MEGASTROBILUS DEVELOPMENT IN

JUNIPERUS ASHEI (CUPRESSACEAE)

THESIS

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by

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Some seeds again are enclosed in a pod,
some in a husk, some in a vessel,
and some are completely naked.

Theophrastus
Enquiry into Plants
300 B.C.

ACKNOWLEDGMENTS

To my committee members: Dr. Paula Williamson and Dr. Robert Koehn I offer my heartfelt gratitude for their patience and encouragement. To Dr. David Lemke, committee chairman, I can only say "a thousand thank you's" for your support, amazing forbearance, and for teaching me botany. To Gerald Farr, computer guru of the Faculty Advancement Center, gratefulness for guiding me through the sometimes arcane geography of PC world. And lastly, a humble recognition of the intrepid and gifted botanists of the 18th, 19th, and early 20th centuries, we stand on the shoulders of the giants who built the foundations of modern botany.

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ABSTRACT

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Juniperus ashei Buchh. (Cupressaceae) is a common gymnospermous tree in central Texas. Its range extends from the mountains of northwestern Coahuila northeast through Texas, eastern Oklahoma, northwestern Arkansas into southern Missouri. Despite the widespread abundance of the species and its importance as a major allergen source, very little is known of its life cycle. This study provides a baseline examination of the life cycle. Megastrobilus initiation occurs in mid October in central Texas. The microscopic megastrobili develop through late

October, November, early December, finally becoming visible just before pollination in late December through early February. Megasporogenesis is concurrent with pollination. Immediately after pollination the micropyle closes and the cone scales fuse to form the berry like cone. As the pollen germinates and pollen tube growth begins, the functional megaspore undergoes mitosis to form a free-nuclear megagametophyte. In early to mid April when the free-nuclear megagametophyte has reached its maximum size, of about 2000 free nuclei, it begins to cellularize. Cellularization is rapid and upon completion an archegonial complex of five to eight archegonia develops at the micropylar end of the megagametophyte. As the archegonial complex matures the pollen tubes reach the neck cells of the archegonia. Fertilization occurs from late April to mid May. One to all the archegonia in a complex may be fertilized generating many potential embryos. The fusion nucleus moves to the bottom of the archegonium where it undergoes three free-nuclear divisions followed by cellularization eventually producing a 12 celled three-tiered proembryo. Initially the suspensor cells and the embryonic suspensor cells of each proembryo do not carry embryo cells at their tips. Embryo cells eventually form as a few or just one line of cells out compete the others and reaches deepest into the gametophyte. The leading embryo rapidly divides giving rise to a secondary suspensor which pushes back any remaining competitors. Embryo competition is completed by late July. The new sporophyte grows and differentiates reaching maturity by late October to early November. The mature megastrobili are shed in November, December, and January. *Juniperus ashei* seeds are primarily bird distributed.

INTRODUCTION

Robert Brown first recognized the distinctiveness of the gymnospermous habit 175 years ago. Gymnosperms are plants that produce seeds but not flowers or fruit. Their seeds are exposed on the surface of the appendages that bear them (Bold 1973) not enclosed in carpels as in angiosperms.

> In my observations on the structure of the female flower in *Cycadeae* and *Coniferae*,..., I endeavored to prove that in these two families of plants the ovulum was in no stage enclosed in an ovarium, but was exposed directly to the action of the pollen (Brown 1825, 1844).

The Coniferophyta arose in the late Paleozoic and attained its greatest diversity and abundance in the middle Mesozoic (Beck 1970; Miller 1977; Stewart and Rothwell 1993). Though diminished in diversity many genera remain abundant and are economically and ecologically important worldwide today (Chamberlain 1935; Biswas and Johri 1997).

Juniperus is the second largest genus in the Coniferophyta and the largest genus in the family Cupressaceae (Eckenwalder 1976; Hart 1987). In contrast to many cupressid genera which are monotypic, the approximately 60 members of this large advanced, actively diversifying genus (Doyle 1963; Page 1990) are arrayed in a contiguous band around the northern hemisphere (Biswas and Johri 1997) with a North American center of distribution in the southwest.

Juniperus ashei J. Buchholz is commonly found in several natural regions

of Texas. It is a dominant plant in the Ashe juniper-oak series of the Edwards Plateau region of Central Texas (Diamond et al. 1987). The range of *J. ashei* is centered on the rocky limestone soils of central Texas and northeastern Mexico (Zanoni y Adams 1979) with outliers to the northeast on limestone soils in the Arbuckle Mountains of Oklahoma (Phillips et al. 1959) and the Ozarks of Arkansas and southern Missouri (Correll and Johnston 1979; Watson and Eckenwalder 1993).

Juniperus ashei is an evergreen tree reaching 8 to 15 m tall with an open rounded crown and dark green to somewhat blue green foliage. The leaves are decussate to whorled, small and scalelike with obvious spherical abaxial resin glands near the leaf base (Vines 1960; Foster and Gifford 1974). The plants are dioecious, the pollen and seed cones found on separate trees (Correll and Johnston 1979). The ovulate cones mature in one year appearing in December or January and dropping from the trees the following late November to January. Glaucous in color, the ovoid megastrobili reach 6 to 7 mm in length and 5 to 6 mm in diameter. In this genus the ovuliferous scale is completely fused with the bract (Eckenwalder 1976) producing a fleshy, aromatic berrylike cone enclosing one and occasionally two brown seeds (Correll and Johnston 1979). The succulent cones are an abundant source of food for wildlife, especially birds. It is likely that the broad distribution of the genus is due to avian transport and deposition of the seeds (Sporne 1965; Holthuijzen and Sharik 1985; Biswas and Johri 1997).

Small green pollen cones appear in March or April and release their pollen during the following December to mid February when the newly formed

megastrobili are receptive. The wingless spherical microstrobili are tiny with ten to twelve overlapping peltate scales that develop three or four microsporangia at their base (Correll and Johnston 1979). Pollen is wind distributed and exceedingly abundant. The golden clouds of pollen, a potent airborne allergen, are the cause of "cedar fever," an allergic reaction that annually plagues millions of people in the south central United States during late December, January and February (Wodehouse 1935; Cox and Leslie 1988; Simpson 1988; Judd et al. 2002). Other juniper species cause similar problems elsewhere in the world.

Although *Juniperus* is an exceptionally large genus for the Coniferophyta, it has been somewhat ignored morphologically. During the flurry of descriptive botanical work in the 19th and early 20th centuries in Europe and North America, botanists such as E. Strasburger (1872), C. Sokolowa (1890), and J. M. Coulter and C. J. Chamberlain (1910) unraveled the general pattern of conifer reproduction. However they primarily worked on *Pinus* species, largely because of the economic importance of that genus and its abundance in the northern hemisphere, and on monotypic exotic genera like *Ginkgo, Athrotaxis* and *Metasequoia*.

By the mid to late 20th century refinements and clarifications were uncovered especially through the work of J. T. Buchholz in the 1920s, 30s, and 40s, J. Doyle in the 1940s and 1950s, and a group of Indian botanists: R.N. Konar, Panchanan Maheshwari and Hardev Singh during the 1960s through 1990s. Currently Singh's *Embryology of Gymnosperms* (1978) is the single best resource on gymnosperm reproduction. He meticulously describes the details of

gymnosperm reproduction using extensive sources encompassing a broad range of gymnosperms including cycads, ginkgo, pines, taxodiaceous genera, *Gnetum,* and *Welwitschia.*

Junipers became a subject of study just after the turn of the 20th century. Several researchers investigated the most common juniper in the northern hemisphere, *Juniperus communis*. C.O. Noren (1907) in Germany, Alice Ottley (1909), and G. F. Nichols (1910) all studied *J. communis*. *Juniperus virginiana*, a species of the southeastern United States, received attention from Ottley (1909) and A. C. Mathews (1939). These four papers, three on a single species *J. communis*, provide virtually all the available information on juniper morphology and reproduction. As a large and actively diversifying genus (Page 1990), *Juniperus* merits further exploration into its reproductive morphology and anatomy.

The gymnosperms, cycads, ginkgo, conifers including junipers, and gnetophytes are a diverse collection of taxa. These various groups are linked by a limited number of characters. Foremost among them is the possession of 'naked seeds.' In the nineteenth century this single trait was considered sufficient to group the various species possessing this character together as the Gymnospermae (Biswas and Johri 1997). Yet even then researchers acknowledged that it was controversial to gather into a single taxa such a large assemblage of diverse organisms using a single character.

As the twentieth century advanced, the obvious morphological and reproductive differences between these diverse plant groups lead researchers to

break the Gymnospermae into smaller taxonomic assemblages. At midcentury a consensus was reached by botanists to separate the gymnosperms into two or three groups (Sahni 1920; Chamberlain 1935; Arnold 1948; Florin 1948). By the 1980s gymnosperm taxonomists divided them into three classes (Stewart and Rothwell 1983): Progymnospermopsida, consisting entirely of fossil forms; Gymnospermopsida, certain fossil forms plus extant cycads, ginkgos, conifers, and Gnetopsida, incorporating the genera *Ephedra*, *Welwitschia* and *Gnetum*. Increasingly, gymnosperms were felt to be a paraphyletic group consisting of heterogeneous taxa (Page 1990; Judd et al. 2002).

Since 1995 numerous molecular phylogenetic studies have been completed using nuclear, mitochondrial and chloroplast DNA. The results of these investigations have brought gymnosperm systematics full circle. Results reported by Bowie et al. (2000) and Chaw et al. (2000) support gymnosperm monophyly. Rydin et al. (2002), using both nuclear and chloroplast DNA, found conifers to be monophyletic and a sister group to the gnetophytes.

The surviving species of gymnosperms are the living members of very ancient lineages. As the representatives of evolutionary histories that diverged long ago the reproductive characters uniting the extant gymnosperms are limited. Among them are: mostly wind pollination (exceptions are some cycads and gnetophytes); a pollination droplet; archegonia; a long time gap between pollination and fertilization; pollen grains landing directly on the nucellar surface; pollen tubes; greatly reduced gametophytes; naked ovules; a prolonged free nuclear stage in megagametophyte development (exceptions are *Sequoia* and

gnetophytes) ; a free nuclear stage in embryo development and seed production (Sporne 1965; Singh 1978; Page 1990; Biswas and Johri 1997).

The conifers, as a monophyletic subset of the gymnosperms, have the following reproductive characters in addition to those listed above: unisexual strobili; anemophily; a reduced nonmotile microgametophyte; compound megastrobili; a lack of neck canal cells; spongy tissue (tapetum) forming around megaspore; a massive, eusporangiate megasporangium; production of multiple embryos in each ovule but only one remaining in the mature seed; and a single integument that differentiates into three layers (Coulter and Chamberlain 1910; Chamberlain 1935; Sporne 1965; Page 1990).

Juniperus ashei, as a member of the family Cupressaceae in the Coniferales, presumably conforms in general to these reproductive characteristics. Cupressids are nested deeply within the Coniferales (Stefanovic et al. 1998; Kusumi et al. 2000) and junipers are a middle to late Mesozoic offshoot of the Cupressaceae (Stewart and Rothwell 1993). As such, how closely does megastrobili development in *J. ashei* correspond to the general conifer pattern and how does it diverge?

This research seeks to illustrate the various morphological stages of *J. ashei* megastrobili development, from the earliest growth of the strobili through pollination, fertilization and seed production, through microscopic examination. Additionally I will produce a timeline for and detailed descriptions of these stages in an attempt to expand the scientific knowledge of reproduction in this genus and to augment the continuing exploration of gymnosperm reproduction. A more complete

understanding of the basic reproductive biology of this species may eventually contribute to the development of enhanced treatments for "cedar fever."

METHODS AND MATERIALS

Samples of megastrobili were initially collected weekly from six specimens of *Juniperus ashei* in the Austin/San Marcos, Texas area from January to megastrobili maturation in November and December. As critical periods of the reproductive cycle were identified, samples were collected every other day or even every day for the requisite periods. Some trees were eliminated, as time passed, due to low seed production or branch accessibility, and eventually two trees provided the bulk of the sampled material. Voucher specimens (*Wakefield 301, 302, 303, 304, 305*) are on deposit at Texas State University herbarium (SWT).

Megastrobili collected at pollination or shortly thereafter were fixed whole in formalin-aceto-alcohol (FAA). As the integuments lignified, the impervious seed coat prevented penetration of the FAA and consequently the nucellus-gametophyte complex was extracted for preservation in FAA. Additionally the juxtaposition of these hard lignified cells with the delicate dividing cells of the gametophyte made successful microtoming impossible.

In an attempt to avoid the time consuming process of seed coat removal, some megastrobili with mature seed coats were treated in 4% or 10% ethylenediamine as per Carlquist (1982a, 1982b). While the ethylenediamine did soften the lignified cell walls of the seed coat, it frequently had adverse effects on the soft tissues of the nucellus and gametophyte. Only a few of the

resulting slides were useable.

Preserved whole megastrobili and nucellus-gametophyte complexes were dehydrated in a standard graded tert-butyl alcohol series then infiltrated with paraffin and embedded in Paraplast® (Johansen 1940; Moseley 1943). The mounted paraffin blocks were microtomed into 12-14 μm thick sections and selected sequences were mounted on glass slides.

Slides were stained in a safranin-fast green series following Johanson (1940). Finally glass cover slips were placed over the sections and sealed with Permount® mounting medium. The slides were photographed on a Nikon HFX-DX light microscope fitted with a Nikon FX-35DX camera using Kodak Elitechrome Extracolor 100 ISO slide film.

RESULTS AND DISCUSSION

In conifers there is a relationship between day length and reproduction (Owens and Hardev 1990). Long days favor the development of microstrobili and short days megastrobili (Owens and Hardev 1990). Pharis and Morf (1968, 1990) found that in *Thuja plicata* full development of the megastrobilus was delayed without a period of short days. *Juniperus ashei* initiates microstrobili in March and April as spring days are lengthening and megastrobili in late fall as day length is decreasing.

Completion of the reproductive cycle of *J. ashei* (Table 1) requires about a year from pollination in late December, January, and February to the shedding of the mature seed cones the following November, December, and January. Most junipers in the southern and southwestern United States have a reproductive cycle of one year (Watson and Eckenwalder 1993) although cycles of more than one year are found in junipers elsewhere, for example *J. communis*, a circumartic species. *Juniperus virginiana*, to the east and northeast, and *J. pinchotti,* to the west, both have annual reproductive cycles. Nonetheless, there is no hybridization between these species and *J. ashei* (Adams 1975). In general interspecific hybrids are rare among gymnosperms (Richards 1986).

In *J. ashei* the megastrobili become visible just before the time of pollination from late December through early February. The megastrobili are located terminally on short axillary shoots (Worsdell 1900; Mathews 1939;

Table 1. Chronology of strobilus development in *Juniperus ashei*

	Stage of Strobilus Development	
Month	**Microstrobilus**	**Megastrobilus**
October		Megastrobilus initiation & development
November		
December	Pollen shed	Pollination & megasporogenesis
January		Free nuclear gametophyte dev
February		
March		
April	Microstrobilus initiation	Gametophyte cellularization Archegonia development
May		Fertilization
June	Microstrobilus development	Proembryogeny
July		Early embryogeny
August		Late embryogeny
September		
October		Mature megastrobilus/seed
November		Megastrobilus shed
December	Pollen shed	

Owens and Molder 1974). Before pollination the microscopic reproductive and vegetative buds can not be distinguished from each other. Because of this complication the exact timing of the initiation of the reproductive primordium is difficult to determine. However in other cupressids it occurs about four to six weeks before pollination (Nichols 1910; Owens and Pharis 1967). In central Texas the megastrobili are most prevalent on new axillary shoots on the side receiving the most sunlight generally the south and west sides of the trees

Among the Cupressaceae the bracts and scales that make up the compound structure of the megastrobilus are completely fused and arise as a single promordium (Aase 1915; Owens and Molder 1974). Conifer ovules are not vascularized (Singh and Johri 1972).

Pollination

Junipers produce abundant wingless pollen. The pollen grains of *J. ashei* are unusually small for the genus, about 20.5 to 22.5 μm in diameter (Wodehouse 1935; Erdtman 1965). The spherical grains consist of a thin single-layered exine covered in orbicules and a thick transparent gelatinous three-layered intine (LaRue 1954; Gullvag, 1966; Rong and Sziklai 1973; Page 1990). There is no germinal furrow or pore (Wodehouse 1935). Juniper pollen is shed in a uninucleate condition, as in all Cupressaceae (Nichols 1910; Mehra and Malhotra 1947; Sterling 1963; Moitra and Bhatnagar 1982).

At pollination a pollination droplet is secreted by the cells at the top of the nucellus and micropylar canal of the megastrobili (Singh 1978). The droplet sits on the micropylar opening of the erect ovule exposed to the copious clouds of windblown pollen grains.

> ...from each orifice there is exuded a minute globule of clear, shinning liquid which rests like an irridescent bubble on the tip, and serves to catch the pollen and conduct it to the nucellus within (Jack 1893).

Secretion of the pollination droplet is cyclic and occurs in the morning under conditions of high humidity. If the ovule is not pollinated, the droplet is withdrawn by midday to be renewed the next morning (McWillam 1958; Singh 1978). The droplet fluid consists of water and sugars: glucose, fructose, and sucrose (McWilliam 1958).

Reception of the appropriate species of pollen on the droplet causes the fluid to be rapidly and permanently withdrawn into the micropyle (Dogra 1964; McWilliam 1958). The pollination droplet is of universal occurrence in the conifers and the action is very uniform across the group: *Callitris* (Baird 1953), *Pinus* (Doyle and O'Leary 1935), and *Cupressus* (Dogra 1964). The retreating droplet ferries the pollen through the micropylar canal and onto the nucellar surface (Coulter and Chamberlain 1910). There is no pollen chamber at the apex of the nucellus in the Cupressaceae (Singh and Johri 1972).

Figure 1 shows the presence of several pollen grains on the distal surface of the nucellus beneath the micropyle giving evidence of pollination in this early February ovule. One is initiating a pollen tube at the nucellar surface. The "naked" ovule is orthotropous with a single beaked integument and a wide open micropyle. The prepollination nucellus is broad with a domed apex. Megasporogenesis, which typically coincides with pollination, is beginning deep in the nucellus. The cone scales, rounded structures on either side of the

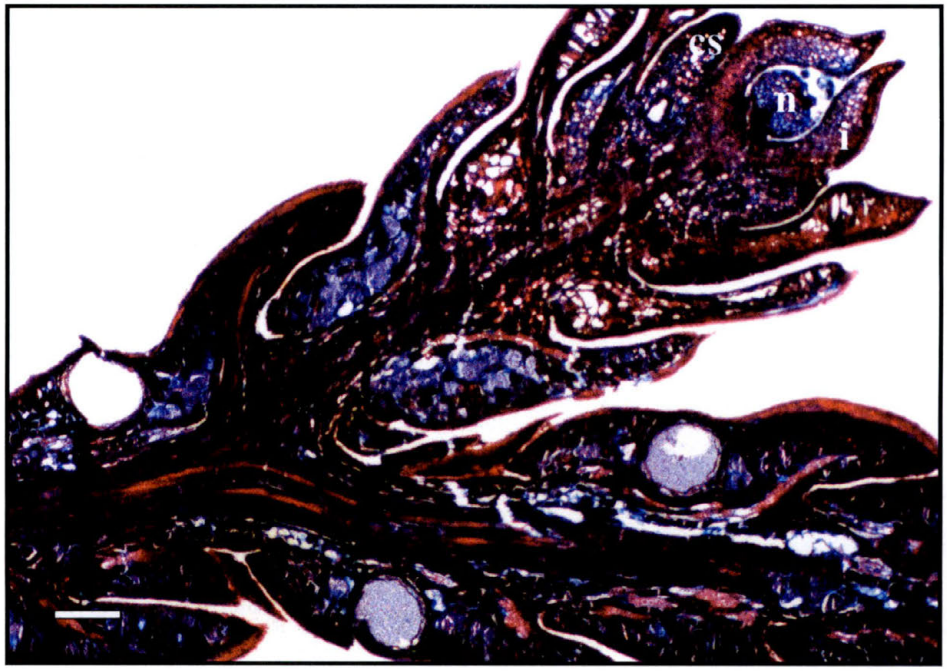

Figure 1. *Juniperus ashei* young megastrobilus. The megastrobili are borne terminally on short axillary shoots. In early February the nucellus is at prepollination maturity and pollination has recently occurred. Two pollen grains lie inside the micropyle at the tip of the nucellus. Cone scales are enlarging. (cs = cone scales, i = integument, n = nucellus; scale = 20 μm)

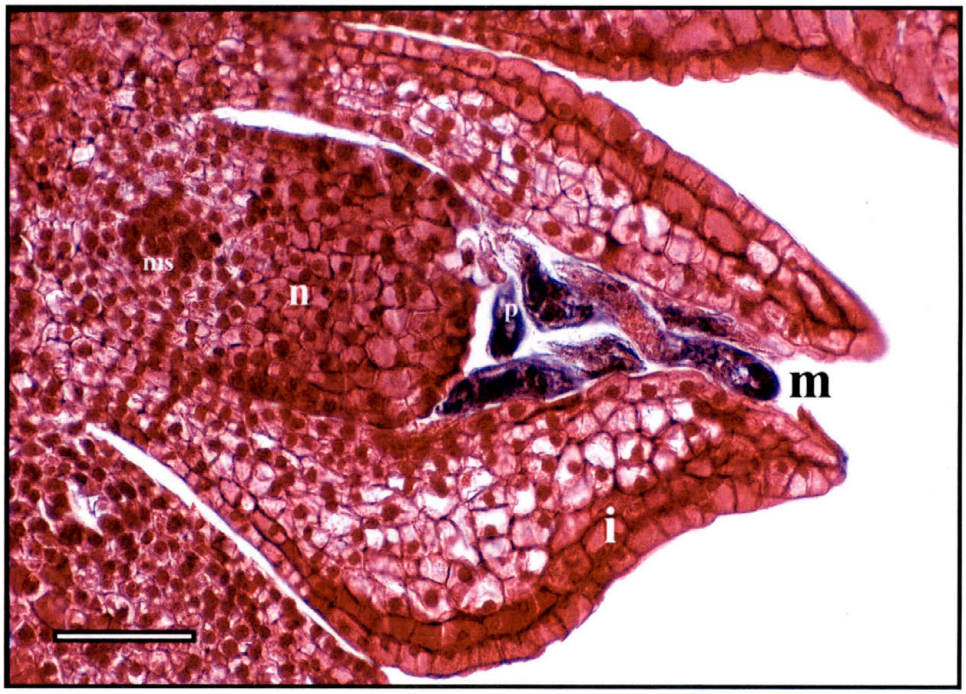

Figure 2. Early ovule with pollen. The open micropyle contains several pollen grains. As the pollination droplet ferries the grains into the micropyle the liquid causes the intine of the pollen to imbibe fluid and swell dislodging the exine. One pollen grain has initiated a pollen tube at the surface of the nucellus. Megasporogenesis is beginning at the chalazal end. (i = integument, m = micropyle, ms = megaspore, n = nucellus, p = pollen grains; scale = 10 μm)

integument, are expanding.

Germination of the pollen grain may begin at once as it sinks through the liquid of the pollination droplet. As it imbibes fluid, the gelatinous intine swells immediately and splits open the thin layer of exine, "which slips off like the skin of a concord grape" (LaRue 1954).

Five or six pollen grains in various stages of germination can be seen in the micropyle and on the nucellus of the ovule in Figure 2. In *J. ashei* pollen grains frequently commence germinating in the micropyle (Fig. 3). After casting off the layer of exine and the two outer layers of intine the innermost intine swells and elongates in the narrow confines of the micropylar canal. Owens and Molder (1975) reported the same germination behavior in *Chamaecyparis nootkatensis*.

The innermost of the three intine layers in the pollen grain gives rise to the pollen tube (Singh 1978). The exine and the two outer layers of intine are discarded in the micropyle or at the surface of the nucellus during germination. Their ridged profiles can often be observed in the micropyle under high magnification (Fig. 3).

The first division of the microspore takes place in the micropyle or upon the grain's arrival at the surface of the nucellus. No prothallial cells are produced in the Cupressaceae (Chamberlain 1935; Sporne 1965). The microspore acts as an antheridial initial and gives rise directly to the generative cell and the tube cell (Sterling 1963; Singh and Johri 1972; Foster and Gifford 1974).

As the pollen tube begins to extend into the nucellus the generative cell divides to form a body cell and a stalk cell (Mathews 1939). The three cells: tube, stalk, and body, migrate towards the tip of the pollen tube where they lie in close proximity as the tube grows towards the distal surface of the gametophyte (Figs. 4).

If an ovulate tree is located in proximity to many staminate trees the number of pollen grains arriving at the nucellus can be very large. It is not unusual to observe six to ten pollen grains in various stages of germination in the micropyle or on the nucellar surface in *J. ashei* (Fig. 2). In *Librocedrus* and *J. communis* as many as 6 to 7 pollen tubes have been observed in a single nucellus (Lawson 1907; Nichols 1910).

In conifers, pollen tubes are not haustorial (Johri 1992). Pollen tubes in cupressids are unbranched and their paths are fairly straight to tortuous (Mehra and Malhotra 1947; Owens and Molder 1975). A pollen tube complex is seen in the nucellus in Fig. 4. A tube cell, possibly a stalk cell, and a body cell can be seen at the tip of one pollen tube. As tubes push through the nucellus, they grow in between the cells. The cells that are pushed aside are crushed and eventually disintegrate (Lawson 1907; Singh and Johri 1972). In *J. ashei* the abundance of pollen tubes (Fig. 4) may hollow out the upper nucellus. Mehra and Malhotra (1974) reported the same phenomena in *Cupressus sempervirens*.

The nucellus continues to enlarge after pollination and reaches its maximum size in the post pollination stages (Singh 1978). Frequently there is a single-celled epidermal layer present surrounding the nucellus (Fig. 2).

Seed coat and cone scale development

After pollination the cone scales and seed coat develop rapidly. The cone scales or sporophylls continue to enlarge until they completely envelop the ovule (Figs. 5, 6). *Juniperus ashei* has two, rarely three, decussate pairs of cone scales (Fig. 7). The cells of the most distal of the converging pairs elongate laterally and dovetail to fuse the opening between them (Quisumbing 1925), sealing in the pollinated ovule. The second pair, which grows in the opposite plane, enlarges and fuses to the first pair slightly below the apex. Occasionally there is an underdeveloped third pair attached to the cone near the base. The two pairs of fused cone scales swell to form a succulent berrylike cone around the ovule (Aase 1915; Mathews 1939; Owens and Molder 1975). The fleshy juniper cone is unique among the Cupressaceae.

Conifer ovules are unitegmic (Quisumbing 1925; Singh 1978). In the cupressids the integument is inserted at the base of the ovule (Singh and Johri 1972) leaving the nucellus largely free from the integument (Vasil and Sahni 1964).

The integument has three layers: a single-celled outer sarcotesta, a middle sclerenchymatous sclerotesta that lignifies to become the stony seed coat, and an inner endotesta that matures into a papery thin layer between the seed coat and the embryo (Lawson 1907; Quisumbing 1925; McWilliam 1958; Vasil and Sahni 1964; Biswas and Johri 1997).

As the micropyle is closing the integument begins to differentiate into the seed coat. Lignification of the middle stony layer (Fig. 8) of the integument

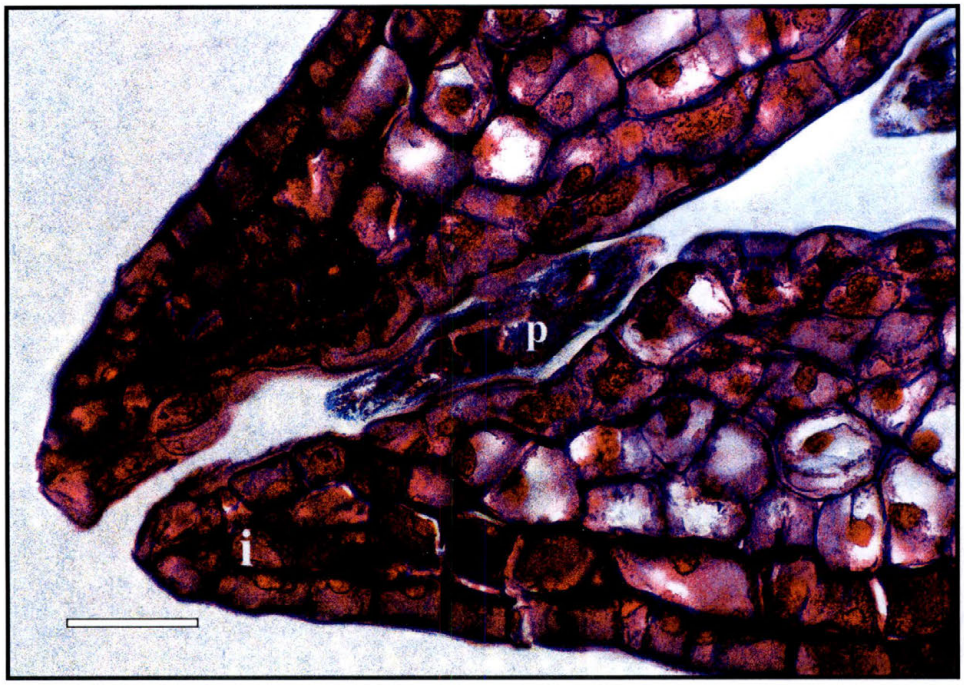

Figure 3. Pollen grain in micropyle. The original spherical shape of the pollen becomes more elongate as germination begins in the cramped fluid environment of the micropyle. (i = integement, p = pollen grain; scale = 5 μm)

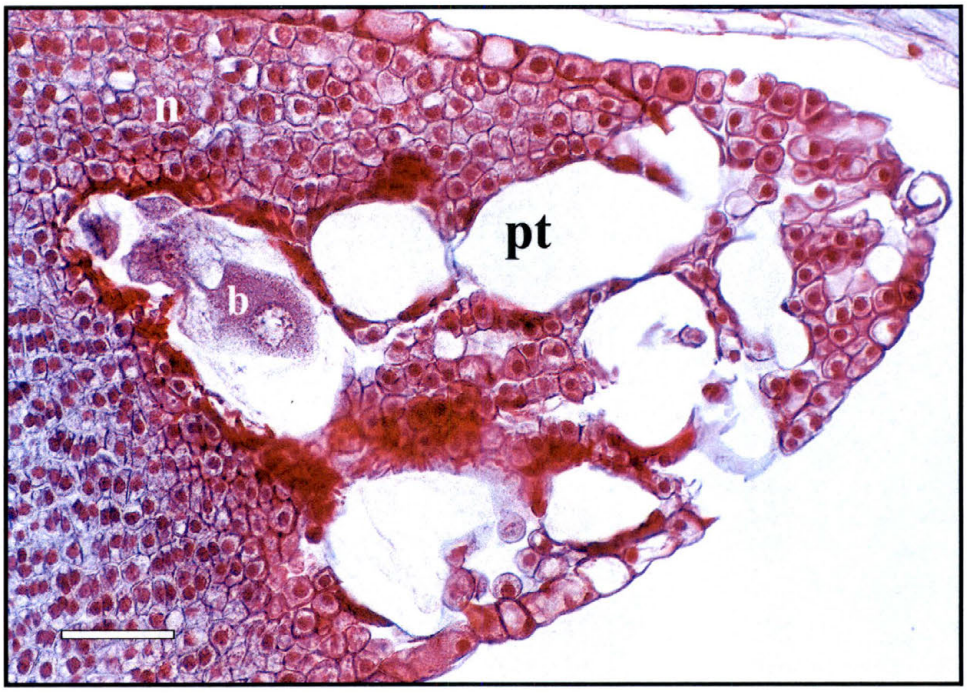

Figure 4. Nucellus riddled with pollen tubes. A body cell and a tube cell are present in the most proximal pollen tube. (b = body cell, n = nucellus, pt = pollen tube; scale = 8 μm)

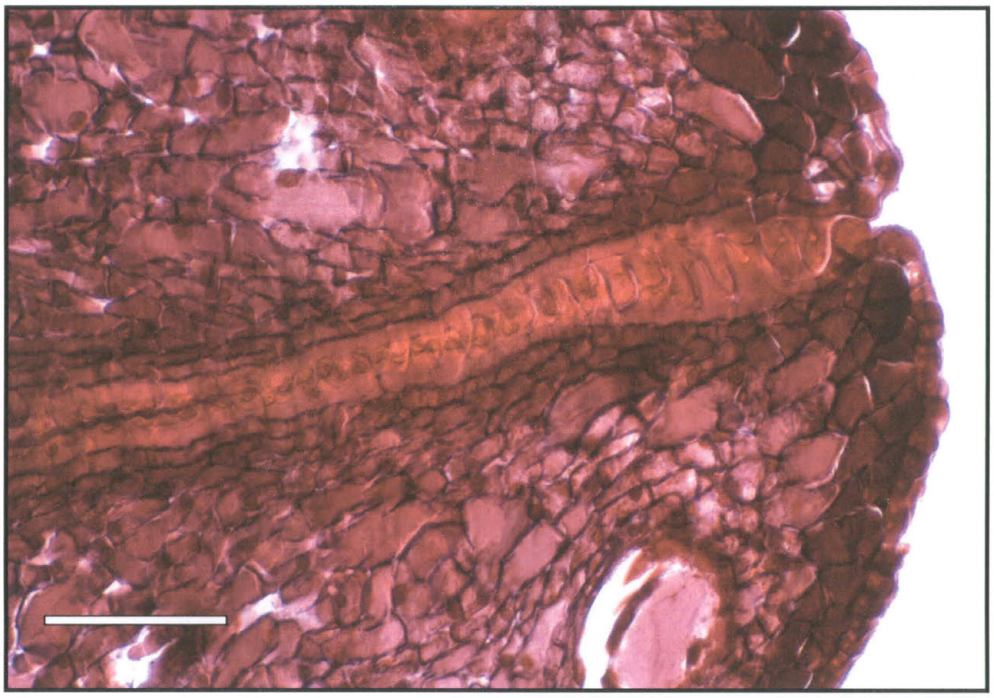

Figure 5. Fusion of opening between cone scales. The epidermal cells lining the aperture elongate inward fusing the opening after pollination. (scale = 10 μm)

Figure 6. Young megastrobili with suture line between fused cone scales. (scale = 1 mm)

Figure 7. Mature megastrobili before shedding. The pointed tips of the individual fused cone scales are recognizable on the mature cones. Mature cones are 5 to 8 mm in diameter.

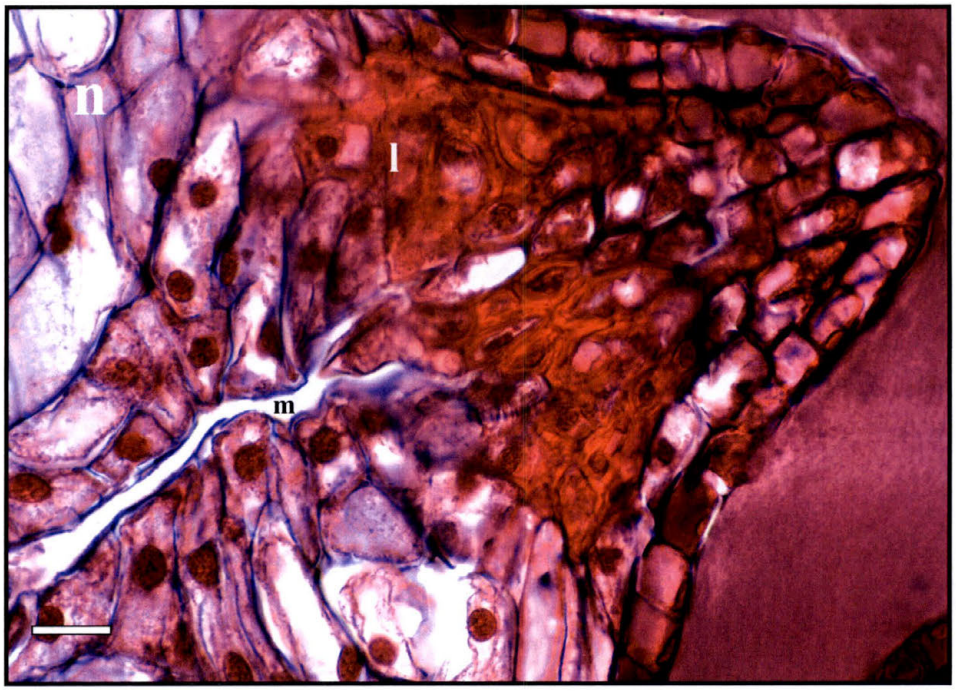

Figure 8. Closure of micropyle. Integument cells elongate to close the micropyle after pollination. The lignification that transforms the integument into the seed coat begins at the micropyle and proceeds to the chalazal end. (l = lignified cells, m = micropyle, n = nucellus; scale = 2.5μm)

arises at the micropyle and proceeds towards the chalazal end. The cells lining the micropyle elongate inwards and seal the channel. The rapid closure of the micropyle enhances pollination by providing high humidity preventing desiccation of the pollen grains (Singh 1978).

The integument in Fig. 9 clearly shows the differentiation process. The single-celled outer layer is present and the cells of the stony layer are lignifying. The lignin reinforcement of these sclerenchymatous cells is laid down in multiple thin layers on the interior surface of their primary wall (Quisumbing 1925; Mathews 1939). Lignification begins near the micropyle and progresses downward towards the cells at the chalazal end, which are the last to entirely lignify. Furthermore cells towards the gametophyte side of the stony layer lignify first then the process moves into the outer cells (Mathews 1939; Singh 1978). Pits are prominent in the thickening secondary cell walls (Fig. 10).

Megasporogenesis and spongy tissue development

Megasporogenesis begins at the time of pollination (Figs. 2, 11). The sporogenous tissue in *J. ashei* becomes recognizable in the nucellus at the level of integument insertion (Figs. 1, 11, 12). Megasporogenesis is monosporic in cupressids (Konar and Moitra 1980). Within the sporogenous tissue one cell enlarges to become the megaspore mother cell (Fig. 11), identifiable by its large size and conspicuous nucleus. The megaspore mother cell divides meiotically to yield a linear tetrad (rarely three) of haploid megaspores (Lawson 1907; Mehra and Malhotra 1947; Maheshwari and Singh 1967). Only one of the megaspores functions, generally the chalazal cell (Chamberlain 1935; Biswas and Johri 1997).

The functional megaspore is the first cell of the haploid female gametophyte generation.

The remaining sporogenous tissue, plus some adjoining nucellar tissue, divides mitotically to become the spongy tissue, which forms a nutritive jacket around the developing megagametophyte (Figs. 11, 12). Spongy tissue is typically well developed in the Cupressaceae (Singh and Johri 1972). The cells of the spongy tissue can be recognized by their dense cytoplasm and large nuclei (Thomson 1905; Singh and Johri 1972; Mehra and Malhotra 1947). Spongy tissue has a tapetal function, providing nutrition to the maturing megagametophyte. It increases in size with the growth of the free nuclear megagametophyte but disorganizes and disappears as the megagametophyte cellularizes (Mehra and Malhotra 1947; Singh 1978).

Free nuclear megagametophyte

The haploid functional megaspore undergoes a number of synchronous free nuclear divisions (Figs. 12, 13, 14) before building cell walls. The number of free nuclear divisions is thought to be constant for a species (Konar and Moitra 1980): 2000 were recorded for *Pinus strobus* (Ferguson 1904) and 6000 for *Cupressus sempervirens* (Mehra and Malhotra 1947). Chamberlain (1935) quotes Noren (1907) as estimating the number of free nuclear divisions in *J. communis* at 2000 just before cell wall formation in the megagametophyte.

In *Chamaecyparis nootkatensis* Owens and Molder (1975) found that initiation of free nuclear divisions of the functional megaspore is closely tied to the initiation of pollen tube growth. *Juniperus ashei* follows this pattern. Megasporogenesis occurs with pollination and free nuclear division in the

gametophyte coincides with the initiation of pollen tube growth (Figs. 12, 13, 14).

As the number of free nuclei increases they are pushed to the periphery of the gametophyte by the enlargement of a central vacuole (Figs. 13, 14). The vacuole keeps the cytoplasm and nuclei in close proximity, through the megaspore membrane, with the surrounding cells of the spongy tissue enabling nutritional substances to be readily absorbed (Lawson 1907; Vasil and Sahni 1964).

The cell membrane of the functional megaspore becomes the megaspore membrane (Figs. 13, 14). It surrounds the female gametophyte, enlarging to accommodate growth of the gametophyte, as free nuclear divisions proceed inside (Coulter and Chamberlain 1910). Initially the megaspore membrane is thin but thickens by fertilization (Thomson 1905; Lawson 1907; Nichols 1910; Maheshwari and Singh 1967). Similarities have long been noted between the megaspore membrane and microspore wall. Both have two layers: a suberized outer layer and an inner cellulose-pectinaceous layer. Moreover sporopollenin is present in both wall and membrane (Thomson 1905; Maheshwari and Singh 1967). Pettitt (1966) suggested that the megaspore membrane includes some spongy tissue in its final form.

In a longitudinal section of an early March ovulate cone (Fig. 13), about five to eight weeks after pollination, the cone scales have enclosed the ovule and begun to enlarge and increase in succulence. The micropyle is sealed; the integument has started to differentiate and lignification of the stony layer is beginning. The nucellus shows pollen tube growth at the micropylar end and has

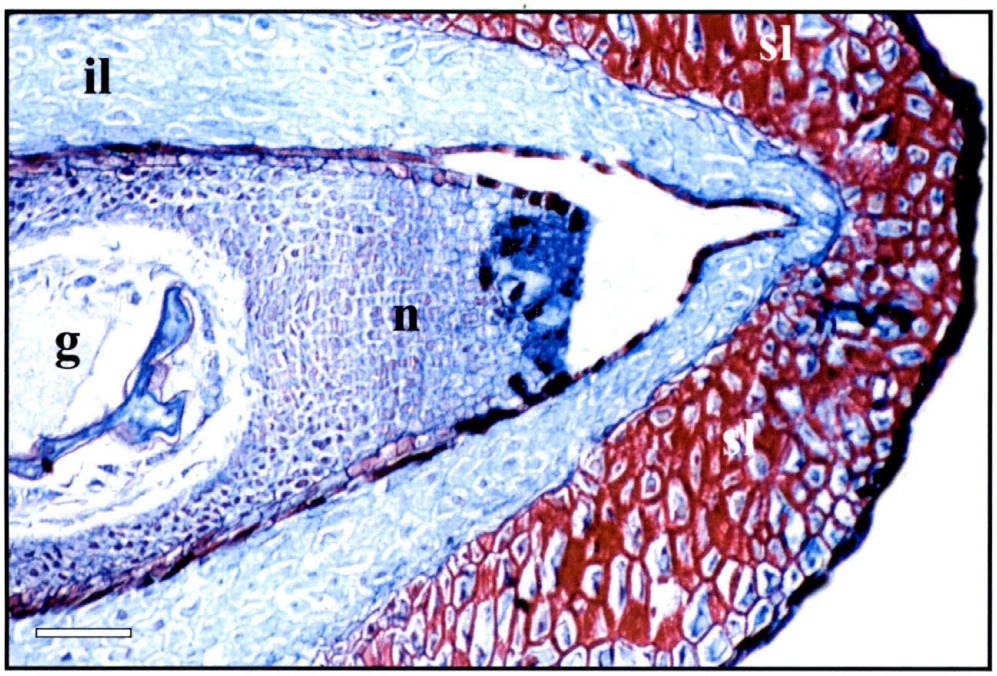

Figure 9. Differentiation of integument. The stony layer lignifies into the seed coat and the inner layer disorganizes to eventually become a paper thin layer between the seed coat and the embryo. A large free nuclear megagametophyte is present in the nucellus. (g = gametophyte, il = inner layer, n = nucellus, sl = stony layer; scale = 10 μm)

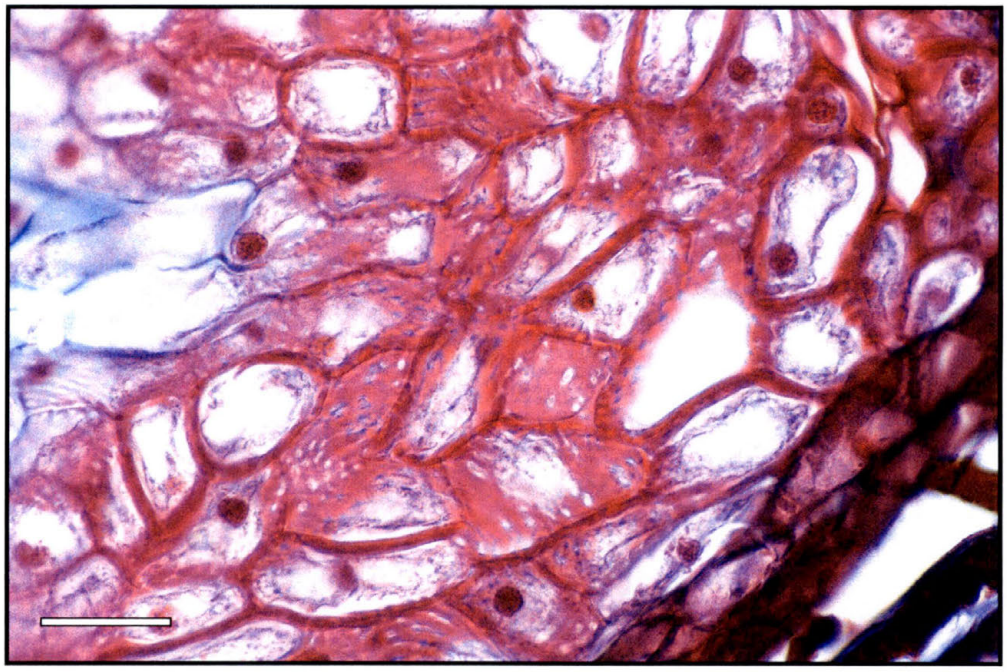

Figure 10. Lignification of cells in the stony layer. Lignin is deposited forming secondary walls inside the primary cell walls. Pits are noticeable in the cell walls. (scale = 10 μm)

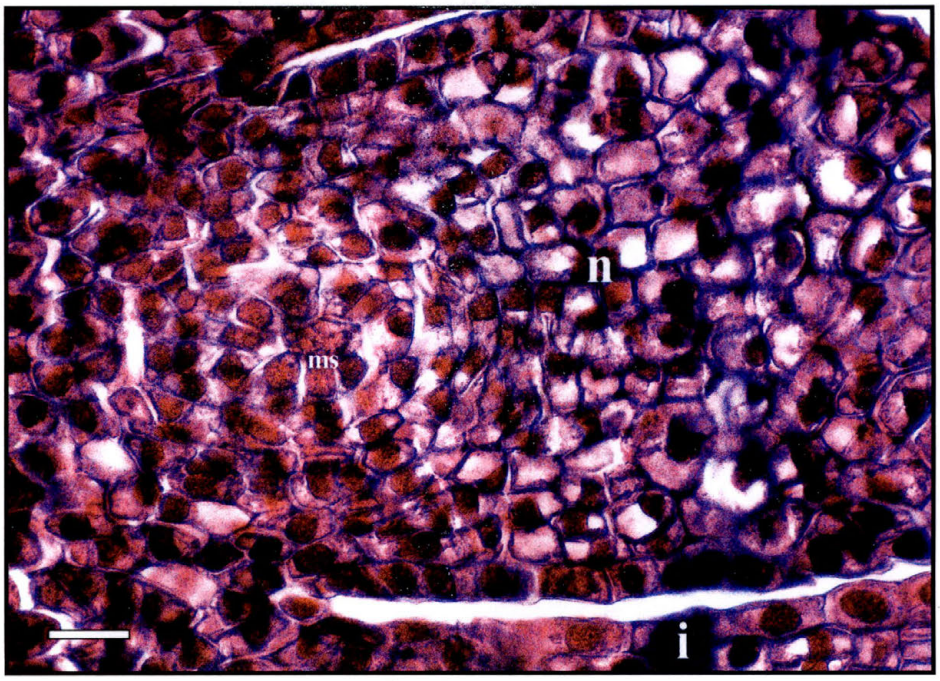

Figure 11. Functional megaspore. Pollination triggers megaspore development at the base of the nucellus. Four megaspores are produced, three degenerate and the remaining cell is the functional megaspore. The surrounding spongy tissue nourishes the developing megaspore.
(i = integument, ms = megaspore, n = nucellus; scale = 2 μm)

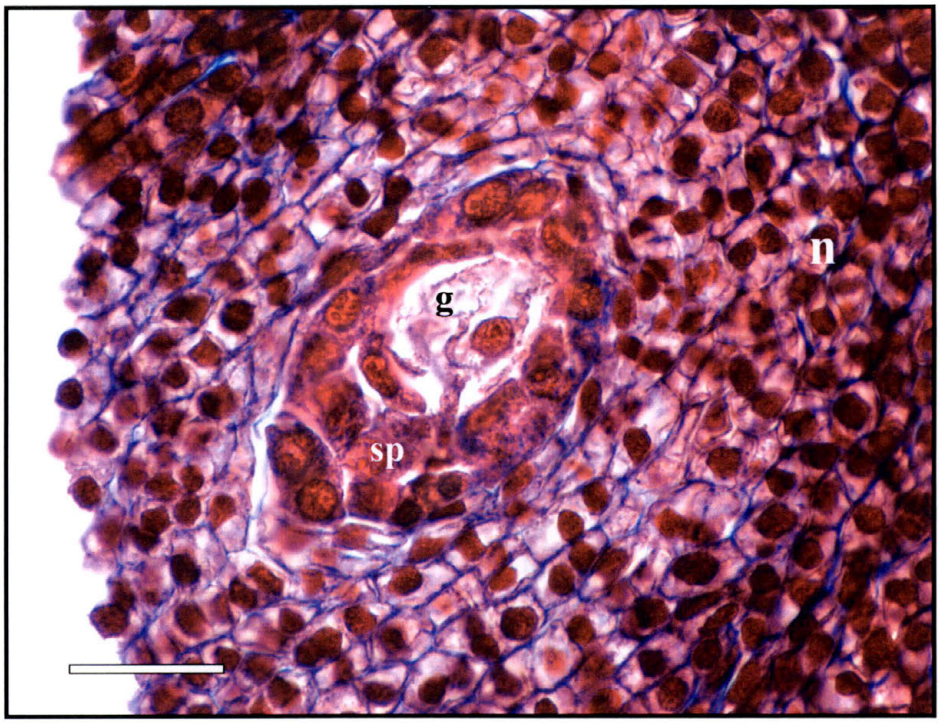

Figure 12. Young free nuclear megagametophyte surrounded by spongy tissue.
(g = megagametophyte, n = nucellus, sp = spongy tissue; scale = 6 μm)

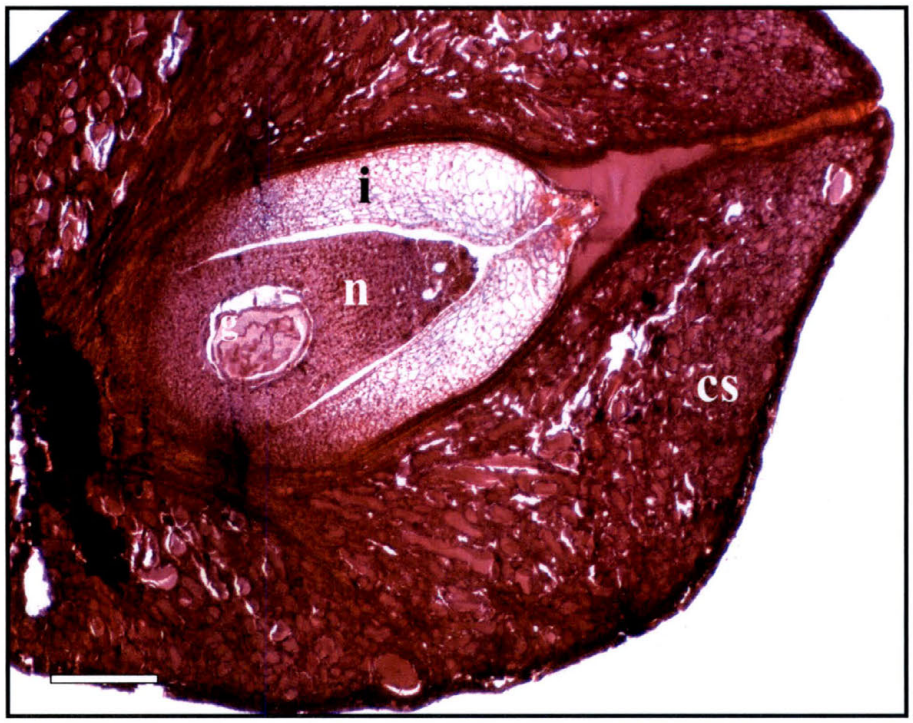

Figure 13. Young megastrobilus. Cone scales envelop the ovule; the integument is sealed and beginning to lignify at the micropyle; the nucellus contains pollen tubes and at the chalazal end a free nuclear megagametophyte. (cs = cone scale, g = gametophyte, i = integument, n = nucellus; scale = 40 μm)

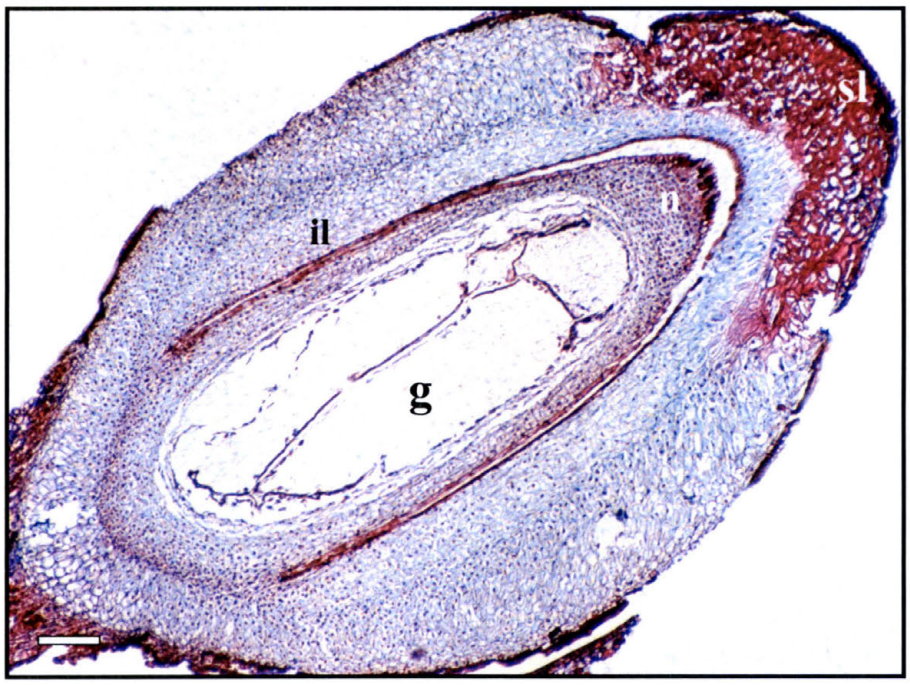

Figure 14. Ovule with large free nuclear megagametophyte. The integument differentiates to become the seed coat. By mid April the free nuclear megagametophyte is near its maximum size. (g = gametophyte, il = inner layer, n = nucellus, sl = stony layer; scale = 24 μm)

an expanding free nuclear megagametophyte at the chalazal end.

By mid to late April the ovule shows further development (Fig. 14); lignification of the integument has progressed, the megagametophyte has enlarged greatly and is approaching its maximal number of free nuclei before beginning cell wall formation.

Cellularization of the megagametophyte

By mid April to early May the free nuclear female gametophyte has reached its maximum size and pollen tubes are approaching its upper surface. When the appropriate number of free nuclei is reached, the gametophyte rapidly undergoes cellularization (Figs. 15, 16).

After the final free-nuclear mitotic division the nuclei, lying in a thin layer of cytoplasm around the periphery, develop secondary spindles so that each nucleus is connected by spindle fibers to as many as six adjacent nuclei. The spindle fibers may be easily seen in Figs. 15 and 16. The spindles lie parallel to the surface of the gametophyte (Singh 1978). Anticlinal walls emerge giving the gametophyte the appearance of a honeycomb. The linked nuclei migrate towards the center of the gametophyte building walls as they move (Figs. 15, 16, 17, 18). The long narrow honeycomblike cells (Figs. 17, 18) are traditionally called alveoli (Looby and Doyle 1940; Brennan and Doyle 1956; Maheshwari and Singh 1967). The alveoli have long side walls and a wall on their outer face towards the megaspore membrane but are open on their inner face near the central vacuole (Singh 1961, 1978). The nuclei remain at the interior open face of the elongating cells. The persistent spindle array seems to guide the laying down of

the anticlinal walls (Maheshwari and Singh 1967; Singh 1978).

The oval shape of the female gametophyte requires that its interior circumference decrease as the cell walls lengthen and approach the center. Fewer cells can be accommodated in this reduced space, so as cellularization progresses some cells close off and do not reach the center of the gametophyte. These cells are called "precociously closed alveoli" (Looby and Doyle 1940) and were observed in *J. ashei* (Fig. 17, 18). Gametophyte configuration dictates that there will be many closed cells especially at the micropylar and chalazal ends of the gametophyte (Singh 1961; Maheshwari and Singh 1967).

As the remaining open cells and their nuclei converge on the center of the gametophyte the spindles reorient and cross-connect permitting the walls to merge and finally all the cells close. The closed alveoli undergo a series of periclinal divisions and the gametophyte looses its honeycomb appearance (Maheshwari and Singh 1967).

Cellularization of the female gametophyte happens quickly, perhaps in as little as 24 to 36 hours. The cells are extremely delicate and do not fix or stain well and it was not possible in this study to observe all stages of this interesting phase as has been the case for many researchers (Singh 1978; Biswas and Johri 1997). The confusing and ephemeral nature of cellularization has led to misinterpretations of this stage of gametophyte development (Maheshwari and Singh 1967). In *J. ashei* cellularization takes place in late April and early May. As cellularization ends the megagametophyte is an irregular, narrow, elongate-oval mass of cells that does not completely fill the nucellus (Singh and

Chatterjee 1963; Owens and Molder 1975).

Archegonia

In conifers the megagametophyte has dual functions: it produces gametes and nourishes the developing embryo (Dogra 1967; Singh and Johri 1972). In *J. ashei* the female gamete is produced in archegonia that develop terminally at the micropylar end of the cellular megagametophyte (Figs. 19, 20). At the center of this area there are several larger undivided cells with noticeable nuclei. These cells are destined to become archegonia (Mathews 1939; Chesnoy 1967). The archegonial initials become distinctive early in the cellularizing of the gametophyte (Ferguson 1904).

In most Cupressaceae, including junipers, archegonia are produced in a single complex of six to ten archegonia with no intervening gametophyte cells (Nichols 1910; Mathews 1939; Sterling 1963). Archegonial complexes are found in those conifers that produce two equal male cells like *Juniperus*. Individual archegonia are found in those conifer genera with two unequal male gametes as in *Pinus* (Sterling 1963; Singh 1978; Moitra and Bhatnagar 1982). The Cupressaceae have two equal sperm cells (Lawson 1907; Singh and Johri 1972) and usually produce one archegonial complex per gametophyte (Maheshwari and Singh 1967). Nichols (1910) reported that maturation of all archegonia in a complex is approximately simultaneous for *J. communis* and Mathews (1939) echoed this description for *J. virginiana*. In *J. ashei* five to eight archegonia per complex is most common and the archegonia display synchronous developmental behavior (Figs. 19, 20, 21).

The archegonia are surrounded by a common jacket of small cells (Maheshwari and Singh 1967). These cells differentiate out from the adjoining gametophyte tissue as the archegonial complex is forming. Their cytoplasm is dense and their nuclei are prominent (Martin 1950; Konar and Moitra 1980). Jacket cells are generally uninucleate but at times binucleate (Singh and Oberoi 1962). Mathews (1939) states they are "usually binucleate" in *J. virginiana*. A well developed jacket layer surrounds the archegonial complex in Fig. 20. The jacket layer cells are distinctive and uninucleate, as was the case for all jacket cells observed in *J. ashei*. Binucleate jacket cells were exceedingly rare.

As the archegonia are maturing the gametophyte cells around the apex of the complex grow and divide rapidly. This tissue expands upward to form a depression or archegonial chamber at the base of which the neck cells of all the individual archegonia are clustered (Lawson 1907; Nichols 1910).

At the beginning of archegonium development an archegonial initial at the apex of the gametophyte divides producing a small neck cell initial and a large central cell (Nichols 1910; Mathews 1939; Singh 1978). The neck initial divides twice periclinally, creating a single tier of four cells (Coulter and Chamberlain 1910; Maheshwari and Singh 1967). The neck cells acquire thick walls and a dense cytoplasm, which may degenerate as the cells reach maturity (Konar and Moitra 1980; Singh 1978). Neck cells are best developed just before fertilization when they facilitate the entry the sperm cells into the archegonium and then rapidly disintegrate (Chamberlain 1935). *Juniperus ashei* has a well developed single tier of four neck cells (Fig. 21). In early May, immediately before

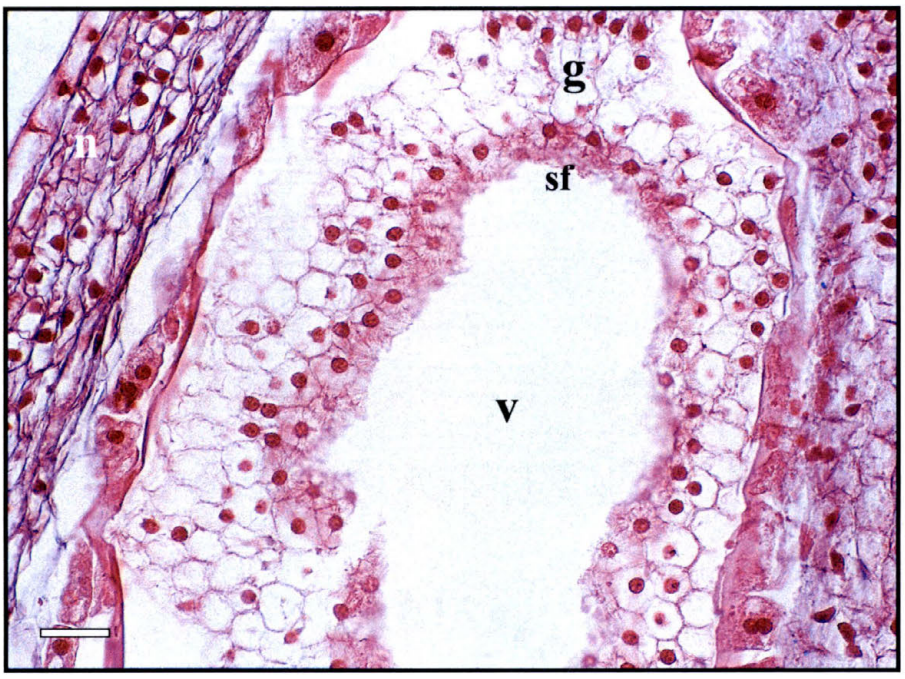

Figure 15. Cellularization of megagametophyte. Each free nucleus links by spindle fibers to the surrounding six nuclei and proceeds in concert to build cell walls while migrating towards the center of the megagametophyte. The inner face of the elongating cell is occupied by the nucleus and remains open. (g = gametophyte, n = nucellus, sf = spindle fibers, v = central vacuole; scale = 6 μm)

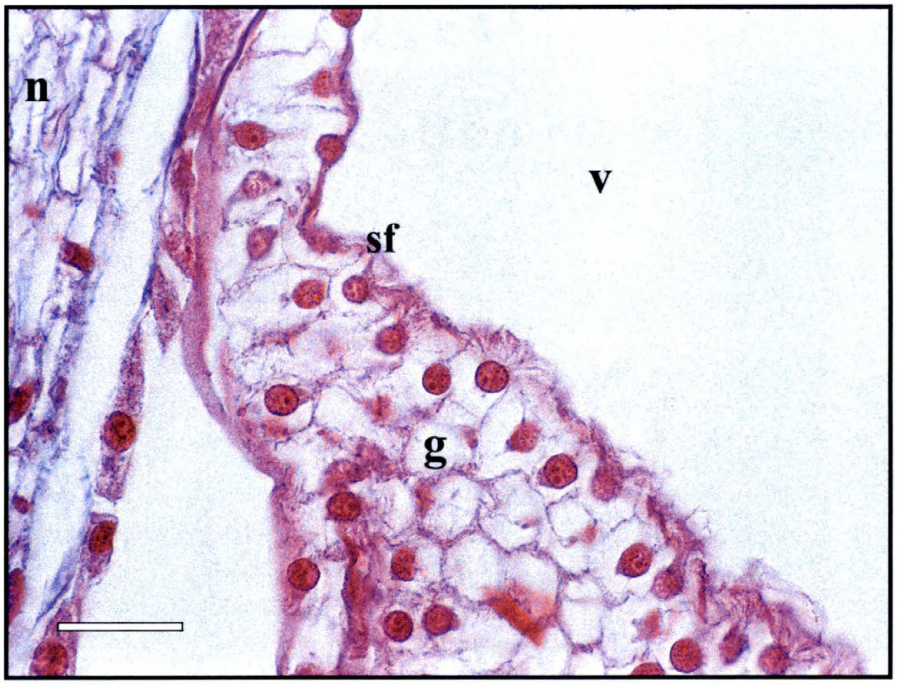

Figure 16. Linked migrating nuclei in cellularizing megagametophyte. (g = gametophyte, n = nucellus, sf = spindle fibers, v = central vacuole; scale = 5 μm)

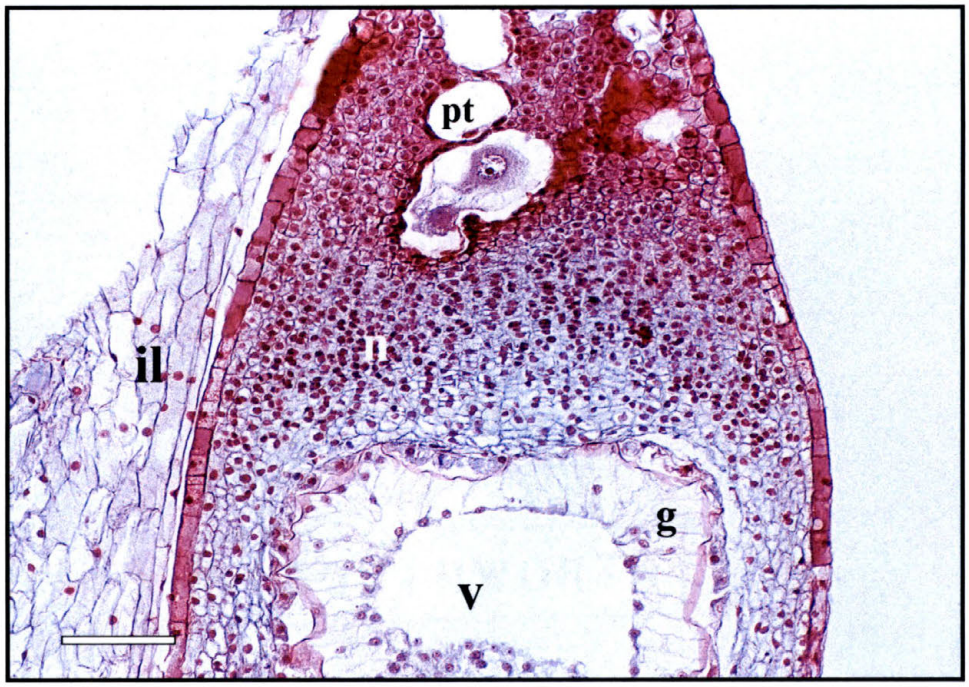

Figure 17. Cellularization of megagametophyte. As the nuclei move they build long open-ended honeycomblike cells called alveoli. Pollen tubes are nearing the upper surface of the gametophyte. (g = gametophyte, il = inner layer of integument, n = nucellus, pt = pollen tube, v = central vacuole; scale = 12 μm)

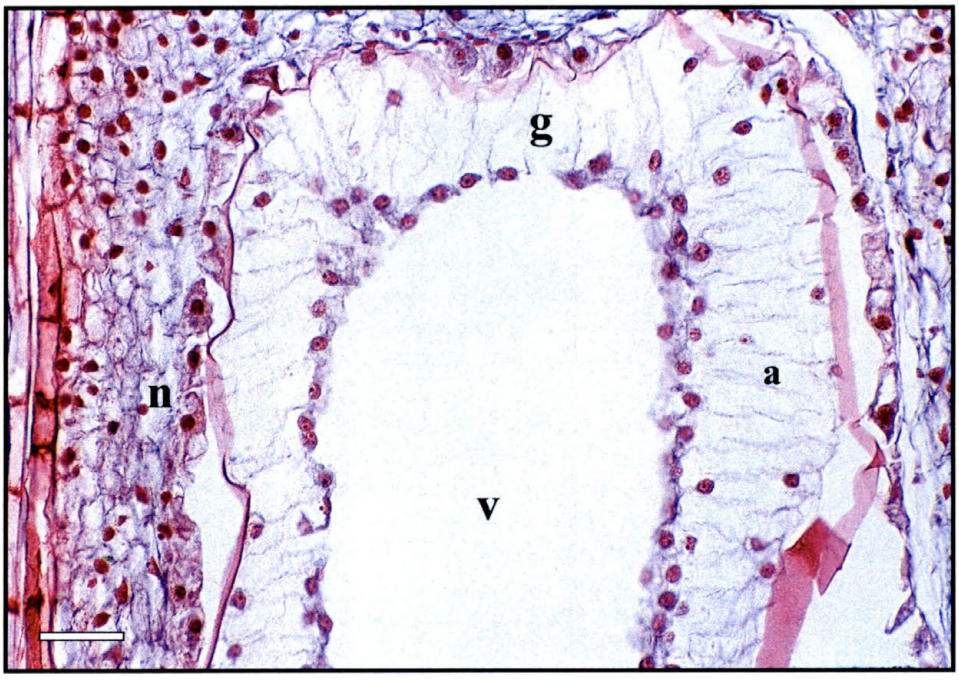

Figure 18. Elongation of alveoli and cell wall formation. Alveoli elongate as their nuclei migrate towards the center of the megagametophyte. Some nuclei are cut off prematurely forming triangular cells called precociously closed alveoli. The small cells are most common at the two ends of the megagametophyte. (a = alveoli, g = gametophyte, n = nucellus, v = central vacuole; scale = 6 μm)

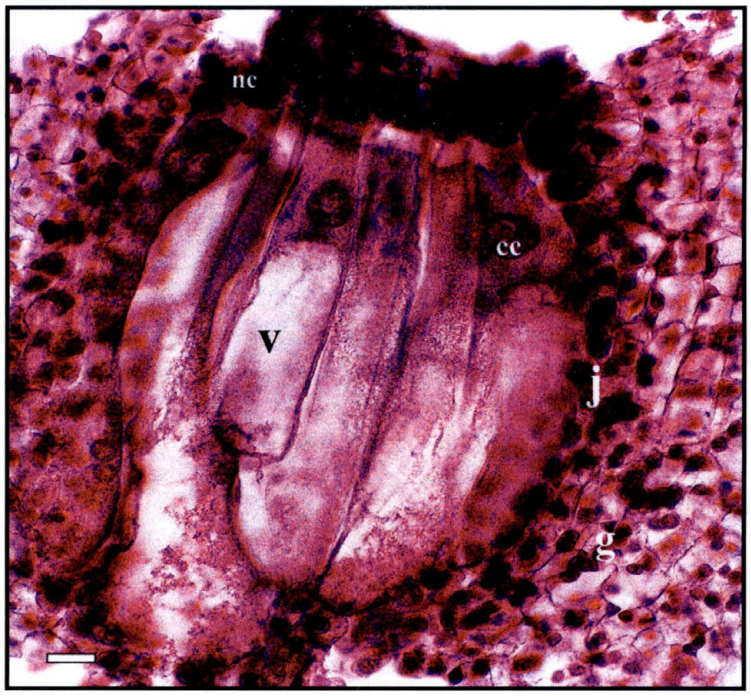

Figure 19. Prefertilization archegonial complex. Neck cells cap each archegonium. The central cell nucleus lies below the neck cells and above the central vacuole. (cc = central cell nucleus, g = gametophyte, j = jacket cell, nc = neck cell, v = central vacuole; scale = 5 μm)

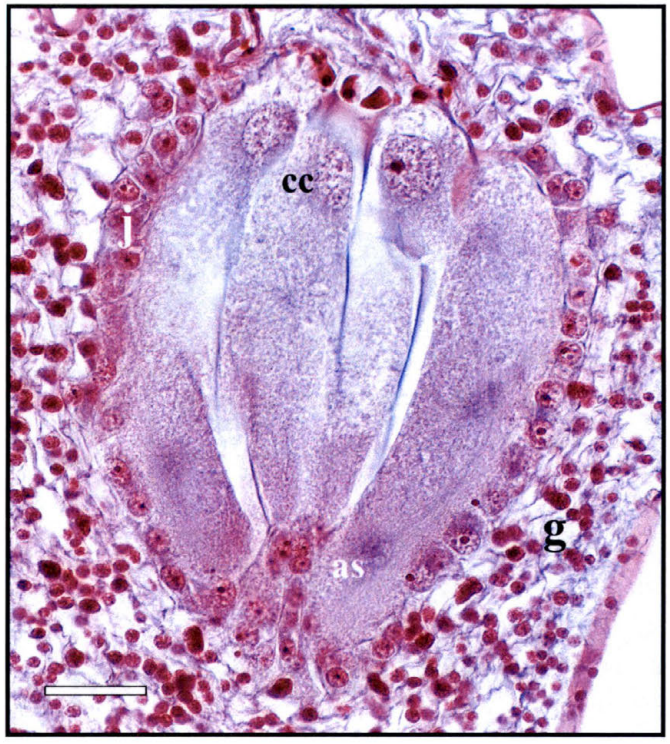

Figure 20. Prefertilization archegonial complex. The central cell nuclei lie below the neck cells. Radiating concentrations of mitochondria and plastids (asteroids) appear as dark areas in the cytoplasm. A jacket of small densely cytoplasmic cells surrounds the archegonial complex. (as = asteroids, cc = central cell nucleus, g = gametophyte, j = jacket cell; scale = 7 μm)

fertilization, the neck cells are very distinct, darkly staining and thick walled (Fig. 21).

As the pollen tubes reach the archegonial chamber the body cell divides forming two equal sperm cells (Johansen 1950; Singh 1964; Sporne 1965; Singh and Johri 1972). This division is approximately concurrent with the division of the central cell nucleus into an egg nucleus and a ventral canal nucleus (Nichols 1910; Chamberlain 1935).

The pollen tube tip breaks down and the two male cells are discharged into the common archegonial chamber where each sperm may enter a different archegonium (Lawson 1907; Nichols 1910; Konar and Moitra 1980). In a single chamber as many as twelve male gametes have been observed (Biswas and Johri 1972).

The central cell

The central cells of the archegonium are narrow and elongate. They reach their maximum length as they differentiate (Chesnoy and Thomas 1971; Singh 1978). Early on their cytoplasm is sparse, thin and highly vacuolate. This has been termed the "foam stage" of the archegonium (Nichols 1910; Chesnoy and Thomas 1971). An archegonial complex has a fusiform shape, widest at the middle and tapering at both ends.

The central cell nucleus lies just below the neck cells in dense cytoplasm (Nichols 1910; Mathews 1939). As the central cell develops, multiple vacuoles coalesce to form a single large central vacuole that occupies the bulk of the cell below the nucleus with the cytoplasm lying in a threadlike layer around the

perimeter of the vacuole (Nichols 1910; Mathews 1939).

"Asteroids," areas of radiating mitochondria and leucoplasts around a center of ribosomes and microtubules (Nichols 1910; Chesnoy 1967; Chesnoy and Thomas 1971; Biswas and Johri 1997), may be seen in the cytoplasm below the nucleus and vacuole (Figs. 20, 22, 23, 24). Organelles organized in this fashion are a distinctive character of some of the Cupressaceae (Chesnoy and Thomas 1971). They have been observed in *Juniperus* (Nichols 1910; Chesnoy 1967), *Biota* (Chesnoy 1969, 1971) and *Taxodium* (Coker 1903), but in no other families of conifers. Chesnoy (1967) suggested that asteroids are centers of organelle replication.

An archegonial complex containing more than five archegonia (Figs. 19) exhibits the characteristic shape with neck cells at the narrow micropylar end of each archegonium. The central cell nuclei are embedded in thick cytoplasm directly below the neck tier and below this large vacuoles occupy the middle of the central cells. A single-celled jacket layer surrounds the archegonial complex. Above the neck cells is the lower border of an archegonial chamber.

Oogenesis

Division of the central cell nucleus into the ventral canal cell nucleus and the egg nucleus occurs just prior to fertilization (Chamberlain 1935; Mathews 1939; Maheshwari and Singh 1967). The smaller ventral canal nucleus is located above the much larger lower egg nucleus. The ventral canal nucleus typically disappears soon after its formation (Mathews 1939). With this final division the vacuole diminishes and disappears and the egg cell fills with cytoplasm

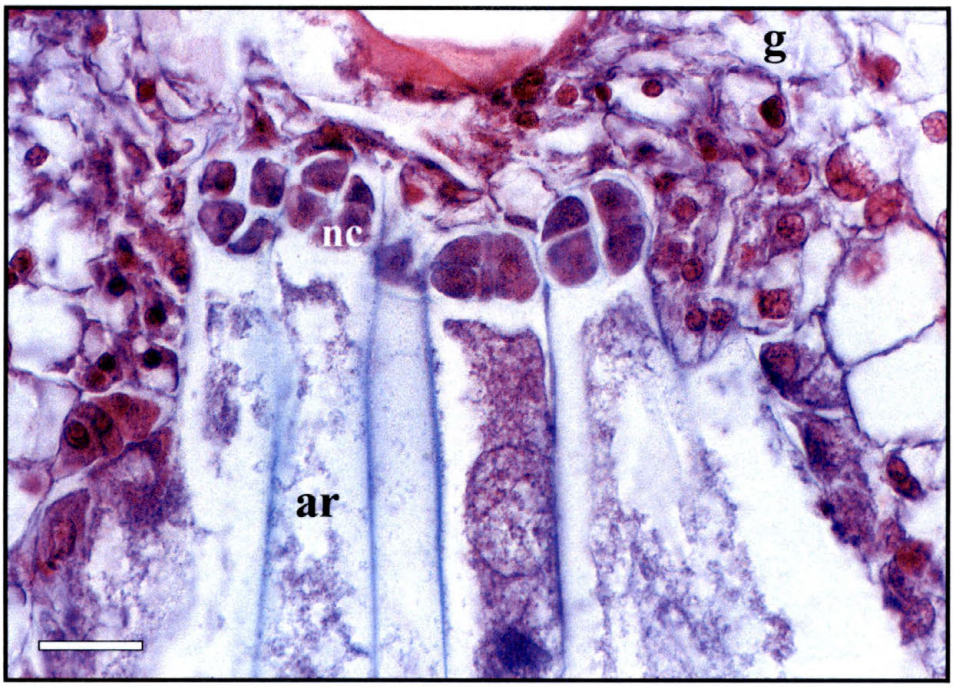

Figure 21. Neck cells at micropylar end of archegonium. Four neck cells are produced by the division of the neck cell initial. (ar = archegonium, g = gametophyte, nc = neck cell; scale = 4 μm)

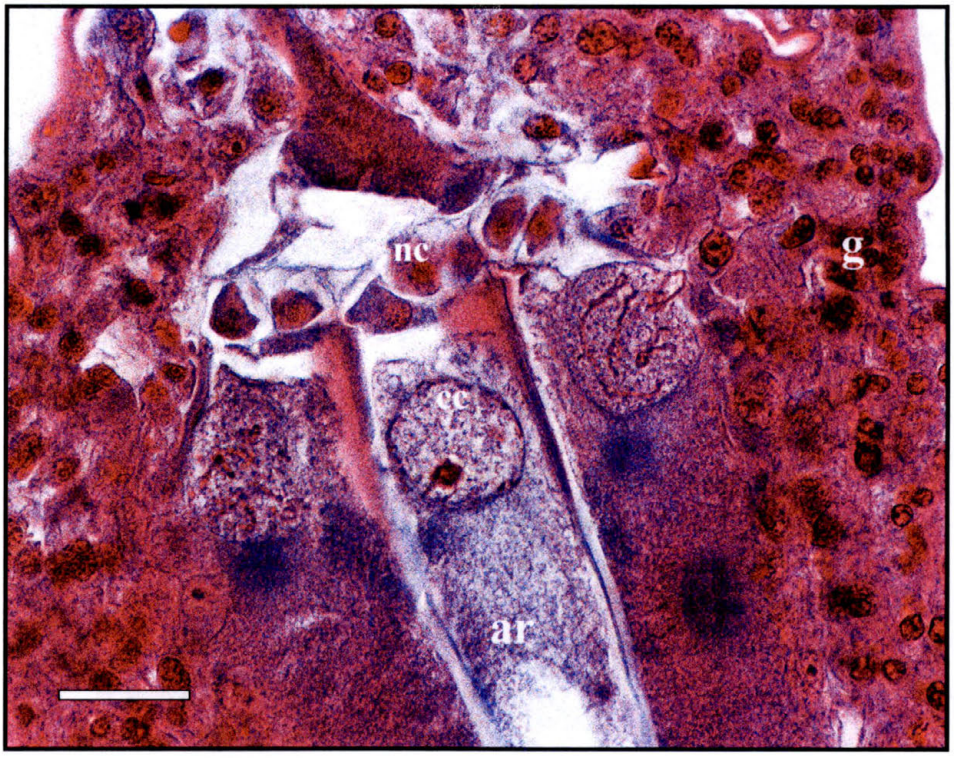

Figure 22. Division of the central cell nucleus. The central cell nucleus divides to produce an egg nucleus and a ventral canal nucleus that degenerates. Chromosomes are present in the dividing central cell nuclei. (ar = archegonium, cc = central cell nucleus, g = gametophyte, nc = neck cell; scale = 4 μm)

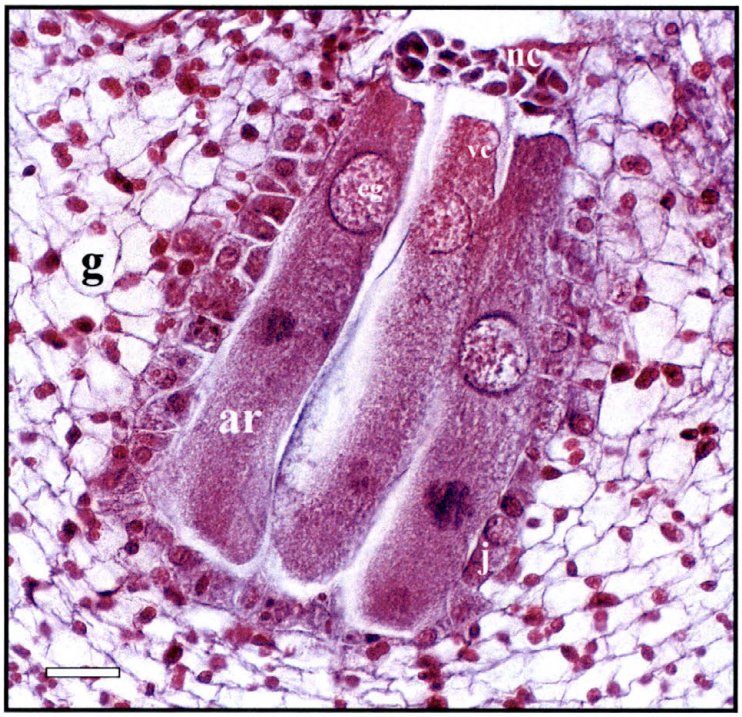

Figure 23. Mature prefertilization archegonial complex. The vacuole disappears and the egg nucleus enlarges and moves towards the center. (ar = archegonium, eg = egg nucleus, g = gametophyte, j = jacket cell, nc = neck cell, vc = ventral canal nucleus; scale = 4 μm)

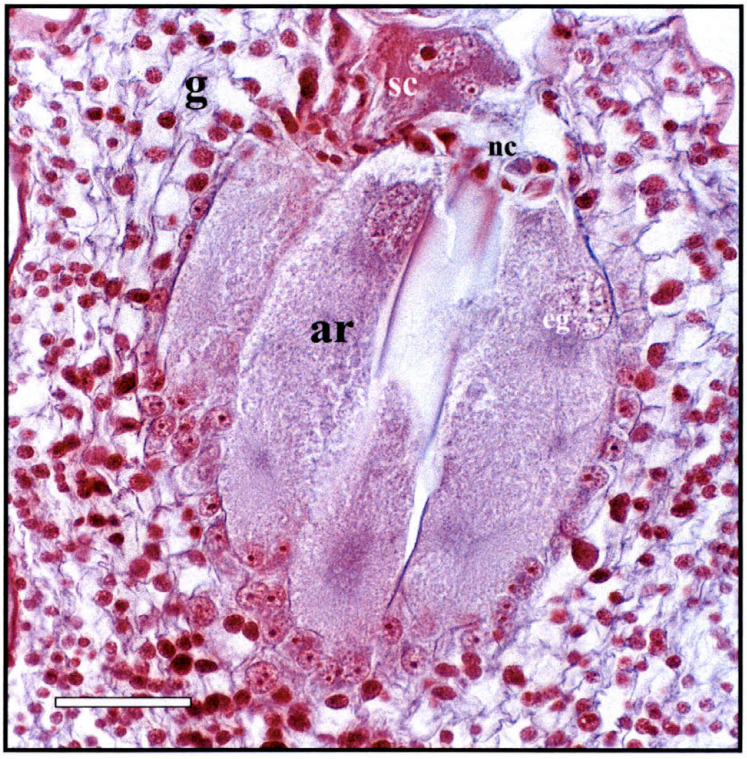

Figure 24. Sperm cell entering archegonium. Neck cells facilitate sperm entry into the archegonium. Sperm nuclei are surrounded by a dense cytoplasmic covering which enters the archegonium. (ar = archegonium, eg = egg nucleus, nc = neck cells, sc = sperm cell; scale=7μm)

(Chesnoy and Thomas 1971).

As the archegonia matures, its cytoplasm becomes increasingly dense and abundant (Mathews 1939; Chesnoy and Thomas 1971). This division occurs rapidly and simultaneously in all the archegonia of a complex (Fig. 22). A mature archegonia (Fig. 23) of *J. ashei* consists of a large egg nucleus near the center of the cell in dense cytoplasm without vacuoles.

Fertilization

Sperm cells are variable in shape with a prominent nucleus and dense cytoplasm (Mehra and Malhotra 1947). As the sperm advances through the neck cells into the archegonium (Figs. 24, 25), its membrane slips off and only the nucleus and its perinuclear cytoplasm enter (Chamberlain 1935; Johansen 1950; Chesnoy and Thomas 1971). Nichols (1910) says that in *J. communis* the sperm nucleus is one fourth the size of the egg nucleus. In *J. ashei* the sperm nucleus is about one half or less the size of the egg nucleus (Fig. 26).

Leaving a vacuolate trail, the sperm nucleus and its attached layer of cytoplasm migrate downward through the maternal cytoplasm (Fig. 26). The paternal perinuclear cytoplasm can be seen as a darkly staining area around the sperm nucleus (Fig. 26).

The egg nucleus rests just above or at the middle of the archegonium (Martin 1950; Konar and Moitra 1980). As the sperm nucleus reaches the egg nucleus, the two nuclei settle into very close proximity. At first a very thin layer of cytoplasm persists between the two nuclei, but it disappears as they rotate, fuse, and sink to the bottom of the archegonium (Sugihara 1938; Looby and Doyle

1940; Chesnoy and Thomas 1971). The perinuclear cytoplasm associated with the sperm nucleus expands and surrounds the fusion nucleus or zygote as it rests at the bottom of the archegonium (Mathews 1939; Chesnoy and Thomas 1971).

In Figure 27 a binucleate embryo surrounded by a halo of darkly staining neocytoplasm rests at the chalazal end of an archegonium. The "neocytoplasm" that envelopes the zygote, and later the free nuclear embryo after mitosis, is largely derived from the perinuclear cytoplasm surrounding the sperm nucleus (Looby and Doyle 1940; Willemse and Linskens 1969; Singh 1978). The remaining maternal cytoplasm in the archegonium degenerates and ultimately disappears (Chesnoy and Thomas 1971).

Working with the cupressid genus *Biota*, Chesnoy and Thomas (1971) determined that the paternal neocytoplasm transmits mitochondria, amyloplasts, cytoplasmic RNA, and other organelles to the embryo. It is probable that junipers follow this pattern, that of paternal origin of most cellular organelles.

Embryogeny

Conifers are polyembryonic (Webber 1940; Singh 1978; Biswas and Johri 1997). They produce multiple embryos within a single ovule but the universal rule is that the mature seed contains only one fully developed embryo (Singh 1978). Two types of polyembryogeny are found among conifers: simple polyembryony where fertilization of more than one egg leads to the development of multiple zygotes and cleavage polyembryony in which a single zygote forms multiple embryos by splitting, lobing, or budding (Buchholz 1926; Thomson 1945;

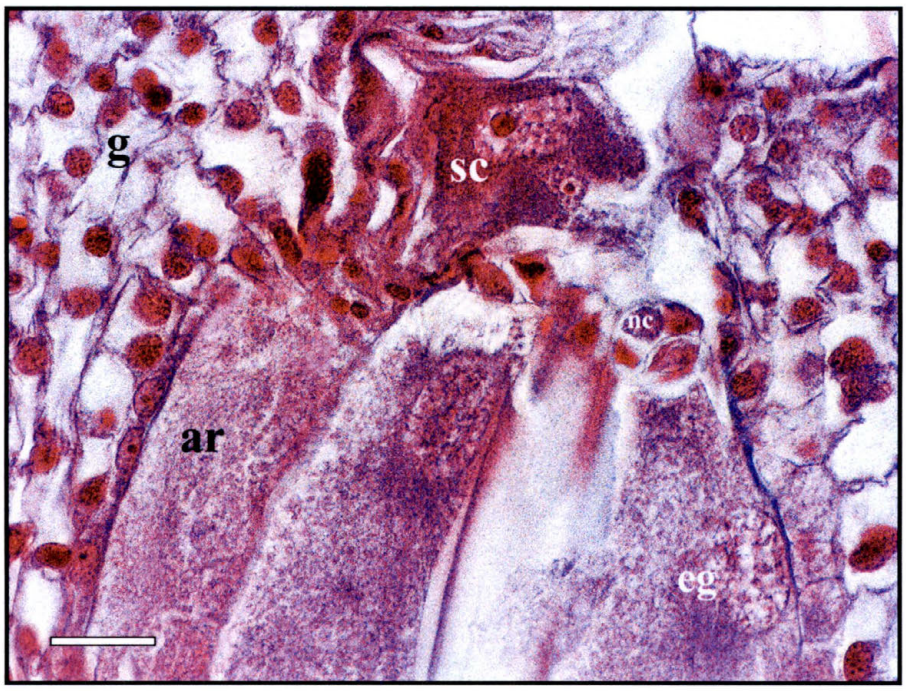

Figure 25. Sperm entering archegonium. (ar = archegonium, eg = egg nucleus, g = gametophyte, nc = neck cells, sc = sperm cell; scale = 7 μm)

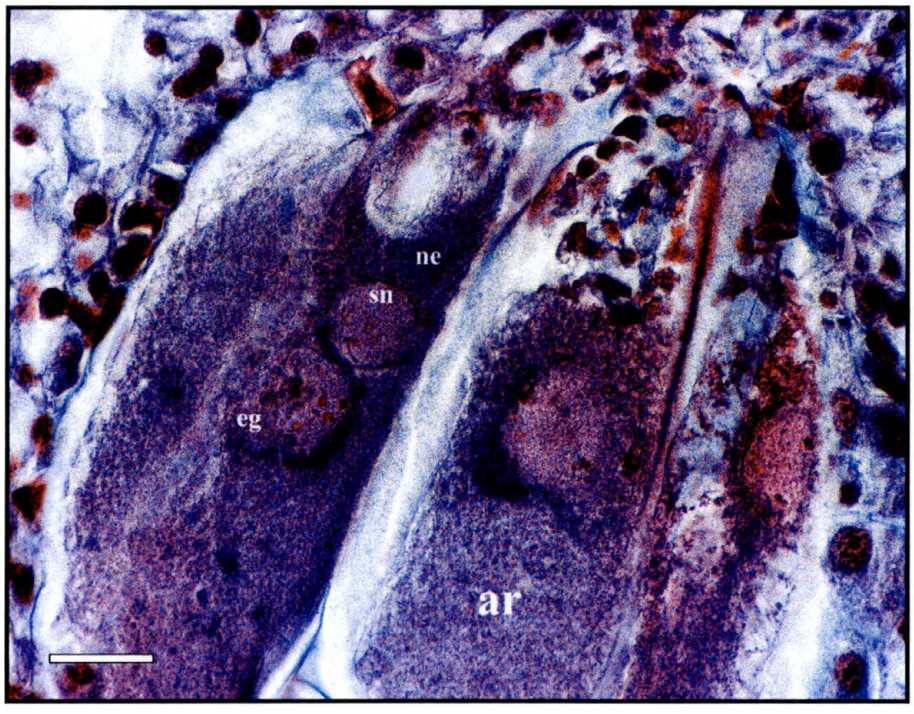

Figure 26. Sperm nucleus near egg nucleus inside archegonium. The sperm nucleus enters the archegonium surrounded by a dense layer of neocytoplasm. (ar = archegonium, eg = egg nucleus, ne = neocytoplasm, sn = sperm nucleus; scale = 3 μm)

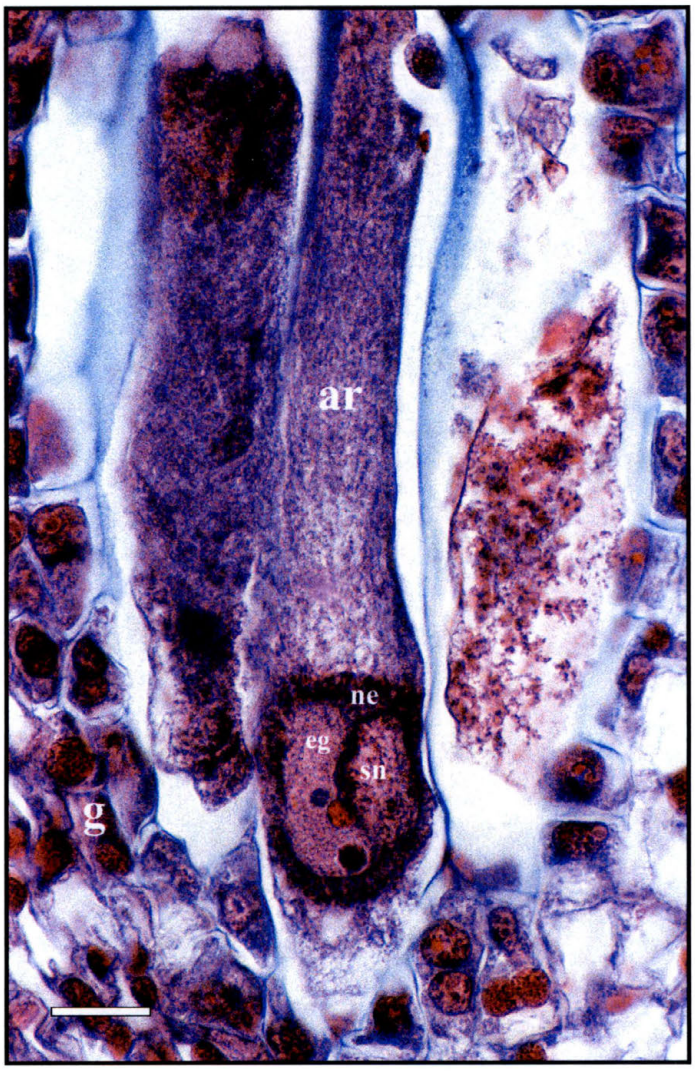

Figure 27. Egg and sperm nuclei at the base of archegonium. The fusing nuclei are located at the bottom of the archegonium surrounded by a dark halo of neocytoplasm. (ar = archegonium, eg = egg nucleus, g = gametophyte, ne = neocytoplasm, sn = sperm nucleus; scale = 3 μm)

Dogra 1967; Singh 1978). The resulting embryos in simple polyembryony may not be genetically identical but in cleavage polyembryony the multiple embryos from a single zygote are clonal.

Junipers have a unique variant of cleavage polyembryony that generates very large numbers of embryos (Cook 1939; Mathews 1939; Doyle and Brennan 1972). Since several to all the archegonia, perhaps as many as eight to ten in junipers, in a complex may be fertilized, the number of zygotes produced is highly variable. With cleavage polyembryony the number of embryonic initials produced per zygote is variable and can be large so the number of embryos an ovule may contain is potentially very great, 2 to 40 or more (Cook 1939; Martin 1950). In junipers each of the eight cells in the suspensor and embryonic tiers of each zygote is a possible embryonic initial (Cook 1939; Mathews 1939).

In either type of polyembrony the embryonic units compete with each other to become the single embryo in the mature seed (Buchholz 1926; Clare and Johnstone 1931). Depending on the type of polyembryogeny, as few as two or three or as many as 30 or 40, embryos may be in competition (Buchholz 1926). As a rule most competitive elimination of embryos occurs in the early stages of development. Competition lessens as development progresses and the number of competing embryos decreases. Finally the leading embryo, the one which has penetrated deepest into the gametophyte (nearest the chalazal end), enlarges and achieves sporophyte status and all other remaining potential embryos degenerate (Buchholz 1926; Clare and Johnstone 1931; Webber 1940; Johansen 1950).

Singh (1978) proposed a three part sequence of embryo development in

gymnosperms: 1. Proembryogeny, all the stages before elongation of the suspensors (Doyle 1963), 2. Early embryogeny, all stages, including elongation of the suspensor and before establishment of the root generative meristem (Sterling 1949), and 3. Late embryogeny, from establishment of the root and shoot meristems through maturation of the embryo.

Junipers adhere closely to the typical basal proembryogenic plan for conifers (Nichols 1910; Doyle 1963) but diverge significantly in their early embryogeny as described by Cook in 1939. Late embryogeny and maturation of the embryo is fairly uniform across all conifer species.

Proembryogeny

The first division of the zygote produces two nuclei which lie close together at the foot of the archegonium (Fig. 27). The nuclei undergo synchronous mitotic divisions (Fig. 28) producing eight coenocytic nuclei lodged at the base of the archegonium surrounded by dense neocytoplasm (Doyle 1963; Chesnoy and Thomas 1971). The eight nuclei are loosely arranged in two tiers, most often four in each but sometimes five in one tier and three in the other (Cook 1939).

Wall fabrication occurs at the eight-celled stage of the proembryo (Cook 1939; Doyle 1963; Vasil and Sahni 1964). After wall formation the proembryo consists of eight cells in two tiers: the cells of the primary upper tier remain open at their micropylar ends to the cytoplasm of the archegonium (Johansen 1950; Dogra 1967), while the three to five cells in the lower primary embryonic tier are fully enclosed (Nichols 1910; Mathews 1939; Dogra 1967).

The cells in the primary upper tier divide again anticlinally yielding an upper open tier and a lower fully enclosed suspensor tier (Johansen 1950; Wardlaw 1955; Doyle 1963). Thus the mature proembryo of *Juniperus* consists of about 12 cells grouped in three ranks: an upper open tier, a middle suspensor tier, and a basal embryonic tier. The upper tier does not participate further in the development of the embryo (Johansen 1950).

Early embryogeny

At the twelve-celled three tier stage the suspensor cells of the proembryo begin to elongate pushing the embryo mass out of the archegonium into the gametophyte tissue (Cook 1939; Mathews 1939; Biswas and Johri 1997). Soon thereafter the cells of the embryonic tier begin to elongate. In junipers these cells elongate like suspensors and are called embryonic suspensors or suspensor tubes (Cook 1939; Singh 1978). They do not, at first, carry embryo initials at their tips.

Due to differential rates of elongation, cells of the suspensor tier can overtake the cells of the embryonic suspensor tier. Thus suspensor tier cells may eventually have the opportunity to generate embryo initials at their tips by lobing or budding if they overtake the embryonic suspensors. However due to their original advantageous position the embryonic suspensor cells generally maintain a competitive advantage and drive rapidly and independently into the gametophyte each contending to produce the final embryo (Cook 1939).

In both suspensor tier and embryonic suspensor tier cells the bulk of the elongate cell is empty with the cytoplasm and nuclei concentrated at the chalazal end (Mathews 1939; Martin 1950; Vasil and Sahni 1964). As the growing

embryonic suspensor cells reach a certain length (15 to 20 microns) (Cook 1939) the nucleus divides and a new cell is cut off at the tip. This new cell may also elongate becoming another embryonal suspensor which will elongate in turn, always pressing deeper into the gametophyte. As many as five or six tiers of embryonic suspensor cells may be added to each autonomous chain of cells (Mathews 1939; Doyle and Brennan 1972). This whole mass, suspensor cells and several tiers of embryonic suspensor cells, twists and digests its way deep into the gametophyte (Owens and Molder 1975). The earlier formed cells, suspensors and embryonic suspensors, collapse as the entire mass lengthens (Johansen 1950). Additionally the embryonic suspensor cells may form several lobes at their tips and each lobe may initiate a new line of embryonic suspensor cells (Cook 1939; Wardlaw 1955).

Corrosion cavity

In the gametophyte tissue below the archegonial complex a rich deposit of starch develops, the corrosion region. It occupies the central portion of the gametophyte (Roy Chowdhury 1962; Dogra 1967). The embryo mass is pushed into this region by the elongation of the various suspensor tiers. If elongation out paces digestion the lengthening cells of the embryo complex will twist forming a coiled suspensor mass (Roy Chowdhury 1962). The embryonic suspensor cells secrete enzymes that disintegrate and liquefy the cells of the corrosion region. The cells of the embryo complex absorb these products (Håkansson 1956; Dogra 1967) creating a corrosion cavity which provides the space in which the final embryo will grow and mature (Mathews 1939; Singh and Johri 1972). The

peripheral tissue of the gametophyte becomes a nutrient storage region utilized during maturation of the embryo and germination of the seed (Dogra 1967).

Eventually a single or a few embryonic suspensor cells delve more deeply than all others into the gametophyte. The leading cell now cuts off an embryo initial at its tip which quickly gives rise to a small multicelled embryo. So in *Juniperus* an embryo initial cell is not created until several, frequently four or more, layers of suspensors/embryonic suspensors have been produced (Cook 1939; Doyle and Brennan 1972; Singh 1978). The cells on the outside and those farthest from the embryo tip elongate backwards forming a large secondary suspensor that pushes back the last few competing embryos (Cook 1939; Mathews 1939; Wardlaw 1955). Ultimately a single embryo emerges from the competition with perhaps as many as 30 of its fellows and begins to enlarge to become the new sporophyte.

The suspensors and embryonic suspensors are elongating and are pushing into the corrosion region of the gametophyte (Fig. 29). Elongation of the suspensor cell mass is more advanced in Fig. 30. Many cells have already fallen behind the leaders. A long section of a complete gametophyte and embryo system (Fig. 31) reveals the irregular structure of the mature gametophyte. The remaining corrosion region is easily identified as an area of darkly staining cells in the center of the gametophyte below the corrosion cavity. The embryo complex is lengthening and hollowing out a corrosion cavity from the corrosion region.

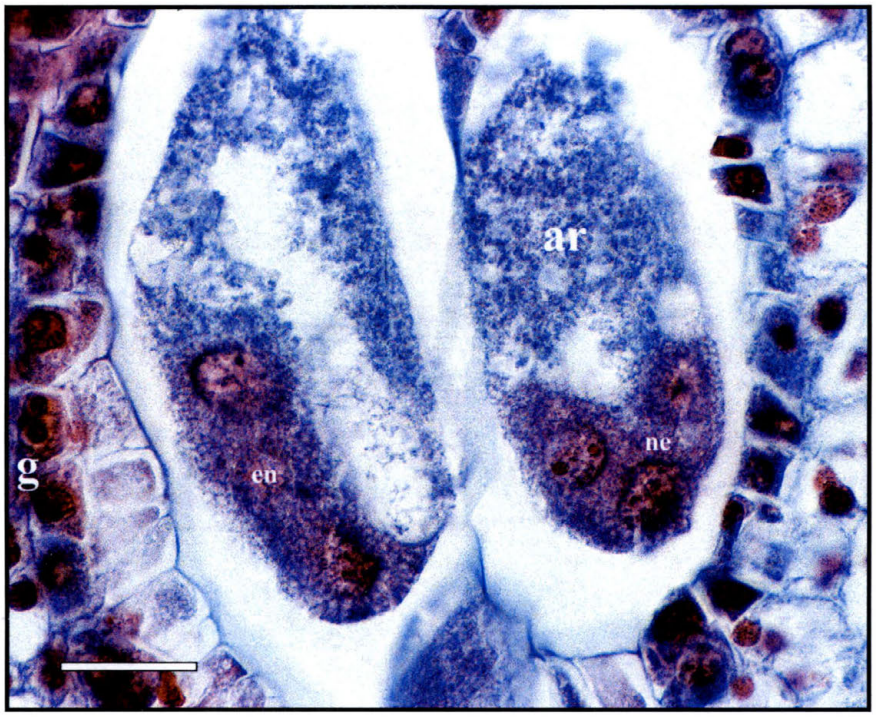

Figure 28. Two four-celled proembryos. Prior to cell wall formation the free nuclei of the proembryo are positioned at the base of the archegonium enveloped in neocytoplasm. (ar = archegonium, en = embryonic nucleus, g = gametophyte, ne = neocytoplasm; scale = 3 μm)

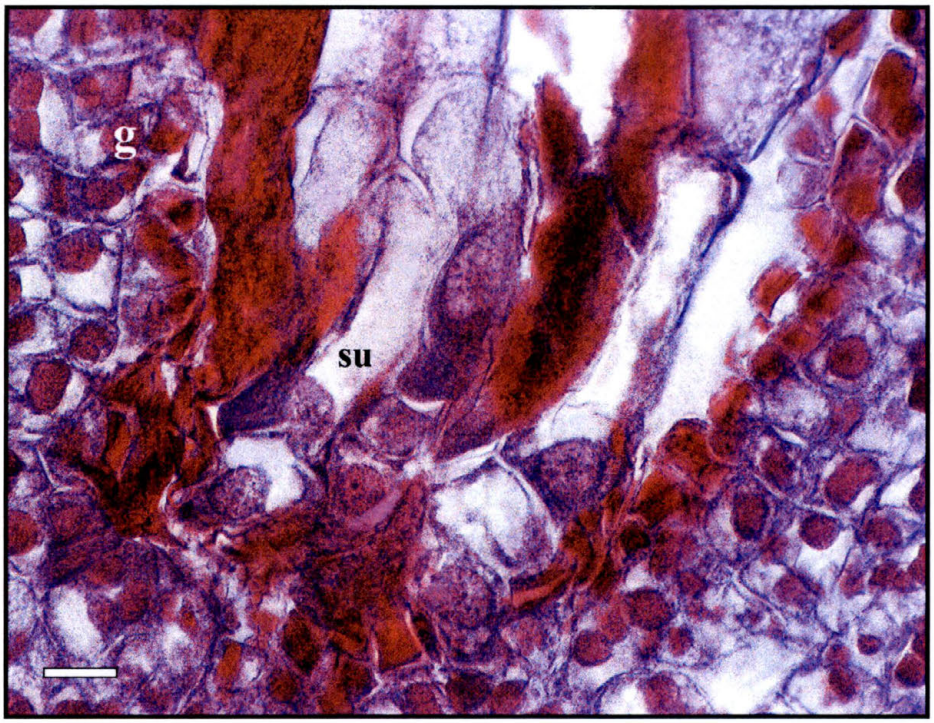

Figure 29. Early suspensor development. After the proembryo cellularizes the suspensor tier begins to elongate pushing out of the archegonium into gametophyte tissue. (g = gametophyte, su = suspensor cell; scale = 3 μm)

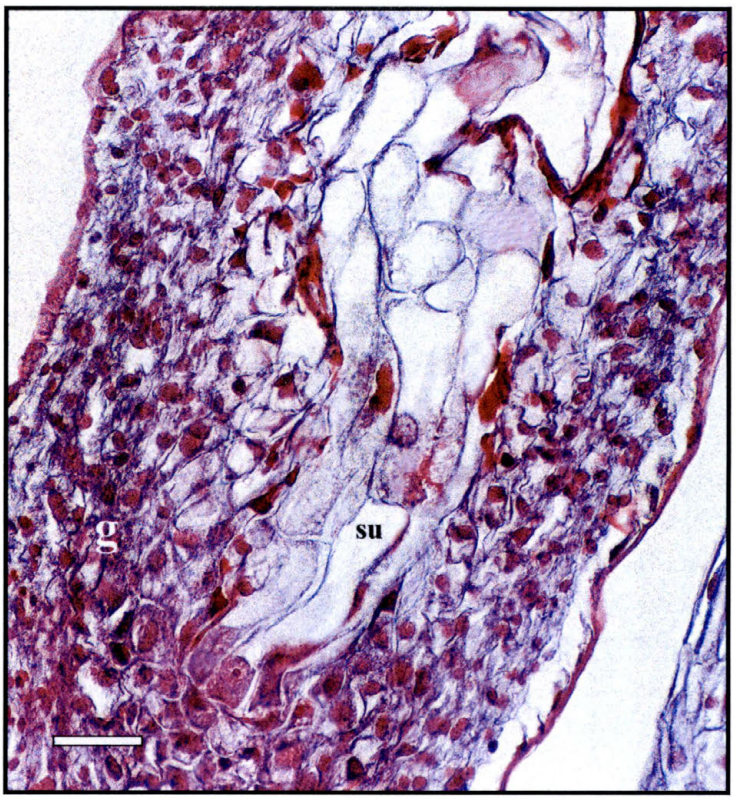

Figure 30. Suspensor development. As suspensors/embryonic suspensors divide and elongate the mass of embryonic cells pushes deeper into the gametophyte tissue. (g = gametophyte, su = suspensors; scale = 5 μm)

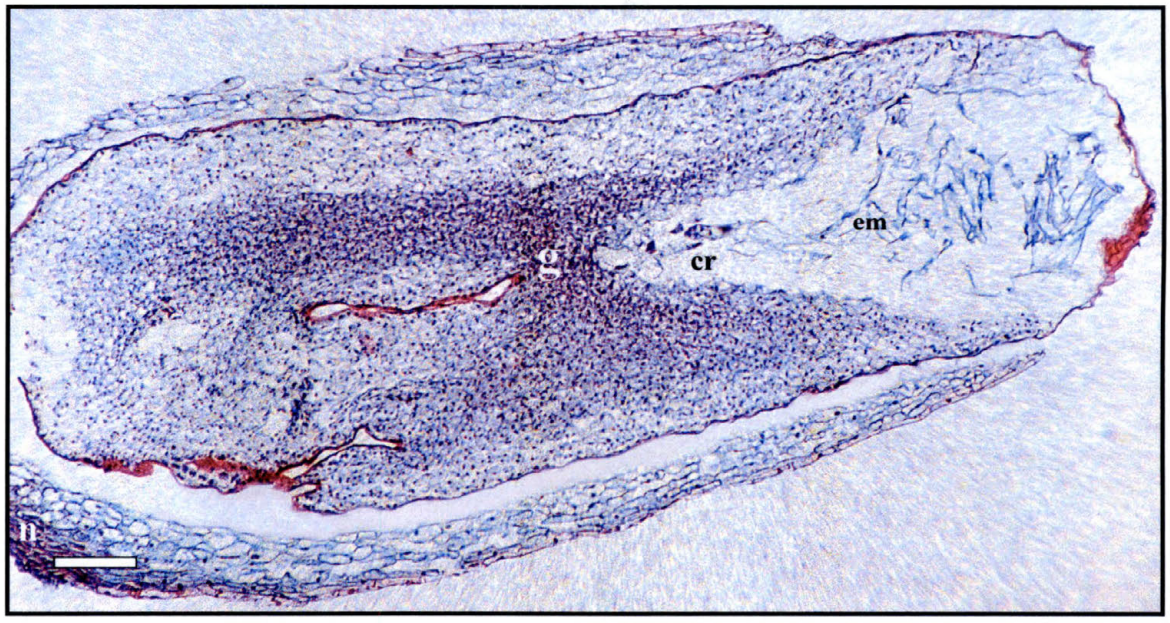

Figure 31. Megagametophyte with embryo/suspensor complex. As the suspensors lengthen and plunge ever deeper into the megagametophyte, gametophyte tissue degenerates to form a corrosion cavity. (cr = corrosion cavity, em = embryo/suspensor mass, g = gametophyte, n = nucellus; scale = 40 μm)

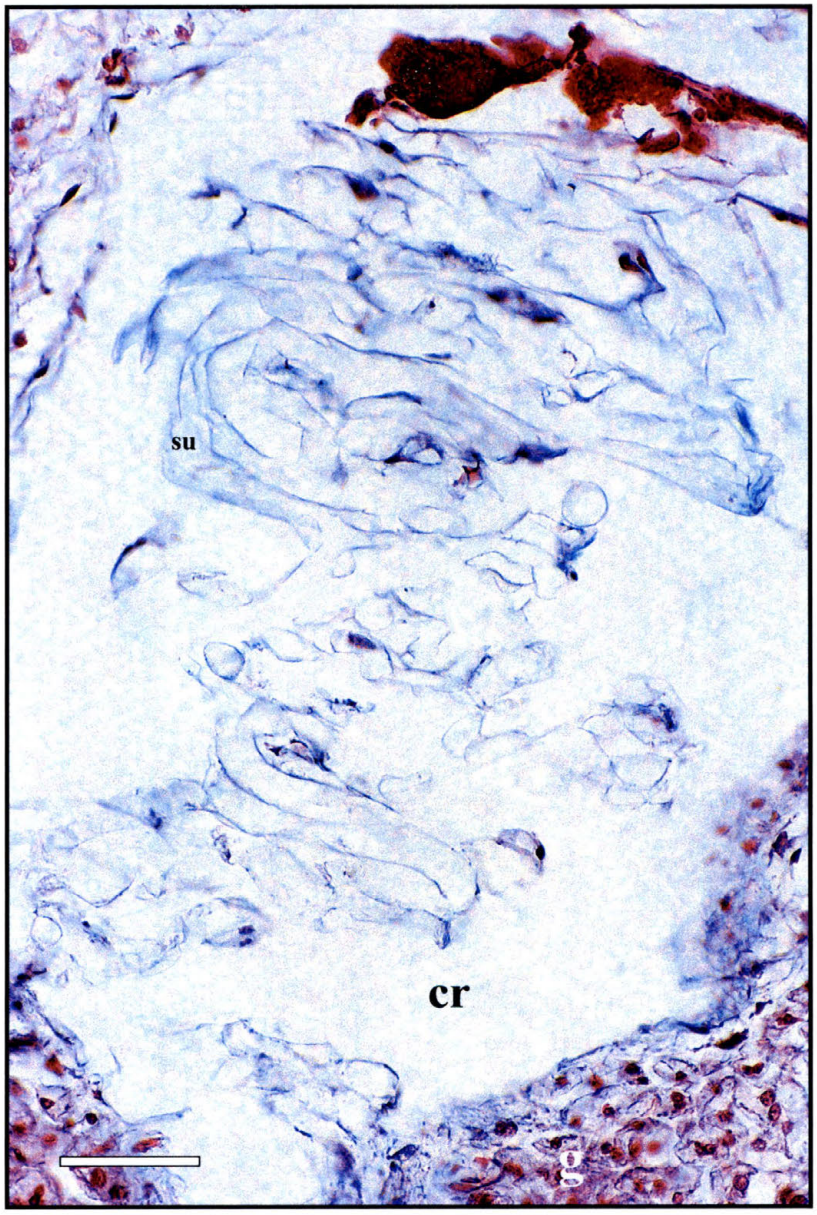

Figure 32. Suspensor/embryonic suspensor system. The elongating suspensors/embryonic suspensors twist and cross as they push into the megagametophyte carving out a corrosion cavity. (cr = corrosion cavity, g = gametophyte, su = suspensor/embryonic suspensor cells; scale = 8 μm)

Most of the length of a suspensor/embryonic suspensor cell is empty (Fig. 32). In the twisted suspensor mass are collapsed suspensor/embryonic suspensor cells and their marooned nuclei. The number of cells in the mass decreases from the archegonial end towards the chalazal, as suspensor/embryonic suspensor cells fall behind the leader and are eliminated. These cells and their potential embryos have lost the struggle.

Figure 33 is the magnified tip of an embryonic suspensor. This suspensor has formed lobes and one lobe has produced an embryo cell. The embryo initial has already established multicellularity.

Late embryogeny

In the undifferentiated young embryo mass, the positions of individual cells determine their eventual fate (Martin 1950). Two regions become established: a proximal region contiguous with the embryonal suspensors which is the forerunner of the root apex and a distal region which will form the hypocotyl-shoot apex (Spurr 1949; Singh 1978).

The distal portion consists of a comparatively homogeneous mass of cells, symmetrical and with a free apex (Spurr 1949). It is organized somewhat like the meristem of a shoot with deeply staining small cells containing relatively large nuclei (Singh 1978). A juncture zone or discontinuity separates the distal region from the proximal region where the rows of cells may early on show hemispherical bending as the root apex is established (Spurr 1949; Martin 1950; Singh 1978). Anchored by the root-suspensor linkage the embryo grows towards the basal or chalazal end of the ovule gradually filling in the corrosion cavity

(Håkansson 1956).

The two regions differentiate simultaneously. The portion above the shoot root juncture, the distal region, begins to differentiate into the hypocotyl-shoot axis (Spurr 1949). The apex broadens and develops shoulders from which two cotyledon primordia arise. In the Cupressaceae most species possess two cotyledons (Butts and Buchholz 1940). The apex between the cotyledons is a flat to conical mound of meristematic tissue – the epicotyl (Singh 1978; Biswas and Johri 1997). In the proximal region the root apex grows and establishes a radicle and a root cap.

Approaching maturity, the embryo thickens, the cotyledons, hypocotyl, and radicle all enlarge, completely filling the corrosion cavity. A single layered epidermis forms around the embryo. Internally, a procambium develops and differentiates into protoxylem and protophloem. Well-developed vascular strands are present in the radicle, hypocotyl, and each cotyledon (Singh 1978).

As the embryo is maturing the stored nutrient materials in the gametophyte fuel embryonic growth. Initially three layers (Håkansson 1956) are present in the gametophyte: Cells bordering the corrosion cavity that contain no starch, a middle layer whose cells contain starch and an outer layer rich in lipids and protein (Singh and Chatterjee 1963; Konar and Moitra 1980).

Konar (1958) suggests that the innermost cell layer is the site of synthetic reactions that provide needed molecules to the rapidly growing and developing embryo. Near maturity the inner two layers disappear. Only the lipid rich outer layer of the gametophyte remains (Owens and Molder 1975). At seed maturity

the lipid and protein content of the embryo and gametophyte are about the same (Konar 1958).

By early September the embryo has established a distal hypocotyl region and a proximal root region (Figs. 34, 35). Indistinct remnants of the suspensor system and the outline of the corrosion cavity can be distinguished. The gametophyte is organized into layers. High levels of metabolic activity and disintegrating cells mark the innermost layer adjacent to the developing embryo as an area of synthesis. The outer layer is rich with nutrient storage products (Fig. 35).

The cotyledons, radicle, and shoot apex are well established by late September (Figs. 36, 37). The gametophyte continues to display zonation but the inner area of high metabolic activity has diminished. The encircling nutrient rich outer layer persists.

The embryo is essentially mature by early to mid October (Fig. 38). Surrounded by an epidermis it has a well developed procambium (Fig. 39) and root cap (Fig. 40). The root cap remains connected to the residue of the suspensor system (Fig.40). The epicotyl/shoot apex is a mound of tissue between the bases of the two cotyledons (Fig. 41). Entirely filling the center of the seed (Fig. 38) the embryo remains enveloped by the persistent outer nutrient rich layer of the gametophyte.

Conclusions

Juniperus ashei completes its reproductive cycle in a single year (Table 1). Pollination occurs between late December and mid February and

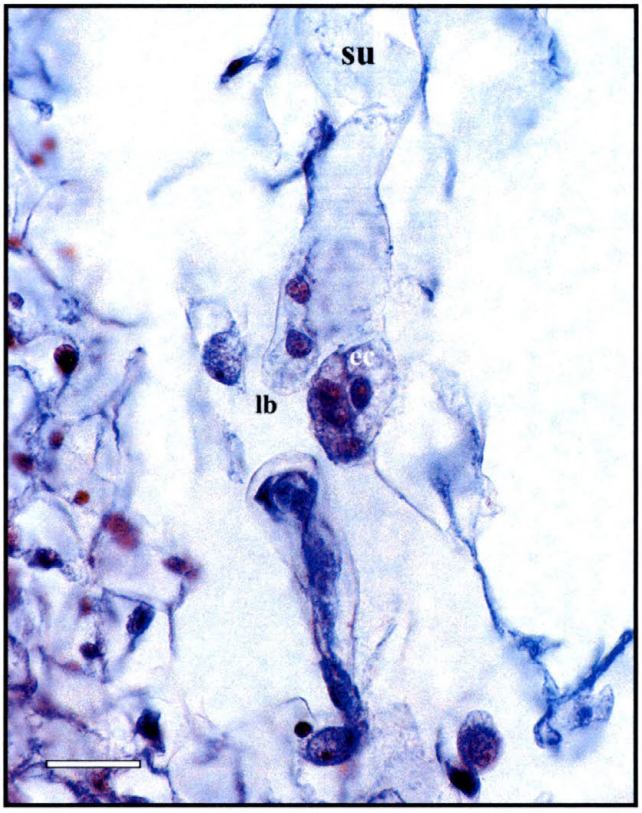

Figure 33. Lobing at tip of suspensor/embryonal suspensor. (ec = embryo cell, lb = lobe, su = suspensor cells; scale = 10 μm)

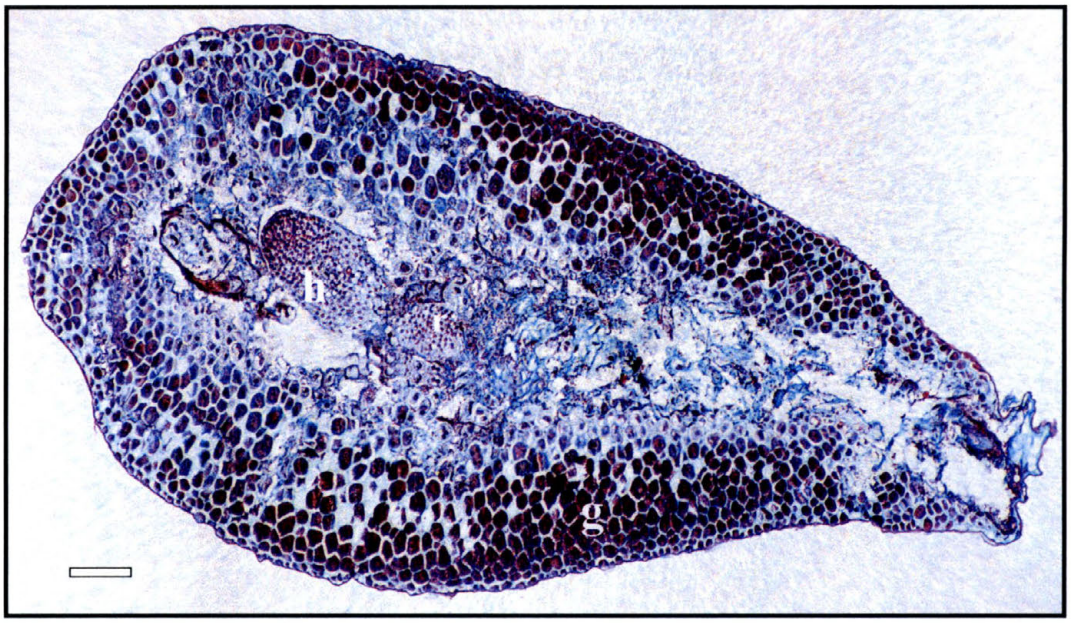

Figure 34. Early embryo development. The cells of the outer layer of the megagametophyte are filled with dark staining lipids. (g = gametophyte, h = hypocotyl, r = radicle; scale = 8 μm)

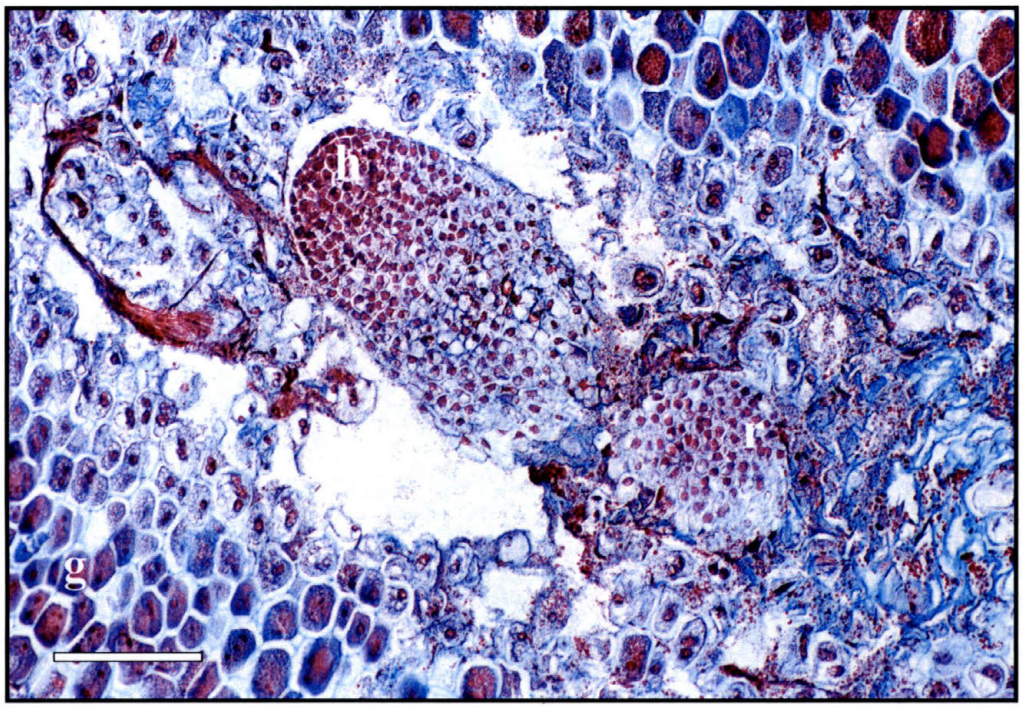

Figure 35. Early embryo development. (g = gametophyte, h = hypocotyl, r = radicle; scale = 8 μm)

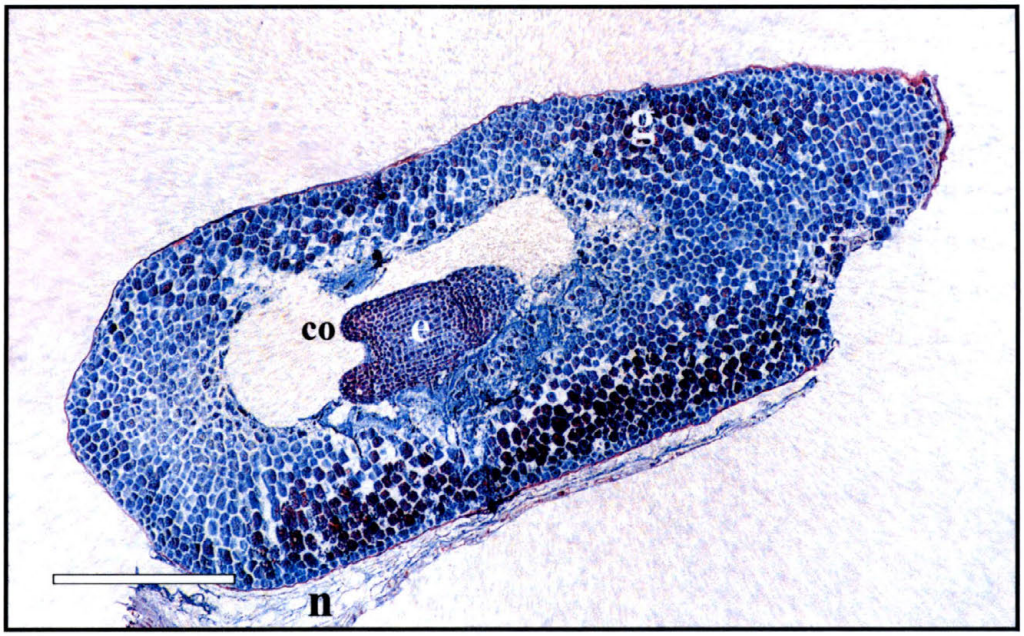

Figure 36. Embryo development. By mid September the embryo is more mature. The two cotyledons are at the chalazal end and the radicle is at the micropylar end of the mega-gametophyte. (co = cotyledon, e = embryo, g = gametophyte, n = nucellus; scale = 26 μm)

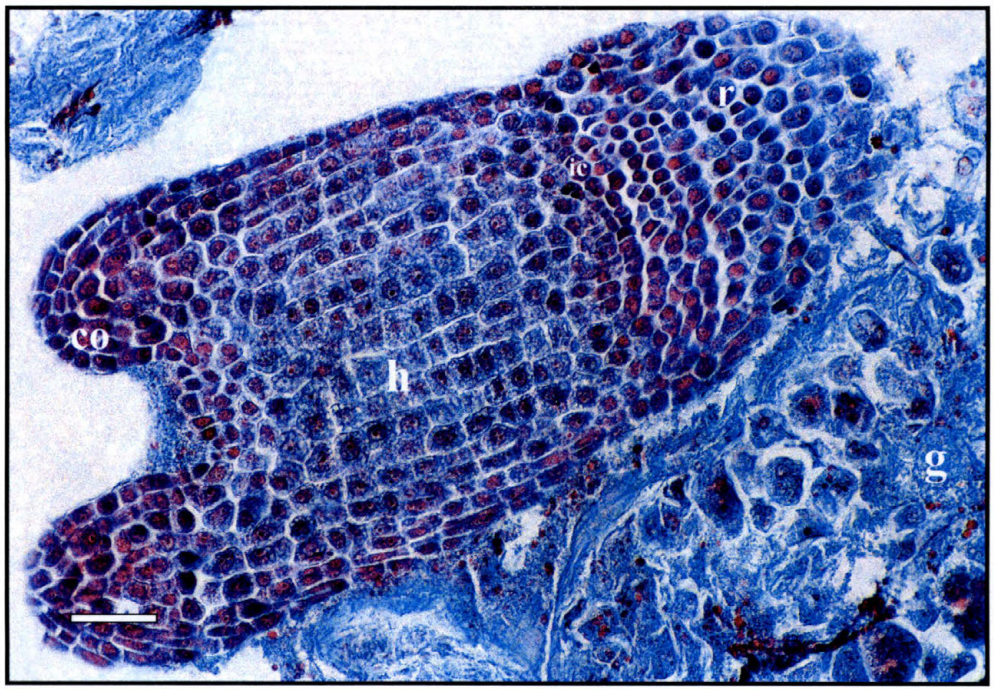

Figure 37. Embryo development. An intercalary meristem separates the hypocotyl from the radicle. (co = cotyledon, g = gametophyte, h= hypocotyl, ic = intercalary meristem, r = radicle; scale = 5 μm)

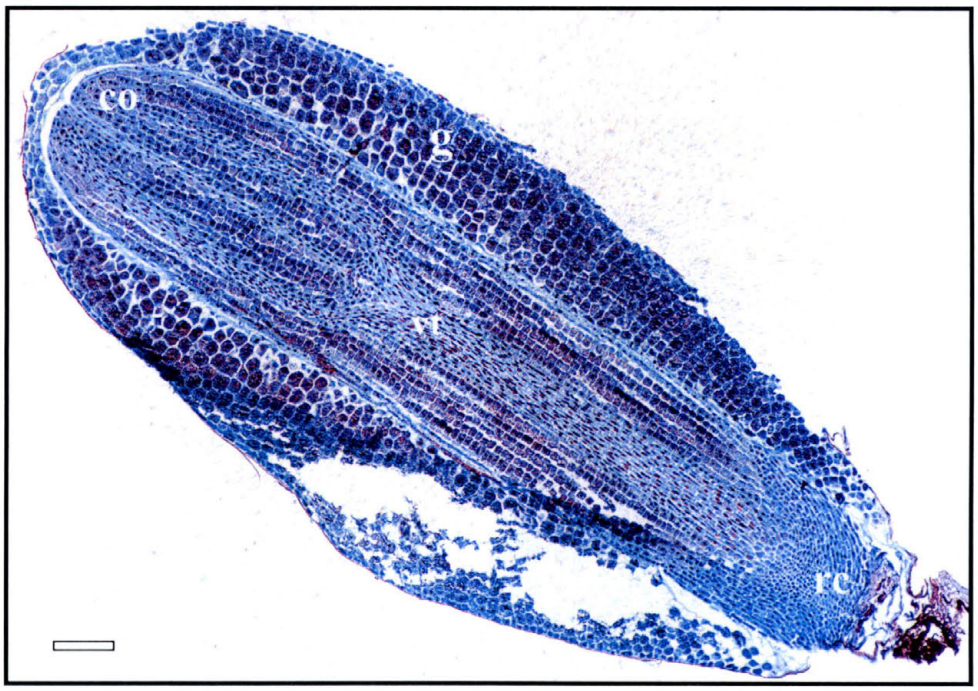

Figure 38. Mature embryo. The embryo is fully differentiated by early October. The apical meristem, two cotyledons, radicle, vascular tissue, and a root cap are present. (co = cotyledon, g = gametophyte, rc = root cap, vt = vascular tissue; scale = 20 μm)

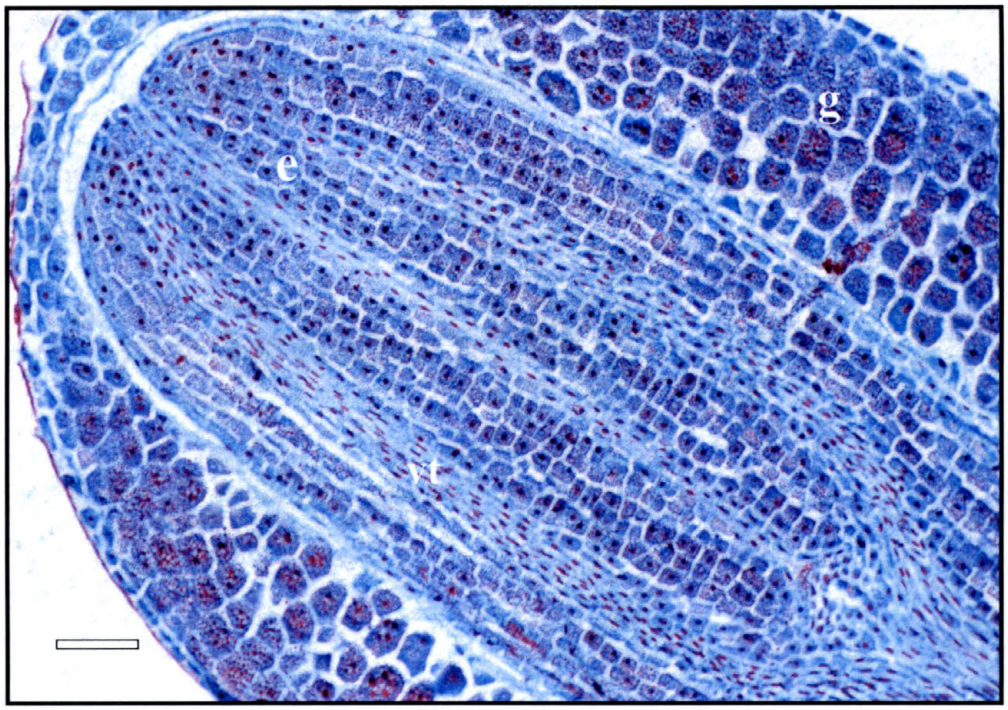

Figure 39. Cotyledons and apical meristem of mature embryo. Epidermal and vascular tissue are present. (g = gametophyte, e = embryo, vt = vascular tissue; scale = 10 μm)

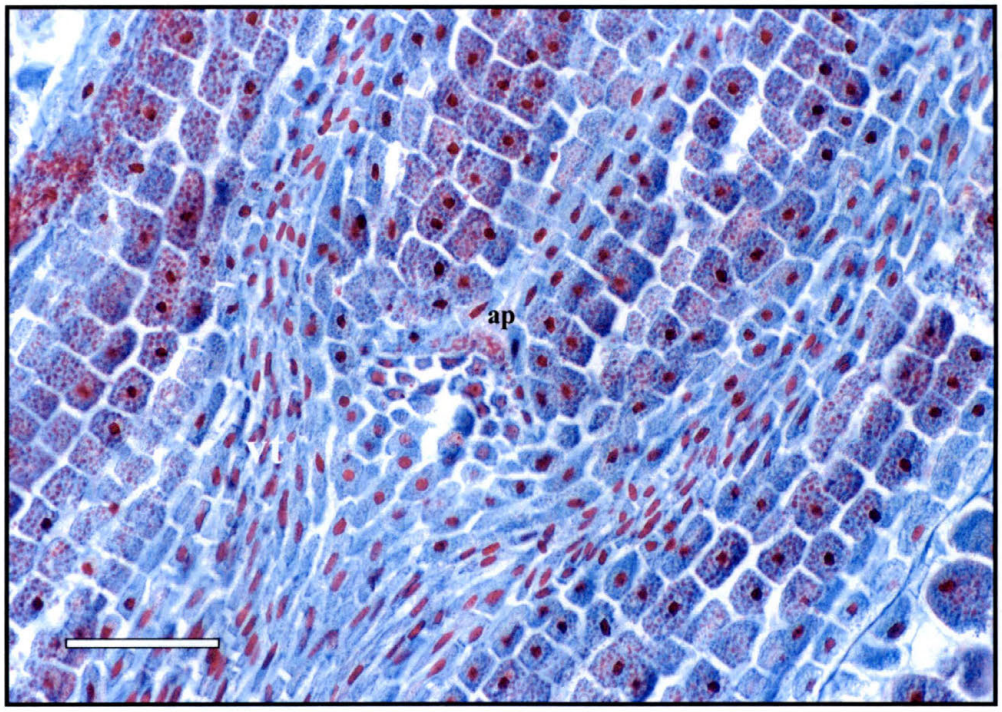

Figure 40. Apical meristem of mature embryo. Just below the apical meristem the vascular strand divides to enter the two cotyledons. (ap = apical meristem, vt = vascular tissue; scale = 10 μm)

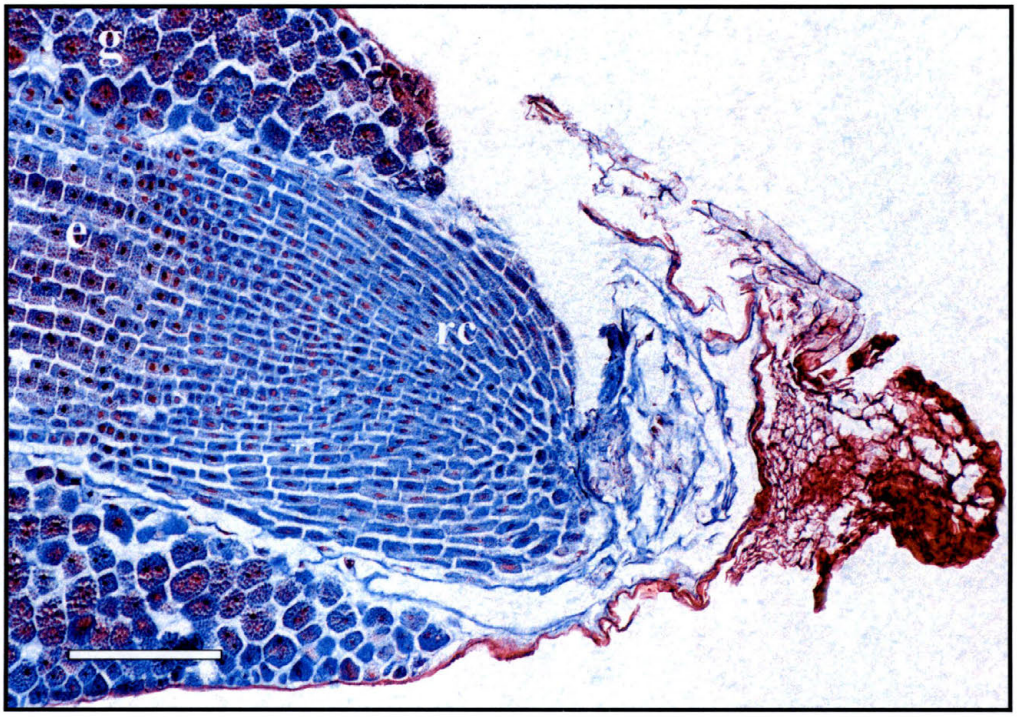

Figure 41. Radicle and root cap of mature embryo. A large and fully developed root cap protects the tip of the radicle. The remains of the suspensor system are still attached to the root cap. (e = embryo, g = gametophyte, rc = root cap; scale = 10 μm)

megasporogenesis coincides with pollination. Fertilization takes place from early April to the middle of May. Embryo development and maturation lasts from mid May to early or mid October when the embryo is fully mature. Megastrobili with mature seeds are shed from late October to January.

The details of *J. ashei* reproduction conform closely to the general plan of conifer reproductive behavior as described by Roy Chowdhury (1962), Doyle (1963), Singh (1978), and Konar and Moitra (1980). As a member of the Cupressaceae it differs in having archegonia arranged in complexes, two equal male gametes, and cleavage polyembryony. Unique to junipers is a distinctive 'juniperoid' type of cleavage polyembryony (Cook 1939; Singh 1978).

Cook mentions that she observed two sorts of embryo complexes, U or V-shaped. *Juniperus ashei* more frequently displays V shaped embryo masses. The V shape is due to a very small number, sometimes just one, of the suspensor lines rapidly outpacing the others and establishing early dominance. In *J. ashei* the period of active embryo competition, proembryogeny and early embryogeny, occurs between early June and late August. So the competition is speedily resolved in about 10 to 12 weeks. Late embryogeny is consistent with that of other conifer species (Singh 1978).

Comparable reproductive data exist on only two other species of North American junipers: *J. communis var. depressa* (Nichols 1910) and *J. virginiana* (Ottley 1909; Mathews 1939). *Juniperus communis* sect. *Juniperus*, the most widespread of all junipers, is found generally at higher latitudes in North America and Eurasia. It has a reproductive cycle of two years.

Juniperus virginiana is in sect. *Sabina* as is *J. ashei*. Both have reproductive cycles of a single year. *Juniperus virginiana* occurs to the east and north of *J. ashei*. The two species do not hybridize but they are clearly more closely related to each other than either is to *J. communis*.

According to Mathews (1939) *J. virginiana* initiates microstrobili in late July and early August at Chapell Hill, North Carolina. The male gametes pass the winter as mature microspores. Ottley (1909) reports that the microsporangia of *J. virginiana* from Wellesley, Massachusetts pass the winter in the mother cell stage. In North Carolina pollen is shed and pollination occurs in late February and early March. Fertilization follows in early June. Mathews states there is a mature embryo by late July. Holthuijzen and Sharik (1985) found that in Virginia the cones of *J. virginiana* ripened from late August through early October.

The reproductive cycles of *J. ashei* and *J. virginiana* are very similar. They differ in the seasonal timing of the various events and the duration of those events. Pollination is from late December to early/mid February in *J. ashei* but begins in late February and lasts through early March for *J. virginana*. Fertilization is from late April to early May in *J. ashei* and in early June in *J. virginiana*. Megastrobili mature by late October in *J. ashei* in central Texas and by August in *J. virginiana* in Virginia.

There are two significant differences in the reproductive cycles of these species: the length of time from fertilization to a mature embryo and the length of time required to produce mature microstrobili. Mathew's report of mature embryos in *J. virginiana* cones by late July, after fertilization in early June, allows

only eight weeks for complete embryonic development. While in *J. ashei* five to six months, May to early October, is needed for embryonic maturation.

In North Carolina *J. virginiana* microsporangia are initiated in July and August and pass the winter as mature microspores and pollen is shed in late February. Further north in Massachusetts they overwinter at the mother cell stage. In contrast *J. ashei* in central Texas initiate microstrobili in late April/early May and shed their mature pollen in early winter, late December to early February. While the relative tempos are different, both species need about eight months to produce mature pollen.

Interestingly, given the several timing differences in reproduction between these two species, each matures its seed in the fall and early winter. Their reproductive cycles seem to slow down or speed up in response to various environmental or chemical signals. Since the seeds of both species are bird distributed, producing mature seed in the fall and early winter allows the trees to take advantage of migratory flocks of birds for dispersal. Due to the general scarcity of food resources in winter, the succulent juniper cones become significant food sources for local and overwintering birds and animals.

These species both have distributions with long north/south axes. Further study of the subtleties of the relationship between the latitude at which a tree grows within its range and the timing of the events of its reproductive cycle is a possible area for future research.

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