubisch bodies is to facilitate the release of pollen at anthesis by decreasing the humidity in the microsporangium, another is the transfer of sporopollenin from the tapetum to the exine (Albertini *et al.* 1987), although there has been debate whether the latter actually occurs (Echlin 1971). It is more likely that the tapetum secretes sporopollenin precursors, some of which are incorporated into the pollen exine, others form the Ubisch bodies (Heslop-Harrison 1972; Dickinson and Bell 1976b). In the present study, few Ubisch bodies were seen apparently fusing with the exine of the pollen, while most remained at the periphery of the loculus associated with the membranous remnants of the tapetal cells. These observations support the idea that the Ubisch bodies may be involved with pollen release, but do not lend strong support to the theory that Ubisch bodies have a prominent role in exine formation, at least in *Picea glauca*. Abnormal Ubisch bodies were formed in microsporangia containing a high proportion of abnormal pollen (Fig.36). At this stage it seems unlikely these would have an effect on pollen development, but they may have affected dissemination of the pollen. However, the presence of abnormal Ubisch bodies may indicate that any sporopollenin precursors produced by the tapetum were also affected. This might affect viability of even apparently normal pollen within the loculus.

5.1.3. Pollen Development

Cyclical dedifferentiation in organelles has been discussed in relation to sporophytic-gametophytic transition in Angiosperms (Heslop-Harrison 1972; Dickinson 1981). Similar dedifferentiation and redifferentiation occurs in *P. glauca*, although the timing of these events differs. Sporogenous cells differentiated into MMCs when the fibrous polysaccharide cell wall was laid down and the cells rounded and separated. Accompanying this was a decrease in ribosome density, as indicated by a decrease in staining intensity of the cytoplasm, and the presence of large pleiomorphic plastids, including some amyloplasts. Amyloplasts were present through pachytene, whereas in *Taxus baccata* starch was present in early leptotene but was gone by pachytene (Pennell and Bell 1985).

In pachytene the MMCs were round but they became irregularly shaped as meiosis progressed, at first becoming crenate, then angular and finally elongate just before the first meiotic division. Before the second meiotic division the nuclei migrated to each pole of the elongate cells and the cells became 4-lobed. Changes in cell shape were also reported in *Picea pungens* (Fechner 1973). These authors used fresh squash preparations and the shapes of the cells were similar but less angular suggesting an effect of fixation on cell shape in the present study. This might indicate the cytoskeleton and the cell walls of meiotic cells were more plastic and susceptible to shrinkage or distortion than at later stages of development.

The pachytene cytoplasm was intensely staining, indicating an increase in ribosome density (Dickinson 1981), and there was an increase in the number of amyloplasts, indicating differentiation of plastids. This was followed by a decrease in starch content just before the first meiotic division. Decrease in starch at this time is common in angiosperms (Bird *et al.* 1983) and has been reported in *T. baccata* (Pennell and Bell 1985).

Singh (1978) reported that the first phase of callose wall formation occurred during the diplotene stage of meiosis, when callose walls formed around the MMC. In *P. glauca* the peripheral callose wall formed after telophase I. The intratetrad callose walls were formed simultaneously after meiosis II, as in most conifers.

Like *Pinus banksiana* (Dickinson 1971) and *P. sylvestris* (Willemse 1971d), PAS-positive fibrillar material was deposited between the callose walls and the plasmalemma, particularly in the areas where the wings would form. This polysaccharide material was obvious through to the late tetrad stage and probably provided precursors for the ectexine and subsequently, the bacula of the wings. This material appeared similar to that produced by the tapetum and secreted into the loculus, however that tapetal product was abundant at a slightly earlier stage and had started to disappear by the late tetrad stage of development, being replaced by the shiny globules.

Saccus development began while microspores were still contained within the tetrad. This was also seen in a previous study of *Picea glauca* (Owens and Molder 1979a), but in *P. sitchensis*, saccus development began after separation of microspores and before microspore mitosis (Owens and Molder 1980a).

Separation of the microspores appeared to result from the physical force applied by saccus expansion after the outer callose wall lost its integrity. The intratetrad callose wall did not loose its aniline blue-induced fluorescence until the microspores separated, supporting the argument that the shiny globules within the loculus contained callases which could not reach the callose while it remained between the microspores.

Once the microspores were released, they appeared to take up fluid quickly, forming large vacuoles. The convoluted appearance of the sacci of the young microspores is probably an artifact of fixation, suggesting a pliable exine at this time, an indication that sporopollenin has not reached its mature state. The chemical constitution of sporopollenin is not known, although it has been suggested to be a polymer of carotenoids or carotenoid esters (Brooks and Shaw 1968). More recently phenols have also been implicated in sporopollenin formation (Osthoff and Wiermann 1987; Herminghaus *et al.* 1988), and waxes and fatty acids have been found to be exine components (Niester *et al.* 1987). It is possible that the latter are produced by the tapetum and deposited on the exine during pollen development.

Pollen of *P. glauca* was shed at the 5-cell stage, as seen in *P. asperata*, whereas *P. sitchensis* pollen is shed at the 4- (Pettitt 1985) or 5-cell stage (Owens and Molder 1980a). Mature pollen of *P. glauca* was similar to that of *Abies* (Lepouse 1969). The lack of typical cell walls between the cells of the pollen grain is similar to that found in *P. asperata* where the plasmalemmae of the cells are separated by a space containing very low levels of polysaccharides (Camefort 1978). Similar atypical walls are seen in *Biota orientalis* (Chesnoy 1975). Cells of dry pollen were similar ultrastructurally. Unlike *Larix* (Goc and Gorska-Brylass 1986) very little starch was found in any of the cells of dry *P. glauca* pollen. Lipid appeared to be the main storage product.

5.1.4. Pollen Forcing

Most studies of temperature effects on pollen development have concentrated on meiotic disturbances (Eriksson 1968; Eriksson *et al.* 1970; Chira 1974; Luomajoki 1977; Andersson 1980). Chira (1974) found that forcing small cut branches of *Pinus cembroides* and *Picea abies* during MMC development and meiosis increased chromosome numbers, resulting in 2n and 4n pollen as well as the normal haploid pollen. Higher ploidy pollen tended to be larger than haploid pollen and to have more than two wings, 3 occurring on 2n pollen and 4 on 4n pollen. Also forcing pollen caused differences in metabolic products, particularly amino acids. Eriksson (1968) found that many abnormalities caused by low temperatures were induced during the active stages of meiosis, and were related to chromosomal irregularities (stickiness, fragmentation) and irregularities in cell division caused by spindle abnormalities. Ho and Owens (1974a) found similar abnormalities in *Tsuga heterophylla* and attributed these to low temperatures during development. The present study used sectioned material, thus stickiness and fragmentation could not be seen if present. However, if spindle abnormalities resulted from problems in microtubule development, phragmoplast and subsequent cell wall formation could also be affected, perhaps explaining the phenomenon of incomplete cell walls seen in Figure 27.

Those instances where exine did form between the microspores, but the tecta were fused (Figs. 28,29) are not as easily explained. "Siamese pollen" was reported at a rate of 1/1000 in *P. sitchensis* (Ho and Sziklai 1972). Ho and Owens (1974) found twin pollen in *T. heterophylla* which they suggested may have resulted from formation of exine before cytokinesis. However, in normal pollen wall development the initial walls formed between the nuclei of a tetrad are callosic. These form a template for subsequent exine formation (Waterkeyn and Bienfait 1970). The exine is deposited inside the callose wall by golgi vesicles and ER (Heslop-Harrisson 1972; Audran 1979). In this study various problems were related to pollen wall formation. The cytoplasmic continuity between cells may be best explained by incomplete cell plate formation, but fusion of exines of adjacent microspores of a tetrad cannot. One possible explanation for such development is that a callose wall was not deposited at all and the phragmoplast vesicles contained exine precursors. This seems plausible, since the bacula of such exines are poorly developed, and their development is thought to be determined by the callose wall.

Abnormal pollen development was found in both forced and control trees, although the proportion was higher in forced trees. Ho and Owens (1974b) studied development in coastal and interior varieties of *Pinus contorta* growing in Victoria, B.C., and found a higher percent of abnormalities in chromosome behaviour, cell wall formation and pollen grains of the interior over the coastal variety. They suggested these could have resulted from low temperatures during meiosis. However since the percent of abnormalities was higher in the interior trees growing in Victoria, it seems more likely that it was a high temperature rather than a low temperature effect. This could also be the case in the present study, since these trees are native to the interior of B.C. which has much lower temperatures in February, the time when meiosis occurred in Victoria. Perhaps growing these trees under ambient temperatures in Victoria is equivalent to a mild forcing treatment.

5.1.4.1. Effects of Forcing on Pollen Physiology

The effect of forcing on carbohydrate levels within the cones is difficult to interpret. It has been found that when stored pollen has lower sucrose content than fresh pollen it also has lower viability (Johri and Vasil 1961). In this study, overall control pollen cones containing 4-cell pollen had higher glucose, sucrose and starch than forced, and those with 5-cell pollen had higher sucrose. These analyses were done on the whole pollen cone, not only on the pollen so all other tissue within the cone could have affected the results.

Ross (1988) found a high proportion of *P. glauca* pollen cones forced in a polyhouse rotted, and suggested humidity may have also affected these cones. The cones chosen for the present study did not include rotted or rotting cones, but it is possible some may have rotted if left to develop on the trees. Ross did not indicate which stage was most susceptible to rotting.

As in other trees subjected to forcing treatments, the rate of pollen development was enhanced in *P. glauca*, and the date of pollen shed was advanced by 1 week to 10 days. However forced pollen generally gave lower seed set than control, and the quality of the resulting seed was not analysed. Forcing in this manner could supply pollen for crosses not otherwise possible, providing seed quality is adequate, however it would not be recommended on a large scale.

5.2. Pollen Hydration, Germination and Tube Growth

In the inaperturate pollen of *P. glauca* the exine at the leptolemma where the pollen tube generally emerged differed from that found over the surface of the rest of the pollen grain. It consisted only of endexine: the footlayer and the nexine.

Under natural conditions, pollen sifts into the cone, is drawn into the micropylar canal by a sugary pollination drop and germinates in the pollen chamber, a cup-shaped depression of the nucellus (Owens *et al.* 1987). The pollination drop probably initiates hydration of the pollen grain.

Upon hydration, the cytoplasm of the tube cell swelled, causing the wings of the pollen grain to separate, exposing the leptolemma. At the leptolemma of *P. glauca*, the foot layer consisted of overlapping shingles which slid apart upon hydration. This was followed by separation of the sporopollenin sheets of the nexine revealing fibrillar material. This fibrillar material may have already been present between the layers of the nexine. Pettitt (1985) found only nexine at the leptolemma. Adjacent nexine units fused at regular intervals to give an infrastructure in *Abies* (Pettitt 1985), also seen here in *P. glauca*. The plasmalemma of the tube cell, which, after 1.5 hours of hydration had been sinuate with deep intrusions into the cytoplasm (Fig.45) became more or less flattened into the intine as swelling of the pollen grain occurred (Fig.48).

Upon hydration of *P. glauca* pollen, only one layer of intine was seen. Electron-dense globules and new cellulosic wall material were non-uniformly distributed between the intine and the plasmalemma. Once germination began, a separate layer was not seen, and the tube wall appeared continuous with the intine. Pettitt (1985) found the intine beneath the nexine at the leptolemma exhibited stratification with 2 distinct layers in *P. sitchensis, Pinus, Abies* and *Cedrus*. In *Lilium*, the pollen tube wall is a distinct layer arising inside and separate from the intine (Miki-Hirosige and Nakamura 1982), whereas in *Crocus*, the tube wall is continuous with the intine (Heslop-Harrison 1979). Back from the tip of the elongated pollen tube of *Lilium*, a callose wall is formed inside the cellulosic tube wall. Callose was not found in *Picea glauca* pollen tube walls, nor has it been reported in pollen tube walls of other conifer species.

Between the plasmalemma and the intine small electron-dense globules and loosely woven cellulosic fibers accumulated. Pettitt (1985) found that acid phosphatase, esterase, protease and amylase were released from the leptolemma during hydration *in vitro*. The present study supports Pettitt's results. Pollen germinated on 1% agar PGM + 0.5% soluble starch (no sugars) contained considerably less starch than pollen grown on media containing sugars. However it did survive and elongate, indicating the ability of *P. glauca* pollen to utilize starch as an energy source. No growth occurred on medium deficient in both starch and sugars. Pollen germinated on 1% gelatin, a soluble protein, then stained with Coomassie blue showed loss of protein from the media indicating a release of protease from the pollen tube (Pettitt 1985).

The rate of pollen germination may be related to the state of the ribosomes in the tube-cell cytoplasm. Free ribosome density of hydrated pollen of *P. glauca* was lower than in dry pollen, and there was an increase in the number of polysomes. Pollen germination of *P. glauca* takes 12 hours or more, compared to minutes in most angiosperms (*Tradescantia* germinates within 10 minutes (Mascarenhas and Bell 1969)). This may be related in part to the lack of polysomes in mature pollen of *P. glauca*, and thus the probable delay in protein synthesis. In some angiosperms there is a decrease in ribosome density associated with increased numbers of polysomes and the onset of protein synthesis (Heslop-Harrison 1987). Hoekstra and Bruinsma (1979) found that pollen with many polysomes in the non-germinated condition (ie. *Tradescantia*) germinated much faster than those with few polysomes (ie. *Typha*). In *Tradescantia*, the mature pollen grain contains all the ribosomes required during pollen-tube growth, of which 38% are in the form of polysomes. During germination, more polysomes are formed. Protein synthesis begins immediately upon imbibition (Mascarenhas and Bell 1969) and continuous protein synthesis throughout tube growth is required for production of vesicles and apical growth of pollen tubes (Picton and Steer 1983). In contrast to *Tradescantia*, the Compositae have few polysomes and low protein synthesis upon hydration, but rapid germination and fertilization, suggesting that protein synthesis is complete before dehiscence (Hoekstra and Bruinsma 1979).

The ultrastructure of the cells of dry pollen was very similar. Starch accumulated in stalk, body and tube cells of pollen germinated *in vitro* within 24 hours of placing the pollen in the medium. This is similar to what Dexheimer (1970) found in *Pinus* pollen germinated *in vitro*. However, he found that in angiosperm pollen germinated *in vitro* the generative cell contained no starch and appeared metabolically inactive as compared to the tube cell. In *Picea glauca*, the body cell of pollen germinated within the ovule contained some amyloplasts but the starch grains were considerably smaller than the large starch grains found in amyloplasts of stalk and tube cells. The body cell cytoplasm was less dense than that of the stalk cell. This may indicate that metabolism of pollen germinated *in vitro* varies from that *in vivo*.

5.2.1. Pollen Tube Zonation

Cytoplasmic zonation in pollen tubes has been reported in angiosperms (Rosen *et al.* 1964; Picton and Steer 1981) and gymnosperms (Pettitt 1985; Pennell and Bell 1986). The growing tip of pollen tubes *in vitro* contain primarily vesicles. In conifers two types of vesicles were reported: small ones with an electron-dense granular phase and larger ones containing dispersed fibrillar material (Pettitt 1985). This area is also reported to contain high RNA, protein and carbohydrate concentrations (Rosen *et al.* 1964). Proximal to this are found mitochondria and

ER, followed by an area with many dictyosomes, golgi vesicles and amyloplasts (Pettitt 1985). In *P. glauca*, pollen tubes grown *in vitro* displayed different zonation. The apical dome contained a high RNA concentration corresponding to extensive network of RER. RER was also abundant in the tips of pollen tubes growing *in vivo*. The other zones were very similar to those described in the literature.

5.2.2. Pollen Tube Growth

Penetration of *P. glauca* pollen tubes into the nucellus is similar to that seen in *Pinus sylvestris* (Willemse and Linskens 1969). Pollen tubes of both species push between degenerating nucellar cells. The shape change from narrow to irregular, rather like a penetration peg, seen in *Picea glauca* was not reported for *Pinus*. *Pseudotsuga menziesii* pollen tubes form as narrow protuberances about one quarter the diameter of the elongated pollen, and are unbranched (Owens and Morris 1990). The tubes of *Pinus* (Willemse and Linskens 1969) and *Picea glauca* have small branches. Willemse and Linskens suggest the tubes implant themselves into the nucellus in this manner. The tips of *Picea* tubes which had penetrated 75% of the nucellus were extremely irregularly shaped, forming projections which appeared to penetrate between degenerating nucellar cells. It is possible that this is the mechanism whereby small peripheral branches originate, and they may have an absorptive role rather than acting as anchors for the pollen tube.

At the distal end of the pollen tubes, small tubule-like projections of the plasmalemma extended into the pollen tube wall. Similar structures have been described in pollen tubes of other species (*Taxus* (Pennell and Bell 1986), *Nicotiana* (Kandasamy *et al.* 1988), in barley scuttlum epithelial cells, primary root tips of germinating peas and the embryo sac of the pea embryo 5 days after fertilization (Harris and Chaffey 1986)). These structures have been termed plasmatubules.

They are found in cells which are involved in a high degree of solute transport (Harris and Chaffey 1986). Kandasamy *et al.* (1988) found they increased the surface area of the plasmalemma of *in vivo* grown pollen tubes 6 to 10 times, probably enhancing nutrient uptake, thus allowing for faster growth. Pollen tubes of *Nicotiana* grown *in vitro* had poorly developed plasmatubules and a slower growth rate. However, *Picea* pollen tubes grew much faster *in vitro* than *in vivo*, but plasmatubules have not been reported and were not seen in *in vitro* grown tubes in this study. Therefore it is much more likely that nutrients supplied *in vitro* are more easily assimilated by tubes without the presence of plasmatubules, or perhaps certain substances (ie. amino-acids, peptides, growth hormones etc.) lacking in the growth media are required to stimulate production of plasmatubules in the plasmalemma of tube cells.

5.2.3. Cytoplasmic Streaming and Cell Movement

Pollen tubes are very dynamic, with active cytoplasmic streaming and extensive migration of nuclei and cells. There has recently been considerable research into the role of microfilaments and microtubules in this movement. The use of specific fluorescent stains (rhodamine-phalloidin and anti-tubulin) in combination or not with cytochalasins and colchicine has indicated that microfilaments rather than microtubules are responsible for cytoplasmic streaming (Franke *et al.* 1972; Partharsarthy *et al.* 1985; Perdue and Partharsarthy 1985; Pierson *et al.* 1986; Heslop-Harrison and Heslop-Harrison 1987; Heslop-Harrison *et al.* 1988; Pierson 1988).

There has been some contradiction as to what is responsible for the movement of cells and nuclei within the pollen tube, but the most conclusive evidence was provided by Heslop-Harrison *et al.* (1988). They found that low concentrations of colchicine eliminated microtubules from the cytoplasm but did not affect pollen-tube shape or extension growth, nor did it stop the vegetative nucleus and generative cell from moving into and through the tube. However it did cause the normally spindle-shaped generative cell to round up. From this the authors concluded that the microtubule cytoskeleton of the vegetative (tube) cell was not responsible for movement of the vegetative nucleus or generative cell in the pollen tube, but that the form and shape changes of the generative cell are correlated with and probably dependent on the microtubule cytoskeleton. In the present study, preservation of microtubules was poor, but they were obvious in the distal extensions of the body cell. This is consistent with the hypothesis that microtubules are related to changes in cell shape.

In *P. glauca*, the separation of the stalk cell from the proximal pole of the pollen grain appeared to occur by dissolution of the intine cap. The plasmalemma of the stalk cell was thus intact as it moved together with the body cell into the tube. The space between the stalk and body cells no longer had any resemblance to a cell wall and the plasmadesmata seen in dry pollen were no longer visible. In the process of intine dissolution any wall material around the stalk or body cells may have also been dissolved.

The tube, stalk and body cells were very closely intertwined at the distal end of the body cell, with elongate portions of the body cell extending around the stalk cell. Similar extensions have also been reported in the body cell of *P. asperata* (Camefort 1978) and *P. abies* (Moshkovich 1988). Moshkovich (1988) reported that this contact remained until the pollen tube reached the female gametophyte and suggested that the stalk cell acts as a "dislocator", causing release of the two male nuclei from the body cell. In *P. glauca* the stalk cell is certainly involved in the initial movement of the body cell from the corpus into the pollen tube and may be a major factor of its movement down the pollen tube. As in *P. glauca*, the body cell of *Pseudotsuga menziesii* is deeply lobed by thin sheets of tube cell cytoplasm, proposed to be involved in pulling the body cell through the tube cell (Owens and Morris 1990). The lobing in the body cell of *P. menziesii* begins before that cell starts to move down the pollen tube. The authors do not mention the fate of the stalk cell.

5.2.4. Male Gametes

Division of the body cell nucleus to form the two male nuclei occurred while the body cell was in the proximal end of the pollen tube. The nucleus was located at the proximal pole of the body cell, away from the stalk cell, and remained there throughout division. Such polarity was also noted in the body cell of *Taxus cuspidata* (Sterling 1948) and *T. baccata* (Pennell and Bell 1986). During division the organelles were grouped in the distal end of the body cell. Between the dividing nucleus and the organelles was a high concentration of polysomes and microtubules. It is probable that this is part of the spindle of the dividing nucleus, and that the section was oblique and did not include the other half of the spindle. After nuclear division, an incomplete cell plate was apparent between the two male nuclei. A similar event was also reported in *T. baccata* (Pennell and Bell 1986), where there was the beginning of a cell plate, but no cell wall was ever formed.

The two male nuclei formed by division of the body cell nucleus were equal-sized, with projections extending into the cytoplasm. Lobing of generative and vegetative G_2 nuclei has been reported in *Hyacinthus* (Bednarska and Gorska-Brylass 1987). This coincided with the disappearance of extranucleolar ribonuclearprotein (RNP) structures and a pronounced decrease of RNA and protein synthesis. Lobing also resulted in increased nuclear surface area which the authors proposed might be connected with RNP transport to cytoplasm.

While the nuclear projections were present, microtubular-like structures were observed within the nucleoplasm just inside the nuclear membrane. Similar structures have been reported in MMC nuclei of wheat (Bennett *et al.* 1974) and in algae (Franke and Scheer 1974). In wheat MMC nuclei bundles of fibrillar material were found between the nuclear membrane and chromatin. Individual fibers measured about 0.02 μm, one third the size of those in *Picea glauca*. They were proposed to be involved in chromosome orientation for meiotic pairing. Those found in algae were associated with the inner nuclear membrane and were thought to be involved in nuclear shaping or in control of intranuclear movement processes (ie. chromosome movement). It is unclear what role these would have in male nuclei of *P. glauca*. Perhaps they are related to movement of the male nuclei through the egg cytoplasm just prior to fertilization, a stage not covered in this thesis.

Chapter 6

SUMMARY AND CONCLUSIONS

This thesis addresses several aspects of pollen biology of *Picea glauca*. It includes a detailed study of pollen and tapetal development from the breaking of dormancy to dehiscence of mature dry pollen. The effects of forcing on a) pollen and tapetal development, b) carbohydrate levels in the pollen cone throughout development, c) rate of pollen and pollen cone development, and d) seed efficiency are described. Pollen germination and pollen tube growth both *in vitro* and *in vivo* are described. The findings are summarized below.

1. *P. glauca* pollen cones overwintered at the sporogenous cell stage. The secretory tapetum differentiated from sporogenous cells after dormancy breaks in the spring, and developmental parallels existed between tapetal cells and MMCs.

2. Developmental anomalies were found in forced as well as control pollen. These included degeneration of microspores within the tetrad; abnormal pollen wall development resulting in a lack of microspore separation; abnormal saccus development; abnormal plane of cell division within the pollen. Secretory products of the tapetum were also affected, some of which may be involved in degradation of the tetrad callose wall. If so, nutrient absorption by the microspores within the tetrads would be affected and microspore degeneration could result. Many of the other abnormalities in pollen development may be attributed to problems in microtubule development. The abnormalities seen in the control pollen may occur because the February temperatures in Victoria are milder than in the natural range for *P. glauca*. Ideally this type of experiment should be carried out with controls

more closely approximating conditions in the natural range of the species.

3. Carbohydrate analysis of pollen cones containing 5-cell pollen showed lower sucrose levels in forced than control pollen cones. The overall seed efficiency resulting from pollination using forced pollen was also lower than control. If this relationship was tested and proved to be consistent, sucrose levels might be a useful tool for pollen testing.

4. Pollen hydration and germination was slower *in vivo* than *in vitro*. Early stages of pollen tube development were similar, although differences in starch accumulation of the body cell and zonation of the tube cell were soon apparent. This probably indicates different metabolic activities, perhaps related to nutrient availability and physical constrictions.

5. When the pollen tube reached the nucellus it formed a pentration peg. The branching of the pollen tubes and the plasmatubules at the tip of the pollen tube may play a role in nutrient absorption.

6. The stalk cell separates from the corpus of the pollen and moves together with the body cell into the pollen tube. It may be a controlling factor in movement of the body cell, but the mechanism is still unclear.

7. The body cell nucleus divided to form the two male gametes 3 weeks after pollination, while the body cell was in the proximal part of the pollen tube. During mitosis the organelles were clumped distal to the dividing nucleus. After mitosis the organelles were distributed around the nuclei and an incomplete cell plate was

apparent between the nuclei. Tubular structures were found within the male nuclei and may be related to nuclear movement.

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