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POLLEN ONTOGENY IN *EPHEDRA AMERICANA* (GNETALES)

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Several investigations have focused on mature pollen of *Ephedra*; however, little is known about pollen ontogeny. This article is the first to describe the complete pollen developmental sequence in the genus. Combined light, scanning electron, and transmission electron microscopy were used to document all major developmental stages in *Ephedra americana*. Microspore mother cells are characterized by an unevenly thickened callose envelope. Both tetrahedral and tetragonal tetrads occur, but the majority of tetrads exhibit tetrahedral geometry. Pollen wall development is initiated in regions that will become characteristic plicae. The majority of exine deposition, including formation of the tectum, infratectum, foot layer, and endexine, occurs throughout the tetrad stage, although endexine deposition continues into the free microspore stage. The intine forms during the late free microspore stage. Two types of infratectal elements occur. Granular infratectal elements are the predominant type and develop throughout ontogeny, whereas columellar elements form in the early tetrad stage but subsequently become indiscernible. Mature grains are elliptic, have a series of longitudinal plicae, and are inaperturate, yet the exine is very thin in the furrow regions between plicae. Pollen grains with straight and undulated furrows co-occur in the same pollen sac, with the straight morphology dominating. Because several key characters are revealed only during an investigation of the full ontogenetic sequence, this study emphasizes the importance of integrating pollen developmental characters into phylogenetic analyses.

Keywords: pollen ontogeny, pollen development, *Ephedra americana*, Gnetales, Coniferales, basal angiosperms, anthophyte.

Introduction

Ephedra is a member of the gymnospermous order Gnetales, a rare clade also including *Welwitschia* and *Gnetum*. The Gnetales are generally regarded as monophyletic, with *Ephedra* basal to the derived *Gnetum* and *Welwitschia* (Arber and Parkin 1908; Doyle 1996; Friedman 1996; Price 1996; Bowe et al. 2000; Ickert-Bond et al. 2003; Rydin et al. 2004). Although the generic relationships within Gnetales are generally well recognized, their phylogenetic position among seed plants has received considerable attention in recent years. Many morphologically based phylogenetic studies have placed Gnetales as the closest living relatives to angiosperms (e.g., Arber and Parkin 1908; Crane 1985; Doyle and Donoghue 1992), and along with the fossil groups Bennettitales and Pentoxylales, Gnetales and angiosperms have comprised the "anthophyte clade" (e.g., Crane 1985; Doyle and Donoghue 1992; Osborn 2000). A series of molecular studies, however, have questioned this phylogenetic interpretation and have indicated that Gnetales are more closely allied with other gymnosperms, specifically, that they are nested within conifers (Samigullin et al. 1999; Bowe et al. 2000; Donoghue and Doyle 2000). Because these molecular data have also been disputed by other molecular studies (e.g., Rydin et al. 2002), developmental and paleobotanical investigations have become increasingly important in reconciling the phylogenetic differences between morphological and molecular data.

Despite the phylogenetic importance of the group, limited information is available about the geologic history of Gnetales. The majority of data about gnetalean evolution through geologic time come from palynological evidence. The record of dispersed, "polyplicate" pollen resembling that of *Ephedra* and *Welwitschia*, at the light-microscope level, extends from the Lower Permian to recent sediments (see Osborn et al. 1993 and references therein; Crane 1996; Osborn 2000; Dilcher et al. 2005). Polyplicate pollen is also produced by several angiosperm groups (e.g., Arales, Laurales, and Zingiberales); however, the pollen walls of these polyplicate pollen types are structurally and chemically different from those of Gnetales (Osborn et al. 1993; Hesse et al. 2000). A limited number of Gnetalean megafossils have been described; examples include *Masculostrobus clathratus* (Ash 1972), *Drewria potomacensis* (Crane and Upchurch 1987), *Piroconites kuesperti* (van Konijnenburg-van Cittert 1992), *Craytonia cotyledon* (Rydin et al. 2003), and *Welwitschiella austroamericana* (Dilcher et al. 2005). Fertile reproductive shoots and seeds containing characteristics of both Gnetales and conifers have also been assigned to the morphospecies *Ephedra archaeorhytidosperma* (Wang 2004). Recently, early Cretaceous seeds and *in situ*

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pollen grains have been assigned to the genera *Ephedra* and *Ephedrispermum* (Rydin et al. 2006).

Ephedra is the largest of the three extant genera of Gnetales. The genus comprises ca. 50 species, which occur primarily in xeric habitats in both the Old and New World. Pollen morphology has been used as a source of important taxonomic characters. Previous studies on mature pollen of *Ephedra* show that the pollen grains are elliptic to elongate and range from 20 to 80 μm in length and from 16 to 50 μm in width. Grains are characterized by a series of longitudinal ribs, or plicae, that typically have a psilate ornamentation. Plicae number ranges from four to 19 (Steeves and Barghoorn 1959). The polyplicate pollen of *Ephedra* is typically considered inaperturate. However, the exine is considerably thinner within the plicae furrows (El-Ghazaly and Rowley 1997; Osborn 2000), and the entire exine is completely discarded before pollen tube germination (El-Ghazaly et al. 1998). Species of *Ephedra* are principally distinguished from one another palynologically by the number of plicae, plica height and degree of slope, and furrow morphology. The furrow may be either straight or undulated/branched. Pollen with both types of furrow morphologies has also been shown to occur within a single species and within a single microsporangium (El-Ghazaly and Rowley 1997; Ickert-Bond et al. 2003).

Although several studies have focused on the morphology of mature pollen in *Ephedra*, no published studies have examined the ultrastructural aspects of pollen ontogeny in the genus. Investigations of pollen development can potentially generate many important characters for assessing phylogenetic relationships that cannot be observed when only mature grains are studied (Blackmore and Barnes 1991; Kreunen and Osborn 1999; Taylor and Osborn 2006).

The primary objective of this article is to comprehensively document pollen wall development in *Ephedra americana* Hum. & Bonpl. ex Willd. Key ontogenetic characters are also compared with those of both saccate and nonsaccate pollen of conifers, as well as with basal-angiosperm pollen, in an effort to help clarify the phylogenetic position of Gnetales.

Material and Methods

Sixty-five pollen cones of *Ephedra americana* were collected from the greenhouse of the Botany Department at Stockholm University in Stockholm, Sweden. Pollen sacs were dissected from the compound strobili, and the sacs were fixed in Karnovsky's fixative plus 1% sucrose (buffered in 0.2 M sodium phosphate buffer; pH 7.2) for 24 h and buffer washed four times. Pollen sacs were postfixed in buffered 1% osmium tetroxide for 3 h, buffer washed four times, and dehydrated in a graded ethanol series.

The pollen sacs were infiltrated and embedded in Spurr epoxy resin and thick- and thin-sectioned on a Leica Ultracut T Ultramicrotome using a diamond knife. Thick sections (850 nm) were collected on microscope slides, stained with Richardson's stain (methylene blue and Azure II) and imaged with bright-field illumination using an Olympus BHS compound light microscope. Thin sections (70 nm) were collected on 1×2-mm slot grids and dried onto Formvar support films. Grids were stained with uranyl acetate and lead citrate and examined/imaged using a JEOL JEM-100SX transmission electron microscope at 80 kV.

Additional specimens were dehydrated in a graded ethanol series, critical-point dried, and macerated using a syringe needle. These specimens were mounted onto aluminum stubs, sputter-coated with a layer of gold, and examined/imaged using a JEOL JSM-6400 scanning electron microscope at 20 kV.

Results

Pollen cones in *Ephedra americana* are compound; the cone axis bears several pairs of bracts, which separate the microsporangiate structures. Each microsporangiate shoot has an axis bearing a pair of bracteoles that enclose a microsporophyll with several pollen sacs.

Pollen sacs within the same cone generally show consistent ontogenetic timing of the pollen grains; however, a limited amount of variation occurs among pollen sacs. Pollen grains within a single pollen sac show consistent and synchronous development. Pollen ontogeny is presented below as it occurs in four major stages, the microspore mother cell, tetrad, free microspore, and mature pollen grain stages. Once the first tectal elements are laid down, each developing plica is characterized by crest and margin regions, with a furrow region between adjacent plicae. These characters are illustrated in figure 1 as they appear in the nearly mature pollen wall, to serve as structural landmarks for comparison with ontogenetic data.

Microspore Mother Cell Stage

Microspore mother cells are large and typically occur clumped together near the tapetum. Each microspore mother cell is surrounded by an unevenly thickened layer of callose ranging from 2.2 to 5.7 μm in thickness (fig. 2*A*–2*C*). The thinner areas of callose, however, are often one-tenth the width of the thick areas, with an average thickness of 0.33 μm and a maximum of only 0.67 μm (fig. 2*C*). The callose is divided into two distinct layers: a thick inner layer that is more electron dense and a thin outer layer that is less electron dense (fig. 2*D*). The outer layer is evenly thickened and is also

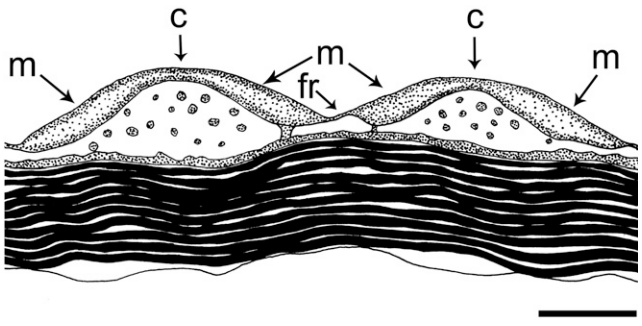

Fig. 1 Illustration of a nearly mature pollen wall in transverse section showing two contiguous plicae. Each plica is characterized by a crest (*c*) and two margins (*m*), with a furrow region (*fr*) between the plicae. Bar = 500 nm.

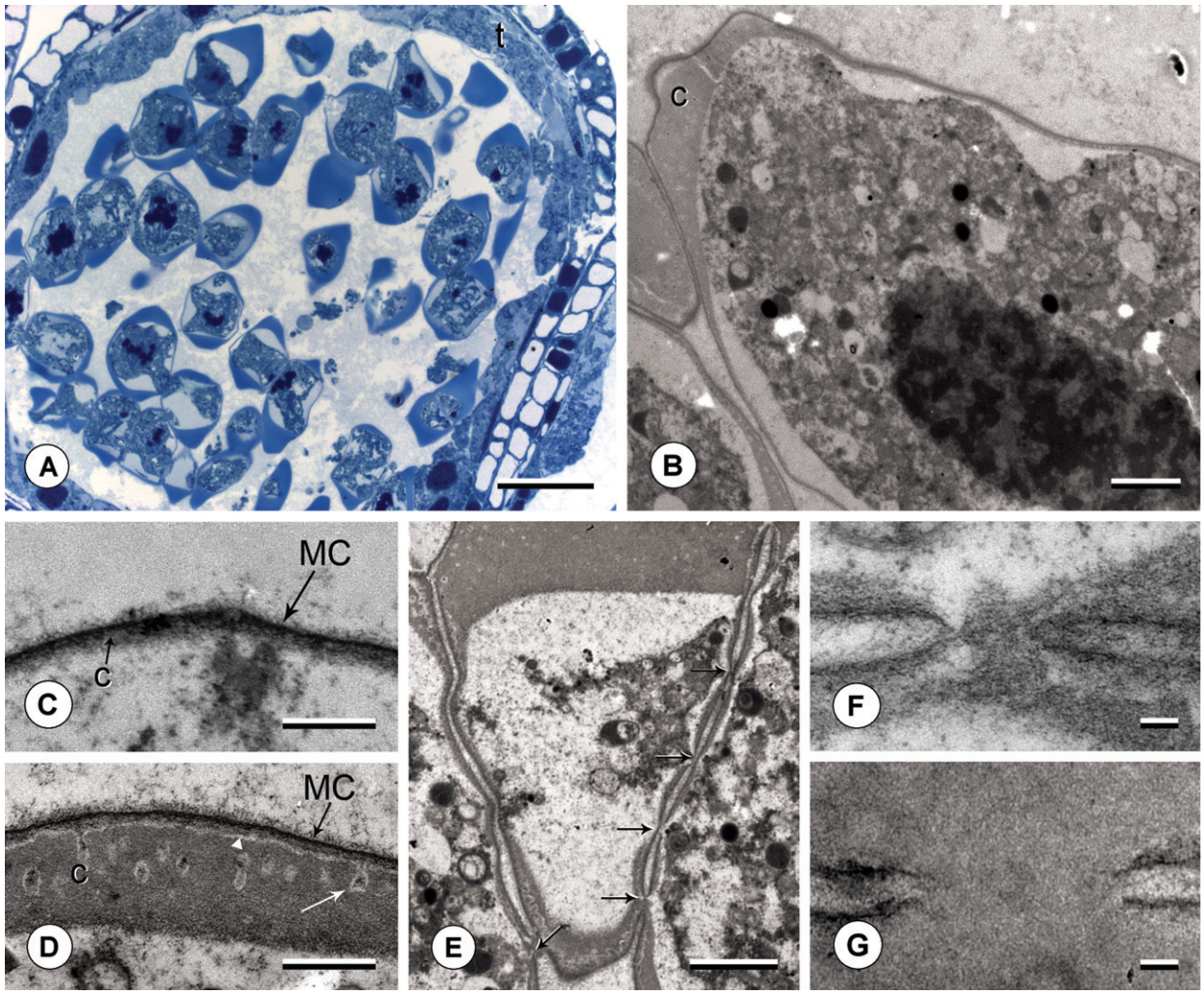

Fig. 2 Microspore mother cell stage. *A*, Section of a pollen sac showing microspore mother cells and tapetum (*t*). Light microscopy; bar = 25 μm. *B*, Section through a single microspore mother cell showing nucleus and unevenly thickened callose (*c*). TEM; bar = 2 μm. *C*, Detail of a microspore mother cell wall with thin callose (*c*) and the microspore mother cell coat (*MC*). TEM; bar = 500 nm. *D*, Detail of a microspore mother cell with thick callose envelope (*c*), surrounded by the microspore mother cell coat (*MC*). Note the two layers of callose (arrowhead) and the spherical infiltrations (arrow) present in the callose. TEM; bar = 500 nm. *E*, Section through adjacent microspore mother cells showing interruptions in the microspore mother cell coat that result in callosic connections (arrows). TEM; bar = 2 μm. *F*, Detail of early adjacent microspore mother cells with limited callosic connection. TEM; bar = 100 nm. G, Detail of late adjacent microspore mother cells showing more extensive callosic connection. TEM; bar = 100 nm.

covered with an electron-dense microspore mother cell coat. Variation in thickness of the inner layer is responsible for the uneven thickening of the callose as a whole. Numerous spherical channels/infiltrations occur in the inner callose layer, and these channels/infiltrations consist of a central electron-dense core surrounded by a less electron-dense layer (fig. 2*D*).

When developing microspores are in close proximity to one another, callosic connections may be established between two or more microspores. These connections result in an interruption of the outer callose wall of each microspore, with the inner layer of the callose wall extending between adjacent microspores (fig. 2*E*–2*G*). As the callose thickens, these intermicrospore connections remain intact and widen (fig. 2*G*).

The tapetum is robust at the microspore mother cell stage. Individual cells comprising the tapetum can be distinguished from one another because of their distinct nuclei (fig. 2*A*). The tapetum extends 80–100 μm into the pollen sac.

Tetrad Stage

Once the microspore mother cells undergo meiosis, the individual microspores are held together in tetrads. A thick layer of callose surrounds the entire tetrad and separates the microspores from one another (fig. 3*A*, 3*B*). A majority of tetrads exhibit tetrahedral geometry, indicating simultaneous microsporogenesis. Occasionally, tetragonal configurations also occur. The earliest tetrads exhibit no exine development, and the microspore plasmalemma is directly adjacent to the callose (fig. 3*B*). During the early tetrad stage, tapetal cells are well defined (fig. 3*A*).

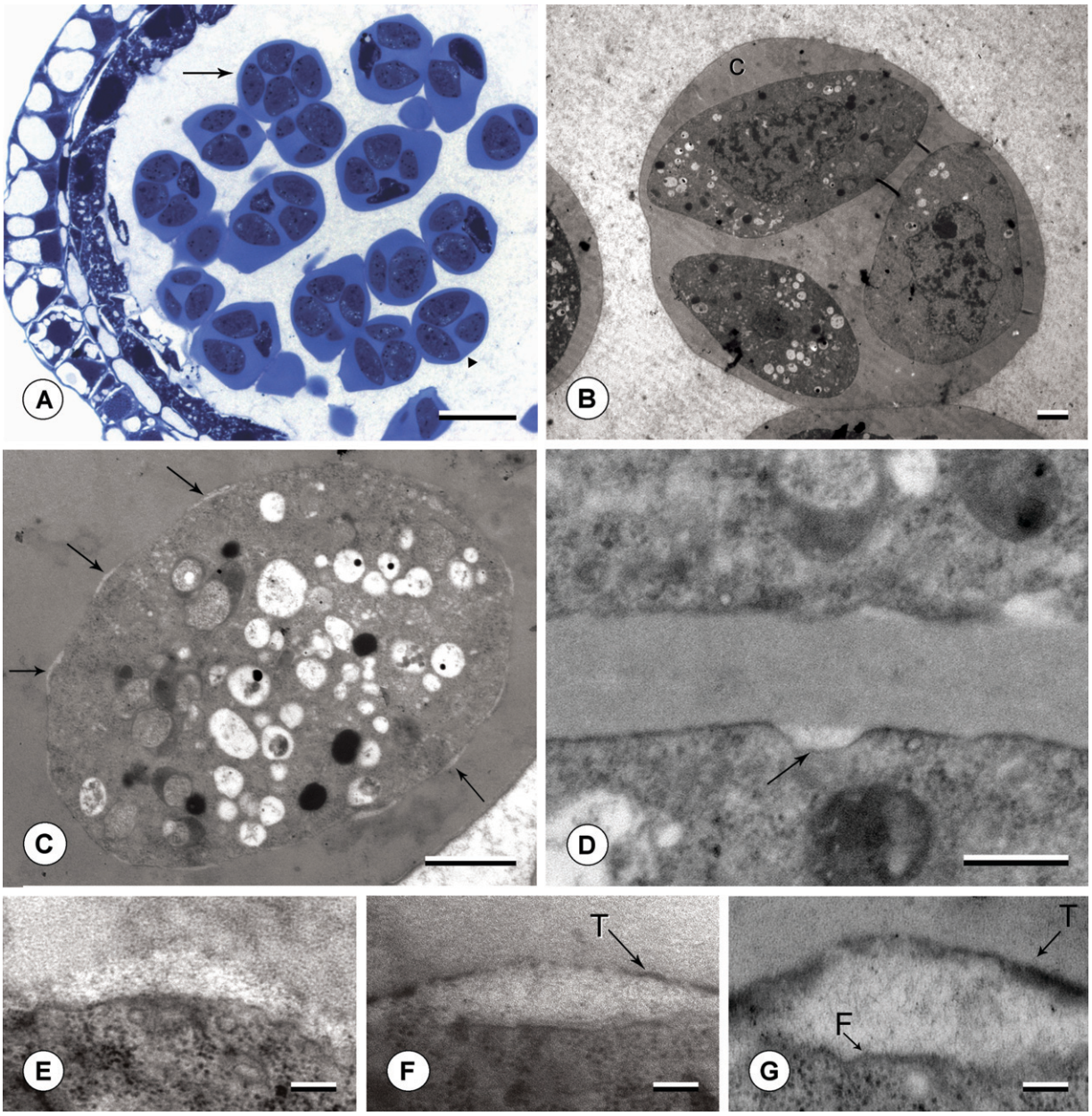

Fig. 3 Early tetrad stage. *A*, Section of a single pollen sac showing both tetragonal (arrow) and tetrahedral (arrowhead) types. Light microscopy; bar = 25 μm. *B*, Section of a single tetrahedral tetrad showing more evenly thickened callose (*c*). Note that there is no detectable microspore wall development. TEM; bar = 2 μm. *C*, Section of a single microspore showing developing primexine in regions where the plasmalemma has pulled back from the callose (arrows). TEM; bar = 2 μm. *D*, Detail of two adjacent microspore walls showing the plasmalemma pulling away from the callose (arrow). TEM; bar = 500 nm. *E*, Detail showing plasmalemma separating from the callose such that the future crest, margin, and furrow regions can be seen. TEM; bar = 100 nm. *F*, Detail showing developing microspore wall. Note that the first tectal elements (*T*) form along the plicae margins. TEM; bar = 100 nm. *G*, Detail showing developing microspore wall. Note that the tectum (*T*) is thicker along the margins and thinner at the crest and that the initial elements of the foot layer (*F*) have formed. TEM; bar = 100 nm.

As development continues, the plasmalemma pulls away from the callose at the crest regions of the future plicae, and an electron-translucent primexine develops (fig. 3*C*–3*E*). A thin, electron-dense layer forms adjacent to the callose, creating the first elements of the tectum (fig. 3*F*). The tectum separates unevenly from the plasmalemma to form plicae before additional pollen wall elements begin to develop. Because of the uneven separation and uneven thickening of the tectum, the crest and margin regions of the plicae can be distinguished in this early tetrad stage (fig. 3*E*). Tectal thickening occurs initially only at the margins of the plicae (fig. 3*F*, 3*G*). The tectum in the crest region of each plica thickens later in development, after the margins are well established (fig. 4*C*). At this stage, no infratectal elements are detectable, and the

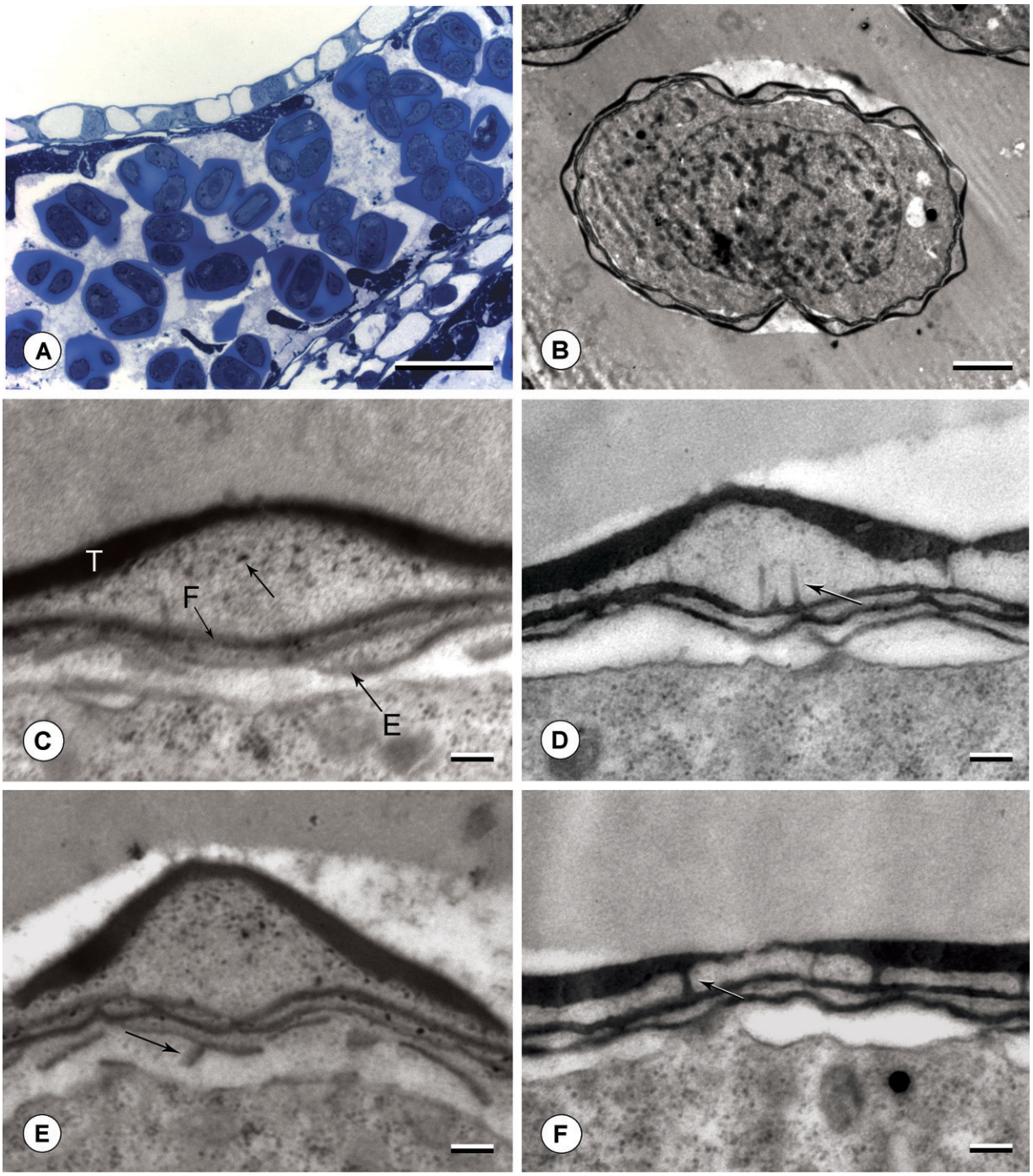

Fig. 4 Middle tetrad stage. *A*, Section of a single pollen sac showing developing tetrads. Light microscopy; bar = 25 μm. *B*, Section of a single microspore showing distinct wall development. TEM; bar = 2 μm. *C*, Detail of the developing wall of a microspore. Note the distinct tectum (*T*), granular infratectal elements (arrow), foot layer (*F*), and initial endexine lamellae (*E*). TEM; bar = 100 nm. *D*, Detail of developing wall of a single microspore. Note the columellar infratectal elements (arrow) extending from the foot layer into the infratectal space. TEM; bar = 100 nm. *E*, Detail of developing wall of a single microspore. Note that the endexine is being laid down as plates (arrow) that fuse to form continuous sheets of lamellae. TEM; bar = 100 nm. *F*, Detail of developing furrow region. Note how the columellar infratectal elements (arrow) extend from the foot layer to the tectum. TEM; bar = 100 nm.

overall thickness of the exine is between 250 and 450 nm. A thin foot layer develops next (fig. 3*G*), directly before the initiation of the endexine.

At the middle tetrad stage (fig. 4), endexine lamellae are initially laid down in discontinuous fragments that later fuse to form continuous sheets (fig. 4*E*). Granulelike units exist initially between adjacent endexine lamellations (fig. 4*C*, 4*E*). As the number of endexine lamellae reaches four to six, the total thickness of the exine ranges from 1.0 to 1.8 μm. Just above the endexine, the foot layer thickens evenly throughout this stage (fig. 4*C*). Small, granular infratectal elements begin to develop in the middle tetrad stage (fig. 4*C*–4*E*). In addition, columellar infratectal elements form that span from the foot layer to the tectum. Although these columellar elements are more abundant near furrows (fig. 4*F*), they are initially present throughout the infratectum, including under the crests (fig. 4*D*).

At the late tetrad stage (fig. 5), the endexine increases in thickness and consists of seven to 10 endexine lamellae. The granulelike units present between the individual lamellae remain distinct (fig. 5*B*). The tectum becomes thicker along the margins of the plicae and remains thinner at the crests (fig. 5*C*). At the furrows, the tectum is considerably thinner (fig. 5*D*). The foot layer remains uniform in thickness throughout the late tetrad stage (fig. 5*B*). The granular infratectal elements become more robust (fig. 5*B*, 5*C*), while the columellar elements become less abundant and persist only near the furrows. At the end of the tetrad stage, the microspores exhibit granular infratectal elements that range from 20 to 40 nm in diameter (fig. 5*B*, 5*C*). The tapetal cells remain intact and attached to the pollen sac wall throughout the late tetrad stage (fig. 5*A*).

Free Microspore Stage

At the end of the tetrad stage, the callose surrounding the tetrads dissociates, and the four microspores separate from

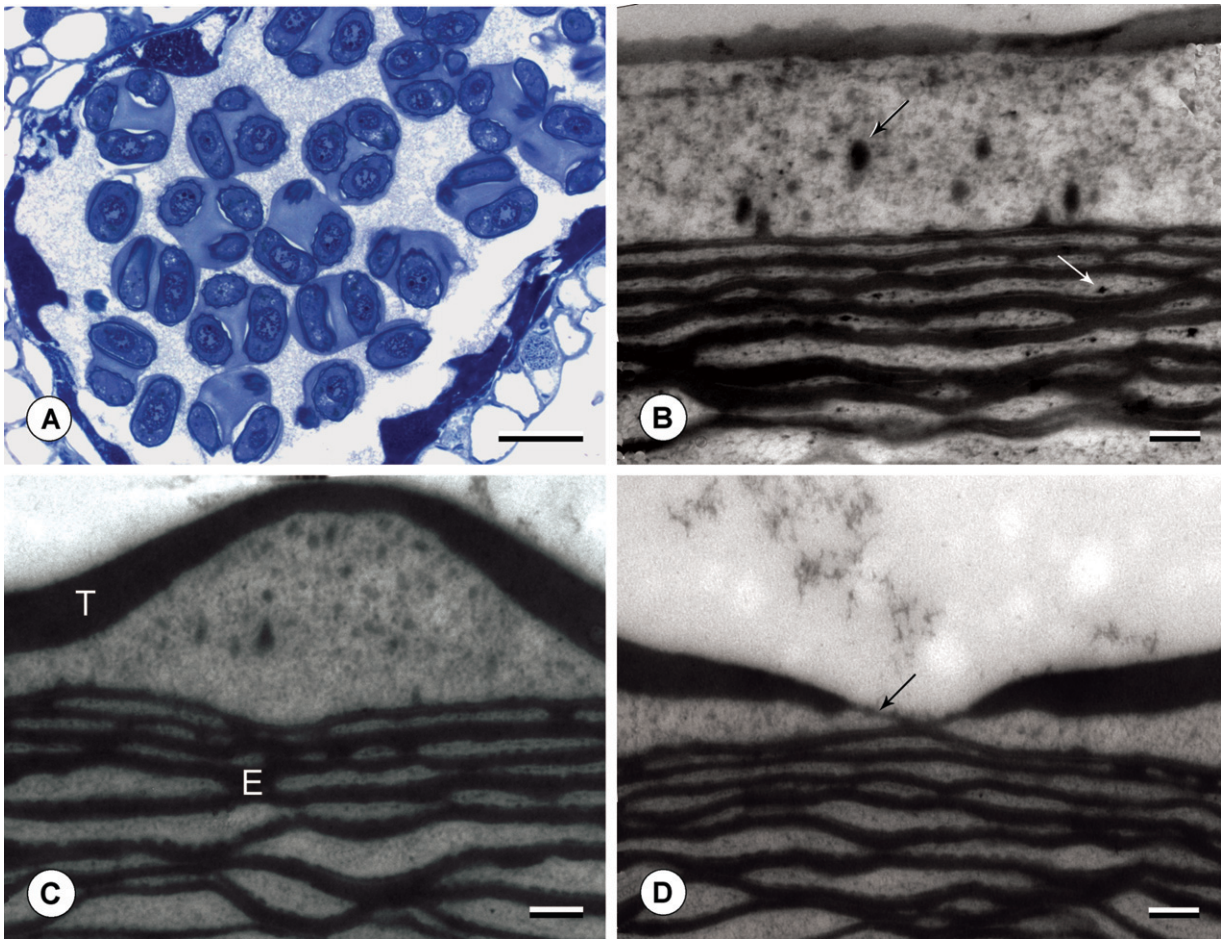

Fig. 5 Late tetrad stage. *A*, Section of a single pollen sac showing developing tetrads. Light microscopy; bar = 25 μm. *B*, Longitudinal section through the developing wall of a single microspore. Note that the granular infratectal elements are increasingly robust (black arrow) and the presence of small, granulelike elements between endexine lamellae (white arrow). TEM; bar = 100 nm. *C*, Section through the developing wall of a single microspore. Note that the tectum (*T*) is thicker along the margins than at the crest. Also note the increasing number of endexine lamellae (*E*). TEM; bar = 100 nm. *D*, Furrow region of the developing wall of a single microspore. Note the reduced thickness of the tectum at the furrow region (arrow). TEM; bar = 100 nm.

one another (fig. 6*A*, 6*B*). At the early free microspore stage, each microspore typically has eight to 10 endexine lamellae, and the average thickness of the exine at the crests is 0.86 μm (fig. 6*D*). The electron-translucent white lines in the center of each endexine lamellation are evident (fig. 6*E*). As soon as the microspores emerge from the callose envelope (fig. 6*B*), tapetal cells are slightly protruded into the locular space (fig. 6*A*) and begin to exocytose orbicules (fig. 6*C*).

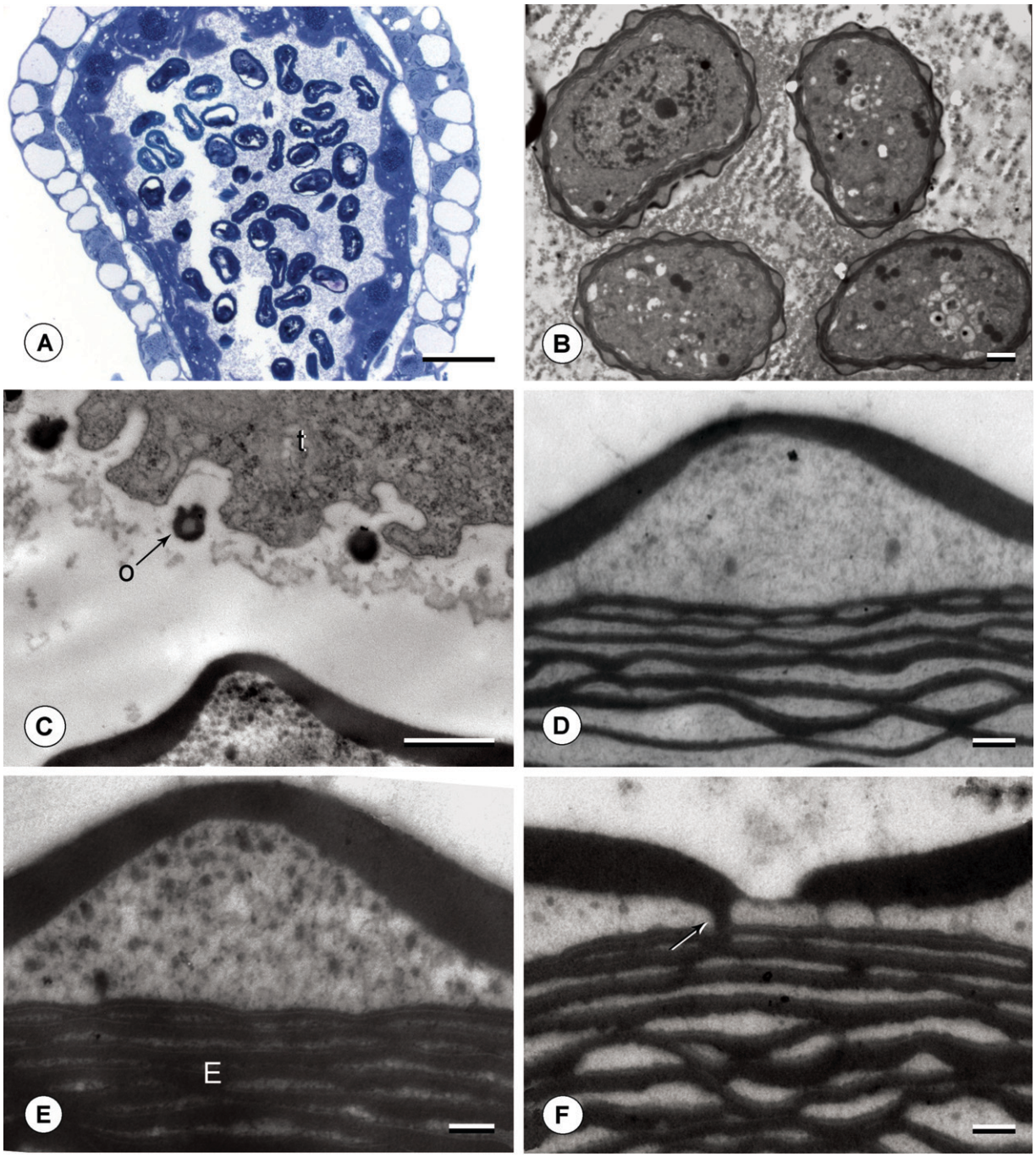

Fig. 6 Early and middle free microspore stage. *A*, Section through a single pollen sac showing developing microspores. Note that the microspores are not surrounded by callose. Light microscopy; bar = 25 μm. *B*, Section through a single tetrad showing individual microspores emerging from callose. TEM; bar = 2 μm. *C*, Section showing the developing wall of a single microspore and the tapetum. Note the emerging orbicules (*o*) that are exocytosed from the tapetum (*t*). TEM; bar = 500 nm. *D*, Section through an early free microspore showing the developing wall. TEM; bar = 100 nm. *E*, Section through a middle free microspore. Note that the endexine lamellae (*E*) are compressed against each other. TEM; bar = 100 nm. *F*, Section through the furrow region of a middle free microspore. Note that the tectum is considerably thinner near the furrow region and that the columellar infratectal elements are concentrated near the furrow (arrow). TEM; bar = 100 nm.

During the middle free microspore stage, the number of endexine lamellae increases to 10–12, and the lamellae become more compressed against each other; thus, the granule-like units previously seen between lamellations can no longer be distinguished (fig. 6*E*). The granular infratectal elements become much more robust and begin to fill the majority of the space in the infratectum. Granules range in diameter from 50 to 80 nm (fig. 6*E*). In contrast, as the tectum concurrently increases in thickness, the columellar infratectal elements become even more reduced in number during the free microspore stage and are only rarely distinguishable at the furrows (fig. 6*F*).

In late free microspores (fig. 7), overall exine thickness ranges from 0.8 to 1.2 μm at the crests (fig. 7*B*, 7*D*). Endexine lamellae, now numbering 12–14, become so compressed that they can be distinguished from one another only because they are white-line centered (fig. 7*D*, 7*E*). The infratectal granules become more robust near the crest, creating a gradient in the infratectum (fig. 7*D*). The foot layer thickens discontinuously during this stage, so that it is unevenly thicker under the margins (fig. 7*D*). The intine begins to be laid down between the endexine lamellae and the plasmalemma. The intine initially appears when there are ca. 11 or 12 endexine lamellae formed (fig. 7*E*), and the intine continues to thicken as the three to five final lamellae are developed. At the end of the free microspore stage, the cytoplasm of each microspore becomes highly vacuolated (fig. 7*A*, 7*C*).

Mature Grain Stage

Mature pollen grains of *E. americana* are ellipsoidal to elongate and range from 30 to 50 μm in length and from 15 to 25 μm in width. Mature grains have between 12 and 17 plicae, with furrow morphology varying between straight and undulated (fig. 8). Grains with different plicae numbers and furrow types occur within the same pollen sac.

Once mature, the plicae are very pronounced, with a thick tectum (fig. 9*B*, 9*C*). The disparity between tectum thicknesses at the crests and margins becomes greatly exaggerated, with the thick portions of the tectum along the margins of the plicae averaging 0.47 μm in thickness and becoming as thick as 0.72 μm (fig. 9*C*). In contrast, the tectum at the crests reaches a thickness of only 0.12 μm, on average (fig. 9*C*). The tectum is also thinner in the furrow regions, where it exists in close proximity to the foot layer (fig. 9*D*).

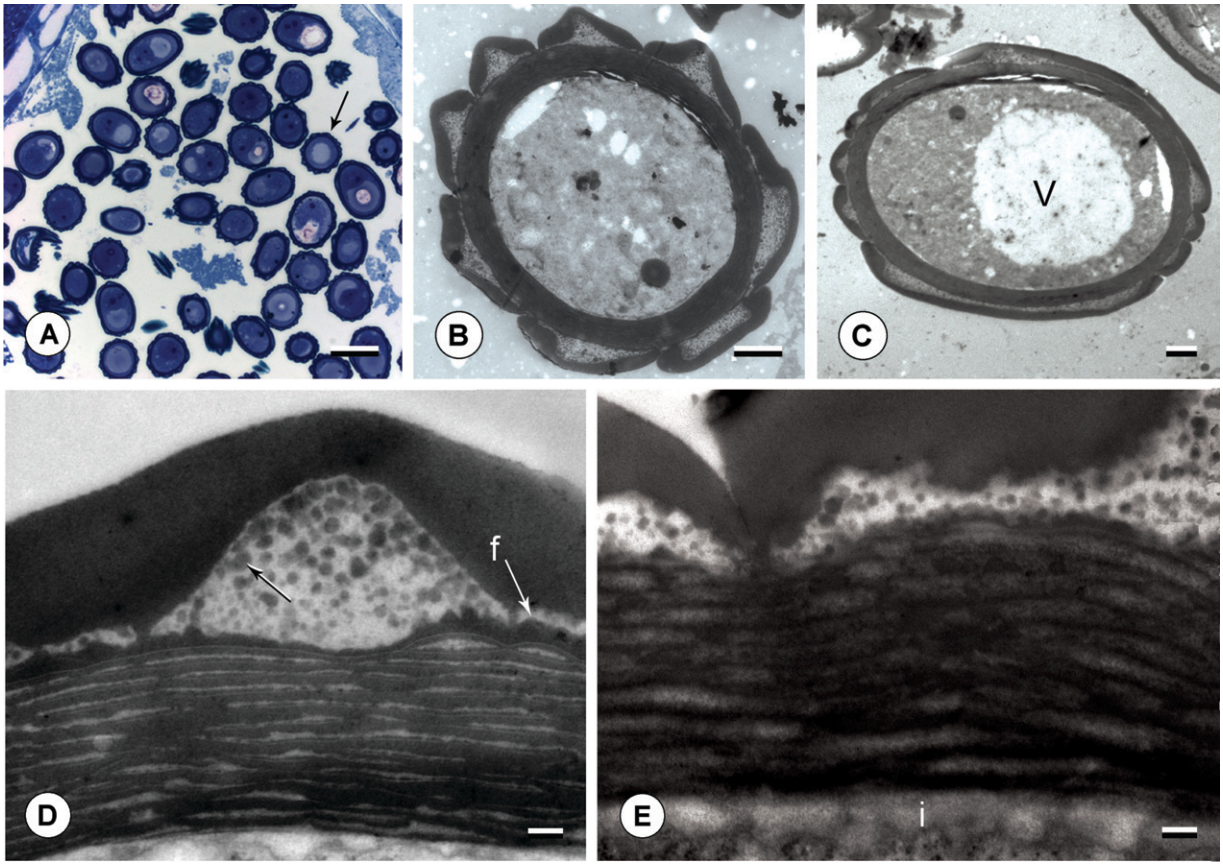

Fig. 7 Late free microspore stage. *A*, Section through a single pollen sac showing developing microspores. Note that many of the microspores are vacuolated (arrow). Light microscopy; bar = 25 μm. *B*, Transverse section through a single developing microspore. TEM; bar = 2 μm. *C*, Longitudinal section through a single developing microspore that is vacuolated (*V* indicates the vacuole). TEM; bar = 2 μm. *D*, Section through the developing wall of a single microspore. Note the robust granular infratectal elements (black arrow) and the unevenly thickened foot layer (*f*). TEM; bar = 100 nm. *E*, Section through the furrow region of a single developing microspore. Note the initiation of the intine (*i*). TEM; bar = 100 nm.

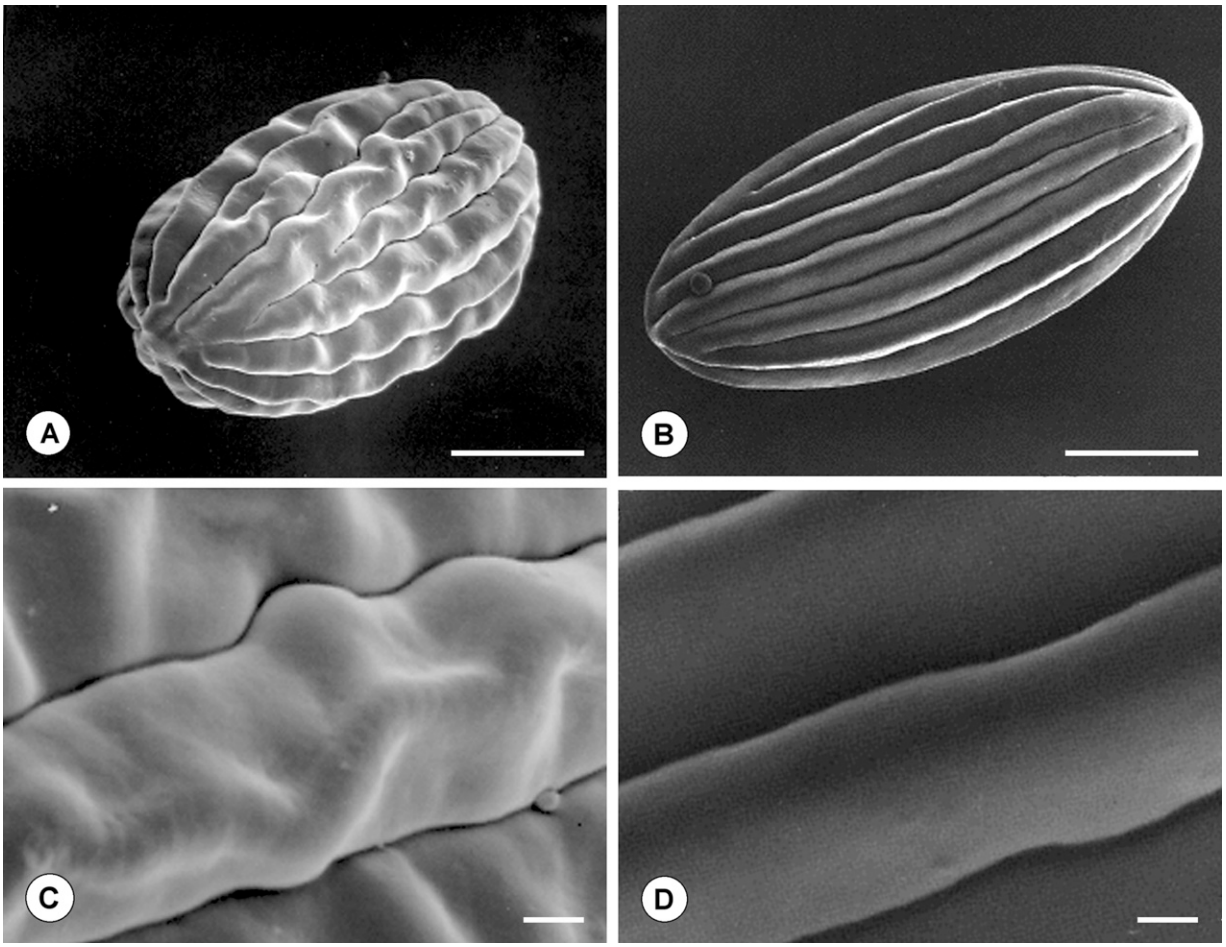

Fig. 8 Mature grain stage. *A*, Single mature grain showing the undulated-furrow morphology. SEM; bar = 10 μm. *B*, Single mature grain showing the straight-furrow morphology. SEM; bar = 10 μm. *C*, Detail of a single mature grain showing the undulated-furrow morphology. SEM; bar = 1 μm. *D*, Detail of a single mature grain showing the straight-furrow morphology. SEM; bar = 1 μm.

The granular infratectal elements become much more robust in mature grains, ranging from 65 to 100 nm in diameter (fig. 9*C*). These elements are not attached to the foot layer or the tectum and are free within the infratectal space. The columellar infratectal elements occur only rarely near the furrows (fig. 9*D*). The foot layer is now unevenly thickened, with thickenings extending into the infratectal space (fig. 9*C*).

Endexine lamellae become even more compressed, so that they no longer appear undulated but instead exist as flat sheets beneath the foot layer (fig. 9*C*). Mature pollen grains typically have between 14 and 17 endexine lamellae. At the crests, the thickness of the overall exine is between 1.0 and 1.9 μm (fig. 9*C*). The intine has thickened in mature pollen, so that it is nearly as thick as the exine. The intine consists of undulating layers that are less electron dense than the adjacent endexine lamellae (fig. 9*B*, 9*C*). The intine is uniformly thick and does not vary between the crest to furrow regions of the wall (fig. 9*B*). The tapetum is typically completely degraded and absent at the mature pollen stage (fig. 9*A*), and orbicules are not obvious within the locular space.

Discussion

This is the first comprehensive study of pollen development in *Ephedra*. Although several studies have examined wall ultrastructure of mature pollen in *Ephedra* (Hesse 1984; El-Ghazaly and Rowley 1997; Osborn 2000; Ickert-Bond et al. 2003), these studies focused on characters of the fully developed exine and did not address ontogeny. Several key characters of microspore and tapetal development are discussed below and summarized in figure 10, and the new ontogenetic data are addressed in a systematic and phylogenetic context.

Callose Ultrastructure and Thickening

The formation of an unevenly thickened callose envelope characterizes the microspore mother cell stage. Although deposition of callose is initially uneven, the callose envelope becomes more uniformly distributed after meiosis throughout the tetrad stage. The callose is bilayered, with a thicker, electron-dense inner layer and a thinner, electron-translucent

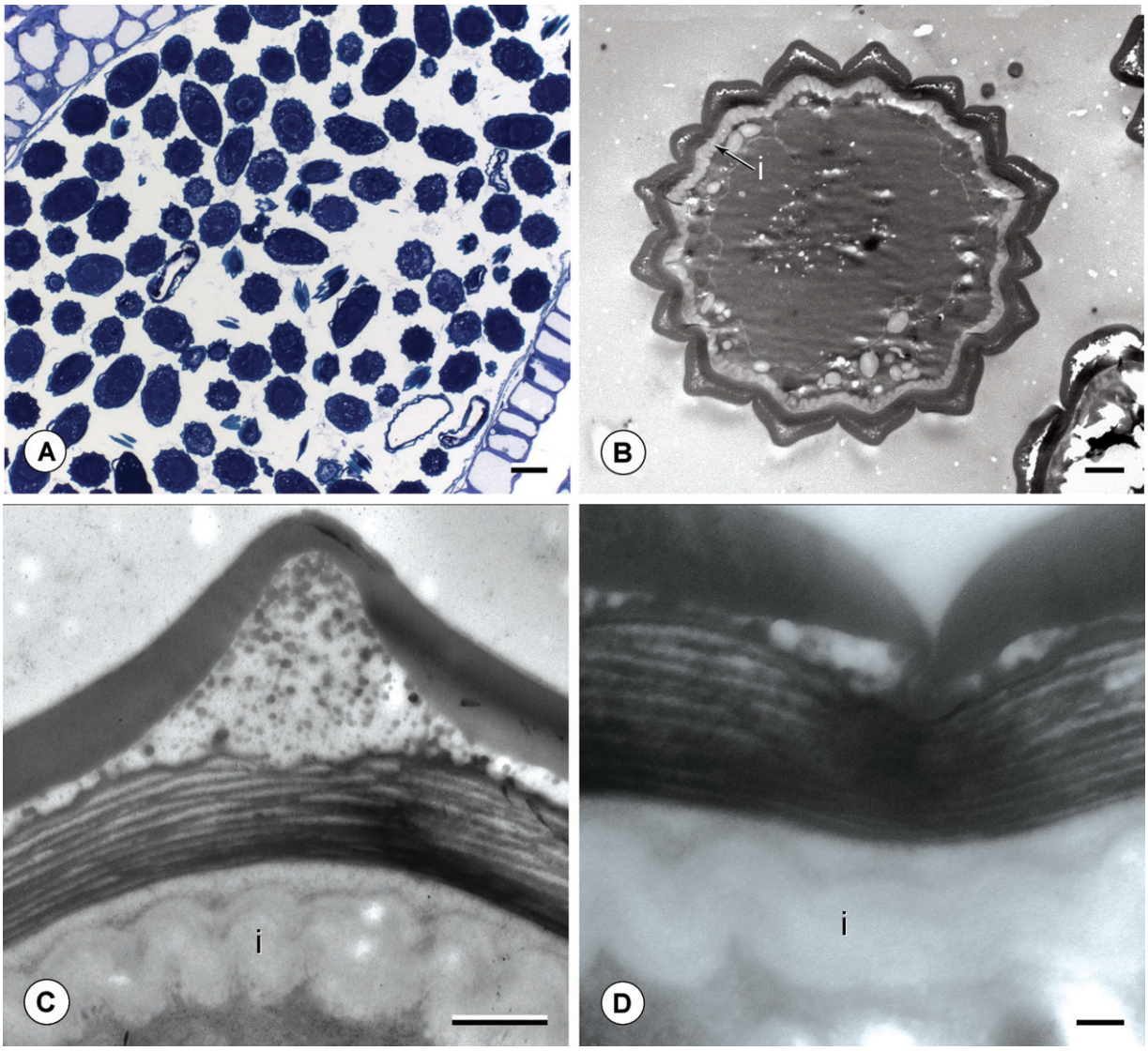

Fig. 9 Mature grain stage. *A*, Section through a single pollen sac. Note the absence of the tapetum. Light microscopy; bar = 25 μm. *B*, Section through a single mature grain showing the evenly thickened intine (*i*). TEM; bar = 2 μm. *C*, Detail through the pollen wall of a single mature grain showing fully developed crest and margin regions, infratectal granules, and endexine lamellae. Note the presence of the intine (*i*) beneath the endexine. TEM; bar = 500 nm. *D*, Detail of the furrow region of the pollen wall of a single mature grain. Note the presence of the intine (*i*) beneath the endexine. TEM; bar = 100 nm.

outer layer. Callosic channels/infiltrations occur in nearly all microspores examined and are more abundant near the microspore mother cell coat. Channels/infiltrations like these have not previously been described. In contrast, callosic protrusions toward the plasmalemma that aid in wall development are known in saccate conifers (Kurmann 1990*a*, 1990*c*; Rowley et al. 2000), but these do not occur in *Ephedra*. The absence of callosic protrusions into the plasmalemma is consistent with gymnosperms that have a granular infratectum (Kurmann 1990*b*). Exine formation does not appear to be greatly mediated by the callose in basal angiosperms either. For example, Zavada (1984) reported the presence of only a special callose wall during the microspore mother cell stage of development and noted that it completely degraded upon the formation of the foot layer in *Austrobaileya maculata*.

Ontogenetic Timing

The majority of exine development occurs during the tetrad stage, with abundant tectum formation and more than half of the endexine lamellae laid down during this stage. This is consistent with pollen wall development in *Welwitschia* (Zavada and Gabarayeva 1991) and similar to development in conifers. In nonsaccate conifers with granular infratecta, such as *Cunninghamia lanceolata* and *Cryptomeria japonica*, ectexine and endexine structure is established in the tetrad stage,

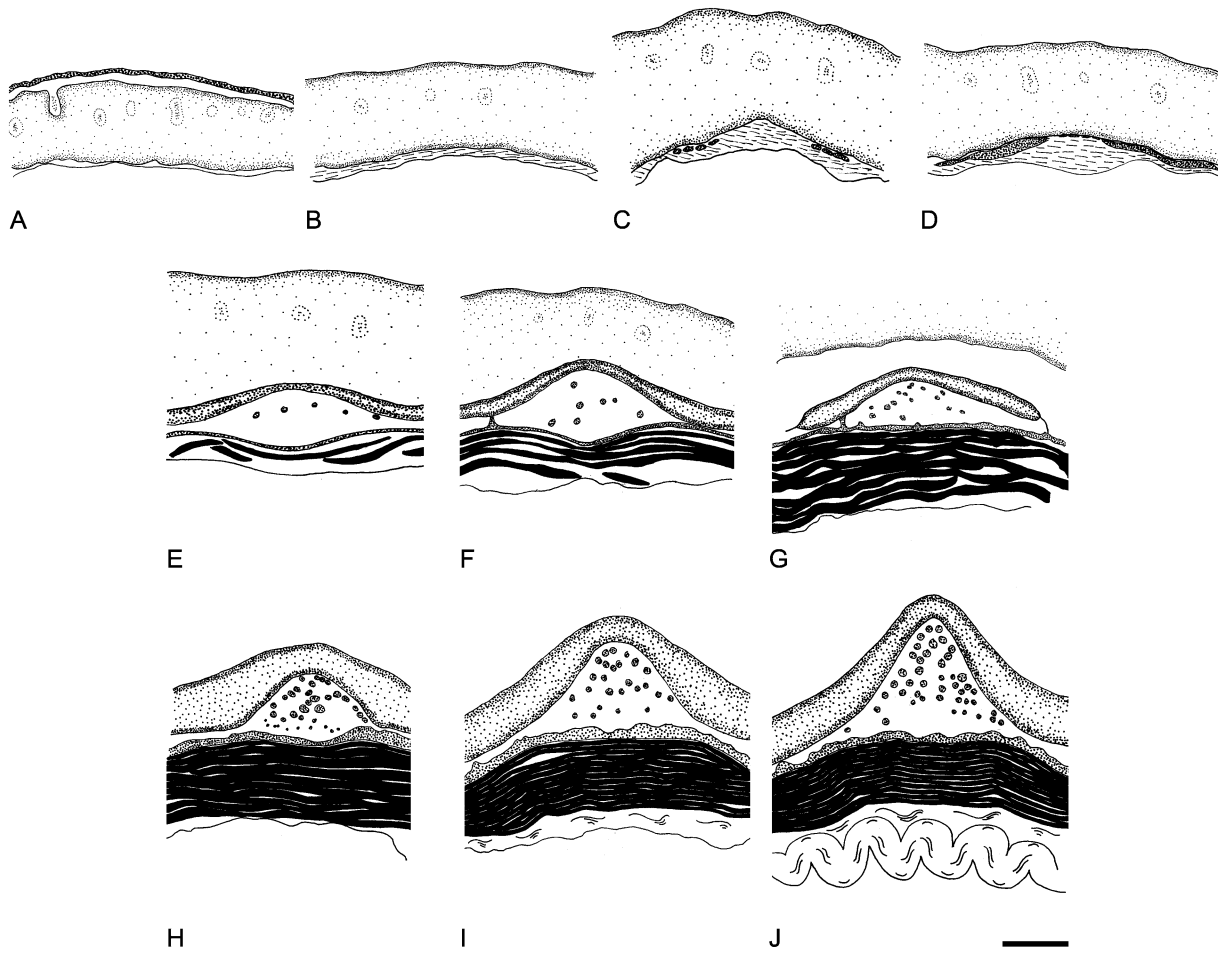

Fig. 10 Summary of the major ontogenetic events during pollen wall development. *A*, Microspore mother cell stage. *B–G*, Tetrad stage. *H*, *I*, Free microspore stage. *J*, Mature grain stage. Bar = 300 nm. Characters include callose (lightly stippled area), primexine (horizontal dashes), ectexine (densely stippled area), endexine (black), and intine (wavy lines).

and development continues through the free microspore stage (Kurmann 1990*b*; Uehara and Sahashi 2000). However, in saccate conifers with alveolar infratecta, timing of endexine formation varies. In *Pinus sylvestris*, endexine formation initiates during the tetrad stage, but thickening continues into the free microspore stage (Rowley et al. 1999, 2000). In *Tsuga canadensis*, formation of endexine lamellae occurs exclusively in the tetrad stage (Kurmann 1990*a*, 1992). In basal angiosperms, however, the majority of endexine development occurs during the free microspore stage (Zavada 1984; Takahashi 1994; Gabarayeva and Grigorjeva 2003; Taylor and Osborn 2006). Zavada (1984) identified the white-line-centered lamellae of many gymnosperms as homologous to the foot layer of basal angiosperms, citing ontogenetic timing. However, the formation and compression of endexine lamellae in *Ephedra* occur well into the free microspore stage, indicating that these characters are in fact part of the endexine and develop independently from the foot layer.

Intine development in *Ephedra* occurs completely during the free microspore stage, with the presence of a thick, fibrillar intine signaling the end of the free microspore stage and the beginning of the mature grain stage. The intine initially develops as a thin, electron-translucent layer. Only after a majority of intine development has occurred does it take on its distinct undulated and fibrillar appearance.

Infratectum Ultrastructure

The infratectum first becomes evident as a distinct layer during the middle tetrad stage, after the tectum has thickened and formed the characteristic plicae of *Ephedra* pollen. Two types of infratectal elements occur throughout development. Granular infratectal elements are evident from the early tetrad to mature stages. These granules increase in diameter as development proceeds, becoming quite large upon maturity. El-Ghazaly and Rowley (1997) reported granular elements grouping together to form columella-like conglomerations within the infratectum of *Ephedra foliata*. This type of granule aggregation was not observed in our study of *Ephedra americana*. However, in the early tetrad stage, distinct columellar elements that

span from the foot layer to the tectum are present throughout the infratectum. These elements develop separately from the granular elements, and, rather than increasing in width and becoming more robust as the grains mature, they become less frequent and almost indiscernible throughout exine ontogeny. At maturity, the columellae are only rarely discernible near furrows. These columellar elements may serve as a remnant from the separation of the foot layer and the tectum, which occurs during the early tetrad stage, or they may aid in foot layer and tectum development. Columellar elements like these have not been reported previously in *Ephedra*.

Overall, the granular infratectum present at maturity is more similar to that of nonsaccate conifer pollen grains (e.g., *Cunninghamia*). Columellar infratectal elements have been reported in several basal angiosperms, including *Illicium religiosum* (Takahashi 1994), *A. maculata* (Zavada 1984), *Schisandra chinensis* (Gabarayeva and Grigorjeva 2003), *Cabomba caroliniana* (Osborn et al. 1991), *Brasenia schreberi* (Taylor and Osborn 2006), and other genera of Nymphaeales (Osborn et al. 2004). *Amborella trichopoda* is reported to have a meandering tectum that extends to the foot layer but is described as lacking true columellae (Hesse 2001).

Overall Exine Thickness

The exine first begins to develop in the earliest tetrad stage and continues its development until the grains are mature. The thickness of the exine continues to increase from initial primexine formation in the earliest tetrad stage until several endexine lamellae are laid down in the middle tetrad stage. During the late tetrad stage and the early free microspore stage, the endexine lamellae become compressed. Because of this, the overall thickness of the exine is actually less in the free microspore stage than in the middle tetrad stage. However, overall exine thickness is greatest when endexine deposition is complete, despite the compression of the lamellae throughout the ontogenetic sequence. This is due primarily to considerable thickening of the tectum and expansion of the infratectal space in the crest regions, as well as modest thickening of the foot layer.

Pollen Shape and Surface Ornamentation

The shape and surface morphology of *E. americana* pollen are typical of ephedroid pollen, in that *E. americana* grains are elliptic to elongate in shape, with distinctive plicae and furrows. Furrow morphology has historically been used as a defining feature in *Ephedra* (Steeves and Barghoorn 1959; Kedves 1987); however, this study has documented variation within *E. americana*. Pollen grains with straight and undulated furrows co-occur in the same pollen sac. The straight morphology dominates, occurring ca. 90% of the time, with undulating furrows present only occasionally. Because varying furrow morphologies have now been described within the same pollen sac in several species (Ickert-Bond et al. 2003; this study), this character must be reexamined as a taxonomically diagnostic tool in *Ephedra*.

Tapetal Ontogeny

Although an array of tapetal types have been described in angiosperms (Pacini et al. 1985; Furness and Rudall 2001), tapetal ontogeny is more consistent among gymnosperms. However, less information is available regarding tapetum biology in gymnosperms. In *E. americana*, the tapetum is distinguishable as a distinct cell layer from the beginning of the microspore mother cell stage. It initially exists as a thick, robust cell layer attached to the middle layers of the pollen sac wall. As the microspores mature, the tapetum begins to disintegrate, and it is completely absent or remains only as a remnant cell layer by the time the grains mature. Because it never becomes separated from the pollen sac wall, the tapetum of *Ephedra* is of the secretory type, like that of most gymnosperms (Pacini et al. 1985). Orbicules, or Ubisch bodies, are typically produced by a secretory tapetum. Orbicules are present in *E. americana*; they become evident in the late tetrad stage and persist into the free microspore stage. Orbicules first emerge from the tapetum as evaginations that become distinct spheres and migrate toward the developing microspores.

Conclusion

Ephedra, along with *Welwitschia* and *Gnetum*, comprises the gymnospermous order Gnetales. Because the systematic and phylogenetic position of the Gnetales has been controversial, both morphological and molecular studies are needed to clarify phylogeny. This study reexamined morphological and ultrastructural characters of the mature pollen wall and described and documented pollen developmental characters in *Ephedra* for the first time. Several key characters, such as endexine developmental timing, ephemeral columellar infratectal elements, and callosic channels/infiltrations, are revealed only during an examination of the full ontogenetic sequence. Some characters, such as tripartite endexine lamellae, endexine developmental timing, and a secretory tapetum, align *Ephedra* with conifers and gymnosperms. However, other characters indicate a possible link between Gnetales and basal angiosperms. These include the brief presence of columellar infratectal elements. This study emphasizes the importance of including developmental pollen characters in systematic studies and the need for integrating ontogenetic data, along with other morphological and molecular characters, in determining phylogenetic relationships.

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