Grana 44: 30–44, 2005

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A histological study of microsporogenesis in *Tarenna gracilipes* (Rubiaceae)

STEFAN VINCKIER and ERIK SMETS

Vinckier, S. & Smets, E. 2005. A histological study of microsporogenesis in *Tarenna gracilipes* (Rubiaceae). – Grana 44: 30–44. ISSN 0017-3134.

We studied the microsporogenesis in *Tarenna gracilipes* (Hayata) Ohwi, with special attention to the mode of exine deposition and tapetum development. We based this research on light (LM), scanning electron (SEM) and transmission electron microscopic (TEM) observations of developing anthers of *T. gracilipes*, from the microspore mother cell stage towards anther dehiscence. Evidence is supplied that the microsporogenesis in *T. gracilipes* can be considered as simultaneous. Columellae, foot layer and tectum develop in a fibrillar matrix. Similar with earlier studies in Rubiaceae species, a single white line formed near the plasmalemma in the extra-apertural region. The developing endexine dilated into several white line centered lamellae at the apertures. An annulus is formed around the inner surface of each pore. In the mature intine two strata can be distinguished. At the apertures thick onci are formed protruding through the apertures thereby forming papillae, a common feature in Rubiaceae. In Rubiaceae species amoeboid as well as secretory tapeta are reported. In *T. gracilipes* it is shown that the tapetum cells possess in all developmental stages characteristics of the secretory type. During microsporogenesis the tapetum cytoplasm undergoes considerable changes which may indicate cycles of hyperactivity. Sporopollenin deposition on the pre-orbicules is mediated by white lines showing correlations with endexine, annulus and columellae ontogeny. These findings corroborate the idea that orbicule wall development can represent a model to study sporopollenin deposition. At anther dehiscence Ca-oxalate crystals are released out of the ruptured septum cells into the locule, providing a possible visual signal for pollinators.

Stefan Vinckier & Erik Smets, Laboratory of Plant Systematics, Institute of Botany and Microbiology, K. U. Leuven, Kasteelpark Arenberg 31, BE-3001 Leuven, Belgium.*
**Corresp. Author*
E-mail: stefan.vinckier@bio.kuleuven.ac.be.

(*Manuscript received 23 February 2004; accepted 30 August 2004*)

In this study the microsporogenesis of *Tarenna gracilipes* (Hayata) Ohwi is studied with special attention to exine and tapetum development. *T. gracilipes* is a shrub with showy white flowers native to Taiwan. *Tarenna* (219 species) is a member of the tribe Pavetteae of the subfamily Ixoroideae (Rubiaceae). It is distributed in Africa, Asia, Malaysia, Oceania and Australia.

The tapetum is the innermost wall layer of the microsporangium, in closest contact with the developing pollen grains. Tapetal cells are often enlarged and may be multinucleate or polyploid (Pacini 1990). This specialised tissue plays an important role in pollen development, because all the nutritive materials entering the sporogenous cells must either pass through it or be metabolised by it (Echlin 1971). Apart from the nutritional role, the tapetum fulfils different other functions, such as production of locular fluid, release of polysaccharides into the loculus, and secretion of callase which dissolves the callosic walls of the tetrads (Pacini 1990). Tapetum cells also play an active role in the formation of exine precursors, viscin threads, an acetolysis-resistant membrane outside the tapetal protoplast, orbicules, sporophytic proteins which may be deposited in the apertural regions of the pollen grains, tryphine, and pollenkitt in entomophilous species (Pacini 1990). There are various types of tapeta in angiosperms, but they can be reduced to two basic types: secretory, also known as parietal or glandular tapetum, and amoeboid tapetum, also known as periplasmodial, invasive, or intrusive (Pacini 1997). However, the distinction between both types has become less evident (Rowley et al. 1992), and transitional types have been described (Hesse & Hess 1993). The predominance of the secretory tapetum among basal angiosperms was proved by Furness & Rudall (2001).

In the anthers of flowering plants, gymnosperms, and seed ferns, tiny (< 4 μm) granules might occur on the radial and innermost tangential wall of secretory tapetum cells, sometimes in close contact with the pollen grains, and are called Ubisch bodies (Kosmath 1927) or orbicules (Erdtman et al. 1961). Orbicules have been observed for the first time by Rosanoff (1865).

Orbicules develop simultaneously with the growing pollen exine and are composed of sporopollenin, similar to the pollen exine. Pre-orbicules, which are produced by the endoplasmic reticulum of secretory tapetal cells, are their progenitors (Echlin & Godwin 1968). The pre-orbicules are extruded through the radial and innermost tangential wall of the secretory tapetum cells, at the beginning of the tetrad stage (Christensen et al. 1972, El-Ghazaly & Jensen 1986, Clément & Audran 1993*a*,*b*,*c*, Vijayaraghavan & Chaudry 1993, Suarez-Cervera et al. 1995). During the past 20 years different hypotheses have been suggested to attribute a function to orbicules. For a summary of some of the

DOI: 10.1080/00173130510010530

proposed functions see Huysmans et al. (1998). Recent studies on wheat anthers demonstrated that orbicules carry a sporophytic structural protein (RAFTIN) that is targeted to the microspore exine and is essential for pollen development in this species (Wang et al. 2003).

In Rubiaceae, only scarce information is available on the pollen and tapetal development. Andronova (1984) investigated the pollen development in several Rubiaceae species, with special attention to the tapetum, and von Teichman et al. (1982) described briefly the microsporogenesis in *Pavetta gardeniifolia*. Only three ultrastructural studies on Rubiaceae species were carried out with special attention to the development of pollen and tapetum (Huysmans 1998, Hansson & El-Ghazaly 2000, El-Ghazaly et al. 2001). Hansson & El-Ghazaly (2000) studied the development and cytochemistry of pollen and tapetum in *Mitriostigma axillare*. In this species no orbicules are developed. The pollen wall, tapetum and orbicule development in *Rondeletia odorata*, were investigated by Huysmans (1998) and El-Ghazaly et al. (2001).

After several years of research on the systematic usefulness of orbicule characteristics in Gentianales (Vinckier et al. 2000, Vinckier & Smets 2002*a,b,c*, 2003, Vinckier 2003), we aim to study the ontogeny of orbicules, tapetum and pollen in the Rubiaceae species *Tarenna gracilipes*, and to compare the results with ontogentical studies in Rubiaceae and other angiosperm taxa. In the present study we trace in detail at the ultrastructural level the developmental sequence of events during sporoderm and tapetum development starting from the microspore mother cell stage towards mature pollen grains at dehiscence.

MATERIAL AND METHODS

Developing anthers of *Tarenna gracilipes* were investigated using light (LM), scanning electron (SEM) and transmission electron microscopy (TEM). Fresh anthers of *T. gracilipes* at different developmental stages were collected at the National Botanic Garden of Belgium (specimen n° 1998 1659–47).

For scanning electron microscopy (SEM), fresh anthers were picked out from the flowers and the tips of the anthers were removed with a razor blade to facilitate the fixation. Anthers were fixed for 24 hours in 2% glutaraldehyde at pH 7.3, and buffered with 0.05 M sodium cacodylate. Transversely cleaved anthers were obtained by fracture in liquid nitrogen. Following dehydration in a graded acetone series, the material was critical point dried (CPD 030, Bal-Tec, Liechtenstein) and mounted on stubs with double-sided adhesive tape. Mature pollen was acetolysed for nine minutes in a heating block at 90°C. Broken pollen grains were obtained by shaking a pollen and glass bead suspension as described by Huysmans et al. (1994). Acetolysed pollen grains for SEM studies were suspended in ethanol (70%), pipetted onto specimen stubs, and air-dried. The stubs were sputter coated with gold (SPI-MODULE™ Sputter Coater, SPI Supplies, West Chester, PA, USA). We used a JSM-6400 SEM (JEOL Inc., Peabody, MA, USA) at 10–20 kV.

For LM and TEM observations, anthers of selected flowers were fixed for 24 hours with 2% glutaraldehyde at pH 7.3 and buffered with 0.05 M sodium cacodylate. Prior to embedding in LR-White Resin (Polysciences Inc., Warrington, PA, USA) the material was dehydrated in a graded ethanol series and block-stained with 1% phosphotungstic acid (PTA) in 100% ethanol (Hayat 1989). PTA is added as a post fixative and histochemical stain for glycoproteins (Benedetti & Bertolini 1963, Marinozzi 1968, Hayat 1989). Semi-thin (±1 μm) sections were cut with a Reichert Jung Ultracut E microtome and stained with 0.1% thionin −0.1% methylene blue. The semi-thin sections were observed with a Leica DM LB light microscope (LM). Calcium oxalate crystals were visualized in the semi-thin sections using a polarizing LM fitted with an ICT/P polarizer and an IC prism. The ultrathin (±70 nm) sections, on copper grids, were stained with uranyl acetate and lead citrate in a LKB 2168 Ultrostainer and were observed in a Zeiss EM 900 transmission electron microscope at 50 kV.

RESULTS

In *Tarenna gracilipes*, a layered tapetum surrounds the sporogenous tissue. The anther wall consists of an epidermis, endothecium, one or two middle layers, and the tapetum (Fig. 1A). Although the microsporogenesis in *T. gracilipes* is a continuous process, for descriptive purposes we distinguished five stages: microspore mother cell stage followed by meiosis, tetrads, free microspores, vacuolate pollen, and mature pollen grains at dehiscence.

Microspore mother cell stage, followed by meiosis

Crystal-bearing tissues

In the microspore mother cell stage crystal-bearing cells surround the vascular bundle, and are present in the septum and the connective tissue between the four loculi (Fig. 1B). The septum consists of the two layers of cells immediately beneath the stomium and is located between the two adjacent loculi. SEM observations of cleaved anthers revealed that the crystals are calcium oxalate druses (Fig. 1C).

Sporogenous tissue

In a cross section of the anther microspore mother cells (MMCs) of *T. gracilipes* are angular in shape and possess a large nucleus with a darkly staining nucleolus (Fig. 1A). In the dyad stage, at the end of the first meiotic division, a thin callosic wall is already formed, enclosing the dyad (Fig. 2A, B). At the beginning of the second meiotic division, dense plastids are very abundant in the cytoplasm, separating both nuclei (Fig. 2A). A spindle of microtubule is clearly visible during anaphase II (Fig. 2B).

Tapetum

The tapetum cells possess a prominent nucleus, nucleolus, rough endoplasmic reticulum and an extensive fibrillar cell wall. At the start of the second meiotic division, when dense plastids are very abundant in the dyad cytoplasm, the tapetal cytoplasm undergoes considerable changes (Fig. 2C). At the same time similar dense plastids appear abundantly in the tapetal cytoplasm (Fig. 2C). In this stage cytoplasmic channels and plasmodesmata are observed between the tapetum cells (Fig. 2C). Chromatin material migrates through the cytoplasmic channels (Fig. 2C). Until the early tetrad stage, no pre-orbicules could be observed inside the tapetal cytoplasm.

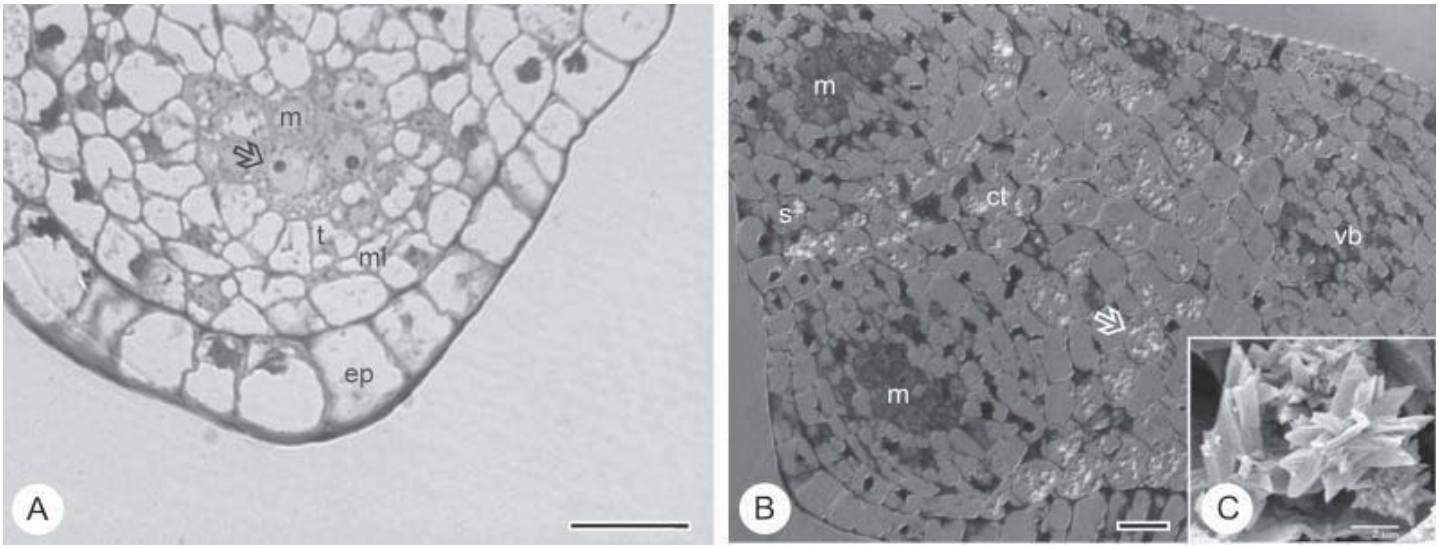

Fig. 1. **Microspore mother cell (*m*) stage**. (A) LM observation of a cross section of an anther with epidermis (***ep***), middle layer (***ml***), and tapetum (***t***). A prominent nucleolus (***arrow***) is present within the nucleus of microspore mother cells (***m***); (B) Polarizing light microscopic (PLM) observation of a cross section of an anther. Crystal-bearing cells (***arrow***) surround the vascular bundle (***vb***) and are present in the connective tissue (***ct***) between the four loculi; (C) Detailed SEM observation of a Ca-oxalate druse present in the connective tissue. Scale bars – 20 μm (A–B); 2 μm (C).
Abbreviations adopted for all figures: an – annulus; **ap** – amyloplast; **c** – callose; **col** – collumella; **ct** – connective tissue; **cw** – cell wall; **cy** – cytoplasm; **ec** – ectexine; **el** – elaioplast; **en** – endexine; **enc** – endocolpus; **end** – endothecium; **ep** – epidermis; **er** – endoplasmic reticulum; **fl** – foot layer; **fm** – fibrillar matrix; **in** – intine; **lo** – locule; **m** – microspore mother cell; **mi** – mitochondria; **ml** – middle layer; **n** – nucleus; **o** – orbicule; **ow** – orbicule wall; **p** – pollen grain; **pa** – papilla; **pan** – pro-annulus; **pc** – pro-columella; **pfl** – pro-foot layer; **pl** – peripheral layer; **pm** – polarizing light microscopy; **po** – pre-orbicule; **ps** – periplasmic space; **pt** – pro-tectum; **rer** – rough endoplasmic reticulum; **s** – septum; **st** – stomium; **t** – tapetum cell; **tec** – tectum; **tet** – tetrad; **tm** – tapetal marker; **v** – vacuole; **vb** – vascular bundle.

Tetrad stage

Tetrads and crystal-bearing tissues

When the second meiotic division is completed, the microspore mother cell is 4-nucleate. The decussate tetrads are enveloped by a callosic wall and fill the entire locule. In the freeze-fractured samples, the edges of the multiangular callosic envelope appear porous (Fig. 3A). At the beginning of the tetrad stage Ca-oxalate druses have accumulated in the septal tissue which is highly enlarged (Fig. 3B). The crystals which were present in the connective tissue adjacent to the septum cells in the microspore mother cell stage seem to have disappeared in the current developmental stage.

In the course of cytokinesis the tetrad partitions form by the process of furrowing (Fig. 3C). In the early tetrad stage a thin glycocalyx layer starts to appear upon the plasma membrane. This glycocalyx layer becomes thicker, and it is evident now that it has the appearance of a multi-layered fibrillar matrix (Fig. 3D). In this fibrillar matrix the primexine starts to develop. Pro-columellae, pro-tectum, and pro-foot layer appear as low electron dense primexine elements within the electron dense fibrillar matrix (Fig. 3D). In a transversal section of the primexine the tubular core parts of the pro-columellae reveal a higher electron density than their outer parts (Fig. 3E). In these core parts a unit of circa 50 nm of diameter may be observed which consists of five electron dense subunits (Fig. 3E). At the borders of the developing apertural region, the primexine is less developed. At the apertural site, the electron dense fibrillar matrix is greatly enlarged, forming an electron dense bulge (Fig. 4A). The bulge is delimited at its sides by low electron dense areas (Fig. 4A). At the late tetrad stage the pro-foot layer, pro-columellae, and pro-tectum have increased considerably in thickness as well as in electron density (Fig. 4B–D). The callose special cell envelope persists until the end of the tetrad stage (Fig. 4B–D).

Tapetum

In the tetrad stage the tapetum cells are enriched in rough endoplasmic reticulum and free ribosomes (Fig. 5A). The fibrillar cell wall has decreased considerably in thickness (Fig. 5A). Pre-orbicules appear in the periplasmic space, surrounded by glycocalyx material (Fig. 5B). The pre-orbicules possess a tail-shaped extension bordered by glycocalyx material, which is sometimes still in close contact with the plasmalemma (Fig. 5B).

Free microspore stage

Pre-orbicules

In this stage clusters of pre-orbicules are located upon the radial and inner tangential tapetal cell walls (Figs 5C, 6B). The tail-shaped extensions of the pre-orbicules are clearly visible (Fig. 5C). At the end of this stage the clusters of pre-orbicules become more embedded and less obvious in the electron dense developing orbicule wall.

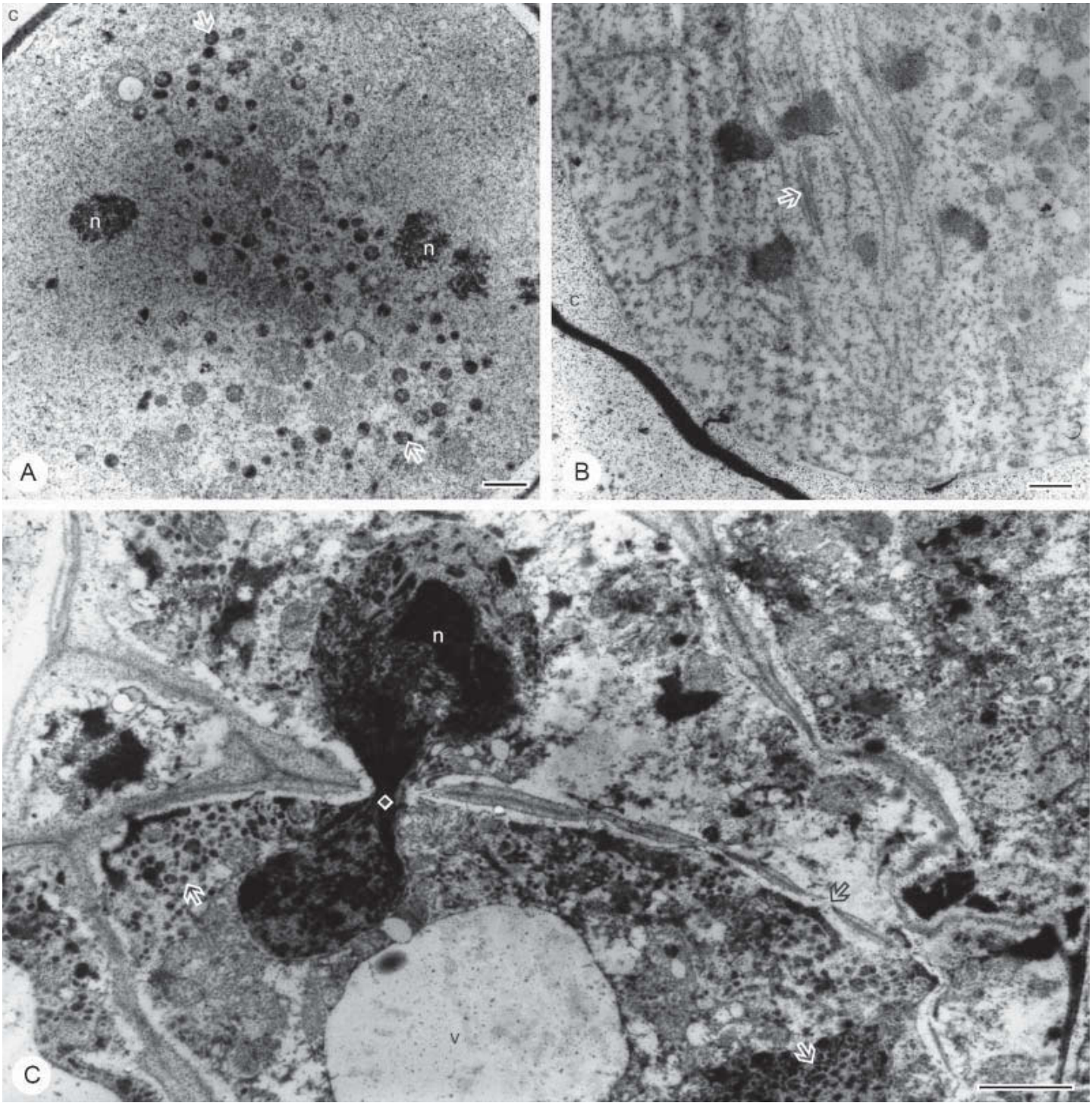

Fig. 2. **TEM observations during dyad stage followed by second meiotic division**. (A) Observation of a dyad. A thin callose (***c***) wall is formed. Dense plastids (***arrows***) are present between both nuclei (***n***); (B) During anaphase II the spindle of microtubuli (***arrow***) is observed; (C) Detailed observation of adjacent tapetum cells. The cytoplasm is enriched with densely staining plastids (***arrows***) and a large vacuole is present. A cytoplasmic channel (◇) is present through which chromatin migrates. Plasmodesmata could be observed (***black outlined arrow***). Scale bars – 1.1 μm (A – B); 2.5 μm (C).

Free microspores

The microspores are released after the callosic envelope disappeared. In the beginning of this stage white line centered lamellae appear first in the electron dense fibrillar matrix of the developing apertures, indicating the position of the future annulus (Fig. 6A). In between the developing apertures a single almost continuous white line appeared, which is the first indication of the developing endexine (Fig. 6A). Near the apertural region the developing endexine dilated into several white line centered lamellae (Fig. 6C). On some sections the white line of the endexine appeared to extent into the base of the columellae (Fig. 6C). The surface of the tectum and infratectum are still covered by electron dense fibrillar material (Fig. 6C). As the free microspore stage progresses the endexine as well as the foot layer and annulus become thicker.

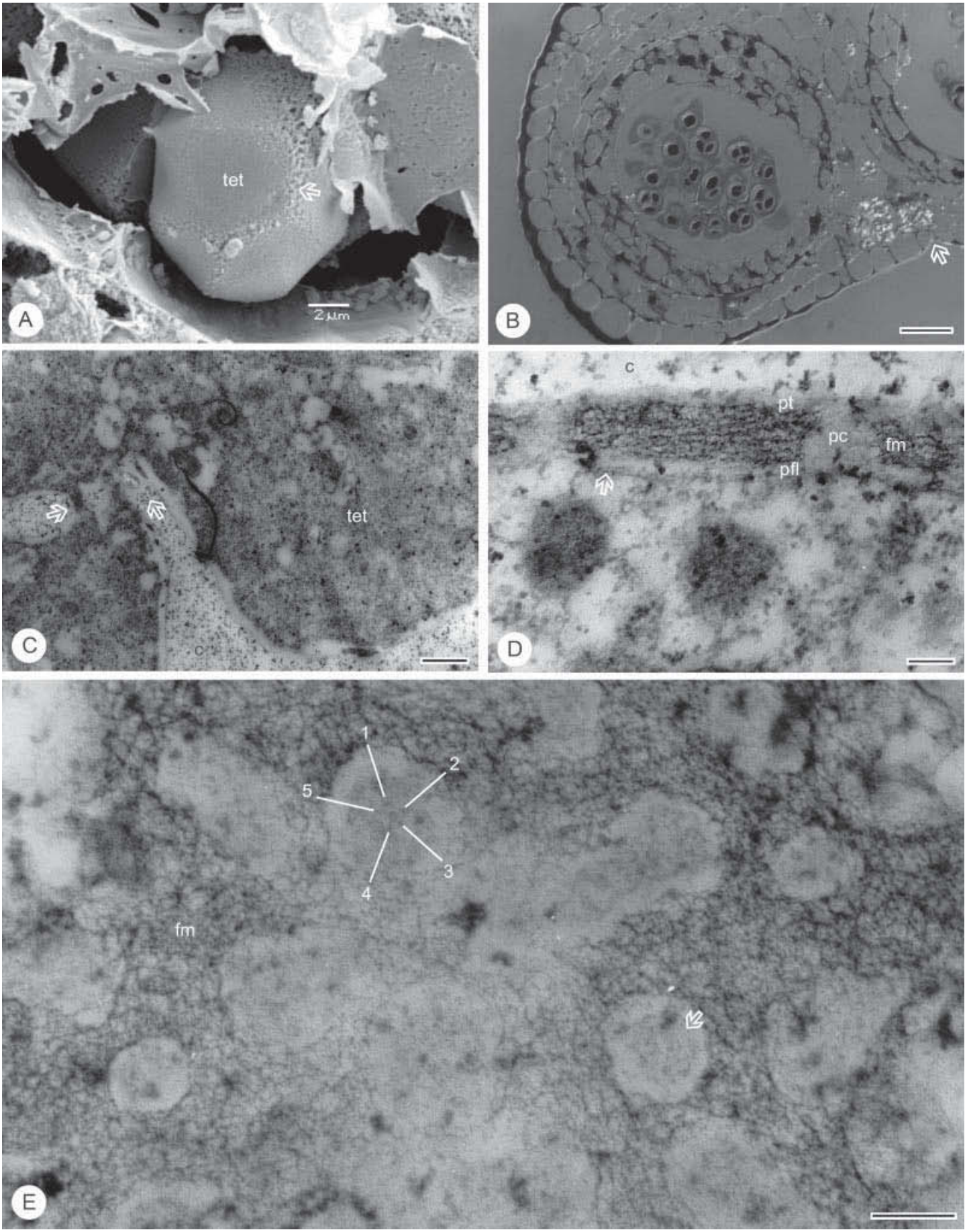

Fig. 3

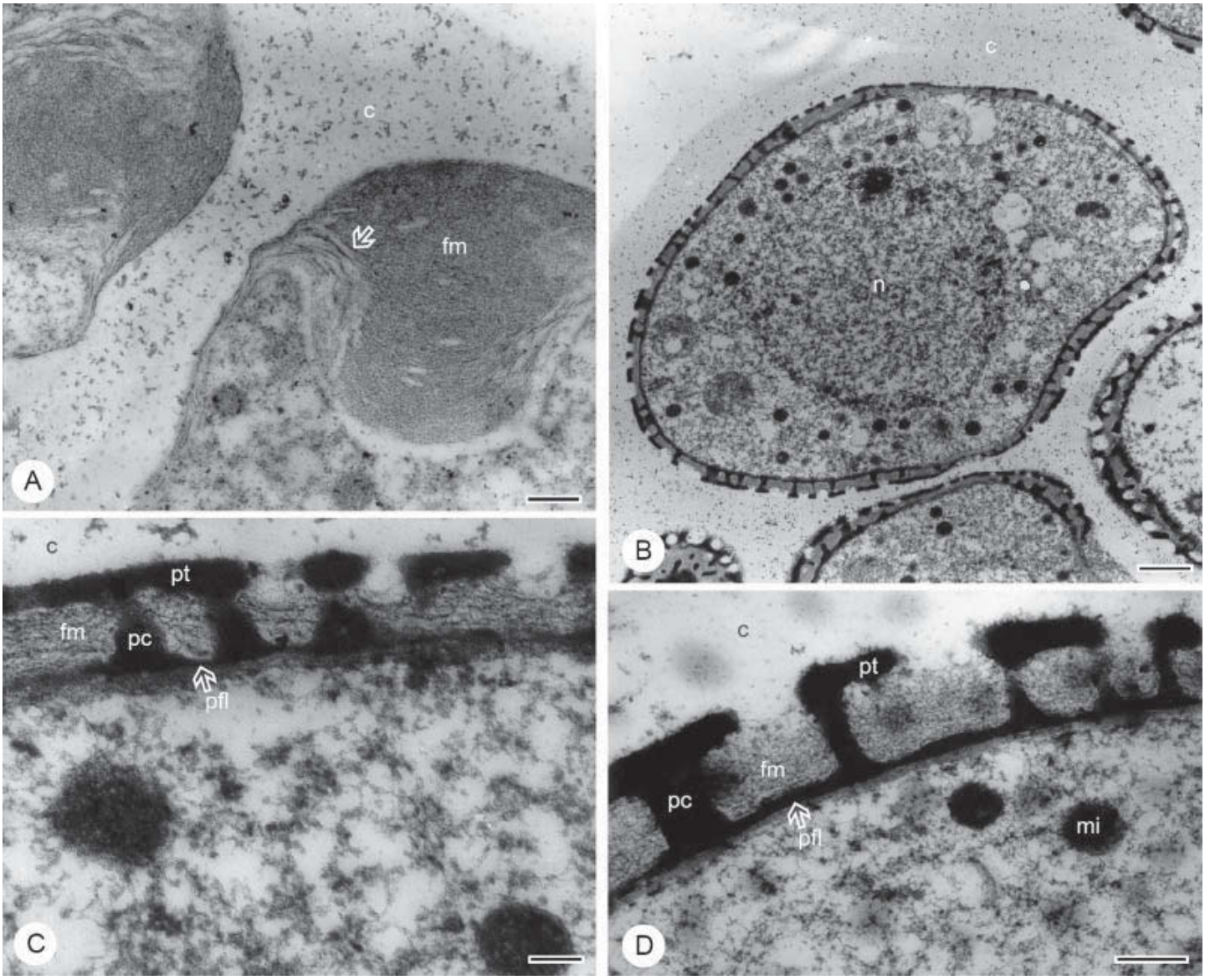

Fig. 4. **TEM observations of the developing microspores in the mid- (A – B) and late tetrad stage (C – D)**. (A) At both sides bordering the developing apertural region, the primexine units are decreased in height. At the apertural site, the electron dense fibrillar matrix (***fm***) is greatly enlarged, forming an electron dense bulge. The bulge is delimited at its sides by low electron dense areas (***arrow***); (B) Overview of a developing microspore in the callose special cell envelope (***c***); (C) The electron density of the pro-foot layer (***pfl***), pro-columellae (***pc***), and pro-tectum (***pt***) have increased considerably; (D) Late tetrad stage. The pro-foot layer (***pfl***), pro-columellae (***pc***), and pro-tectum (***pt***) have enlarged considerably compared to the primexine elements shown in Fig. 4C. Scale bars – 0.4 μm (A); 1.1 μm (B); 0.15 μm (C); 0.4 μm (D).

Vacuolate microspore stage

Crystal-bearing septum

Ca-oxalate druses are accumulated in the septal tissue which is highly enlarged (Fig. 7A).

Tapetum

In this stage the tapetal cell cytoplasm is enriched by endoplasmic reticulum and abundant elaioplasts presenting spherical inclusions (Fig. 7B, C). Orbicules are abundant on the inner tangential and radial walls of the tapetum cells, and have

Fig. 3. **Early tetrad (*tet*) stage.** (A) SEM observation of a freeze-fractured anther, the edges of the multi-angular callosic envelope appear porous (***arrow***); (B) PM of a cross section of an anther. Ca-oxalate druses have accumulated in the septal tissue which is highly enlarged. In this stage the formation of the stomium is initiated (***arrow***); (C) TEM observation of a young tetrad. In the course of cytokinesis the tetrad (***tet***) partitions form by the process of furrowing (***arrows***); (D) Detailed TEM observation of a young tetrad. Upon the plasmamembrane (***arrow***) a multi-layered fibrillar matrix (***fm***) consisting of a glycocalyx is formed. In this fibrillar matrix the primexine starts to develop. Pro-columellae (***pc***), pro-tectum (***pt***), and pro-foot layer (***pfl***) appear as low electron dense primexine elements within the electron dense fibrillar matrix; (E) TEM observation of a transversal section of the primexine. The tubular core parts (***arrow***) of the pro-columellae reveal a higher electron density to their outer parts. In these core parts a unit of circa 50 nm of diameter may be observed which consists of 5 electron dense subunits (***no. 1 to 5***). Scale bars – 20 μm (B); 0.6 μm (C); 0.15 μm (D – E).

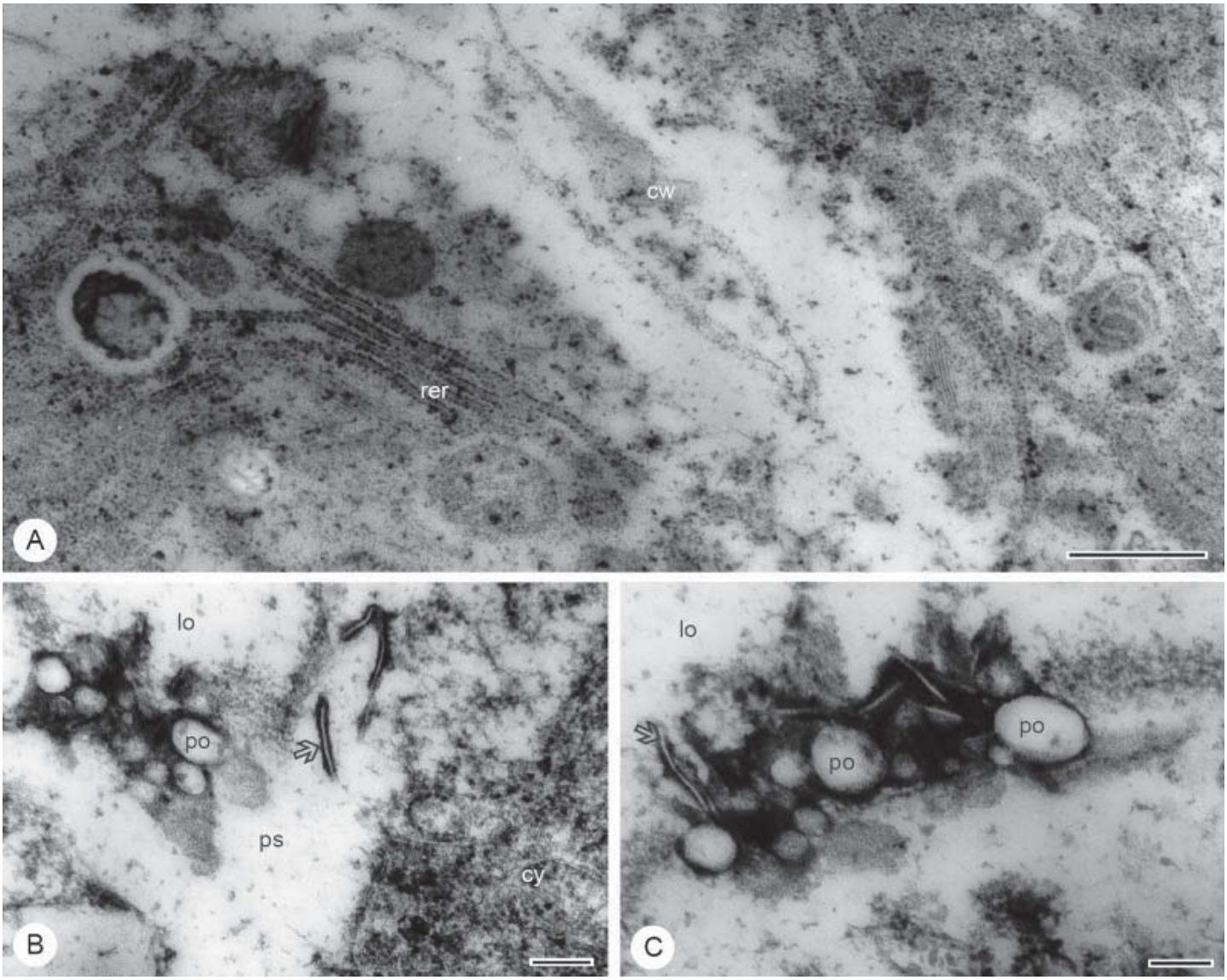

Fig. 5. **TEM observations of the tapetum and pre-orbicules in the tetrad (A – B) and free microspore (C) stage**. (A) Observation of the tapetum cells during the tetrad stage. The cytoplasm is enriched in rough endoplasmic reticulum (***rer***) and the fibrillar cell wall (***cw***) has decreased considerably in thickness; (B) Pre-orbicules (***po***) appear in the periplasmic space (***ps***), surrounded by glycocalyx material (***arrow***); (C) Free microspore stage. Clusters of pre-orbicules (***po***) are located upon the radial and inner tangential tapetal cell walls, the tail-shaped extensions of the pre-orbicules are clearly visible (***arrow***). Scale bars – 0.6 μm (A); 0.25 μm (B – C).

reached their final size (Fig. 7B). A thin layer of electron dense fibrillar material lines the homogenous electron dense orbicule wall. The electron density of this wall has decreased in comparison with the free microspore stage and is very similar to the exine electron density (Fig. 7B, D & E). The position of the tail-shaped extensions protruding into the orbicule wall is still visible (Fig. 7B). At the end of the vacuolate stage the tapetal cells are more flattened and shrunken towards the endothecium, and as a consequence they are covering each other (Fig. 7D). They are completely disorganized and the cell organels are not recognisable anymore (Fig. 7D). Due to the shrinkage of the tapetal cells, the orbicules approach each other, creating a more continuous layer.

Pollen

In the early vacuolate stage the intine is absent (Fig. 7E). The foot layer can be disitinguished from the early deposits of endexine when a single white line is present (Fig. 7E). The deposition of intine will take place in the periplasmic space (Fig. 7D). The fibrillar matrix that covered the tectum and infratectum in earlier stages retracts in this stage and lines now the infratectum and tectum (Fig. 7E). The electron density of the exine elements has decreased in comparison with the free microspore stage.

Mature pollen grain

Pollen

In the mature intine two strata can be distinguished, a thin white inner one and a corrugated outer one (Fig. 8A, B). The generative cell is associated with the vegetative one and the electron dense cytoplasm of the vegetative cell is full of endoplasmic reticulum, amyloplasts and mitochondria (Fig. 8A, B). The plasmalemma is undulating and close to

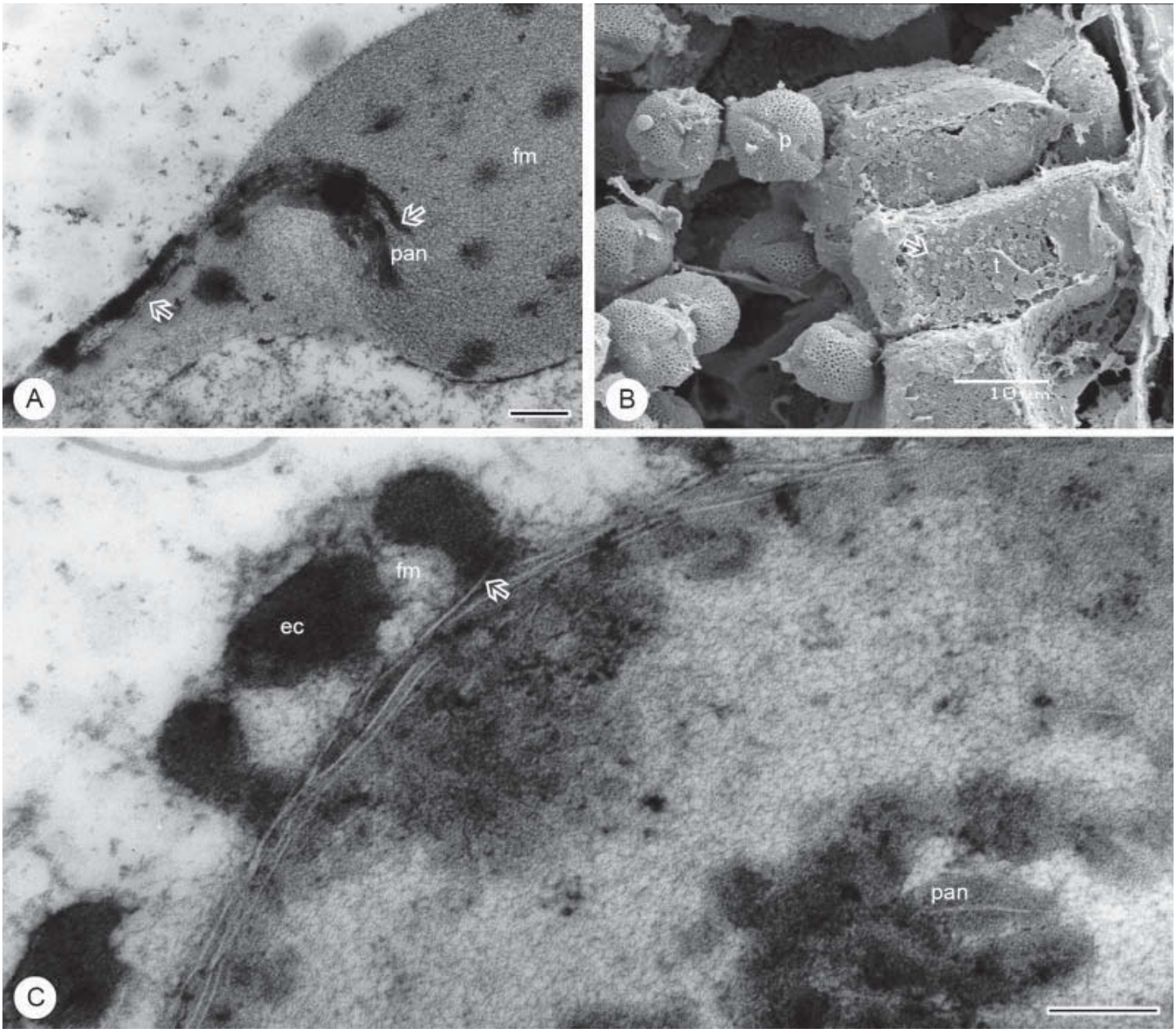

Fig. 6. **Free microspore stage**. (A) TEM observation of the apertural region. White line centered lamellae (***arrows***) appear first in the electron dense fibrillar matrix of the developing apertures, indicating the position of the future annulus (***pan***); (B) SEM observation of a freeze-cracked anther. Orbicules (***arrow***) are present on the radial and inner tangential tapetum (***t***) cell walls; (C) Detailed TEM observation of the apertural region. The developing endexine dilated into several white line centered lamellae. On some sections the white line of the endexine appeared to extend into the base of the columellae (***arrow***). The white line is apparent in the developing annulus (***pan***). Scale bars – 0.4 μm (A); 0.25 μm (C).

the apertural regions stacks of rough endoplasmic reticulum are conspicuous (Fig. 8A). The exine is totally structured. A thin layer of electron dense fibrillar material covers the tectum and lines the infratectum (Fig. 8A, B).

The mature pollen grains are small tri-colporate monads (E 15.3–(16.2)–17.3 μm, P 13.7–(14.3)–15 μm), oblate spheroidal (P/E 0.88 on average) in equatorial view and sub-triangular in polar view (Fig. 8C, D). They possess a micro-reticulate exine, which appears perforate at the polar regions (Fig. 8D, F). In the apertural region an annulus is formed (Fig. 8E). The granular annulus around the inner surface of each pore is clearly visible when the inside ornamentation of broken acetolysed pollen is observed (Fig. 8E). A short equatorial endocolpus with 'fish tail' ends is present beneath each pore (Fig. 8E). It is 2 μm wide and at the porus up to 4 μm wide. The inside ornamentation of the acetolysed pollen grains is granular-psilate (Fig. 8E). Under the apertures, the intine is enlarged in thickness forming thick onci (Fig. 8G). The intine protrudes through the apertures and forms thereby papillae (Fig. 8F, G).

Release of crystals in locule

At dehiscence the stomium opens and Ca-oxalate druses are released out of the ruptured septal cells into the locules, and cover the pollen grains and/or the locule surface (Figs 8F, 9A).

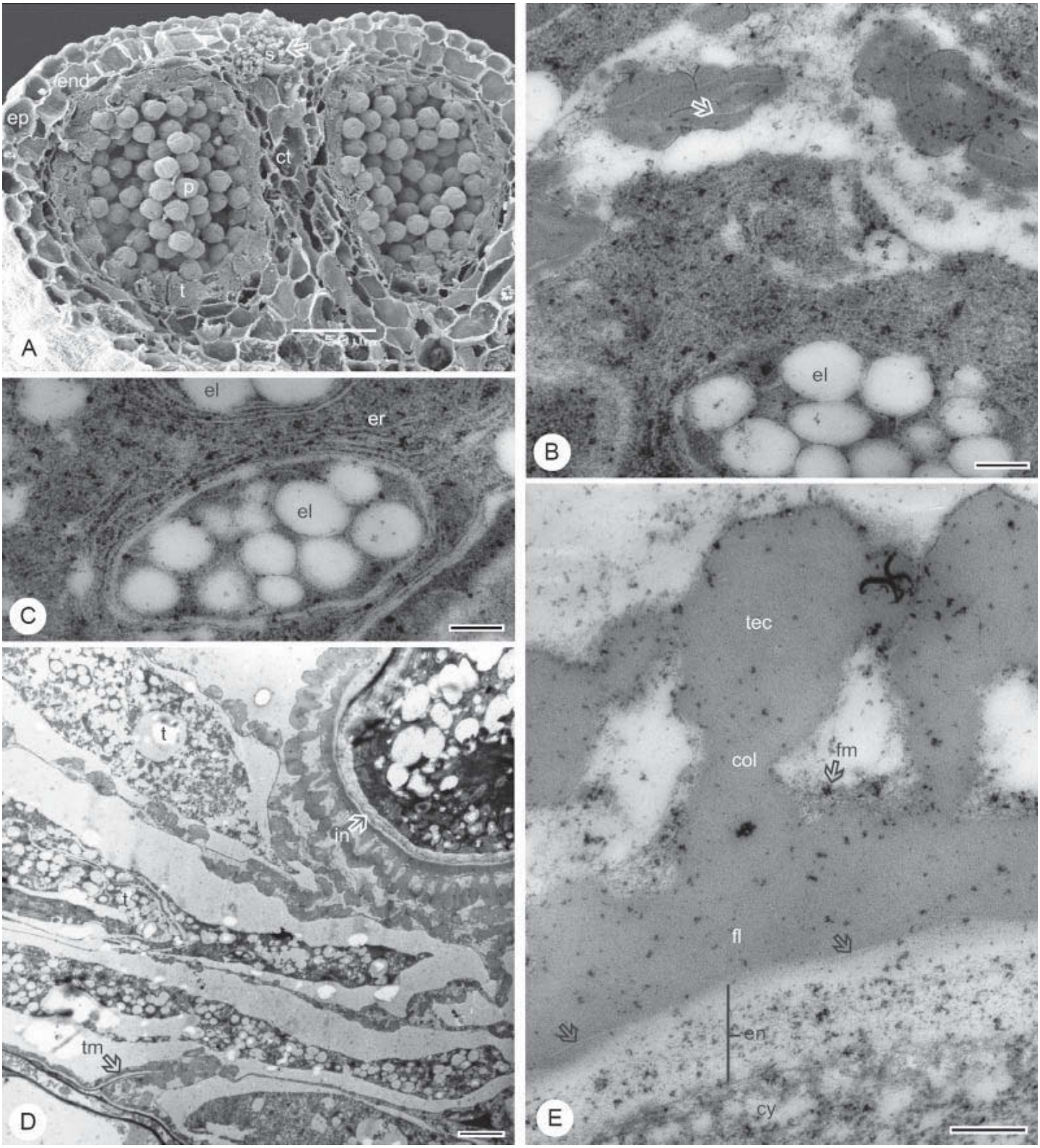

Fig. 7. **Vacuolate microspore stage**. (A) SEM observation of two locules of a freeze-cracked anther. The septal (***s***) cells form a crystal filled cavity (***arrow***) between the two adjacent locules; (B) General TEM view of orbicules present upon the tapetal cell wall. The white line tail-shaped extensions protruding into the orbicule wall are clearly visible (***arrow***); (C) Detailed TEM observation of the cytoplasm of a tapetum cell. Elaioplasts (***el***) are surrounded by endoplasmic reticulum (***er***); (D) TEM observation of tapetum (***t***) cells at the end of the vacuolate stage. The tapetal cells are more flattened and shrunken towards the endothecium. They are completely disorganized and the cell organels are not recognisable anymore. The tapetal marker (***tm***) reveals the position of the radial cell wall of the tapetum cells; (E) Detailed TEM observation of the developing pollen wall in the early vacuolated stage. The fibrillar matrix (***fm***) that covered the tectum (***tec***) and infratectum retracts and lines the infratectum and tectum. The foot layer (***fl***) can be disitinguished from the early deposits of endexine (***en***) when a single white line is present (***arrows***). Scale bars – 0.4 μm (B, C, E); 2.5 μm (D).

Tapetum

At this stage the tapetal cells are completely degenerated and the remains are compressed to form the tapetal membrane, densely covered with orbicules (Fig. 9B – D). The orbicules possess an irregular and granular shape (Fig. 9B). Between the orbicules and/or between the orbicules and the tapetal membrane thin interconnecting threads may occur (Fig. 9B). The tapetal membrane sometimes consists of different layers of tapetal cell walls covered with orbicules (Fig. 9C). The tapetal markers are still visible, indicating the previous position of radial tapetal cell walls (Fig. 9C). At all stages of development, the tapetum cells maintained their individuality and position. These observations indicate that the tapetum cells in *T. gracilipes* are of the secretory type. In the mature orbicules the tail-shaped extensions of the pre-orbicules are still visible (Fig. 9D). A thin layer of electron dense fibrillar material, the peripheral layer, lines the homogeneous electron dense orbicule wall (Fig. 9D).

DISCUSSION

This is the first study describing the pollen, tapetum and orbicule development in *Tarenna gracilipes*.

Pollen ontogeny

After the first meiotic division, no ephemeral cell plate was formed, and the second meiotic division soon followed. Microsporogenesis in *T. gracilipes* can thus be considered as simultaneous. At the beginning of the second meiotic division, dense plastids are very abundant in the cytoplasm; presumably preventing coalescence of meiosis II spindles (Rodkiewicz et al. 1986, Bednara et al. 1986) (Fig. 2A, B). Sometimes more than one developmental stage was present in one anther, suggesting that the early events in microspore development occur rapidly.

The columellae are the first pollen wall components to be deposed in the fibrillar matrix in early tetrad stage, followed by tectum and foot layer development (Fig. 3D). In tangential sections from the early tetrad stage, tubular core parts of the pro-columellae are revealed which represents 'tufts' (Rowley & Flynn 1968, Flynn & Rowley 1971, Rowley et al. 1981, Hideux & Abadie 1985) and are composed of five electron dense subunits (Fig. 3E). These observations corroborate the exine substructure model described by Abadie et al. (1986). From the early tetrad stage onwards the deposition of sporopollenin occurs in two periods: in the first period accumulation of sporopollenin precursors results in an increase of the electron density of primexine elements (Figs 3D, E, 4A – D, 6A & C), while in the second period the maturation of sporopollenin decreases the electron density of primexine elements (Figs 7D, E, 8A & B) (Abadie et al. 1986). These phenomena occur both in the developing exine and orbicules (Figs 5B, C, 7B, 9C & D) indicating the fundamental relationships existing between both structures.

At the beginning of the endexine formation, a single white line is observed near the plasmalemma in the extra-apertural region and the developing endexine is dilated into several white line centered lamellae in the apertural regions (Fig. 6A, C). A comparable lamellated endexine in apertural regions is observed in *Olea europaea* (Fernández & Rodríguez-García 1988), *Lycopersicon esculentum* (Fernández et al. 1992), *Epilobium* and *Poinciana* (Rowley 1995), *Nelumbo* (Kreunen & Osborn 1999), *Artemisia* (Rowley et al. 1999*a*) and *Rondeletia odorata* (El-Ghazaly et al. 2001). These lamellae consist of a trilamellated structure in early development: an electron translucent white line surrounded by material positively staining for glycoproteins (PTA), indicating a possible glycocalyx nature (Fig. 6C). Similar to the columellae formation, the first endexine components form on strands of glycocalyx elements (Rowley et al. 1999*b*). When the pollen is mature this white line is only vestigial, comparable with observations in *Xiphidium* (Simpson 1989), *Asimina* (Gabarayeva 1993) and *Aristea* (Le Thomas et al. 2001). The foot layer can be distinguished in mature pollen grains when remnants of a single white line appear separating the inner ectexinous layer from the initial deposition of the endexine (Fig. 8B). The white line was described under different names in mature pollen grains, e.g. "junction plane" (Xi & Wang 1989, Rowley & Dunbar 1996, Rowley & Rowley 1996) and "commissural line" (Simpson 1983). During maturation and increase in volume of the pollen, the white line centered lamellae are compressed and adhere to each other, as well as to the foot layer, which makes the white line centered lamellae partly invisible in mature pollen grains (Gabarayeva 1993).

Rowley et al. (1999*b*) suggested that the formation of ectexine and endexine is similar or even the same although the morphology and ultrastructure of both layers is different. This hypothesis is corroborated by this study: the annulus (ectexine) and endexine develop in a similar way on a white line (Fig. 6A, C), although both structures at maturity differ in composition resulting in differences in electron density (Fig. 8B). These differences in electron density between the mature endexine and annulus are also observed in the rubiaceous species *Mitriostigma axillare* (Hansson & El-Ghazaly 2000). The ultrastructural appearance of the mature annulus present in *T. gracilipes* also strongly resembles the annulus described in *Mitriostigma axillare* (Hansson & El-Ghazaly 2000).

From the vacuolated stage onwards the footlayer became more pronounced and had a similar stainability as the ectexine, i.e., darker than endexine (Figs 7E, 8A & B). Comparable results are reported by El-Ghazaly et al. (2001) for the developing footlayer in the rubiaceous species *Rondeletia odorata*, and *Mitriostigma axillare* (Hansson & El-Ghazaly 2000).

According to the species concerned the intine layer may develop differently in the number of strata observed. A single stratum was recorded in *Olea europaea* (Fernández & Rodríguez-García 1988) and *Lycopersicon esculentum* (Fernández et al. 1992). An intine consisting of two strata, as we found in *Tarenna gracilipes* (Fig. 8A, B), is reported in *Anigozanthus viridi* (Rowley & Rowley 1996), and *Hypecoum imberbe* (Romero et al. 2003). In a number of studies more elaborate intines were distinguished (Heslop-Harisson & Heslop-Harisson 1991, Marquez et al. 1997*a*,*b*, Suarez-Cervera et al. 2000, Suarez-Cervera et al. 2001*a*,*b*). The

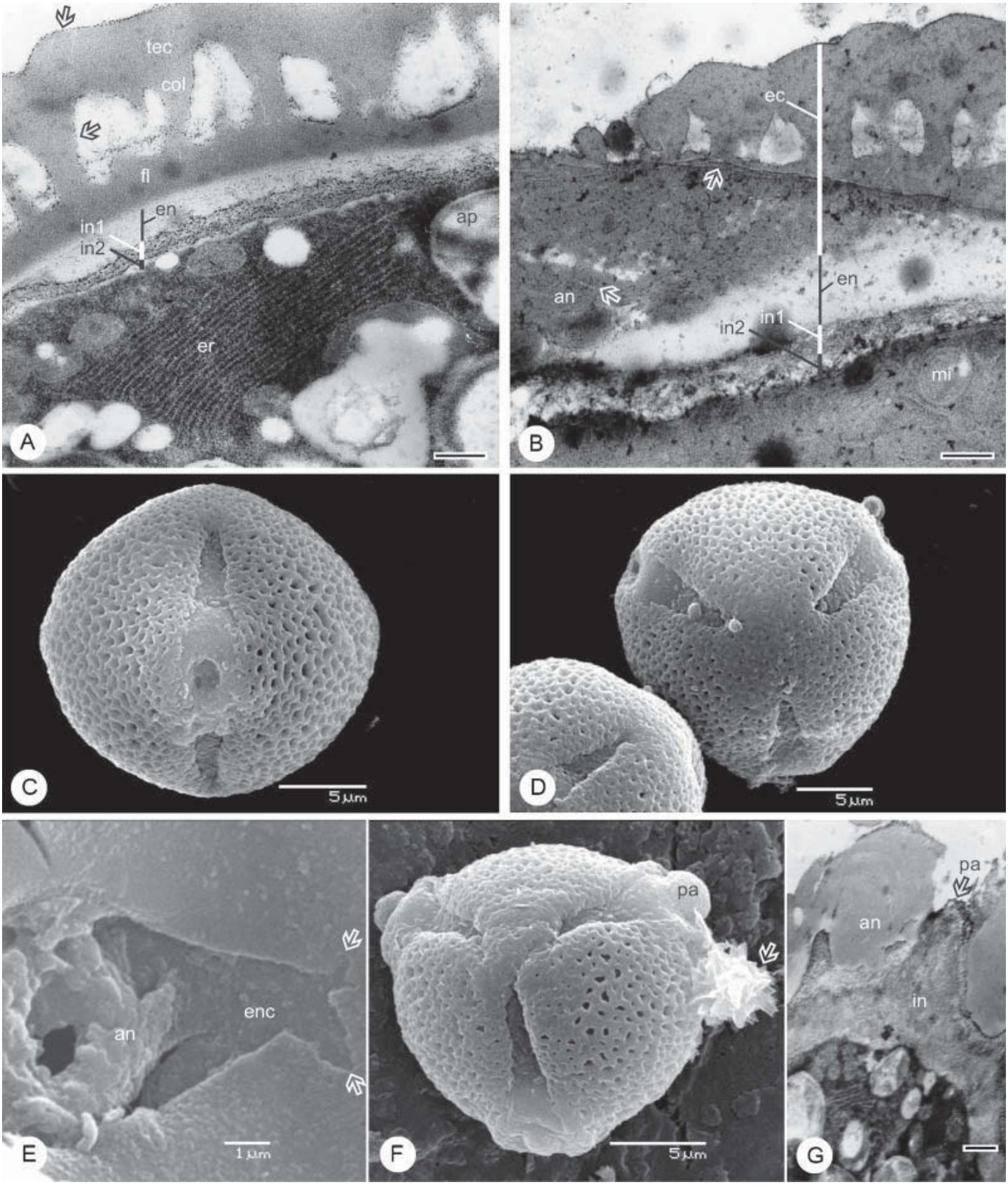

Fig. 8

intine formed thick onci often protruding through the apertures and thereby forming papillae (Fig. 8F, G). Similar papillae are reported in several Rubiaceae species: *Oldenlandia nudicaulis* (Farooq & Inamuddin 1969), *Stephegyne parviflora* (Ramam 1954), several species of the genera *Canthium*, *Keetia*, and *Psydrax* (Tilney & Van Wyk 1997), and *Mitriostigma axillare* (Hansson & El-Ghazaly 2000).

Tapetum and orbicule development

In Rubiaceae species amoeboid as well as secretory tapeta are reported, for a review see Hansson & El-Ghazaly (2000). In *Rondeletia odorata* (Rubiaceae) the tapetal cells show non-invasive amoeboid characteristics only during the free microspore stage (Huysmans 1998, El-Ghazaly et al. 2001). However, in *Mitriostigma axillare* (Hansson & El-Ghazaly 2000) and *T. gracilipes* it is shown that the tapetum cells possess in all developmental stages characteristics of the secretory type. The tapetum cytoplasm undergoes quite considerable changes (Figs 2C, 5A, 7B, C & D), such as change in volume densities of rough endoplasmic reticulum, plastids, and occurrence of plasmodesmata, which may indicate that the tapetal cells went through cycles of hyperactivity (Rowley 1993). The presence of cytoplasmic channels and plasmodesmata between tapetum cells (Fig. 2C) represent an important means of cell to cell communication (McLean et al. 1997). The migration of chromatin material from one cell to another (Fig. 2C) indicates the occurrence of cytomixis (Malallah & Attia 2003). At the end of the tetrad stage, pre-orbicules appear in the periplasmic space of tapetum cells and possess 'white line' tail-shaped extensions (Fig. 5B, C) which can be distinguished until maturity (Fig. 9D). Similar white line tail-shaped extensions are observed in *Platanus acerifolia* (Suarez-Cervera et al. 1995); in *Rondeletia odorata* these are apparent in the late free microspore stage (Huysmans 1998, El-Ghazaly et al. 2001), and in *Pinus sylvestris* in metaphase-I to prophase-II (Rowley & Walles 1993). The material surrounding the pre-orbicules gave positive reactions for glycoproteins (PTA) and can be interpreted as a glycocalyx positioned upon the membrane delimiting the pre-orbicule (Clément & Audran 1993*c*) (Fig. 5B – C). All the enzyme systems which participate in the maturation of pro-sporopollenin are thought to be located at this site (Clément & Audran 1993*c*). As for the orbicule development in *Platanus acerifolia* (Suarez-Cervera et al. 1995), the setting of pro-sporopollenin from the tapetum cells on the pre-orbicules is related with the deposition of pro-endexine and the thickening of pro-ectexine at the beginning of microspore liberation.

At the late vacuolated state tapetal cell degradation is initiated and orbicules have reached their final size (Fig. 7B, D). The changes in electron density during the development of the orbicule wall take place simultaneously and similar with the changes in the ectexine electron density (see discussion above). During the development of the orbicule wall upon the pre-orbicules, the electron density of the orbicule wall decreased considerably (Figs 5C, 7B & 9D), except for the peripheral layer (Fig. 9D). This observation can be explained by the fact that pro-sporopollenin maturation occurs from the base towards the top of the orbicule wall. The accumulation of sporopollenin in the orbicule wall gives an explanation for the decreasing stainability of the glycocalyx filaments, because they become embedded within sporopollenin. The glycocalyx filaments at the top of the orbicule wall, the peripheral layer, are not yet totally embedded with sporopollenin and therefore still can be revealed with PTA (Clément & Audran 1993*b*) (Fig. 9D).

Anther dehiscence and development of Ca-oxalate druses

The anther dehiscence of *T. gracilipes* involves differentiation of the stomium, accumulation of Ca-oxalate crystals in septal cells (Figs 3B, 7A), endothecium formation and the programmed cell death of the septum and stomium, ultimately leading to pollen release (Fig. 9A). An association between the accumulation of calcium oxalate crystals in the septum and development of the stomium was observed in different Solanaceous species and a function in anther dehiscence was proposed (Bonner & Dickinson 1989, Horner & Wagner 1992). Calcium oxalate crystals not only develop in the septum of Solanaceous species, but they are also reported in septal tissue of different plant families (Horner 1977, D'Arcy et al. 1996). At dehiscence the crystals are in close contact with the pollen grains of *T. gracilipes* (Fig. 8F), and may play a role in enhancing pollination by providing a visual signal for pollinators (D'Arcy et al. 1996). In addition to *T. gracilipes*, similar observations illustrated the occurrence of calcium oxalate druses in the anther locule at the stage of dehiscence in the following Rubiaceae species studied: *Coffea pseudozanguebariae*, *Rutidea membranacea*, *Aulacocalyx caudata*, *Pavetta abyssinica*, *Blepharidium mexicanum* (Vinckier et al. 2000, D' hondt 2002, *pers. observ.*). Twin raphides with one dovetailed end, are present in the anthers of *Cosmibuena grandiflora*, *Hillia panamensis*, *Coptosapelta hameliaeblasta*, *C. montana*, *C. tomentosa* (D' hondt 2002, Verellen 2002). The different calcium oxalate crystal types in Rubiaceae are clearly characteristic of several taxonomic groups, e.g. twin raphides seem to be the usual raphide type in Hillieae-Hamelieae (Cinchonoideae) and in Rubioideae (Jansen et al. 2002).

CONCLUSION

Our observations in *Tarenna gracilipes* clearly show that sporopollenin deposition on the pre-orbicules is mediated by

Fig. 8. **Mature pollen grains at dehiscence**. (A) TEM observation of the totally structured exine close to the apertural region. Inside the cytoplasm endoplasmic reticulum (***er***) and amyloplasts (***ap***) are observed. A thin layer of electron dense fibrillar material (***arrows***) covers the tectum (***tec***) and lines the infratectum. In the intine the two strata can be made out, a thin white inner one (***in2***) and a corrugated outer one (***in1***); (B) TEM observation of the annulus at the apertural region. At this site the intine is enlarged in thickness. White lines (***arrows***) are visible in cross sections of the granular annulus (***an***); (C) SEM observation of an acetolysed pollen grain in equatorial view; (D) SEM observation of an acetolysed pollen grain in polar view; (E) SEM observation of the inside ornamentation of broken acetolysed pollen. The granular annulus (***an***) around the inner surface of a pore is clearly visible. A short equatorial endocolpus (***enc***) with 'fish tail' ends (***arrows***) is present beneath each pore. The inside ornamentation is granular-psilate; (F) General SEM observation of a pollen grain lying on the locule wall surface at dehiscence. A calcium oxalate druse (***arrow***) is attached to the exine of the pollen grain. The papillae (***pa***) at the aperture, formed by protruding onci are clearly visible; (G) TEM observation of the oncus formed by the intine (***in***). Scale bars – 0.4 μm (A – B); 0.6 μm (G).

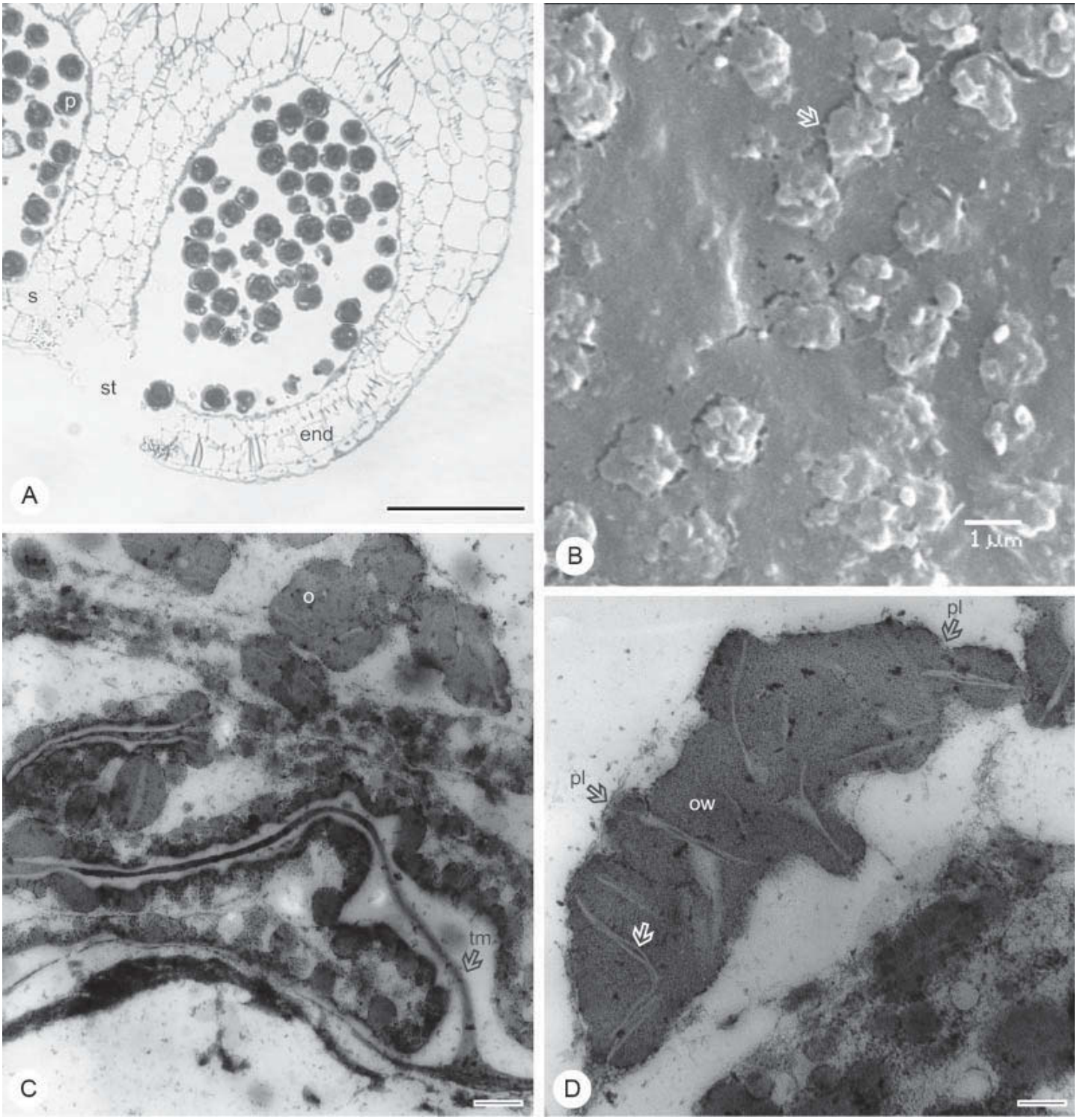

Fig. 9. **Mature orbicules at dehiscence**. (A) LM observation of a cross section of an anther at dehiscence. The stomium (***st***) opens and the septal (***s***) cells are ruptured; (B) SEM observation of irregular and granular shaped orbicules. Between the orbicules and/or between the orbicules and the tapetal membrane thin interconnecting threads may occur (***arrow***); (C) TEM observation of completely degenerated tapetal cells. The remains are compressed to form the tapetal membrane, densely covered with orbicules (***o***). The tapetal membrane consists of different layers of tapetal cell walls covered with orbicules. A tapetal marker (***tm***) indicates the previous position of the radial tapetal cell wall; (D) TEM observation of a mature orbicule. The tail-shaped extensions of the pre-orbicules are still visible (***white arrow***). A thin layer of electron dense fibrillar material, the peripheral layer (***pl***), lines the homogeneous electron dense orbicule wall (***ow***). Scale bars – 100 µm (A); 0.4 µm (C); 0.25 µm (D).

white lines, showing a correlation with endexine, annulus as well as columellae ontogeny which was in the latter indicated by extending white lines into the base of developing columellae. In *Helleborus* a similar correlation between endexine and orbicule development was demonstrated by Echlin & Godwin (1968). These findings corroborate the idea proposed by Clément and Audran (1993*b*,*c*) that the orbicule wall development can represent a

model to study sporopollenin deposition in the anther since orbicules are extracellular structures, independent of cytoplasmic control, as opposed to the pollen exine. The fact that often striking analogies are found between the ornamentation of the pollen exine and that of the orbicule wall (Nilsson & Robyns 1974, El-Ghazaly & Jensen 1986, Hesse 1986, Vinckier & Smets 2002*a*, *b*, *c*, 2003) may indicate that in depth studies on orbicule wall formation may clarify the underlying factors determining exine and orbicule wall patterning.

ACKNOWLEDGEMENTS

The director of the National Botanic Garden of Belgium (BR) is greatly acknowledged for the supply of flowering buds. We thank Koen Collart (TEM) and Marcel Verhaegen (SEM) for technical assistance. This work has been financially supported by research grants of the K. U. Leuven (OT/01/25, PDM/03/145) and the Fund for Scientific Research - Flanders (Belgium) (F.W.O. - Vlaanderen) (G.104.01). Dr. Stefan Vinckier is a postdoctoral fellow of the Fund for Scientific Research - Flanders (Belgium) (F.W.O. - Vlaanderen).

REFERENCES

Abadie, M., Hideux, M. & Rowley, J. R. 1986. Ultrastructural cytology of the anther. II Proposal for a model of exine considering a dynamic connection between cytoskeleton, glycolemma and sporopollenin synthesis. – Ann. Sci. Nat., Bot. (Paris) 13(8): 1 – 16.

Andronova, N. N. 1984. The structure of the anther and pollen development in the Rubiaceae. – Bot. Zeitschr. 69: 43 – 54.

Bednara, J., Gielwanowska, I. & Rodkiewicz, B. 1986. Regular arrangements of mitochondria and plastids during sporogenesis in *Equisetum*. – Protoplasma 130: 145 – 152.

Benedetti, E. L. & Bertolini, B. 1963. The use of phosphotungstic acid as a stain for the plasma membrane. – J. R. Microsc. Soc. (London) 81: 219 – 222.

Bonner, L. J. & Dickinson, H. G. 1989. Anther dehiscence in *Lycopersicon esculentum* Mill. I. Structural aspects. – New Phytol. 113: 97 – 115.

Christensen, J. E., Horner, H. T., Jr & Lersten, N. R. 1972. Pollen wall and tapetal orbicular wall development in *Sorghum bicolor* (Gramineae). – Am. J. Bot. 59: 43 – 58.

Clément, C. & Audran, J.-C. 1993*a*. Cytochemical and ultrastructural evolution of orbicules in *Lilium*. – Plant Syst. Evol. 7: 63 – 74.

Clément, C. & Audran, J.-C. 1993*b*. Orbicule wall surface characteristics in *Lilium* (Liliaceae). – Grana 32: 348 – 353.

Clément, C. & Audran, J.-C. 1993*c*. Electron microscope evidence for a membrane around the core of the Ubisch body in *Lilium* (Liliaceae). – Grana 32: 311 – 314.

D'Arcy, W. G., Keating, R. C. & Buchmann, S. L. 1996. The calcium oxalate package or so-called resorption tissue in some angiosperm anthers. – In: The anther: form, function and phylogeny (ed. W. G. Keating, R. C. Keating), pp. 159 – 191. – Cambridge Univ. Press, Cambridge U. K.

D' hondt, C. 2002. Pollen- en orbiculemorfologie van Hillieae (Rubiaceae). – Lic. Diss. Bot. Dept., K. U. L., Leuven (In Flemish).

Echlin, P. 1971. The role of the tapetum during microsporogenesis of angiosperms. – In: Pollen development and physiology (ed. J. Heslop-Harrison), pp. 41 – 61. – Butterworths, London.

Echlin, P. & Godwin, H. 1968. The ultrastructure and ontogeny of pollen in *Helleborus foetidus* L. I. The development of the tapetum and Ubisch bodies. – J. Cell Sci. 3: 161 – 174.

El-Ghazaly, G. & Jensen, W. A. 1986. Studies of the development of wheat (*Triticum aestivum*) pollen. – Grana 25: 1 – 29.

El-Ghazaly, G., Huysmans, S. & Smets, E. 2001. Pollen development of *Rondeletia odorata* (Rubiaceae). – Am. J. Bot. 88: 14 – 30.

Erdtman, G., Berglund, B. & Praglowski, J. 1961. An introduction to a Scandinavian pollen flora. – Almqvist & Wiksell, Stockholm.

Farooq, M. & Inamuddin, M. 1969. The embryology of *Oldenlandia nudicaulis* Roth. – J. Ind. Bot. Soc. 48: 166 – 173.

Fernández, M. C. & Rodríguez-García, M. I. 1988. Pollen wall development in *Olea europaea* L. – New Phytol. 108: 91 – 99.

Fernández, M. C., Romero, A. T. & Rodríguez-García, M. I. 1992. Aperture structure, development and function in *Lycopersicon esculentum* Miller (Solanaceae) pollen grains. – Rev. Palaeobot. Palynol. 72: 41 – 48.

Flynn, J. J. & Rowley, J. R. 1971. Wall microtubules in pollen grains. – Zeiss Inform. 76: 40 – 45.

Furness, C. A. & Rudall, P. J. 2001. The tapetum in basal angiosperms: Early diversity. – Int. J. Plant Sci. 162: 375 – 392.

Gabarayeva, N. I. 1993. Sporoderm development in *Asimina triloba* (Annonaceae). II. The developmental events after callose dissolution. – Grana 32: 210 – 220.

Hansson, T. & El-Ghazaly, G. 2000. Development and cytochemistry of pollen and tapetum in *Mitriostigma axillare* (Rubiaceae). – Grana 39: 65 – 89.

Hayat, M. A. 1989. Principles and techniques of electron microscopy: Biological applications (ed. M. A. Hayat). – McMillan, London.

Heslop-Harrison, J. & Heslop-Harisson, Y. 1991. Structural and functional variation in pollen intines. – In: Pollen and spores: Patterns of diversification (ed. S. Blackmore & S.H. Barnes), pp. 331 – 343. – Syst. Assoc. Vol. 44. Clarendon Press, Oxford.

Hesse, M. 1986. Orbicules and the ektexine are homologous sporopollenin concretions in Spermatophyta. – Plant Syst. Evol. 153: 37 – 48.

Hesse, M. & Hess, M. W. 1993. Recent trends in tapetum research. A cytological and methodological review. – Plant Syst. Evol. Suppl. 7: 127 – 145.

Hideux, M. & Abadie, M. 1985. Cytologie ultrastructurale de l'anthère des principeaux types exiniques du genre *Saxifraga* L. 1. Période d'initiation des précurseurs des sporopollénines. – Can. J. Bot. 63: 97 – 112.

Horner, H. T. 1977. A comparative light- and electron-microscopic study of microsporogenesis in male-fertile and cytoplasmic male-sterile sunflower (*Helianthus annuus*). – Am. J. Bot. 64: 745 – 759.

Horner, H. T. & Wagner, B. L. 1992. Association of four different calcium crystals in the anther connective tissue and hypodermal stomium of *Capsicum annuum* L. (Solanaceae) during microsporogenesis. – Am. J. Bot. 79: 531 – 541.

Huysmans, S. 1998. Palynology of the Cinchonoideae (Rubiaceae). Morphology and development of pollen and orbicules. – Ph. D. Thes. Bot. Dept., K.U. L., Leuven.

Huysmans, S., El-Ghazaly, G. & Smets, E. 1998. Orbicules in angiosperms. Morphology, function, distribution, and relation with tapetum types. – Bot. Rev. 64: 240 – 272.

Huysmans, S., Robbrecht, E. & Smets, E. 1994. Are the genera *Hallea* and *Mitragyna* (Rubiaceae-Coptosapeltaea) pollen morphologically distinct? – Blumea 39: 321 – 340.

Jansen, S., Robbrecht, E., Beeckman, H. & Smets, E. 2002. A survey of the systematic wood anatomy of the Rubiaceae. – IAWA J. 23: 1 – 67.

Kosmath, L. 1927. Studie über das Antherentapetum. – Österr. Bot. Zeitschr. 76: 235–241.

Kreunen, S. S. & Osborn, J. M. 1999. Pollen and anther development in *Nelumbo* (Nelumbonaceae). – Am. J. Bot. 86: 1662–1676.

Le Thomas, A., Suarez-Cervera, M. & Goldblatt, P. 2001. Ontogeny of the exine in pollen of *Aristea* (Iridaceae). – Grana 40: 35–44.

McLean, B. G., Hempel, F. D. & Zambryski, P. C. 1997. Plant intercellular communication via plasmodesmata. – Plant Cell 9: 1043–1054.

Malallah, G. A. & Attia, T. A. 2003. Cytomixis and its possible evolutionary role in a Kuwaiti population of *Diplotaxis harra* (Brassicaceae). – Bot. J. Linn. Soc. 143: 169–175.

Marinozzi, V. 1968. Phosphotungstic acid (PTA) as a stain for polysaccharides and glycoproteins in electron microscopy. – In: Electron Microscopy 1968. 4th Eur. Reg. Conf. El. Microsc. Rome 1968. Pre-Congr. Abstr. (ed. D. S. Bocciarelli), pp. 55–56. – ERCEM Org. Comm./Tip. Poligl. Vaticana, Rome.

Marquez, J., Seoane-Camba, J. A. & Suarez-Cervera, M. 1997*a*. Allergenic and antigenic proteins released in the apertural sporoderm during the activation process in grass pollen grains. – Sex. Plant Reprod. 10: 269–278.

Marquez, J., Seoane-Camba, J. A. & Suarez-Cervera, M. 1997*b*. The role of the intine and cytoplasm in the activation and germination processes of Poaceae pollen grains. – Grana 36: 328–342.

Nilsson, S. & Robyns, A. 1974. Pollen morphology and taxonomy of the genus *Quararibea* s.l. (Bombacaceae). – Bull. Jard. Bot. Natl Belg. 44: 77–99.

Pacini, E. 1990. Tapetum and microspore function. – In: Microspores, evolution and ontogeny (ed. S. Blackmore, R. B. Knox), pp. 213–237. – Acad. Press, London.

Pacini, E. 1997. Tapetum character states: analytical keys for tapetum types and activities. – Can. J. Bot. 75: 1448–1459.

Ramam, S. S. 1954. Gametogenesis and fertilization of *Stephegyne parviflora* Korth. – Agra Univ. J. Res. 3: 343–348.

Rodkiewicz, B., Bednara, J., Mostowska, A., Duda, E. & Stobiecka, H. 1986. The change in disposition of plastids and mitochondria during microsporogenesis and sporogenesis in some higher plants. – Acta Bot. Neerl. 35: 209–215.

Romero, A. T., Salinas, M. J. & Fernández, M. C. 2003. Pollen wall development in *Hypecoum imberbe* Sm. (Fumariaceae). – Grana 42: 91–101.

Rosanoff, S. 1865. Zur Kenntniss des Baues und der Entwicklungsgeschichte des Pollens der Mimosaceae. – Jahrb. Wissensch. Bot. 4: 441–450.

Rowley, J. R. 1993. Cycles of hyperactivity in tapetal cells. – Plant Syst. Evol. 7: 23–37.

Rowley, J. R. 1995. Are the endexines of pteridophytes, gymnosperms and angiosperms structurally equivalent? – Rev. Palaeobot. Palynol. 85: 13–34.

Rowley, J. R. & Flynn, J. J. 1968. Tubular fibrils and the ontogeny of the yellow water lily pollen grain. – J. of Cell Biol. 39: 159a.

Rowley, J. R., Dahl, A. O. & Rowley, J. S. 1981. Substructure in exines of Artemisia vulgaris (Asteraceae). – Rev. Palaeobot. Palynol. 35: 1–38.

Rowley, J. R., Gabarayeva, N. I. & Walles, B. 1992. Cyclic invasion of tapetal cells into loculi during microspore development in *Nymphaea colorata* (Nymphaceae). – Am. J. Bot. 79: 801–808.

Rowley, J. R. & Walles, B. 1993. Cell differentiation in microsporangia of *Pinus sylvestris*. V. Diakinesis to tetrad formation. – Nord. J. Bot. 13: 67–82.

Rowley, J. R. & Dunbar, A. 1996. Pollen development in *Centrolepsis aristata* (Centrolepidaceae). – Grana 35: 1–15.

Rowley, J. R. & Rowley, J. S. 1996. Pollen wall structure in *Anigozanthos viridis* (Haemodoraceae). – Acta Soc. Bot. Pol. 65: 83–90.

Rowley, J. R., Claugher, D. & Skvarla, J. J. 1999*a*. Structure of the exine in *Artemisia vulgaris* (Asteraceae) a review. – Taiwania 44: 1–21.

Rowley, J. R., Skvarla, J. J. & Walles, B. 1999*b*. Microsporogenesis in *Pinus sylvestris*. VII. Exine expansion and tapetal development. – Taiwania 44: 325–344.

Simpson, M. G. 1983. Pollen ultrastructure of the Haemodoraceae and its taxonomic significance. – Grana 22: 70–103.

Simpson, M. G. 1989. Pollen wall development of *Xiphidium coeruleum* (Haemodoraceae) and its implications. – Ann. Bot. 4: 257–269.

Suarez-Cervera, M., Marquez, J. & Seoane-Camba, J. 1995. Pollen grain and Ubisch body development in *Plantanus acerifolia*. – Rev. Palaeobot. Palynol. 85: 63–84.

Suarez-Cervera, M., Le Thomas, A., Goldblatt, P., Marques, J. & Seoane-Camba, J. A. 2000. The channeled intine of *Aristea major*: Ultrastructural modifications during development, activation and germination. – In: Pollen and spores: Morphology and biology (ed. M. M. Harley, C. M. Morton & S. Blackmore), pp. 57–71. – R. Bot. Gards, Kew.

Suarez-Cervera, M., Guillespie, L., Arcalis, E., Le Thomas, A., Lobreau-Callen, D. & Seoane-Camba, J. A. 2001*a*. Taxonomic significance of sporoderm structure in pollen of Euphorbiaceae: Tribes Plukenetieae and Euphorbieae. – Grana 40: 78–104.

Suarez-Cervera, M., Arcalis, E., Le Thomas, A. & Seoane-Camba, J. A. 2001*b*. Pectin distribution pattern in the apertural intine of *Euphorbia peplus* L. (Euphorbiaceae) pollen. – Sex. Plant Reprod. Published online: *DOI 10.1007/s00497-001-0121-5*.

Tilney, R. M. & Van Wyk, A. E. 1997. Pollen morphology of *Canthium*, *Keetia* and *Psydrax* (Rubiaceae: Vanguerieae) in southern Africa. – Grana 36: 249–260.

Verellen, J. 2002. Palynologische studie en revisie van *Coptosapelta* (Rubiaceae). – Lic. Diss. Bot. Dept., K. U. L., Leuven.

Vijayaraghavan, M. R. & Chaudhry, B. 1993. Structure and development of orbicules in the tapetum of *Prosopsis juliflora* (Leguminosae, Mimosoideae). – Phytomorphology 43: 41–48.

Vinckier, S. 2003. Orbicules: morphology, ultrastructure, and development in Gentianales and their possible role as vector of allergens. – Ph. D. Thes. Bot. Dept., K.U. L., Leuven.

Vinckier, S. & Smets, E. 2002*a*. Morphology, ultrastructure and typology of orbicules in family Loganiaceae s.l. and related genera, in relation to systematics. – Rev. Palaeobot. Palynol. 119: 161–189.

Vinckier, S. & Smets, E. 2002*b*. Morphological and ultrastructural diversity of orbicules in relation to evolutionary tendencies in Apocynaceae s.l. – Ann. Bot. 90: 647–662.

Vinckier, S. & Smets, E. 2002*c*. Systematic importance of orbicule diversity in Gentianales. – Grana 41: 158–182.

Vinckier, S. & Smets, E. 2003. Morphological and ultrastructural diversity of orbicules in Gentianaceae. – Ann. Bot. 92: 657–672.

Vinckier, S., Huysmans, S. & Smets, E. 2000. Morphology and ultrastructure of orbicules in the subfamily Ixoroideae (Rubiaceae). – Rev. Palaeobot. Palynol. 108: 151–174.

von Teichman, I., Robbertse, P. J. & van der Merwe, C. F. 1982. Contributions to the floral morphology and embryology of *Pavetta gardeniifolia* A. Rich. Part 3. Microsporogenesis and pollen structure. – S. Afr. J. Bot. 1: 28–30.

Wang, A., Xia, Q., Xie, W., Datla, R. & Selvaraj, G. 2003. The classical Ubisch bodies carry a sporophytically produced structural protein (RAFTIN) that is essential for pollen development. – Proc. Natl Acad. Sci. 100 (24): 14487–14492.

Xi, Y. - Z. & Wang, F. H. 1989. Pollen exine ultrastructure of extant Chinese gymnosperms. – Cathaya 1: 119–142.