SEXUAL REPRODUCTION OF INTERIOR SPRUCE (PINACEAE). I. POLLEN GERMINATION TO ARCHEGONIAL MATURATION

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Stages of normal sexual reproduction between pollen germination and egg maturation are described for interior spruce. These prezygotic stages were studied by light and electron microscopy in more detail than was possible in previous studies, and new observations have been made. Sperm (male gametes) formation and the organelles that accompany the sperm within the pollen tube are described. The pollen tube grows toward a lipid secretion that originates in the neck region of the archegonium. Megagametophyte development was followed from the early cellular stage through the series of mitoses that result in neck cells, the ventral canal cell, the central cell, and ultimately the egg. Maturation of the egg includes formation of modified plastids (formerly described as large inclusions) and small inclusions that are membrane-bound regions of cytoplasm. At egg maturation, modified plastids are excluded from the mitochondria-rich perinuclear zone that surrounds the nucleus. Small inclusions occupy the periphery of the cell so that the egg nucleus and perinuclear zone are centrally located. A second article in this series describes pollen tube entry into the archegonium, delivery of sperm to the egg, gamete fusion, and proembryo and embryo formation.

Keywords: development, Picea, spruce, sexual reproduction, pollen, megagametophyte, archegonium, conifer.

Introduction

White spruce (Picea glauca [Moench] Voss) and Engelmann spruce (Picea engelmannii Parry) hybridize freely at intermediate elevations (600–1500 m) in the interior of British Columbia and along the eastern slopes of the Coast Range mountains (Coates et al. 1994). Collectively, these species and their hybrids are known as interior spruce. In excess of 90 million interior spruce seedlings will be required annually for reforestation in British Columbia by the year 2000. More than half of these seedlings should be from genetically improved seed produced by repeated selection on parental growth form. As a component of a larger study to identify reasons for relatively low yields in seed production orchards, we have completed a more detailed description of development. Many observations have been made utilizing paraffin-embedded tissues. In this study, resin-embedded tissue was used for higher resolution light microscopy and ultrastructural investigation, allowing a more detailed description of development. Many observations are included here for the first time.

Reports describing sexual reproduction in conifers are numerous from the mid-nineteenth century onward. Reviews of early literature are available in more modern sources. Camefort (1968) acknowledges the pioneering work of Hofmeister, Strasburger, and others during the mid to late nineteenth century as the basis for our present understanding of gymnosperm reproduction. Maheshwari and Singh (1967) and Konar and Oberoi (1969) include comprehensive bibliographies of work published in the twentieth century. The most recent comprehensive text on gymnosperm embryology is by Singh (1978) and includes sporogenesis to seed maturation. Since Singh's book in 1978, much work has been done on sexual reproduction in conifers. A detailed account of pollination and fertilization that includes a review of the literature on cytoplasmic inheritance was provided by Chesson (1987). Pennell (1988) has reviewed sporogenesis. Cone production periodicity, reproductive cycles, floral induction, and all stages from pollination through seed development were reviewed by Owens (1991). Misra (1994) has reviewed zygotic embryogenesis and described biochemical and molecular changes within developing and germinating conifer seed.

Pollination occurs in Picea when, through interaction with the pollination drop, pollen float into the pollen chamber in the apical part of the nucellus within the ovule (Doyle 1945; Runions and Owens 1996). Pollen germinates after a variable amount of time within the ovule: within 2 d in white spruce (Dawkins and Owens 1993) or after as long as 2 wk in interior spruce (Owens and Molder 1984). Picea pollen tubes grow through the nucellus with no further delay after germination.

This article describes postpollination development within the ovule of interior spruce. Figure 1 summarizes the stages of pollen and ovule development covered between pollen germination and archegonial maturation. At maturation, the archegonium is ready to be fertilized. The second article in the series (Runions and Owens 1999, in this issue) describes de-
Fig. 1  Summary of developmental stages within the pollen and ovule of interior spruce during the period described in this article. Pollen germinates and pollen tubes containing body cells penetrate the nucellus (top). Mitosis of the body cell produces two sperm in each pollen tube while the archegonial initial divides to form the central cell and primary neck cell (middle). When archegonia are mature they are composed of a large egg cell, a smaller ventral canal cell and several tiers of neck cells (bottom).

Development between the time sperm are released from the pollen tube and early embryos form.

Material and Methods

Tree Selection and Pollination

Trees used in this study were established as grafts in a clonal seed orchard at the Kalamalka Seed Centre near Vernon, British Columbia, in 1982. In April 1992, two ramets from each of eight clones (QL orchard clones 1830, 1879, 4700, 4709, 4757, 4768, 4777, and 4835) were used in a survey of development during fertilization. Seed cones were collected from these trees, and ovules were embedded in paraffin. In April 1993, beginning 1 wk after the final pollination, ovules were collected and prepared for the developmental study. Every 2 d for 18 d, two seed cones were collected per treatment per tree. Median longitudinal sections <1 mm thick were cut from 10 ovules from the center region of each cone, fixed in phosphate buffered glutaraldehyde, postfixed in OsO₄, dehydrated, and embedded in Spurr’s resin. Fifty-five ovules were sectioned. Ovules were serially sectioned at 0.8 μm with a Reichert Ultracut E microtome and stained with 0.01% toluidine blue (CI 52040) at pH 11.0 for light microscopy (O’Brien and McCully 1981). Whole, living megagametophytes were stained with fluorescein diacetate and observed by confocal microscopy. Fluorescein diacetate was made by first dissolving 2.0 mg/mL in acetone and then mixing this stock solution in 0.05 M phosphate buffer at pH 5.8. For transmission electron microscopy, sections were cut at 0.06 μm, picked up on 300-mesh or 75-mesh formvar-coated copper grids, stained with uranyl acetate and lead citrate and examined with an Hitachi TEM. All of the developmental stages described occurred during the 8-d period from 13 to 20 d after the final pollination.

Results

Pollen Tube

Each tree had an abundant supply of developing pollen- and seed-cone buds. In 1992, seed cones were left open to natural pollination. In 1993, pollen-cone buds were removed, and isolation bags were placed over branches bearing seed-cone buds before the pollination period. Fresh pollen was collected from 12 clones that were not otherwise included in the study and mixed. Pollen was tested to ensure that it was between 5% and 10% moisture content before artificial pollination. Pollination on May 6 and 7 was accomplished by compressed air injecting 0.5 mL aliquots of pollen into each bag daily during the period when seed cones appeared maximally receptive. Once seed cones had become postreceptive, the paper bags were replaced with mesh bags to prevent insectivory of the developing ovules.

Anatomical Study of Fertilization Period

In 1992, the natural pollination period extended from April 18 to May 6. Postreceptive seed cones were collected from trees on May 12, 19, 27, and June 2 and placed into Navashin’s fixative (Sass 1958) for storage. Twenty ovules per clone per collection that had been embedded in paraffin were sectioned for a total survey of 640 ovules.

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Fig. 2  A–G, Pollen tube (nu = nucellus). A, Body cell division occurred shortly after pollen germination to form the sperm (male symbol i and male symbol ii). Cell plate appears to form across the phragmoplast (arrowhead). DIC. Bar = 50 μm. B, Pollen tube (pt) has grown through the nucellus. Sperm (*) differ in staining intensity. LM. Bar = 10 μm. C, Body cell cytoplasm surrounding one of the sperm (male symbol) contains plastids (p) and mitochondria (m). TEM. Bar = 2.5 μm. D–G, Details of the nucellus (nu). D, Cells in the central part of the apical nucellus that the pollen tube grows through are small and angular with thin primary cell walls. Plastids contain abundant starch. LM. Bar = 4 μm. E, Outer one to two layers of nucellar cells accumulate lipids (l) between plasmalemma and cell wall. Lipid deposits are greatest in the outer tangential walls. LM. Bar = 10 μm. F, Lipid (l) deposits in cells of the outer layer of the nucellus occur between plasmalemma (arrowhead) and the cell wall (cw). TEM. Bar = 0.5 μm. G, After the pollination period, cells in the central apical region of the nucellus (dark staining region) become metabolically more active than surrounding cells as indicated by intense staining with toluidine blue. This type of staining is not as intense in unpollinated ovules. LM. Bar = 200 μm. H, Inner one to three layers of nucellar cells surrounding the megagametophyte (mg) are tapetal. Tapetal (t) deterioration occurs as the archegonia mature within the megagametophyte. LM. Bar = 20 μm. I, Tapetal cells are secretory in nature and as they deteriorate, the ektexine of the megaspore wall (mw) is deposited around the megagametophyte. TEM. Bar = 2 μm.
sperm is densely packed with plastids and mitochondria (fig. 2C). Plastids are ovoid or elongate with a darkly staining stroma and a sparse thylakoid membrane system. Mitochon-
dria are spherical with well-defined inner and outer membranes and cristae. The stalk cell retains its thin cell wall as it migrates along with the sperm in the tube cell.

**Nucellus**

Cells in the central micropylar region of the nucellus are thin walled, have few vacuoles, and contain amyloplasts with abundant starch grains (fig. 2D). The peripheral one to three layers of nucellar cells in the micropylar region have an accumulation of lipids between their cell walls and plasmalem-
as. This accumulation, which stains positively for lipids with toluidine blue (Yeung 1990), is greatest inside the outer tangen-
tial cell walls but occurs, as well, in radial and inner tangen-
tial cell walls (fig. 2E). Lipid bodies vary in size and the smallest ones impregate the inner layer of the cell wall (fig. 2F). The site of lipid production is either within these nucellar cells or in the adjacent cells of the inner layer of the seed coat, which appears deteriorated, suggestive of secretion earlier in development.

Cytoplasm of nucellar cells in the central region stains heav-
ily with toluidine blue (fig. 2G). Staining is most intense in the region near a pollen tube and is light in unpollinated ovules.

**Megagametophyte**

At pollination, the megagametophyte is cellular. Prothallial cell walls are very thin, and the cells contain only a thin pe-
ripheral cytoplasm. Cells, thought to be tapetal, in the inner two to three layers of the nucellus surrounding the megaga-
metophyte, begin to breakdown as the megagametophyte be-
gins to enlarge (fig. 2H). Lipids liberated by this breakdown accumulate on the megaspore wall around the megagame-
rophyte (fig. 2I). The megaspore wall is thick and elaborated into short, outward surface projections between the pro-
clonal cells in the megagametophyte. The peripheral one to three layers of surrounding megagametophyte cells that are continuous with the archegonial jacket enlarge and appear metabolically active while nucellar cells in several layers de-
teriorate so that a depression, the archegonial chamber, forms above each neck. The megaspore wall remains intact between the nucellus and neck cells. At maturity, there are three to four tiers of four neck cells each so that the ventral canal cell and egg are deep within the megagametophyte tissue (fig. 3F). Ma-
ture neck cells have very thick, primary cell walls (fig. 3G). At maturation, the neck cells begin to deteriorate and to sep-
rate while surrounding cells remain intact. Deterioration of neck cells was observed in pollinated and unpollinated ovules. As deterioration proceeds, lipids accumulate in the archegonial chambers (figs. 3G, H). Cells surrounding the neck cells are secretory in appearance. They have undulating plasma mem-
branes and abundant dictyosomes. Deteriorating neck cells and the healthy appearing surrounding cells contain lipid bodies, and lipids were visible in the lamellar space between their cell walls (fig. 3I, J).

**Mature Archegonium**

Eggs become mature just before pollen tubes reach the neck (fig. 4A). Eggs are large, ca. 500 × 250 μm, and ovoid. The egg nucleus is spherical and ca. 70–80 μm in diameter. Nu-
cleoplasm is homogeneous and contains several nucleoli. By the time pollen tubes contact the megaspore wall, the egg nu-
cleus has migrated into a central position (fig. 4B). Egg cy-
toplasm increases in volume after central cell division, and most of the large vacuoles disappear. Cytoplasm of the mature egg has, at most, one to two small vacuoles.

Egg cytoplasm is generally described as containing large and small inclusions. Modified plastids (large inclusions) are the regions of cytoplasm surrounded by darkly staining mem-
branes (fig. 4C). Formation of modified plastids begins at cen-
tral cell division. All of the plastids in the cell become elongate and distorted and eventually enclose areas of cytoplasm (fig. 4C, D). No organelles are included in the modified plastids. Modified plastids are concentrated toward the chalazal end and periphery of the mature egg (fig. 4E). They become very robust with concentric systems of membranes that compart-
mentalize areas of cytoplasm (fig. 4F). Formation of modified plastids occurs in all cells of the megagametophyte except the prothallial cells (fig. 4G).

Small inclusions are more difficult to describe. They are the smaller, spherical or elongate regions of cytoplasm that are conspicuous because they are slightly more dense than sur-
rounding cytoplasm and contain no organelles (figs. 4F, H, I). They are bound by a single membrane (fig. 4H). Mitochondria
Fig. 3  

A, Mitosis of the archegonial initial produces a vacuolate central cell (cc) with a large nucleus (n) and a smaller primary neck cell. Mitosis of the primary neck cell gives rise to a single tier of neck cells (nc). LM. Bar = 25 \( \mu \)m.  

B, Before central cell division, the large prophase central cell nucleus remains at the micropylar pole of the cell isolating a small region of cytoplasm (arrowhead) that forms the ventral canal cell cytoplasm (v = vacuole). LM. Bar = 25 \( \mu \)m.  

C, Archegonial jacket cells (aj) are small and angular and completely surround the central cells and early eggs (ec). TEM. Bar = 5 \( \mu \)m.  

D–J, Neck cells (nc). D, Neck cells, and surrounding cells, become very metabolically active as they mature, as indicated by fluorescein diacetate staining. Confocal. Bar = 20 \( \mu \)m. E, Central cell division produces the egg (ec) and the smaller ventral canal cell (vc). Continued mitosis of the neck cells produces a second tier. Cells of the inner layer of the nucellus (nu) deteriorate above each neck forming a depression. LM. Bar = 20 \( \mu \)m. F, Mature archegonium has a neck composed of three to four tiers of cells. Proliferation of cells in the apical region of the megagametophyte (mg) surrounding each neck results in mature archegonia becoming embedded deeply in the megagametophyte. LM. Bar = 50 \( \mu \)m. G, Mature neck cells have thick primary cell walls and the middle lamellae sometimes deteriorates. Lipid deposits (l) accumulate in the depression between each neck and the nucellus before fertilization. LM. Bar = 25 \( \mu \)m. H, Lipid deposits occur in the depression between neck cells and the megaspore wall (mw). TEM. Bar = 1 \( \mu \)m. I, Neck cells deteriorate but adjacent cells of the megagametophyte, which may represent an extension of the archegonial jacket (aj), appear healthy and secretory with abundant lipid bodies, plastids, mitochondria, and dictyosomes. TEM. Bar = 2 \( \mu \)m. J, Lipids are abundant in the cell walls between neck cells and the adjacent cells of the megagametophyte. Lipids occur in the cell walls between most cells in the neck. TEM. Bar = 1 \( \mu \)m.
of the egg are excluded from small inclusions (fig. 4H). A large proportion of egg mitochondria are concentrated into many small groups around the egg nucleus (fig. 4I). Not all mitochondria move into this perinuclear zone; many are stranded at a distance from the nucleus as small inclusions enlarge and seal off the perinuclear cytoplasm (fig. 4J). Upon formation of the perinuclear zone, the egg nuclear membrane becomes convoluted (fig. 4K). At this point, the egg is mature and ready to be fertilized.

Discussion

Many stages of development in the ovule of interior spruce as described in this article were similar to those published by other investigators of *Picea* (Mergen et al. 1965; Owens and Molder 1979; Dawkins and Owens 1993 for white spruce; Singh and Owens 1981 for Engelmann spruce; and Owens and Molder 1984 for interior spruce). However, certain features were observed here for the first time or at higher resolution than in previous studies. The results reported in this article represent an overview of normal development from the time of pollen germination until archegonial maturation.

Pollens Tube

In interior spruce, pollen germination and pollen tube growth in the nucellus are coincident with archegonium development, and pollen tubes arrive at the megagametophyte as eggs become mature. Pollen germinates within 13 d after pollination. Body cell division occurs once the pollen tube has penetrated a short way into the nucellus. This was also observed in Engelmann spruce by Singh and Owens (1981) and unusual as, in conifers, sperm generally form later, in the vicinity of the megagametophyte (Owens and Molder 1977). Pollen of white spruce remained in the pollen chamber at the tip of the nucellus for 2–3 wk before germination in an earlier study (Owens and Molder 1979) but for only 2 d in the study of Dawkins and Owens (1993). Time to germination may depend on temperature. Germination required 2–3 wk when trees were cultured in vitro at room temperature (Dawkins and Owens 1993) or when pollen is cultured in vitro at room temperature (Webber 1991) or warmer (28°C; deWin et al. 1996).

Small branches observed in the pollen tube may serve an anchoring or haustorial function (Johri 1992), but a single main tube always forms and grows directly toward the megagametophyte. In culture, 10%–50% of the pollen tubes of Scots pine (*Pinus sylvestris* L.) ramified (deWin et al. 1996), and we speculate that this may be a result of lack of a chemical signal from the megagametophyte. Takaso et al. (1996) describe a chemotactic response elicited in pollen tubes of *Pseudotsuga menziesii* when a homogenate of the megagametophyte was supplied in the culture medium.

Sperm of interior spruce were described as male nuclei in Dawkins and Owens (1993). These nuclei were approximately similar in size and remained in a common cytoplasm with no surrounding cell wall formation following body cell mitosis. Mitochondria of the body cell cytoplasm surrounded the sperm and were distinguishable from those within the archegonium because they possessed better developed cristae. Plastids surrounding the sperm were orthodox in appearance and clearly distinguishable from those of nucellar cells or the incipient modified plastids in the central cell.

Nucellus

Cells in the central column of the apical region of the nucellus contain abundant starch, which is hydrolyzed in cells proximal to the growing pollen tube. Cytoplasm of all cells in the central nucellar apex stains strongly with toluidine blue during pollen tube growth. This is characteristic of increased levels of RNA, polycarboxylic acid, and related compounds of cellular metabolism (O’Brien and McCully 1981). Nucellar cells seem to increase their metabolism and begin starch hydrolysis and production of compounds necessary for pollen tube growth throughout the apical region, and heightened metabolism does not require physical contact with the growing pollen tube. Nucellar cells are not, however, apoptotic (Bell 1996; Havel and Durzan 1996) in that they do not spontaneously deteriorate and die before physical contact with the pollen tube. Pollen tube growth is intercellular in the nucellus. Deterioration of nucellar cells in contact with the pollen tube tip suggests that substances released from the tube mediate cell wall or middle lamella dissolution. Pettit (1985) determined...
that many proteins with hydrolytic properties were released from the pollen tube tip of conifers.

A layer two to three cells thick at the margin of the nucellar apex has abundant lipid accumulation between the plasma-lemma and cell wall. These lipids impregnate the cell walls and may have a waterproofing function. The nucellar apex is a small, exposed tissue that must remain hydrated during development and during pollen germination and growth. Particularly when the micropyle is open before pollination, the nucellus would be susceptible to desiccation. Lipids in the outer cell walls of these marginal cells may slow evaporation. Tillman-Sutela et al. (1996) described lipids in the collapsed nucellar layers of mature seeds of Scots pine and ascribe to them a function in regulating germination. Perhaps the origin of the lipids they describe is in the nucellar apex before pollination.

**Megagametophyte**

By the time pollen germinates, the megagametophyte is cellular and at least at the division stage of the archegonial initial. Development during the following 5–7 d resulted in archegonia, which are mature and ready for fertilization.

**Megaspore wall.** Nucellar cells in the inner one to three layers surrounding the megagametophyte have a tapetal function. Their degeneration results in release of a lipidic substance that has the same staining properties as the megaspore wall. In the early cellular megagametophyte, the megaspore wall is composed of an inner layer that is pectic/cellulosic and an outer layer that stains positively for lipids (Favre-Duchartre 1956). These layers were called “intine” and “exine,” respectively, and described as functionally similar to the same layers in pollen (see Kurmann 1990). The origin of the intine layer, whether gametophytic, as described for *Ephedra* by Moussel and Moussel (1973), or from the nucellar tapetum, as described for several gymnosperms by Pettitt (1966), was not determined here as it was in place before the first specimen collection. Deposition of material liberated from deteriorating tapetal cells results in thickening and elaboration of the ek-texine layer of the megaspore wall in interior spruce so the wall can be described as, at least partly, sporophytic in origin. In the fully developed megagametophyte of interior spruce, the megaspore wall is thickest in the chalazal region and thinnest at the site of pollen tube penetration near the neck cells.

**Cell divisions in the archegonia.** Development of archegonia occurs by a series of cell divisions common to all genera of Pinaceae (Singh 1978; Singh and Owens 1981). Megagametophytes of interior spruce have two to three archegonia. Uneven division of an archegonial initial produces a large central cell and a small, distal primary neck cell. Uneven division of the central cell produces a large egg and a small ventral canal cell that subtend the archegonial neck. Pollen is not required for normal megagametophyte or archegonial development and is not required as a signal for initiation of megagametophyte development in most conifer genera (Owens and Blake 1985).

Archeogonial initials are vacuolate. Rapid enlargement of the central cell is accompanied by a reduction in vacuole size and number, and the mature egg is almost completely filled with cytoplasm. There is approximately an order of magnitude difference in length between the archegonial initial and egg (40–400 μm). This represents a change in volume that approaches three orders of magnitude in a very short time. Asymmetric mitosis of the central cell is preceded by isolation of a small area of cytoplasm between the nucleus and neck cells. A similar situation was observed in *Abies grandis* Lindley (Singh and Owens 1982). This area of cytoplasm is in position to form the ventral canal cell cytoplasm after central cell mitosis. The mechanism, which is probably cytoskeletal, by which the central cell nucleus is held in place at the micropylar pole, was not observed. Upon division, a cell wall forms between the ventral canal cell and egg before the egg nucleus begins to migrate into the central region.

**Neck cells and the archegonial jacket layer.** Primary neck cell division and subsequent neck cell mitosis result in a three- to-four-tiered neck. Very often the actual number of neck cell tiers was impossible to determine as the cells were not symmetrically oriented. Reports of two neck cell tiers in Engelmann spruce (Owens and Molder 1984) and three tiers in white spruce (Owens and Molder 1979) probably represent natural variation in planes of cell division.

Archeogonial jacket cells differentiate from prothallial cells of the megagametophyte surrounding each archegonial initial. They are densely cytoplasmic and divide synchronously during central cell enlargement. The increase in archegonial jacket-cell number compensates for size increase in the egg. Individual archegonia are entirely enclosed by an archegonial jacket layer as a rule; however, rapid enlargement of the egg periodically resulted in separation of jacket cells and direct contact between neighboring eggs, as reported by Maheshwari and Singh (1967). The archegonial jacket does not enclose the neck of the archegonium but proliferates into a two to three cell thick zone around the mature neck cells. Singh (1978) cited the work of Maugini and Fiordi (1970), who reported that storage products, starch and protein, of the megagametophyte cells are solubilized and translocated into the central cells and egg cells through the archegonial jacket cells. This was not verified in interior spruce, but very thin, primary pit fields form between the archegonial jacket cells and the egg. No breakdown of cell walls between the archegonial jacket and the egg, as reported in *Pinus* (Singh 1978), was observed, nor has this breakdown been reported for any other taxa in more recent literature (Owens and Morris 1990). Such reports in older literature may have resulted from poor fixation or processing of tissue. Archegonial jacket cells do, however, become vacuolated and appeared secretory in nature during egg-cell maturation.

As the egg nears maturity, lipid secretions from the neck cells and adjacent archegonial jacket cells accumulate between the neck and megaspore wall in the archegonial chamber. This secretion could not be attributed to one cell type because lipid bodies were observed within the cell walls between neck cells and archegonial jacket cells, and even in the cell walls between neck cells and the ventral canal cell. Neck cell cytoplasm deteriorates before pollen tube arrival. Singh (1978) noted this deterioration in neck cells of *Cephalotaxus* and attributed it to production of the very thick, primary cell walls as observed here in interior spruce. Archegonial jacket cells surrounding the neck remain densely cytoplasmic and secretory with many lipid bodies and dictyosomes. Fluorescein diacetate staining of the megagametophyte at this stage indicates esterase activity in all of the archegonial jacket cells, but the highest intensity
is in the neck and surrounding cells. Lipid accumulation in the archegonial chamber occurs at exactly the site to which pollen tubes must grow. Takaso and Owens (1996) described similar lipid-like secretions in the walls of prothallial cells in the apical portion of the megagametophyte of *Pseudotsuga menziesii* before fertilization.

A central question in the study of conifer reproductive biology is, how do the pollen tubes find the neck? Mascarenhas (1993) discussed chemotropism of the angiosperm pollen tube. Pollen tubes of pearl millet (*Pennisetum glaucum*) were chemotropic to a variety of chemicals, including glucose, calcium, and low molecular weight proteins (Reger et al. 1992). This list does not include lipids, however. Wolters-Arts et al. (1998) have recently demonstrated that lipids are essential for pollen tube penetration of the stigma in various angiosperms. Lipid accumulation in the archegonium neck might be an analogous situation of chemical signaling in the pollen tube pathway of a gymnosperm.

**Mature Archegonium: Cytoplasmic Inclusions and the Perinuclear Zone**

Archegonia are considered mature when the egg nucleus has migrated to the middle of the cell and is surrounded by a perinuclear zone containing clusters of maternal mitochondria. At this stage, vacuolization of the egg cytoplasm is minimal and modified plastids (large inclusions) and small inclusions are prominent features.

Chesnoy and Thomas (1971) reviewed large inclusion form and function as interpreted by earlier workers. They cite the pioneering electron microscope studies of Camefort (e.g., Camefort 1959) as the first to give evidence of large inclusion formation as it is now known to occur. Modified plastids form in interior spruce when plastids of the central cell elongate and encircle regions of cytoplasm. This deformation of plastids continues during egg maturation, resulting in highly convoluted membrane systems that fully enclose regions of cytoplasm. Plastid modification in the archegonial jacket cells and ventral canal cell occurs coincidentally with that in the central cell, while plastids in nucellar cells do not change in appearance.

Interpretation of small inclusion formation is more difficult. Small inclusions are easily seen in the light microscope because of what they do not contain. They are regions of cytoplasm devoid of organelles and bound by a simple membrane. Cytoplasm within small inclusions is sometimes more densely stained than surrounding, nonincluded cytoplasm. The cytoplasmic fraction not included in small inclusions contains all of the maternal mitochondria and so appears granular by comparison. Earlier reports show vacuolization within the bounding membrane of small inclusions during egg maturation (Singh 1978). This was not observed in interior spruce. Fixation and dehydration of cytoplasm can result in shrinkage, and apparent vacuolization may have been artifactual.

Nuclei and mitochondria move within the egg by a mechanism that is not understood. Small inclusions are positioned such that they could mediate or, at least, regulate movement of nuclei and organelles. How the organelles and nuclei might move along the small inclusion membrane system is open to conjecture. Actin-myosin-mediated movement of organelles and sperm has been demonstrated in angiosperm pollen tubes (Heslop-Harrison and Heslop-Harrison 1989) and postulated but not conclusively demonstrated in angiosperm eggs (Russell 1993; Huang and Russell 1994). Terasaka and Niitsu (1994) have demonstrated filaments of F-actin and myosin-coated cytoplasmic components in the pollen tubes of *Pinus sylvestris* L. This actin-myosin association may also occur in the egg of pinaceous species.

Small inclusions seem to play a role in the formation of the perinuclear zone. They partition the egg cytoplasm in such a way that the nonincluded cytoplasm is relatively small in volume. Small inclusions increase in number toward the periphery of the egg as it matures and may simply squeeze the cytoplasmic fraction containing mitochondria into the center. Certainly, when the immature egg nucleus has recently migrated to the center of the cell, elongate small inclusions fill the area in its wake so that nonincluded cytoplasm is displaced toward the nucleus.

Questions of small inclusion form and function may be resolvable if the egg cytoplasm can be studied in the absence of artifacts induced by chemical fixation. This is true in any case where deduction of cell function is attempted by interpretation of chemically fixed structures. Freeze fixation, if possible in a cell so large, or confocal microscopy after staining with fluorescent vital stains may help resolve some of these issues.

At maturity, the egg has a large centrally located nucleus surrounded by clusters of maternal mitochondria in a perinuclear zone. Small inclusions partition the egg cytoplasm in such a way that modified plastids are held toward the periphery where they had formed. Formation of the small inclusion-surrounded perinuclear zone and the undulating appearance of the egg nuclear membrane are good indices of egg maturation and competence for fertilization.

The second article in this series covers pollen-tube penetration of the archegonium, sperm delivery to the egg, gamete fusion, and proembryo and early embryo formation.

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