

COMPARATIVE POLLEN DEVELOPMENT IN DIOSCOREALES

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Pollen and orbicule ontogeny in representatives of three genera of Dioscoreales—*Narthecium ossifragum* Huds. (Nartheciaceae), *Tacca artocarpifolia* Seem. and *Tacca chantrieri* André (Dioscoreaceae), and *Dioscorea communis* (L.) Caddick & Wilkin (Dioscoreaceae)—is described and illustrated using LM, SEM, and TEM. The main difference is in microsporogenesis, which is successive in *Narthecium* Huds. and simultaneous in *Tacca* J.R. & G. Forst. and *Dioscorea* L. This is reflected in the tetrad configuration but not in the apertures: *Narthecium* and *Tacca* are monosulcate, while *Dioscorea* has two equatorial apertures. Other features of pollen development are similar in all three genera. Exine development starts at the proximal pole, and during the tetrad stage, the exine remains thicker at this pole. Intine development begins before mitosis. A conspicuously channeled intine forms beneath the aperture(s) in all three genera, and in *Tacca* it also occurs in nonapertural regions. After mitosis, the generative cell is situated at the proximal pole in all genera. Orbicules are present in *Narthecium* and *Dioscorea* but not in *Tacca*, although it has a secretory tapetum. In the tetrad stage, pro-orbicules appear on the tapetum in *Narthecium* and *Dioscorea*, and pro-sporopollenin is consequently deposited to form the orbicule wall.

Keywords: intine, microsporogenesis, monocotyledons, orbicules, pollen ontogeny, systematics, tapetum, tetrads.

Introduction

The current circumscription of the order Dioscoreales, comprising three families (Nartheciaceae, Burmanniaceae, and Dioscoreaceae), is relatively recent, following analysis of new morphological and molecular data (Caddick et al. 2000, 2002a). Data on microsporogenesis and pollen morphology have proved crucial to the resolution of relationships within lilioids (Rudall et al. 1997), Dioscoreales (Caddick et al. 1998), and Dioscoreaceae (Schols et al. 2001, 2003, forthcoming). These articles provided significant palynological data and indicated the need for pollen ontogenetic studies within Dioscoreales. In this order (fig. 1), a change from successive to simultaneous microsporogenesis occurs (Caddick et al. 1998) as well as a switch from monosulcate to the less frequent disulcate pollen type (Schols et al. 2003, forthcoming), and examination of pollen ontogeny could offer some insight into the mechanisms causing these changes. Additionally, such a study could also provide more data on the development of the distinctive intine observed by Schols et al. (2001), the tapetum type, and the development of orbicules, small sporopollenin bodies reported to be abundant within *Dioscorea* (Schols et al. 2001, 2003). To date, however, pollen ontogeny has not been studied in detail in Dioscoreales apart from obser-

vations of microsporogenesis (Caddick et al. 1998; Furness and Rudall 1999).

This article aims to document the pollen ontogeny of three genera of Dioscoreales using *Narthecium ossifragum* Huds. (Nartheciaceae), *Tacca artocarpifolia* Seem. and *Tacca chantrieri* André (Dioscoreaceae), and *Dioscorea communis* (L.) Caddick & Wilkin (Dioscoreaceae) as exemplar taxa. These species were chosen because they represent different parts of the Dioscoreales phylogeny and because they encompass the range of pollen characters found in the order (fig. 1). Nartheciaceae are sister to the rest of Dioscoreales and are successive and monosulcate, the most common combination within monocots (Caddick et al. 1998, 2002a; Furness and Rudall 1999). *Dioscorea* L. and *Tacca* J.R. & G. Forst. both have simultaneous microsporogenesis (Caddick et al. 1998). *Dioscorea communis* is disulcate, the most common aperture type in *Dioscorea*, which is a synapomorphic character (fig. 1), while *Tacca* is monosulcate (Schols et al., forthcoming). In this article, the development of the pollen grains, tapetum, and orbicules in the three selected genera is described and compared both between these taxa and with other monocots. Burmanniaceae, the third family in the order, is not represented here. They have successive microsporogenesis, like Nartheciaceae (Chakrapani and Raj 1971; Caddick et al. 1998). They are mycoheterotrophs, which cannot be grown outside their natural habitat among the leaf litter in moist tropical forests, making it extremely difficult to obtain material for ontogenetic observations.

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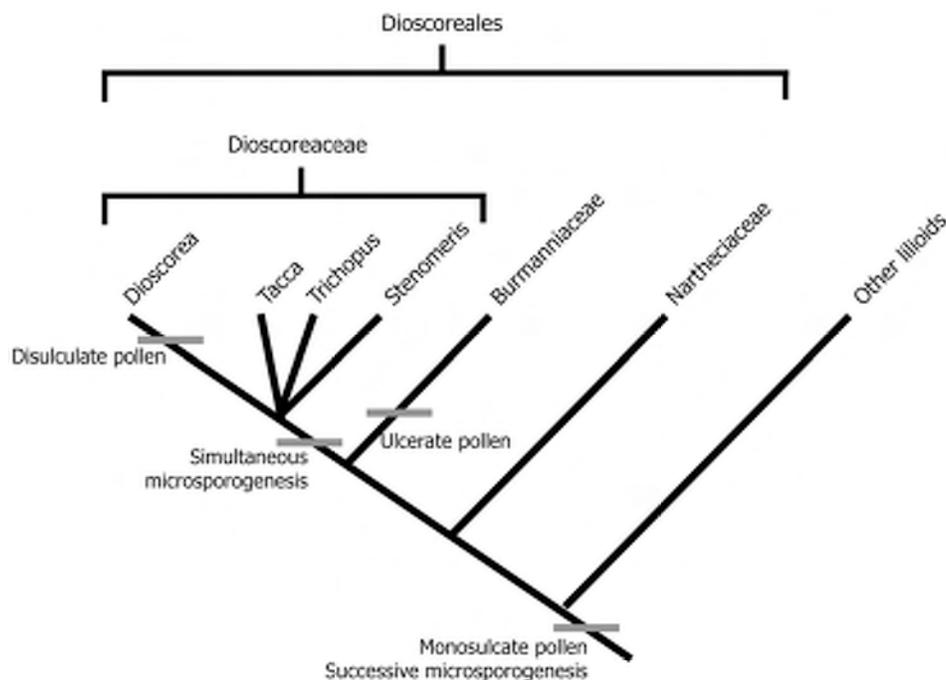


Fig. 1 Evolution of pollen characters in Dioscoreales, based on Caddick et al. (2002b). *Dioscorea* section *Stenophora*, sister to other *Dioscorea* species, is also monosulcate (Schols et al., forthcoming).

Material and Methods

Material

Material of *Nartheicum ossifragum* was collected in the wild, and a voucher specimen was deposited in the herbarium of the Royal Botanic Gardens, Kew (K: Furness and Rudall 1). Material of *Tacca* was collected from specimens in the living collections of the Royal Botanic Gardens, Kew (*Tacca artocarpifolia*: HK: 1996-946; *Tacca chantrieri*: HK: 1986-329). For *Dioscorea communis*, inflorescences were obtained from a specimen grown in the botanical garden of the Institute of Botany and Microbiology, Katholieke Universiteit Leuven. Voucher specimens have been deposited in the herbarium of Leuven.

Methods

SEM. Fresh flowers were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 24 h and dehydrated through an acetone series. They were critical-point dried in a Balzers CPD 030. Following critical-point drying, anthers were removed, and pollen grains were mounted on stubs with carbon strip tape. Micrographs were taken using digital imaging on a JEOL JSM 5800 scanning electron microscope at 80 kV.

LM and TEM. Anthers of *N. ossifragum*, *T. artocarpifolia*, and *T. chantrieri* were placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), deaerated under vacuum for 1 h, and fixed for 16–20 h at 4°C. They were washed in cacodylate buffer, postfixed in 1% buffered osmium tetroxide for 3 h at room temperature, and washed again. Tissues were dehydrated through an ethanol series followed by three

changes of 100% ethanol and embedded in LR White resin (London Resin, Reading, UK) in gelatin capsules. Fresh whole anthers of *D. communis* were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 24 h and postfixed in 2% osmium tetroxide for 1 h. Anthers were bloc stained with uranyl acetate (1%) for 10 min, dehydrated through an acetone series followed by propylene oxide, and embedded in araldite. For all taxa, semithin sections were stained with thionin (0.1%) and methylene blue (1%) and examined using a Labophot light microscope and a Nikon AFX-II camera attachment. Ultrathin sections on copper grids were stained with uranyl acetate and lead citrate. Electron micrographs were taken using a Zeiss EM906 transmission electron microscope at 80 kV.

Measurements. Measurements were carried out on digital SEM and TEM images using Carnoy 2.1 for Mac OS X (Schols et al. 2002).

Terminology. Terminology follows the international glossary (Punt et al. 1998). Although anther and pollen development is a continuous process, we have organized the descriptions of developmental events and cytological changes in four distinct stages—microsporocytes (microspore mother cells), tetrads, free microspores, and mature pollen grains—following the usual convention for pollen development (e.g., Kreunen and Osborn 1999).

Results

Nartheicum

Microsporocyte stage (fig. 2A–2D). The anther wall typically consists of an epidermis; an endothecium; one, two, or

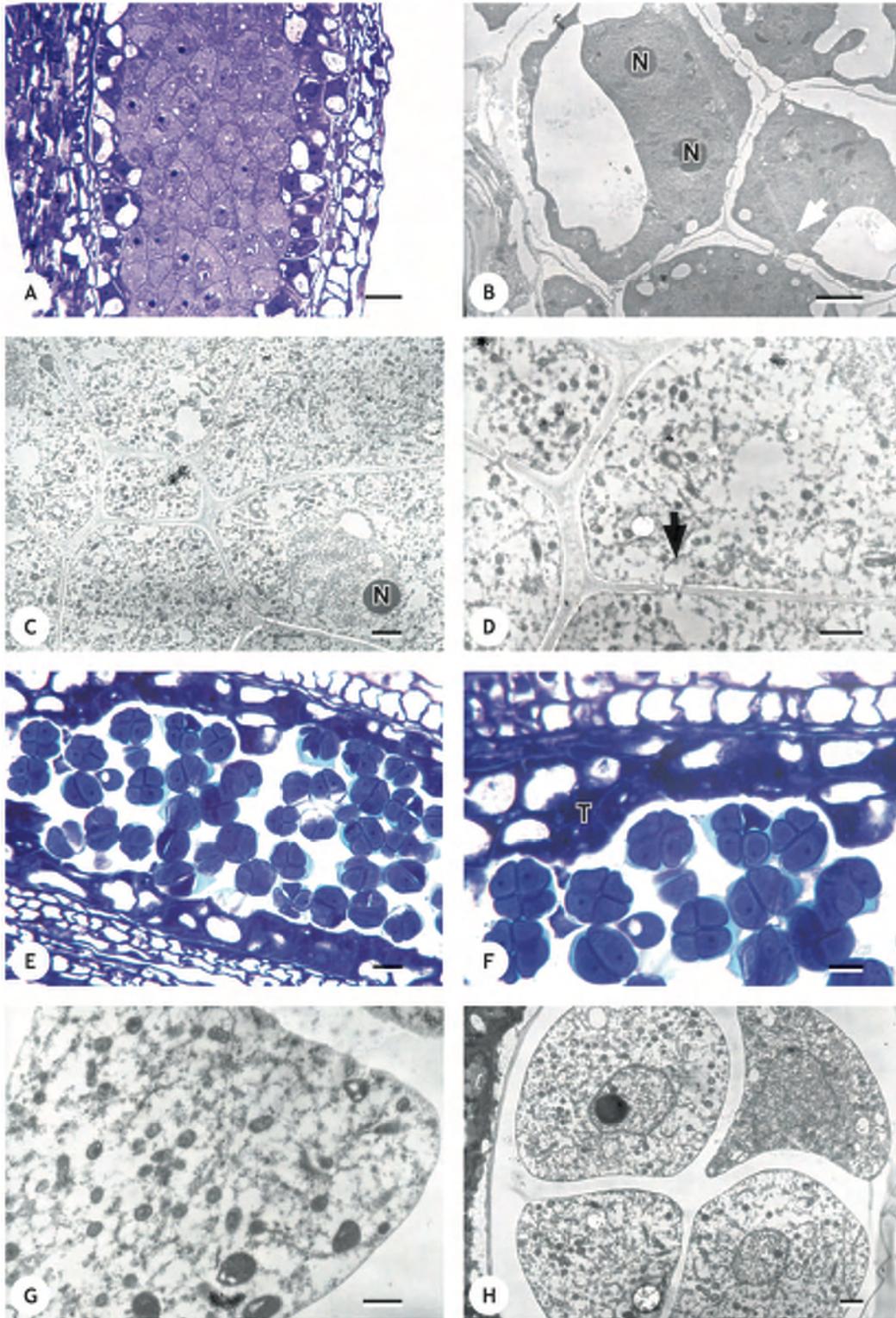


Fig. 2 *Narthecium ossifragum*, microsporocyte stage and tetrad stage. *A–D*, Microsporocyte stage. *A*, Section through a single locule showing early microsporocytes and densely staining tapetum (LM). *B*, Section through a tapetal cell with two nuclei (N) (TEM). *C*, Section through microsporocytes surrounded by callose (TEM). *D*, Detail of *C* showing a cytoplasmic channel (arrow) (TEM). *E–H*, Tetrad stage. *E*, Section through a locule filled with tetragonal tetrads (LM). *F*, Detail of *E*. The tapetum (*T*) contains many vacuoles and densely staining substances (LM). *G*, Section through one microspore in a tetrad at the early tetrad stage (TEM). *H*, An early tetragonal tetrad, with no indication of wall formation (TEM). Bars = 10 μm in *A*, *E*; 1 μm in *B–D*, *G*, *H*.

rarely three middle layers; and a tapetum. The tapetum is mostly uninucleate, sometimes binucleate, and one or two layered (fig. 2A, 2B). Microsporocytes are ca. 25 μm long and 15 μm wide, angular in shape with large nuclei (fig. 2C). They are interconnected through cytomictic channels of ca. 0.5 μm diameter (fig. 2B, 2D), which allow intercell communication.

Tetrad stage (figs. 2E–3D). Following meiosis, tetragonal tetrads of haploid microspores randomly fill the entire space of the anther locule. Each microspore in a tetrad is surrounded by a thick callose envelope (fig. 2E–2H). A fibrillar surface coat develops between the callose and the plasmalemma of each microspore. This primexine matrix has about the same thickness over the entire surface, with no visible differentiation between the proximal pole and the distal pole, where the aperture will be formed (fig. 2G, 2H). This is the most productive stage for the tapetal cells, and they stain very densely (fig. 2E, 2F; fig. 3C). Lipid droplets and pro-orbicules, originating by exocytosis, are visible in depressions of the tapetal plasmalemma (fig. 3D). In microspores in slightly older tetrads, T-shaped electron-dense material is radially oriented in the proximal part of the plasmalemma surface coat; these elements are the initiation of the columellae and tectum. Wall deposition seems more developed at the proximal pole: at the late tetrad stage, the exine is ca. 0.4 μm thick at the proximal pole and 0.2 μm at the distal pole (fig. 3A). An incomplete tectum starts to form. The aperture sites become visible for the first time: they lack columellae, and the exine elements above the aperture are very thin or even nonexistent. One elongated aperture develops at the distal pole, leading to monosulcate pollen grains (fig. 3B). We could not find any intermediate stages between early and late tetrads, indicating that the deposition of the first exine elements occurs very rapidly and the foot layer originates soon thereafter. Entire tetrads in the late tetrad stage have a diameter of ca. 17 μm . In this phase of anther development, the tapetal cells are still in a state of high metabolic activity, indicated by their many organelles and cytoplasmic inclusions.

Free microspore stage (figs. 3E–4B). Breakdown of the callose wall marks the transition from the tetrad stage to the free microspore stage. In the earliest free microspore stage, the anthers contain microspores still in a tetrad arrangement, with residual callose present between them (fig. 3E). By the time the callosic envelope has completely dissolved and the microspores are released from the tetrads, their exines have increased considerably in thickness to ca. 0.8 μm (fig. 3G, 3H). Microspores at this stage are characterized by the presence of one or more large vacuoles and have a diameter of ca. 20 μm (fig. 3F). In the late free microspore stage, each microspore still contains many vacuoles as well as starch and lipid droplets. The intine develops underneath the aperture and protrudes together with part of the protoplasm (fig. 4A). The intine is almost exclusively present in the apertural region and contains radially oriented channels. Intine initiation precedes mitosis since the intine layer is clearly present at the late free microspore stage. Rather large orbicules of ca. 0.8 μm diameter line the surface of the tapetal cells (fig. 4B) on the inner and outer tangential walls as well as on the radial surfaces. These orbicules have an electron-lucent core of

0.2 μm diameter (fig. 4B). At this stage, the tapetum starts to degenerate.

Pollen grain stage (fig. 4C–4H). In mature pollen grains, the exine is ca. 1.2 μm thick. The foot layer, tectum, and columellae are now well differentiated (fig. 4C, 4D). *Nartheicum ossifragum* has a perforate sexine. The intine develops further and also becomes visible at nonapertural sites. Two intine layers are observed. A channeled layer is very conspicuous in the apertural region and very thin in nonapertural regions (fig. 4D, 4E). Seen in tangential section, the channels are arranged in a characteristic polygonal structure (fig. 4F). The second layer is a thin endintine covering the entire pollen grain (fig. 4D, arrows). After mitosis, the generative cell is situated at the proximal pole at the opposite side of the aperture (fig. 4C, 4D). Starch seems to be the main cytoplasmic storage product, since the pollen contains many compound starch grains. The tapetum has almost entirely degenerated, leaving cytoplasmic remnants together with orbicules on the locule walls (fig. 4G, 4H). The orbicules have a diameter of ca. 1.3 μm and an electron lucent core of ca. 0.5 μm .

Tacca

Microsporocyte stage (fig. 5A, 5B). An epidermis, endothecium, one middle layer, and one layer of tapetal cells surround the microsporocytes. The tapetum is mostly uninucleate or sometimes binucleate. The microsporocytes are angular in shape (fig. 5A), and late microsporocytes are surrounded by callose walls (fig. 5B).

Tetrad stage (fig. 5C–5F). The tetrad stage in *Tacca* is very similar to that in *Nartheicum*. The main difference is the presence of tetrahedral tetrads in *Tacca* (fig. 5C) that are correlated with simultaneous microsporogenesis. A thick callose envelope surrounds each tetrad unit (fig. 5D). The cytoplasm of the microspores contains many vacuoles and Golgi vesicles. Between the callose and the plasmalemma, a fibrillar surface coat develops (fig. 5F). As in *Nartheicum*, T-shaped electron-dense material is radially oriented in the plasmalemma surface coat. This is the initiation of the columellae and tectum. Wall deposition is more developed at the proximal pole at this stage. As in *Nartheicum*, the exine is ca. 0.2 μm thick at the proximal pole and 0.1 μm thick at the distal pole. Where the plasmalemma is pressed directly against the callose wall at the distal pole, exine is completely lacking, and this region will become the apertural site (fig. 5E). Entire tetrads have a diameter of ca. 34 μm . This is about twice the size of tetrads in *Nartheicum* and reflects the larger size of pollen in *Tacca* (ca. 50 μm compared with ca. 20 μm in *Nartheicum*; Schols et al., forthcoming). In this phase of anther development, the tapetal cells are still in a state of high metabolic activity, indicated by their many organelles and cytoplasmic inclusions. Orbicules or pro-orbicules were not observed.

Free microspore stage. Unfortunately, observations of the free microspore stage in *Tacca* are not available since pollen development often aborted (cytoplasm was lacking) after the tetrad stage in the material of *Tacca chantrieri* examined. Observations of the pollen grain stage are based on sections through a mature anther of another *Tacca* species (*Tacca artocarpifolia*).

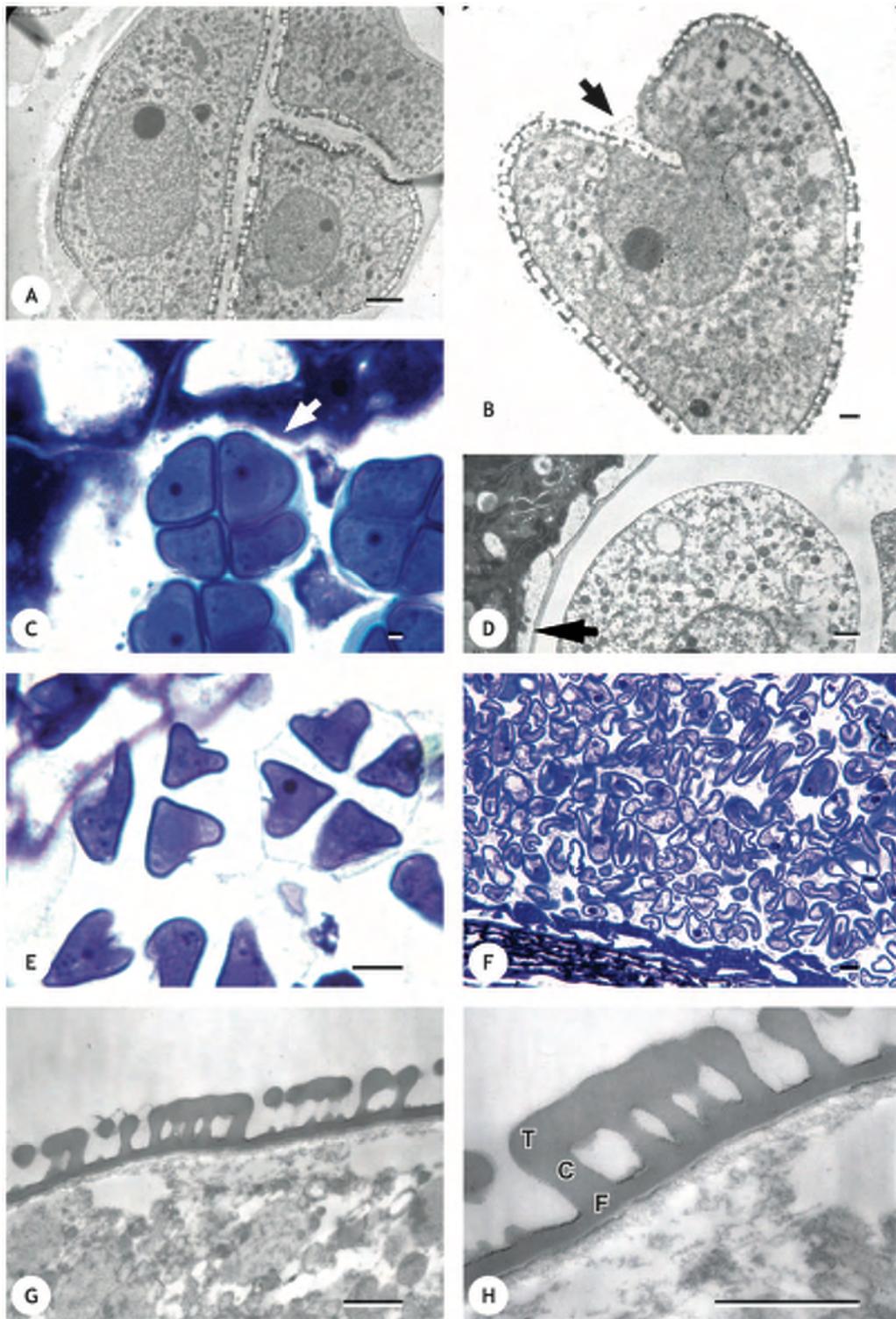


Fig. 3 *Narthecium ossifragum*, tetrad stage (continued) and microsporocyte stage. A, Late tetrad with T-shaped elements forming the pro-columellae and the pro-TECTUM (TEM). B, Detail of one microspore from a tetrad with exine and aperture site (arrow) (TEM). C, Tetragonal tetrads. The single sulci are visible at the distal poles (arrow) (LM). D, Detail of the tapetum releasing pro-orbicules (arrow) in cuplike depressions of the plasma membrane (TEM). E–H, Free microspore stage. E, Early free microspores being released from the tetrads as the callose walls dissolve (LM). F, Anther locule with microspores in the vacuolate stage, with one or more large vacuoles occupying a large part of each microspore. The microspores appear wrinkled (LM). G, Developing exine during the vacuolate stage: tectum, columellae, and foot layer are already present (TEM). H, Detail of G showing tectum (T), columellae (C), and foot layer (F) (TEM). Bars = 1 μm in A–D, G, H; 10 μm in E, F.

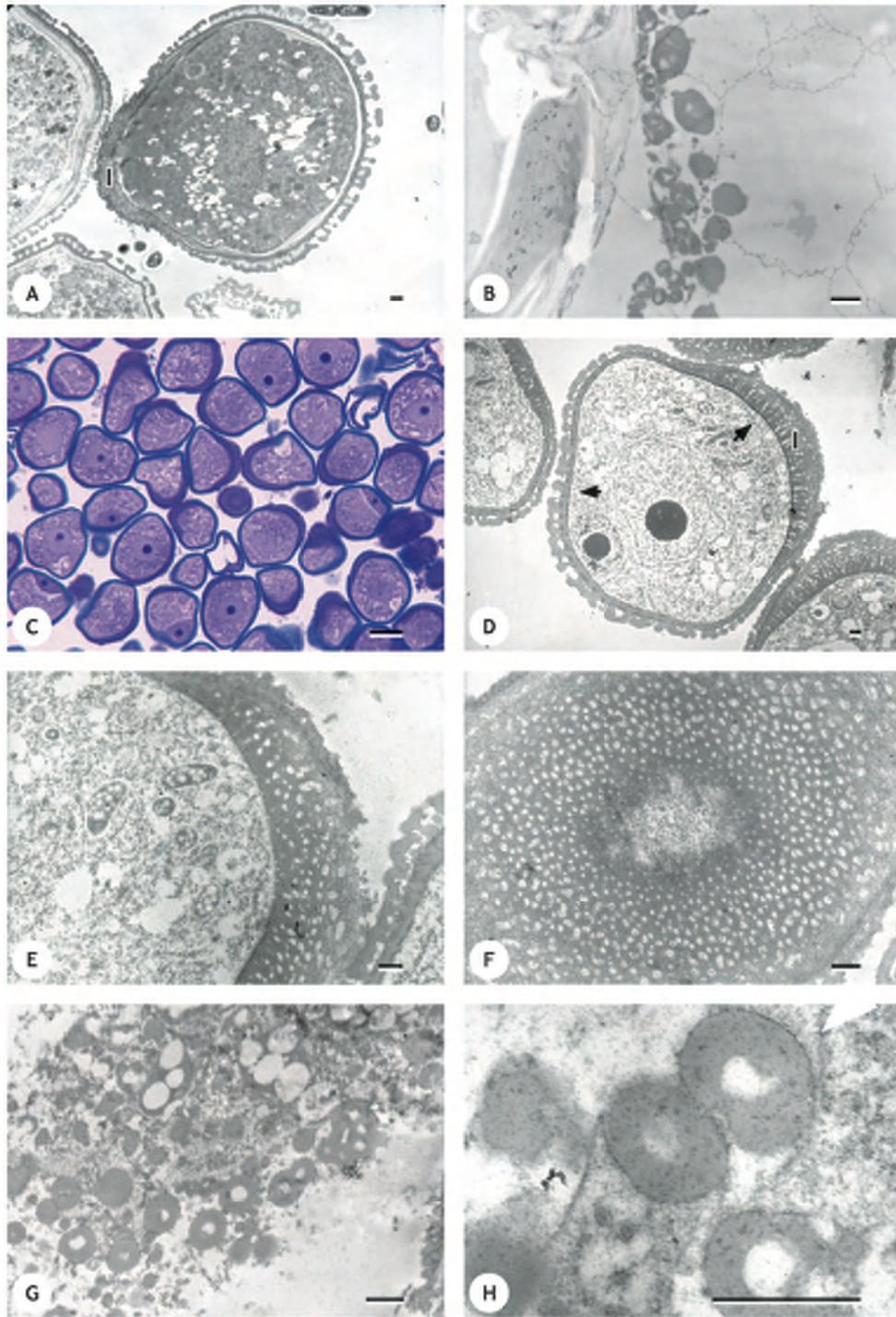


Fig. 4 *Narthecium ossifragum*. Free microspore stage (continued) and mature pollen grain stage. *A, B*, Free microspore stage. *A*, Late microspore with a well-developed exine. Intine (*I*) is already present beneath the sulcus, and the cytoplasm contains many starch grains (TEM). *B*, Orbicules at the late microspore stage with electron-lucent cores and electron-dense walls. The tapetum is degenerating. *C-H*, Mature pollen grain stage. *C*, Pollen grains in an anther locule. Both the vegetative and generative cells are visible in some grains. The generative cell is always located at the proximal pole, opposite the aperture. The intine stains densely beneath the aperture (LM). *D*, Section through a mature pollen grain. The generative cell is located opposite the aperture as seen in *A*. Arrows indicate the difference in intine thickness between the apertural and nonapertural sites. The intine (*I*) beneath the aperture is thick and channeled (TEM). *E*, Transverse section through the apertural intine of a mature pollen grain (TEM). *F*, Tangential section through the apertural intine of a mature pollen grain. The electron-lucent channels are arranged in polygonal structures (TEM). *G*, Orbicules on the tapetal remnants. The electron-dense wall has thickened compared with the free microspore stage (TEM). *H*, Detail of orbicules with a darker peripheral layer on the outside (arrow) (TEM). Bars = 1 μm in *A, B, D-H*; 10 μm in *C*.

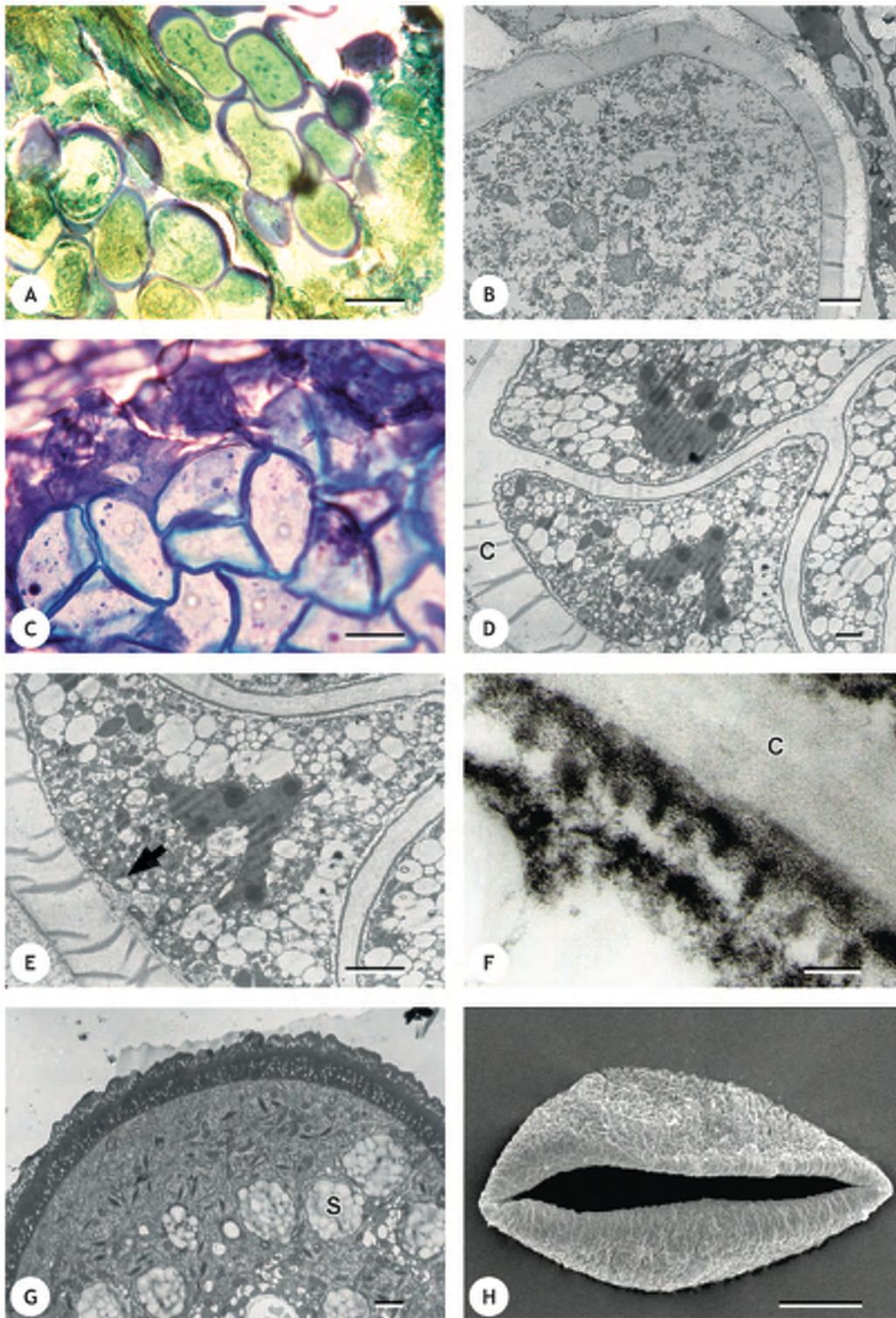


Fig. 5 *Tacca chantrieri*, microsporocyte stage, tetrad stage, and mature pollen grain stage. *A, B*, Microsporocyte stage. *A*, Section through an anther locule with microsporocytes surrounded by densely staining callose walls (LM). *B*, Detail of a microsporocyte surrounded by callose (TEM). *C–F*, Tetrad stage. *C*, Section through a locule filled with tetrahedral tetrads (LM). *D*, A tetrahedral tetrad with a highly undulating plasma membrane and thick callose wall (C) (TEM). *E*, Detail of *D* showing the future aperture site at the distal pole (arrow) where no exine deposition occurs (TEM). *F*, Exine synthesis at the proximal pole, underneath the callose (C) (TEM). *G, H*, Mature pollen grain stage. *G*, Young pollen grain containing many compound starch grains. The exine is well developed, and a thick, channeled intine is present around the entire pollen grain (TEM). *H*, Mature pollen grain with a distal sulcus (SEM). Bars = 10 μm in *A, C, H*; 1 μm in *B, D–G*.

Pollen grain stage (figs. 5G–6C). After mitosis, the generative cell is situated at the proximal pole at the opposite side of the aperture (fig. 6A). In mature pollen grains, the exine is ca. 1.4 μm thick with a well-differentiated foot layer, tectum and columellae. *Tacca artocarpifolia* has monosulcate pollen (fig. 5H) and rugulate sexine ornamentation (fig. 6B). The intine develops further and also becomes visible at nonapertural sites. One channeled intine layer forms, and this is ca. 1.1 μm thick in both apertural and nonapertural regions (fig. 5G), although this pollen grain is not fully mature. The intine channels are arranged in a polygonal structure. Each pollen grain contains many compound starch grains, and starch seems to be the main cytoplasmic storage product, as in *Narthecium*. At this stage, the tapetum has almost entirely degenerated, and there are no orbicules present on the locule wall (fig. 6C).

Dioscorea

Microsporocyte stage (fig. 7A–7E). In addition to the epidermis and endothecium, one middle layer and one layer of tapetal cells surround the microsporocytes. The tapetum is mostly uninucleate or sometimes binucleate and contains many vacuoles and densely staining substances (fig. 7A, 7B). The microsporocytes are angular in shape and ca. 25 μm long and 20 μm wide (fig. 7B, 7C). They are surrounded by callose and contain many vesicles (fig. 7C–7E).

Tetrad stage (fig. 7F–8H). After meiosis, tetrahedral tetrads of haploid microspores fill the anther locules (fig. 7F–

7H). Each tetrad unit is surrounded by a callose envelope. The cytoplasm of the microspores is rich in vacuoles, ribosomes, and Golgi vesicles. Golgi vesicles are abundant near the plasmalemma in particular and are probably involved in the initiation of the primexine, which is not yet present. In slightly older tetrads, a fibrillar surface coat develops between the callose and the plasmalemma (fig. 7I), and T-shaped electron-dense material is radially oriented in the proximal part of the plasmalemma surface coat. These elements are the initiation of the columellae and have a length of ca. 0.2 μm (fig. 8A–8C). Exine deposition clearly starts at the proximal pole, and the exine elements are thicker and better developed here (figs. 7I, 8E). The aperture sites are equatorial, positioned between the proximal and distal pole and forming an angle of 180°, and they lack deposition of the T-shaped elements. The apertures are parallel to the longest axis of the pollen grain. The cytoplasm of the microspores contains many proplastids (fig. 8D–8F). Entire tetrads in the late tetrad stage have a diameter of ca. 30 μm . Some tetrads are still connected via cytomictic channels that seem to persist into the late tetrad stage (fig. 8G). This is the most productive stage for the tapetal cells, which stain very densely and contain endoplasmic reticulum and electron-dense sporopollenin precursors. Lipid droplets and pro-orbicules are visible in depressions of the tapetal plasmalemma, originating by exocytosis (fig. 8H).

Free microspore stage (fig. 9). Residual callose is still present between the free microspores in the early free

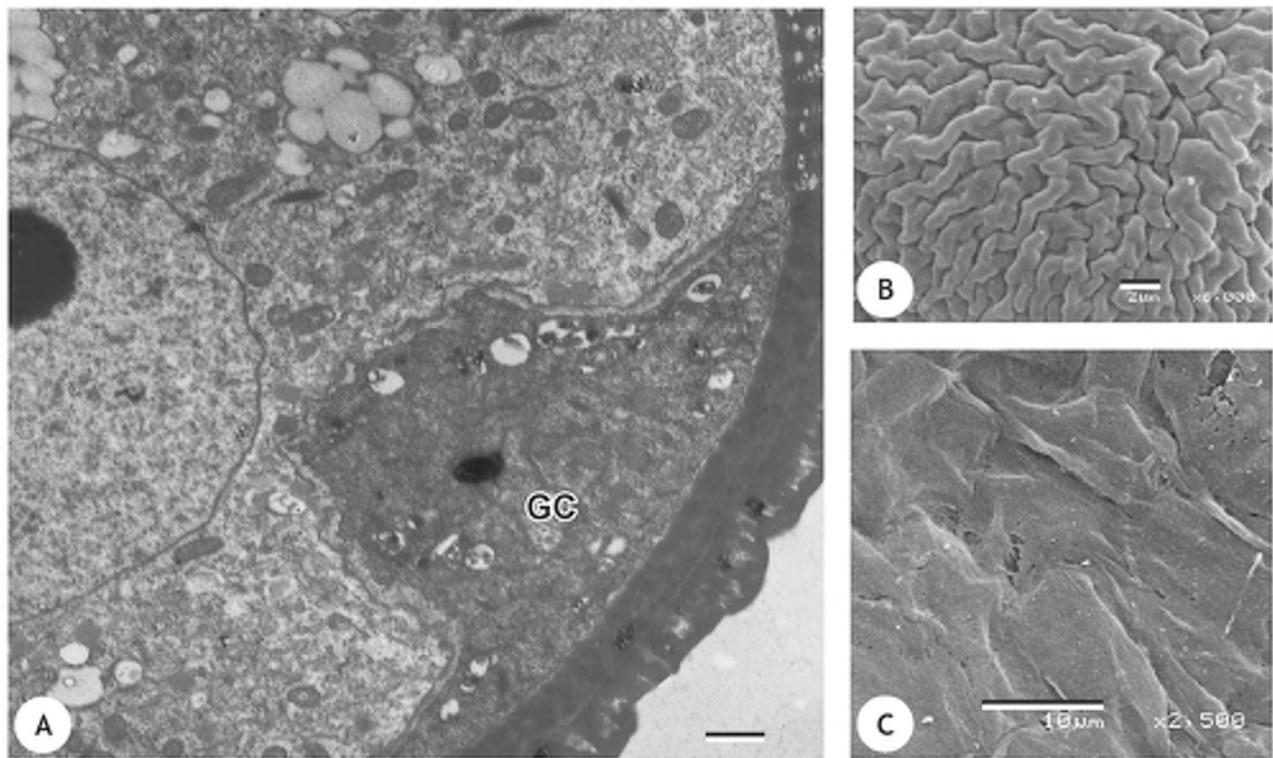


Fig. 6 *Tacca chantrieri*, mature pollen grain stage. A, Section through a mature pollen grain with the generative cell (GC) situated at the proximal pole, opposite the aperture (TEM). B, Detail of the sexine in a mature pollen grain (SEM). C, Tapetal remnants without orbicules (SEM). Bars = 1 μm in A; 2 μm in B; 10 μm in C.

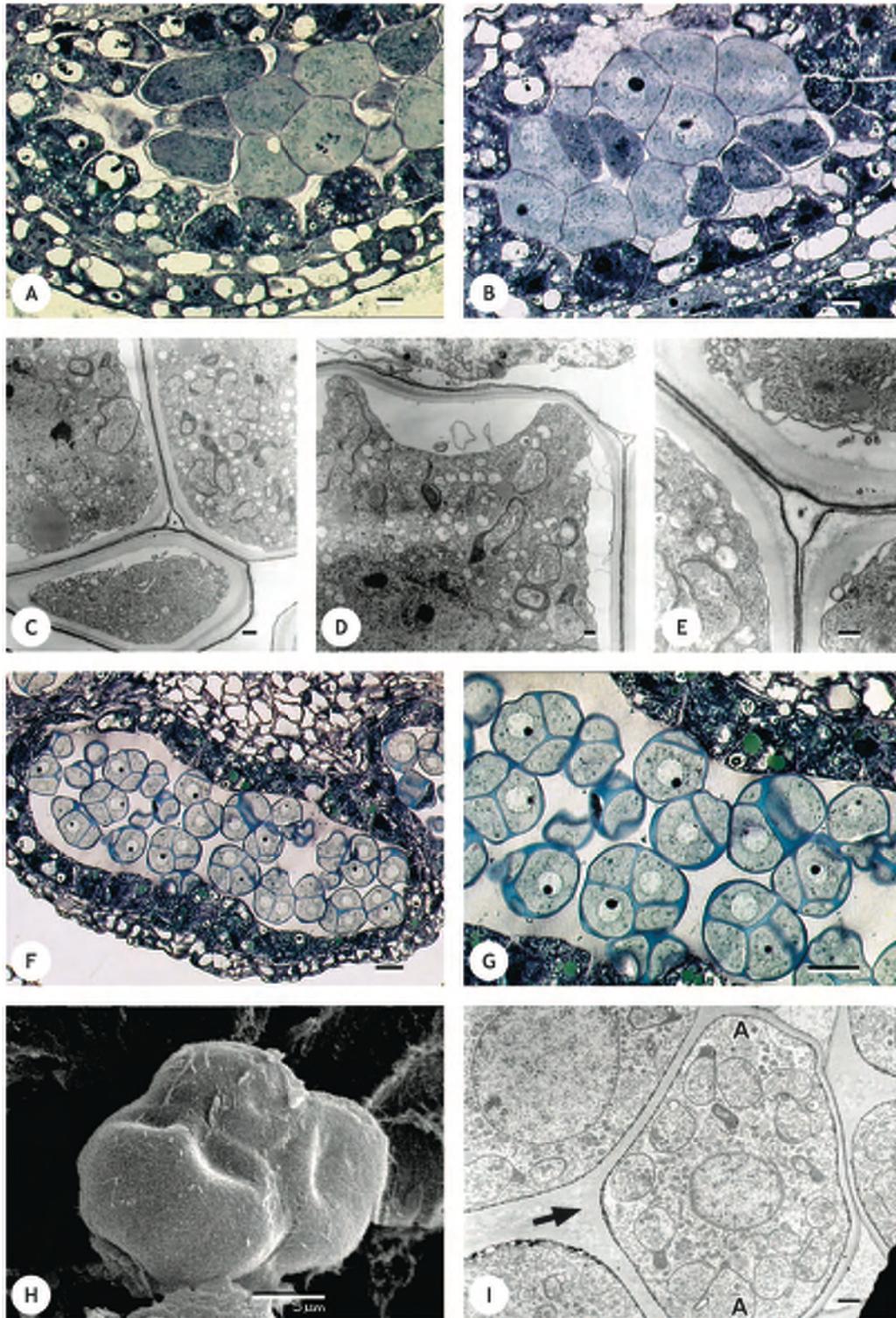


Fig. 7 *Dioscorea communis*, microsporocyte stage and tetrad stage. *A–E*, Microsporocyte stage. *A*, Section through an anther locule with early microsporocytes and densely stained tapetum (LM). *B*, Section through an anther locule with either mono- or binucleate tapetal cells (LM). *C*, Section through microsporocytes surrounded by thick callose walls (TEM). *D*, Detail of a microsporocyte with an undulating plasmalemma (TEM). *E*, Detail of microsporocytes surrounded by thick callose walls. The cytoplasm contains many vesicles (TEM). *F–I*, Tetrad stage. *F*, Section through an anther locule filled with tetrahedral tetrads (LM). *G*, Detail of *F*. The tapetum contains many densely staining substances (LM). *H*, A tetrahedral tetrad surrounded by callose (SEM). *I*, An early tetrad with a microspore showing one of the two future aperture sites (*A*) lacking exine synthesis, which is most advanced at the proximal pole (arrow) (TEM). Bars = 10 μm in *A*, *B*, *F*, *G*; 1 μm in *C–E*, *I*; 5 μm in *H*.

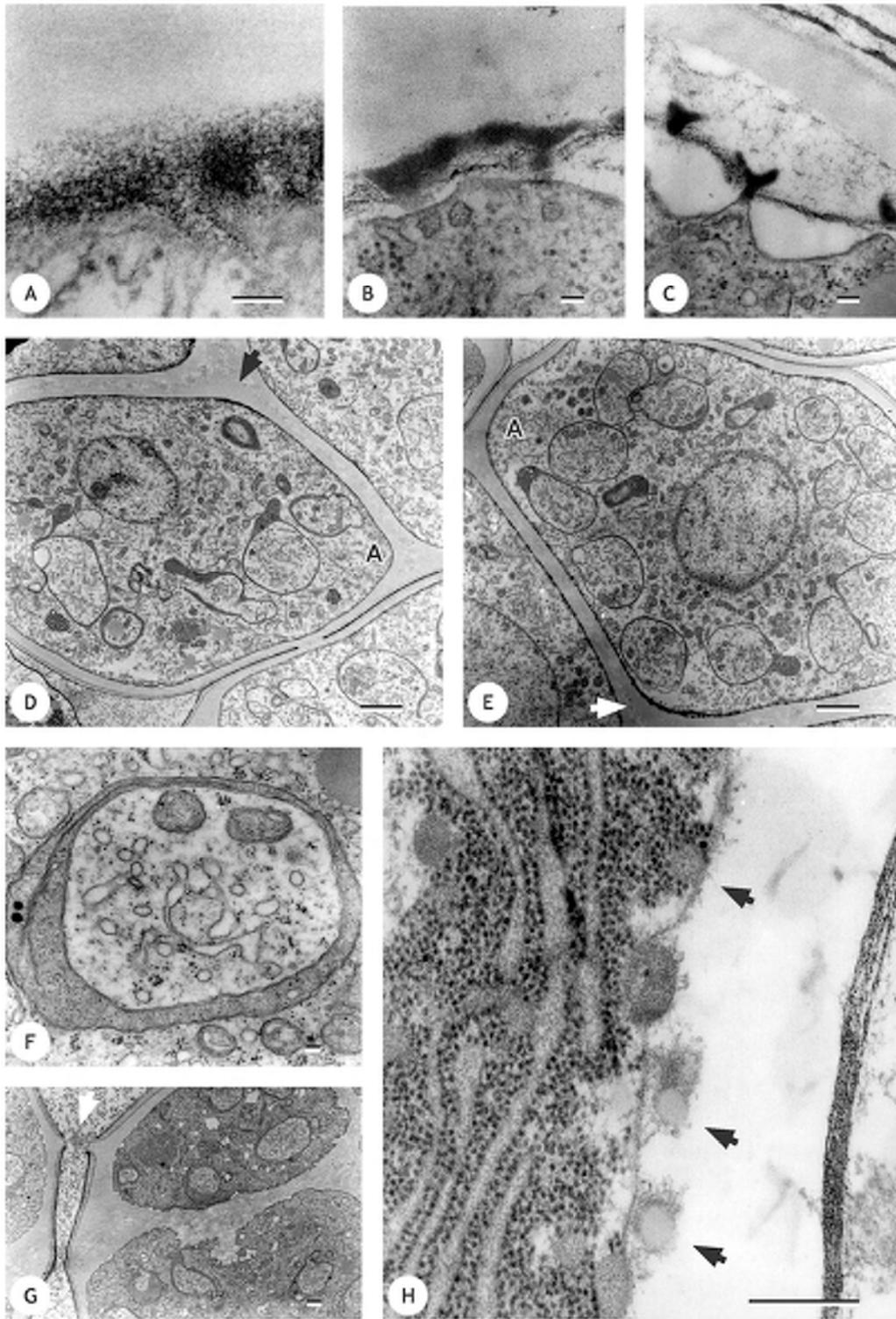


Fig. 8 *Dioscorea communis*, tetrad stage. *A*, Section with the primexine matrix developing in the early tetrad stage (TEM). *B*, Deposition of T-shaped elements in the primexine matrix (TEM). *C*, Slightly later stage than in *B*. The plasma membrane is highly undulated, and the T-shaped elements are clearly visible (TEM). *D*, A microspore in a tetrad. The primexine deposition clearly starts at the proximal pole (arrow). Cytomictic channels are still present between adjacent tetrads. (*A* = aperture) (TEM). *E*, Another microspore with primexine deposition at the proximal pole (arrow) and no exine deposition near the aperture (*A*). The microspore contains many plastids (TEM). *F*, Detail of a plastid in the cytoplasm of a microspore (TEM). *G*, Detail of cytomictic channel (arrow) (TEM). *H*, Section through the tapetum containing many rough endoplasmic reticulum strands. Pro-orbicules are visible at the tapetal plasmalemma (arrows) (TEM). Bars = 0.1 μm in *A*–*C*, *F*; 1 μm in *D*, *E*, *G*, *H*.

microspore stage (fig. 9A). Each microspore then develops a large vacuole comprising up to 70% of the volume of the microspore and with a diameter of ca. 21 μm (fig. 9B–9D). The exine is well developed (fig. 9E). The two sulci are now clearly visible because of their minimal amount of exine deposition (fig. 9F, 9G). Orbicules of ca. 0.3–0.5 μm diameter line the surface of the tapetal cells on the inner and outer tangential walls as well as on the radial surfaces. The orbicules have an electron-lucent core of 0.1–0.2 μm . At this stage, the tapetum starts to degenerate.

In the late free microspore stage, the microspores still contain many vacuoles as well as starch grains and lipid droplets. The outer, channeled layer of the intine is present only beneath the apertures (fig. 9H). As in *Nartheicum*, intine initiation precedes mitosis.

Pollen grain stage (fig. 10). Mature *Dioscorea communis* pollen is disulcate, with two elongated apertures situated between the proximal and distal poles (fig. 10A–10C). In outline, the pollen grains have a curved proximal pole and a flattened distal pole (fig. 10B). The exine is ca. 1.1 μm thick at anther dehiscence, and the foot layer, tectum, and columellae are well differentiated. The ultrastructure of the tectum results in a perforate sexine as observed using SEM (fig. 10C, 10E). The intine has increased in thickness, and two layers are present. The first is a channeled layer with channels arranged in a characteristic polygonal structure (fig. 10G), which is very conspicuous in the apertural regions (up to 1 μm thick; fig. 10F) and very thin to nonexistent in the nonapertural regions (fig. 10D, 10F). The second layer is a thin endintine (ca. 0.3 μm thick) covering the entire pollen grain (fig. 10D). The generative cell is situated at the proximal pole as far away as possible from both apertures (fig. 10B). As in *Nartheicum* and *Tacca*, the pollen contains many compound starch grains, and starch seems to be the main cytoplasmic storage product. The tapetum has almost entirely degenerated, leaving cytoplasmic remnants together with orbicules on the locule walls (fig. 10H). The orbicules have a diameter of ca. 0.3–0.4 μm .

Discussion

Anther Wall Layers and Number of Tapetal Nuclei

The structure of the anther wall appears very similar across all species in this study. It consists of an epidermis; an endothelial layer; and mostly one, sometimes two, and (in *Nartheicum*) rarely three middle layers. This anther wall structure is the most common within angiosperms (Pacini 1994), and within lilioids a similar structure has been found in other Dioscoreaceae (e.g., in *Stenomeris*; Caddick et al. 1998) and Liliaceae (Takahashi 1987). The tapetum is mostly one cell layer thick and uninucleate, although it is sometimes binucleate.

Microsporocytes and Cytomictic Channels

Cytomictic channels have been found in all three genera examined. These channels are present in many families such as Fabaceae (Xi 1991) and Malvaceae (Sarvella 1958) and are probably a feature of all angiosperm microsporocytes (Whelan et al. 1974). In Orchidaceae, they persist until the mature pollen grain stage (Shukla et al. 1998). At least in *Di-*

oscorea, cytomictic channels persist into the tetrad stage. These channels facilitate cell to cell communication (McLean et al. 1997). Cytomictic channels have also been reported to enable transfer of chromatin between adjacent microsporocytes in Brassicaceae (Malallah and Attia 2003). According to Heslop-Harrison (1971), formation of channels between microsporocytes enables them to share a common cytoplasm and effectively function as a syncytium, enabling synchronization of meiosis.

Microsporogenesis and Tetrad Configuration

Tetrad configuration is correlated with microsporogenesis type (Rudall et al. 1997; Furness and Rudall 1999; Furness et al. 2002). *Nartheicum*, with successive microsporogenesis, has tetragonal tetrads, while *Tacca* and *Dioscorea* have simultaneous microsporogenesis and tetrahedral tetrads, confirming the results of Caddick et al. (1998) and Furness and Rudall (1999). The shift from successive microsporogenesis in Nartheciaceae (and Burmanniaceae; Caddick et al. 1998) to simultaneous microsporogenesis in Dioscoreaceae (including *Tacca*) did not affect the aperture type directly. Both *Nartheicum* and *Tacca* have monosulcate pollen with one aperture at the distal pole. This indicates that tetrad configuration is not the sole factor determining aperture number and position, although it may play an important role in aperture development. On the other hand, it might be that the shift from successive to simultaneous cytokinesis has enabled the shift to occur from monosulcate apertures in *Tacca* and *Dioscorea* section *Stenophora* (sister to other *Dioscorea* species) to the disulcate type found in the rest of *Dioscorea* (fig. 1; Schols et al., forthcoming).

Aperture Formation

Apertural regions are characterized by their lack of exine deposition in the early to midtetrad stage. Ressayre (2001) summarized several mechanisms inducing the formation of apertures, such as the prevention of primexine deposition by shields of endoplasmic reticulum, callose knobs, or interstitial bodies or through another mechanism in which exine outgrowth breaks up during microspore expansion to expose the intine. None of these mechanisms were observed in the species we have studied. The presence of shields of endoplasmic reticulum or callose knobs should be detectable with TEM. The only unusual cellular activity beneath the future aperture sites in early tetrads is the presence of a high number of Golgi vesicles. The aperture site for both *Nartheicum* and *Tacca* is positioned at the distal pole, and one distal sulcus is the most common condition within monocots. In *Dioscorea*, two elongated apertures appear in the midtetrad stage, positioned between the proximal and distal poles. Apertures in *Dioscorea* are equatorial and parallel to the longest axis of the grain and are referred to as sulci (Punt et al. 1998). As discussed above, simultaneous microsporogenesis might be necessary to develop this kind of aperture configuration, although disulcate pollen has also been reported in taxa with successive microsporogenesis, e.g., Pontederiaceae species (Ressayre 2001).

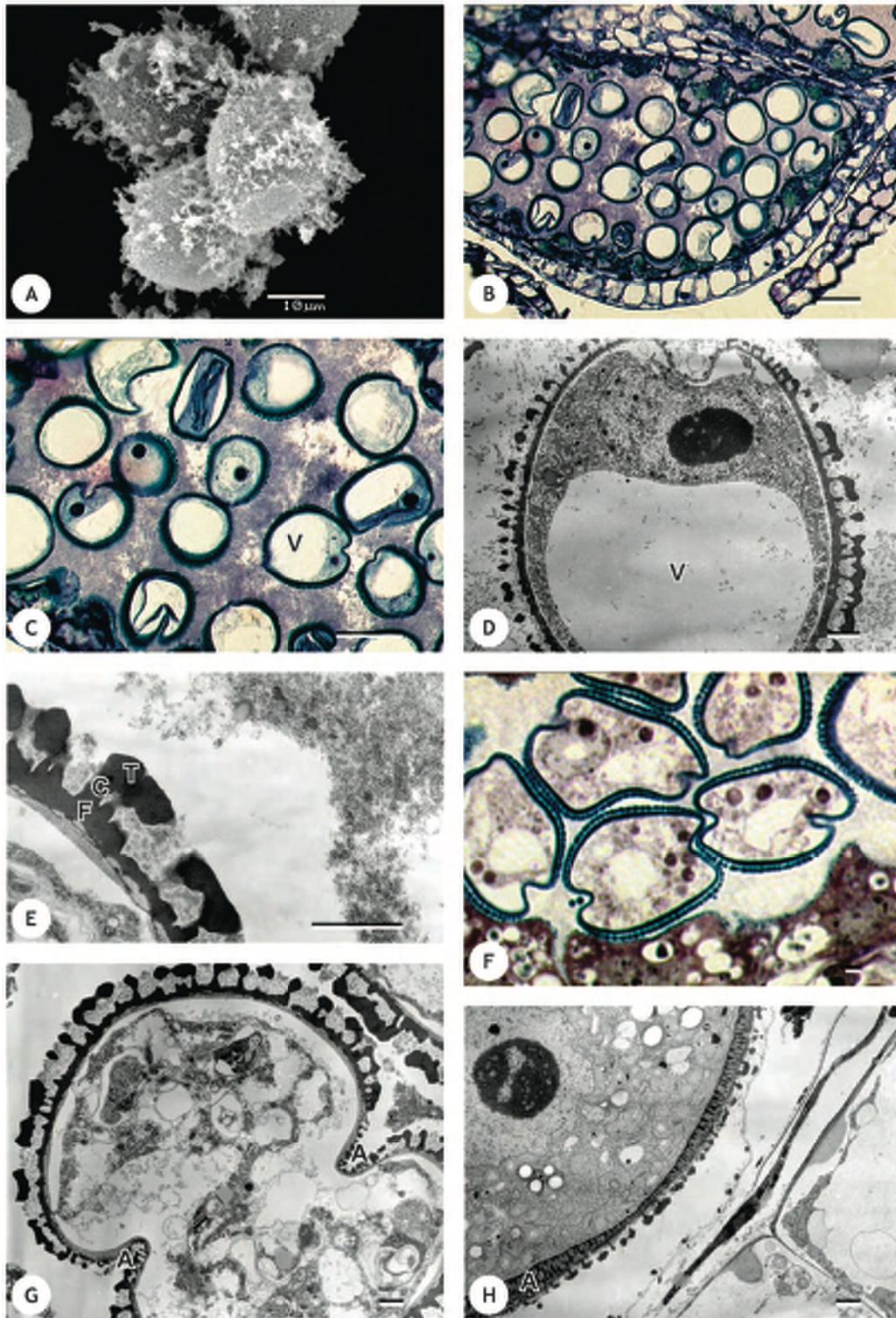


Fig. 9 *Dioscorea communis*, free microspore stage. *A*, Individual microspores released from a tetrahedral tetrad by the dissolving callose (SEM). *B*, An anther locule with free microspores at the vacuolate stage. The vacuole takes up a very large proportion of each microspore, and the cytoplasm is pressed against the microspore wall (LM). *C*, Detail of *B* (*V* = vacuole) (LM). *D*, Section through a vacuolate microspore (*V* = vacuole) (TEM). *E*, Exine ultrastructure at the vacuolate stage. Tectum (*T*), columellae (*C*), and foot layer (*F*) are present (TEM). *F*, Section through microspores at the late free microspore stage. The disulcate apertures can be seen (LM). *G*, Section through a microspore at the late free microspore stage. The two apertures (*A*) can be recognized by their reduced amount of exine deposition (TEM). *H*, Detail of a section through a late free microspore showing the thin, channeled intine present in this stage (*A* = aperture) (TEM). Bars = 10 μm in *A*–*C*; 1 μm in *D*–*H*.

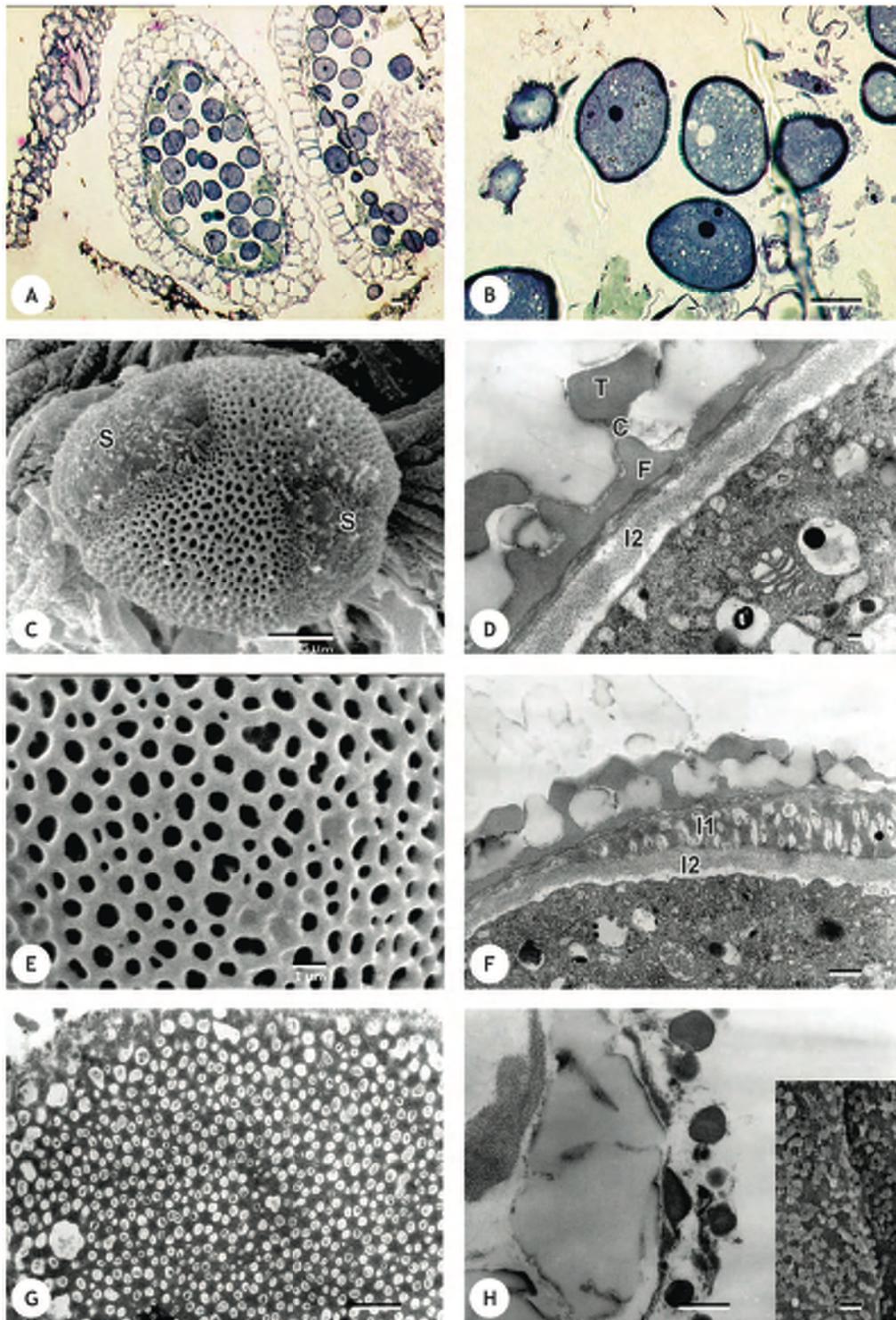


Fig. 10 *Dioscorea communis*, mature pollen grain stage. *A*, Section through the anther locules just before anthesis (LM). *B*, Pollen grains at anthesis with both the vegetative and generative cells visible in some grains. The generative cell is always located at the proximal pole, as far away from the apertures as possible. The intine stains densely beneath the sulculi (LM). *C*, Overview of a mature pollen grain showing the sulculi (S) (SEM). *D*, Exine ultrastructure showing tectum (T), columellae (C), and foot layer (F). A thin endintine (I2) is present (TEM). *E*, The perforate sexine surface (SEM). *F*, Wall ultrastructure near a sulculus. The channeled intine (I1) is only present beneath the apertures while the endintine (I2) surrounds the entire pollen grain. *G*, A tangential section through the intine. The electron-lucent channels are arranged in polygonal structures (TEM). *H*, Mature orbicules covering the tapetal remnants. (main image, TEM; inset, SEM). Bars = 10 μm in *A*, *B*; 5 μm in *C*; 0.1 μm in *D*; 1 μm in *E*, *G*; 0.5 μm in *F*.

Exine Formation

The first T-shaped exine elements, consisting of pro-columellae and a pro-tectum, are deposited in the primexine matrix around the midtetrad stage. In the late tetrad stage, these elements become thicker, and a foot layer becomes visible. In Dioscoreales, the exine ultrastructure is completely determined during the tetrad stage. In the free microspore stage, the exine thickness increases significantly, becoming up to three times thicker. All species in this survey have a perforate or rugulate sexine lacking the supraterectal elements found in striate *Dioscorea* species (Schols et al. 2001). No indication of endexine deposition was observed in any of the taxa examined in this study, although Schols et al. (2001) described white lines beneath the foot layer in mature pollen of *Dioscorea sylvatica* Eckl.

Intine Ontogeny and Ultrastructure

All three genera possess a well-developed intine consisting of two layers: a thick, channeled outer layer and a thin inner layer. The outer layer (or exintine) has electron-lucent channels arranged in a polygonal structure, as seen in tangential sections (figs. 4F, 10G). This layer forms beneath the apertures in all three genera, but in *Tacca* it also occurs in the nonapertural regions. A well-developed intine in general and these channels in particular seem to characterize many monocot genera; for example, they have been reported in Liliales (Nakamura 1979), Asparagales (Suárez-Cervera et al. 2000), Zingiberales (Kress and Stone 1982), and Poaceae (El-Ghazaly and Jensen 1986, 1987).

Suárez-Cervera et al. (2000) carried out a detailed ontogenetic study of the intine in *Aristea major* (Iridaceae) and concluded that three distinct layers were present: a pectic exintine, a pectic proteinic channeled middle layer, and a fibrillar cellulosic endintine. The two intine layers observed in Dioscoreales in our study correspond to the middle intine layer and the endintine of Suárez-Cervera et al. (2000), and the pectic exintine layer on top of the channeled intine is absent. The same two layers were also found in *Canna* (Rowley and Skvarla 1986) and *Zingiber* (Theilade and Theilade 1996), which also lack exintine. Possible roles of the thick, channeled intine include prevention of gametophyte desiccation during pollination, release of enzymes related to pollen tube growth, and control of the enlargement of the apertural region (Suárez-Cervera et al. 2000). As in *Aristea*, the intine in Dioscoreales develops during the free microspore stage, after the vacuolate microspore stage. Intine generally develops

after mitosis in angiosperms (Shukla et al. 1998). In Dioscoreales, however, intine is clearly present during the free microspore stage, before microspore mitosis. This has also been demonstrated in *Lilium* and *Silene* (Heslop-Harrison 1968).

Position of the Generative Cell

The generative cell is located at the opposite side of the pollen grain to the aperture in the monosulcate species examined (*Narthecium ossifragum* and *Tacca artocarpifolia*) and as far away from both apertures as possible in the disulcate *Dioscorea communis* pollen. In both cases, the generative cell is located at the proximal pole. A similar position for the generative cell is described in *Haemanthus* (Amaryllidaceae: Shukla et al. 1998).

Orbicule Ontogeny and Ultrastructure

Orbicules are usually correlated with secretory tapeta (Huysmans et al. 1998, 2000). They occur in *Narthecium* and *Dioscorea* but not in *Tacca*, although it has a secretory tapetum as in other Dioscoreales (Furness and Rudall 1998). This confirms previous observations by Schols et al. (forthcoming). Within Dioscoreales, orbicules occur in *Dioscorea*, *Trichopus* (L. R. Caddick, personal communication), and Nartheciaceae (V. Mercx, personal communication), while they are absent in *Tacca* and in Burmanniaceae (P. Schols, unpublished data). No orbicule data is available for *Stenomeres*. The absence of orbicules in taxa with secretory tapeta is not uncommon within the angiosperms, since it occurs in Brassicaceae, Cactaceae, Cucurbitaceae, Fabaceae, Geraniaceae, Lamiaceae, and Orchidaceae (Huysmans et al. 1998). Why orbicules are lacking in some taxa with secretory tapeta is still unclear.

Pro-orbicules appear on the outside of the tapetal plasmalemma in *Narthecium* and *Dioscorea* while primexine polymerization is taking place in the microspores. Soon afterward, they acquire a thin sporopollenin coat that increases in thickness continuously until pollen maturity. In both *Narthecium* and *Dioscorea*, the orbicules have an electron-lucent core that is the remains of the pro-orbicule, and this does not appear to increase in size during orbicule development. Around this core is the main electron-dense orbicule layer, referred to as the orbicule wall (Clément and Audran 1993), and outside this is a very electron-dense peripheral layer that can be seen only at high magnifications (fig. 4H, arrow). This orbicule ultrastructure in Dioscoreales is very similar to that found in *Lilium* (Liliales; Clément and Audran 1993). Orbicules are significantly larger in *Narthecium* than in *Dioscorea*, ca. 1.3 μm compared with 0.4 μm .

Table 1

Summary of Ontogenetic Characters

	<i>Dioscorea</i>	<i>Tacca</i>	<i>Narthecium</i>
No. of middle layers	1–2	1–2	1–2 (3)
Microsporogenesis	Simultaneous	Simultaneous	Successive
Tetrad configuration	Tetrahedral	Tetrahedral	Tetragonal
Aperture position	Equatorial	Distal	Distal
Aperture type	Disulcate	Monosulcate	Monosulcate
Channeled intine beneath apertures	Present	Present	Present
Channeled intine in nonapertural regions	Absent	Present	Absent
Orbicules	Present	Absent	Present

Conclusions

The general pollen ontogenetic pattern described for all three genera of Dioscoreales is relatively similar (table 1). This supports the close relationship between the three genera. Nartheciaceae, sister to other Dioscoreales, is characterized by successive microsporogenesis and tetragonal tetrads, while Dioscoreaceae are simultaneous and have tetrahedral tetrads. In *Narthecium* and *Tacca*, one distal sulcus develops during the tetrad stage, while in *Dioscorea* there are two equatorial sulculi. Exine deposition starts in the midtetrad stage in all three genera with the formation of T-shaped elements. All Dioscoreales examined are characterized by a thick intine that consists of two layers. The channeled outer layer is present only beneath the aperture except in *Tacca*, where it occurs beneath the entire exine. Intine synthesis clearly begins before mitosis in the free microspore stage. Orbicules are present in *Dioscorea* and *Narthecium* but lacking in *Tacca*. They are formed around pro-orbicules that originate from

the tapetum at the tetrad stage. Further work is required to determine why pro-orbicules are not produced in *Tacca* and to understand the mechanisms influencing the shift in microsporogenesis type and aperture number within Dioscoreales.

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