

## Pollen, Tapetum and Orbicule Development in *Modiolastrum malvifolium* (Malvaceae)

BEATRIZ G. GALATI\*, FEDERICO MONACCI, MARINA M. GOTELLI and SONIA ROSENFELDT  
Depto. Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pab. II, Ciudad Universitaria, C1428EHA, Buenos Aires, Argentina

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- **Background and Aims** Although orbicular functions are still a matter of debate, they are considered by most authors to be exclusively formed by a secretory tapetum. However, the presence of orbicules on a peritapetal membrane associated with a plasmodial tapetum has been described for *Abutilon pictum* (Malvaceae) in a previous study. Thus, studies on other species of Malvaceae are necessary to corroborate the presence of such bodies in other members of the family. Pollen and microsporangium development of *Modiolastrum malvifolium* has been studied in this work.
- **Methods** Anthers at different stages of development were processed for transmission electron microscopy and light microscopy. Membranes and pollen walls resistant to acetolysis were isolated from whole anthers.
- **Key Results** Microspore tetrads have a tetrahedral arrangement. Pollen grains are shed at the bicellular stage. The tapetum is invasive, non-syncytial and a peritapetal membrane with orbicules is formed.
- **Conclusions** This is the first report of the presence of orbicules on a peritapetal membrane in a species with a tapetum of an invasive, non-syncytial type. Taking into consideration all the information on the subject, it can be concluded that the presence of orbicules is not a stable criterion to differentiate between a secretory or plasmodial, or intermediate invasive, non-syncytial tapetum.

**Key words:** *Modiolastrum malvifolium*, invasive non-syncytial tapetum, orbicules, peritapetal membrane.

### INTRODUCTION

A previous ultrastructural ontogenetic study on pollen development of *Abutilon pictum* has revealed the presence of a peritapetal membrane with Ubisch bodies or orbicules in the mature anther (Strittmatter *et al.*, 2000). In this species, orbicules as well as the membrane are formed from a plasmodial-type tapetum. The presence of orbicules associated with a peritapetal membrane had not been described before for any other species of Angiospermae.

Although the functions of orbicules are still under discussion, they are considered by most authors to be exclusive of secretory tapeta (Raghavan, 1997; Huysmans *et al.*, 1998; Furness and Rudall, 2001).

Ultrastructural studies on other species are required in order to ascertain whether orbicules are present in other members of the Malvaceae family. Since nothing was known about the embryology of *Modiolastrum* (Johri *et al.*, 1992), pollen and microsporangium development of *M. malvifolium* has been studied under light and transmission electron microscope.

### MATERIALS AND METHODS

Samples of *Modiolastrum malvifolium* (Griseb.) K. Schum, were collected from garden populations in Zárate, Province of Buenos Aires, and were fixed in FAA (formalin : ethanol : acetic acid : water, 10 : 50 : 5 : 35 voucher : B. G. Galati. 691. Department of Biodiversidad y Biología Experimental, FCEyN, UBA).

\* For correspondence. E-mail galati@bg.fcen.uba.ar

For transmission electron microscopy (TEM), anthers at different developmental stages were pre-fixed in 3 % glutaraldehyde in phosphate buffer (pH 7.2) at 2 °C for 2 h and then post-fixed in OsO<sub>4</sub> at 2 °C in the same buffer for 3 h. They were dehydrated in an ethanol series and embedded in Spurr's resin (O'Brien and McCully, 1981). Fine sections were prepared using a Sorval ultramicrotome, and stained with uranyl acetate and lead citrate. They were observed and photographed using a JEOL 100C TEM (JOEL USA, Inc. Peabody, MA).

For light microscopy, 1.5-mm thick sections of resin-embedded tissue were prepared and stained with toluidine blue. Anthers were also fixed in FAA, dehydrated in an ethanol series and embedded in paraffin. Sections were cut with a rotatory microtome, stained with safranin-fast green or crystal violet, mounted in synthetic resin and photographed with a Zeiss photomicroscope.

The callosic walls were stained with 0.01 % aniline blue, which imparts a yellow fluorescence to callose (O'Brien and McCully, 1981).

Membranes and pollen walls resistant to acetolysis were isolated from whole anthers. The acetolysis was carried out by a modification of Erdtman's method (1960; Genise *et al.*, 1990). Structures resistant to acetolysis were washed with water and mounted in glycerine-jelly. A Zeiss fluorescence microscope was used to study autofluorescence.

### RESULTS

Anther ontogeny of *M. malvifolium* presents six stages of development, thus allowing further comprehension of the

ultrastructural details of the formation of pollen grains, the tapetum and the Ubisch bodies.

*Stage 1: microspore mother cell (MMC)*

The young anther wall consists of an epidermis, endothecium, one middle layer and a tapetum with

uninucleate cells (Fig. 1A). Tapetal cells show a conspicuous nucleus and a cytoplasm with small vacuoles, a few endoplasmic reticula of rough type (ERr), plastids, mitochondria and lipid globules (Fig. 2A, B). Cells that will form the endothecium and the middle layer tissues present larger vacuoles and a cytoplasm poorer in organelles (Fig. 2A). Epidermal cells show an

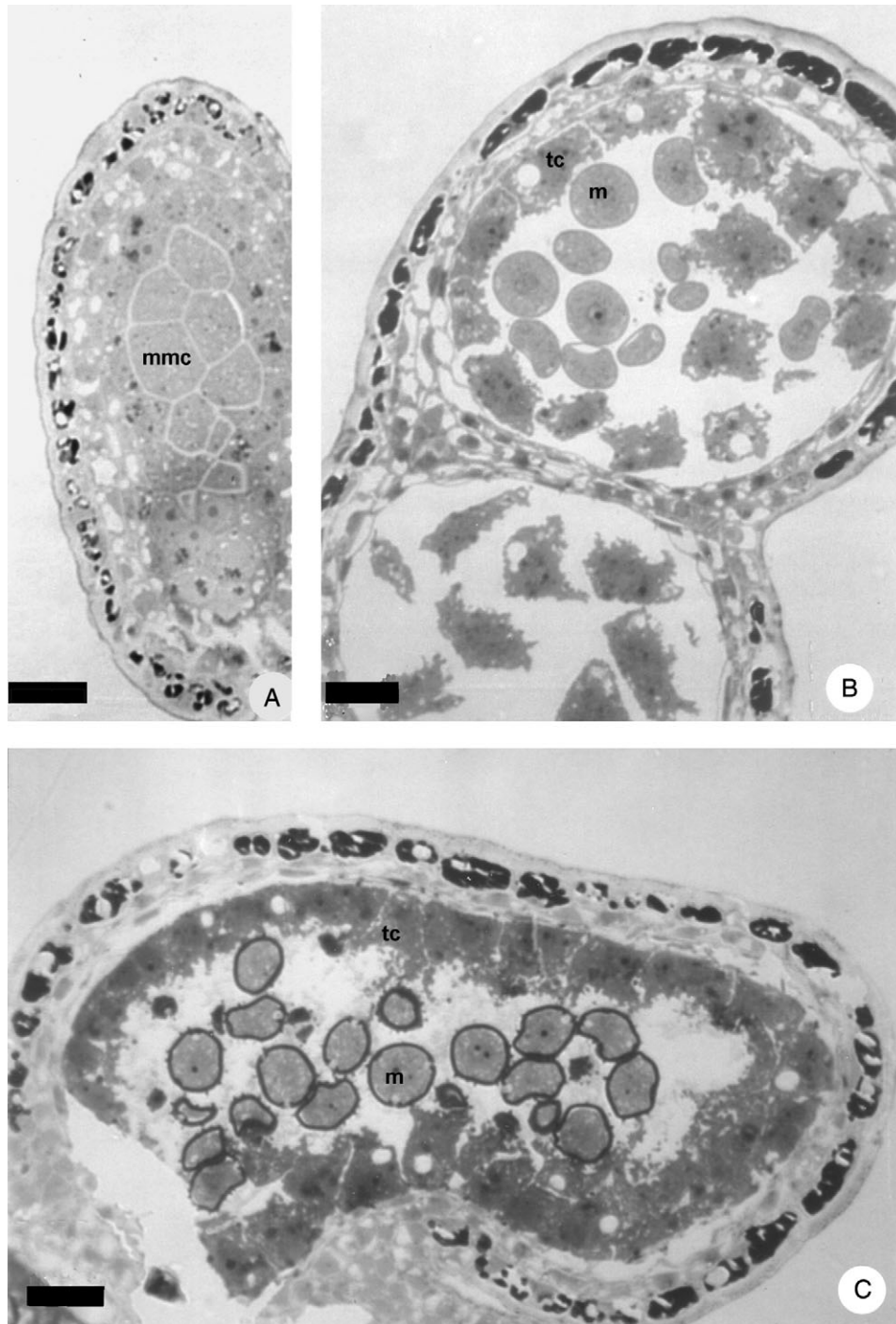


FIG. 1. *Modiolastrum malvifolium*, anther transversal sections under a light microscope. (A) Microspore mother cell stage (mmc). (B) Free, young microspores (m) stage: tapetal cells (tc) with lobules are towards the interior of the locule. (C) Mature microspore (m) stage. The exine wall is formed, tapetal cells (tc) with lobules are more conspicuous. Scale bars = 20  $\mu$ m.

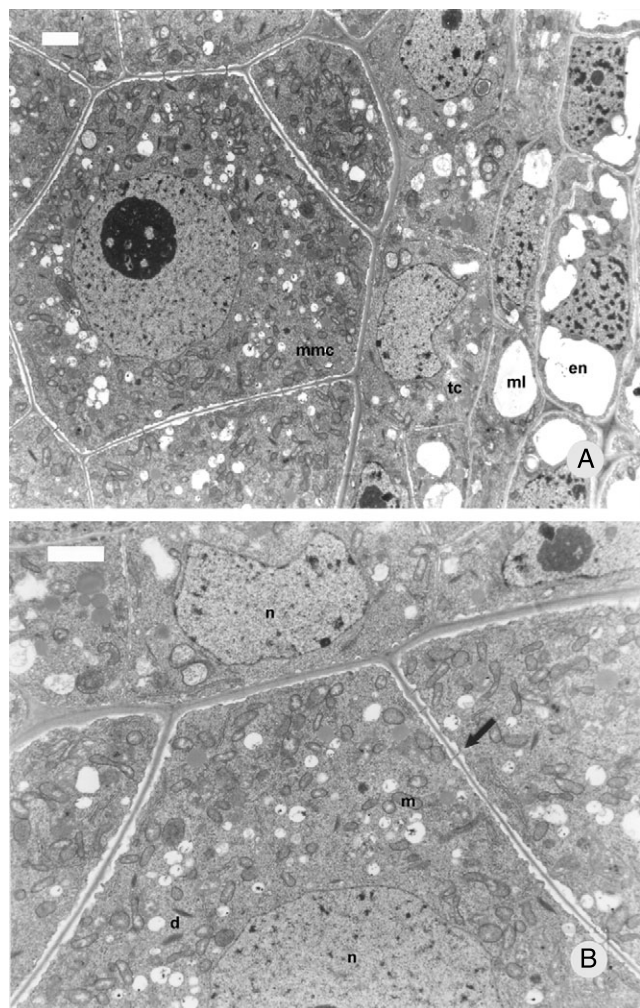


FIG. 2. Microspore mother cell stage observed with a TEM. (A) Microspore mother cell (mmc), tapetal cells (tc), middle layer (ml), endothecium (en). (B) Detail of the microspore mother cell: cytoplasmic connections (arrow), mitochondria (m), nucleus (n) and dictyosomes (d). Scale bars = 2  $\mu$ m.

electron-dense cytoplasm and the tangential external wall is thickened (Fig. 1A).

The microspore mother cell possesses a nucleus of great volume with a conspicuous nucleoli and a dense cytoplasm (Fig. 2A). The latter is filled with numerous small vacuoles and mitochondria, some of these in division. Abundant ER and some dictyosomes are also observed. There are numerous cytoplasmic connections between these cells and a callosic wall is beginning to form between the plasmalemma and the primary wall (Fig. 2A, B).

#### Stage 2: microspore tetrads

Tapetal cells walls are degraded and the tapetal nuclei divide. The middle layers are compressed (Fig. 3A).

The ultrastructure of the cytoplasm of tapetal cells is similar to that presented in the previous stage.

Microspore mother cells undergo simultaneous meiosis, forming tetrads with a tetrahedral arrangement (Fig. 3A).

These are surrounded by a thick callosic wall that is fluorescent when stained with aniline blue and irradiated with 365-nm light (Fig. 3B). The cytoplasm of microspores shows numerous free ribosomes, some mitochondria, and abundant plastids and dictyosomes (Fig. 4A, B). At this stage, the primexine fibrillar matrix is starting to develop. On it, the future basal layer, probacula and protectum can be seen as more electron-dense zones (Fig. 4C).

#### Stage 3: free, young microspores

At this stage, in the inner tangential faces of the tapetal cells an incipient lobe starts to grow towards the locule (Fig. 1B). The tapetal cytoplasm shows a great amount of ER, whose cisterns appear in a parallel disposition. Abundant dictyosomes are also observed (Fig. 5A, B).

A membrane is formed over the outer tangential wall of the tapetal cells and in touch with the middle layer. On its inner surface, future orbicules are observed as electron-dense corpuscles (Fig. 5A).

Once the callosic wall disintegrates, microspores are freed. An electron-dense fibrillar substance fills the locule and deposits over the probaculae and protectum (Figs 5B, 6C). There are no differences between the cytoplasm of the microspores at this stage and at the previous stage (Fig. 6C).

#### Stage 4: microspores with a developed exine wall

The lobe of the tapetal cell grows towards the anther locule (Fig. 1C); however, the ultrastructural characteristics of the tapetum remain the same.

The exine wall of the microspores is formed by a basal layer, numerous columellae close to each other, and a thick tectum (Fig. 6D). At this stage, the apertures of the future pollen grains and the intine oncus are formed.

#### Stage 5: young pollen grain

Tapetal cells do not lose their individuality as they invade the anther locule and surround pollen grains (Fig. 7A).

The peritapetal membrane is folded and very electron-dense corpuscles are observed on it (Fig. 5C).

The generative cell formed by a mitotic division of the microspore occupies a parietal position. Pollen grains show a very thick wall with an echinate tectum (Fig. 7A).

#### Stage 6: mature pollen grain

At this stage, tapetal and middle layer cells are mostly degraded (Fig. 7B). The peritapetal membrane is no longer folded, due to an increase in volume of the anther, and it is in direct contact with the endothecium (Figs 7B, 6A). In the latter, cells develop lignified thickenings of the secondary wall, formed as bands through their radial and tangential walls (Figs 7B, 6A).

Solid orbicules, without a central core and of piriform aspect are found on the peritapetal membrane (Fig. 6B).



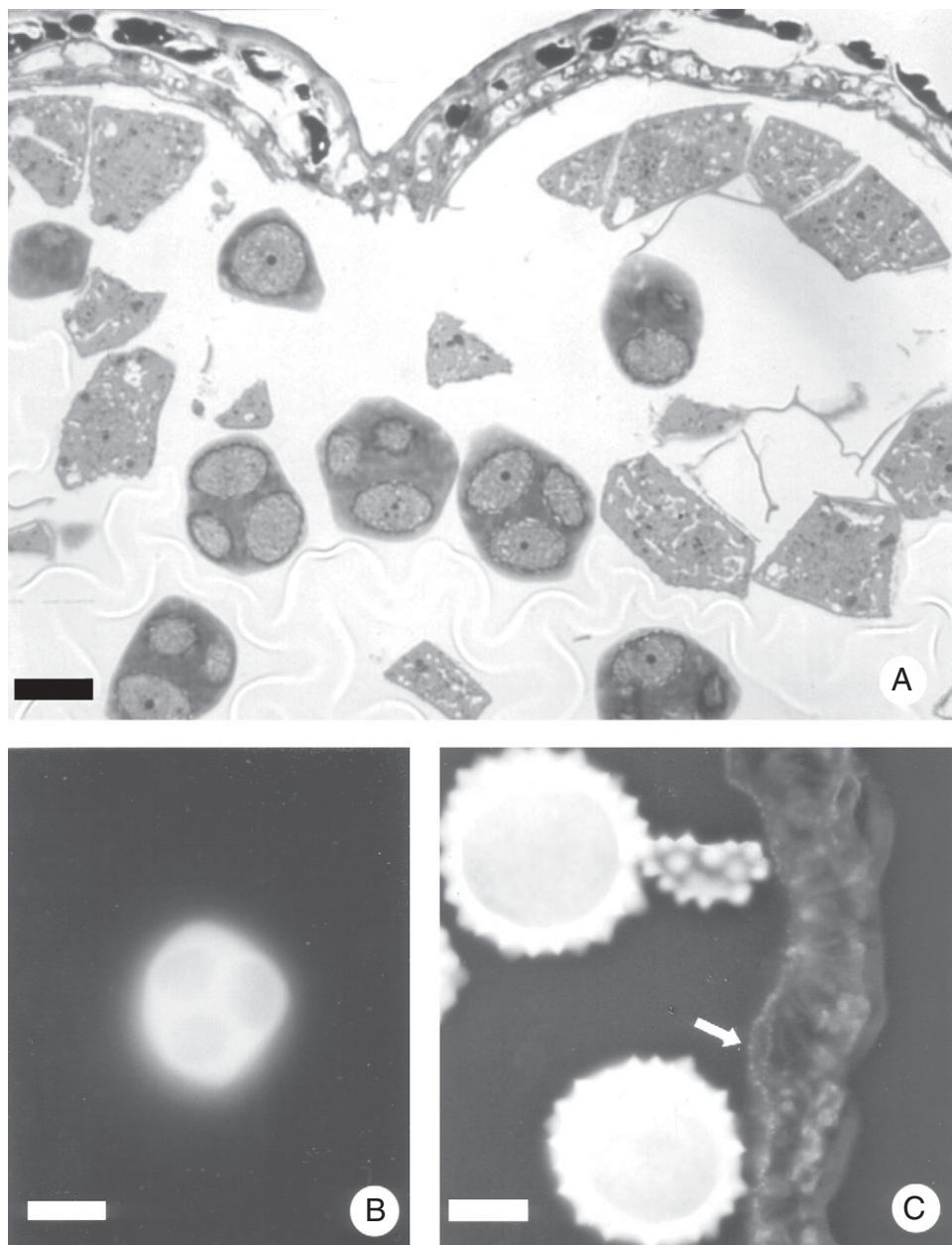


FIG. 3. (A) Microspore tetrad stage, under a light microscope. (B, C) Observations under an epifluorescence microscope. (B) Microspore tetrad, with fluorescence induced by the callose stained with aniline blue. (C) Autofluorescence of the sporopollenin of the exine wall and orbicules (arrow). Scale bars = 20  $\mu\text{m}$ .

These are fluorescent and react to stains (Crystal violet and toluidine blue) in the same way that the pollen grain exine does (Figs 3C, 7B) and are resistant to acetolysis, as the peritapetal membrane.

The pollen grain wall becomes thinner and collumella separate due to its increase in volume. The exine is more compacted. It has collumella on a thick basal layer and conspicuous supratectum spines. The exine has a very thin endexine layer, and a fibrillar intine with numerous invaginations coated by the plasmalemma can be observed.

Some tapetal derivatives are left between the collumella (Fig. 6E).

Inside the vegetative cell cytoplasm there can be found extensive cisterns of the ERr, numerous mitochondria and abundant amyloplasts (Fig. 8A, B). The generative cell is completely enclosed by the cytoplasm of the vegetative cell. The former possesses a conspicuous nucleus and a reduced cytoplasm with scarce mitochondria and bodies with concentric intravacuolar membranes. It presents a thin, irregular wall that is transparent to electrons (Fig. 8B).

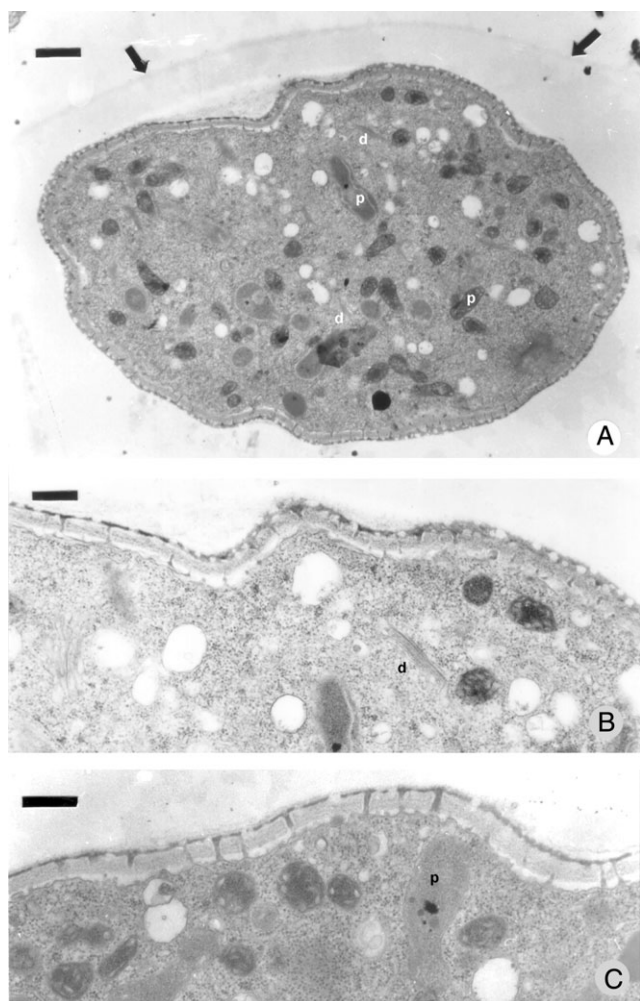


FIG. 4. Microspore tetrad stage observed under a TEM. (A) General aspect of a microspore surrounded by callose wall (arrows). (B) Detail of the microspore cytoplasm with abundant ribosomes and dictyosomes (d). (C) Detail of the beginning of microspore wall formation, with primexine, probacules and protectum. Numerous plastids (p) are observed in the microspore cytoplasm. Scale bar A: 1  $\mu$ m, B–C: 500 nm.

The generative cell is surrounded by numerous vesicles with a fibrillar content of low electron density in contact with more and smaller vesicles (Fig. 8B). Both kinds of vesicles are also regularly dispersed throughout the rest of the cytoplasm of the vegetative cell.

## DISCUSSION

At the microspore mother cell (MMC) stage numerous cytoplasmic connections are observed while the callose wall starts to form and the primary wall is still present. The existence of such connections has been reported for many angiosperms (Heslop-Harrison, 1964, 1966a, b). *Lycopersicum* presents some plasmodesmata, the diameter of which may allow the passage of small plastids (Pacini and Cresti, 1978; Polowick and Sawhney, 1992). It seems that only small particules could pass through these connections in *M. malvifolium*. According to Heslop-Harrison

(1964, 1966a, b), these connections allow a synchronic development of the archesporium.

The mitochondria and dictyosomes found on the cytoplasm of the MMC suggest great metabolic activity. Due to the activity of the dictyosomes, numerous vesicles in the cytoplasmic periphery are present. This indicates the possible role of dictyosomes in the production of callose (Heslop-Harrison, 1966a).

The generative cell of the mature pollen grain is enclosed by the vegetative one, and is surrounded by lots of low-electron-density vesicles, which are associated with a great number of small vesicles. The former are reminiscent of 'p-particles' described for pollen grains of some species of gramineae; these particles are bodies that possess polysaccharide precursors of wall reserves derived from dictyosome activity (Heslop-Harrison and Heslop-Harrison, 1992; Heslop-Harrison and Heslop-Harrison, 1993; Heslop-Harrison *et al.*, 1997).

Due to the presence of a myosinic cover of the p-particles and of actin fibrils in the cytoplasm of the vegetative cell of some species, its function seems to be related to cellular motility based on the interaction between actin and myosin (Heslop-Harrison *et al.*, 1997). The vesicles observed in *M. malvifolium* may have a similar function, related in this case to the movement of the generative cell during pollen-tube germination. An immunocytochemic study of the presence of myosin and actin in these vesicles would be necessary to corroborate its function.

At the free microspore stage, a material with a similar electron density to sporopollenin fills the whole locule and deposits over the pollen grain wall. Similar observations have been recorded in *Jacaranda mimosifolia* (Galati and Strittmatter, 1999), *Passiflora ssp.* (Amela García *et al.*, 2002) and *Oxalis articulata* (Rosenfeldt and Galati, 2005). Such material might be considered to be one of the sporopollenin precursors originated by the tapetum (Heslop-Harrison, 1971).

In *M. malvifolium*, tapetal cells loose their walls and become multinucleate at the tetrad stage, and their intrusion towards the interior of the locule begins at the microspore stage. This sequence has been observed in most of the dicotyledons that have tapeta of the plasmodial type (Pacini *et al.*, 1985). However, in *M. malvifolium* tapetal cells were observed to invade and occupy the entire locule, but they did not fuse and never developed to form a true plasmodium, as each protoplast kept its individuality. This type of tapetum has been described as invasive non-syncytial, or intermediate, and according to Pacini *et al.* (1985) it corresponds to 'type six' of their classification. It has been cited for few families of the Angiosperms: Gentianaceae, Dipsacaceae, Asteraceae, Heliconiaceae (Pacini *et al.*, 1985), Nymphaeaceae (Furness and Rudall, 2001), Cannaceae (Tiwari and Gunning, 1986; Furness and Rudall, 1998), Triuridaceae (Furness *et al.*, 2002), Velloziaceae (Furness and Rudall, 2006) and Brassicaceae (Murgia *et al.*, 1991).

According to Pacini (1990), Pacini and Franchi (1993) and Huysmans *et al.* (1998), one of the main characteristics of secretory tapeta is the production of orbicules. Pacini *et al.* (1985) considered such structures not to



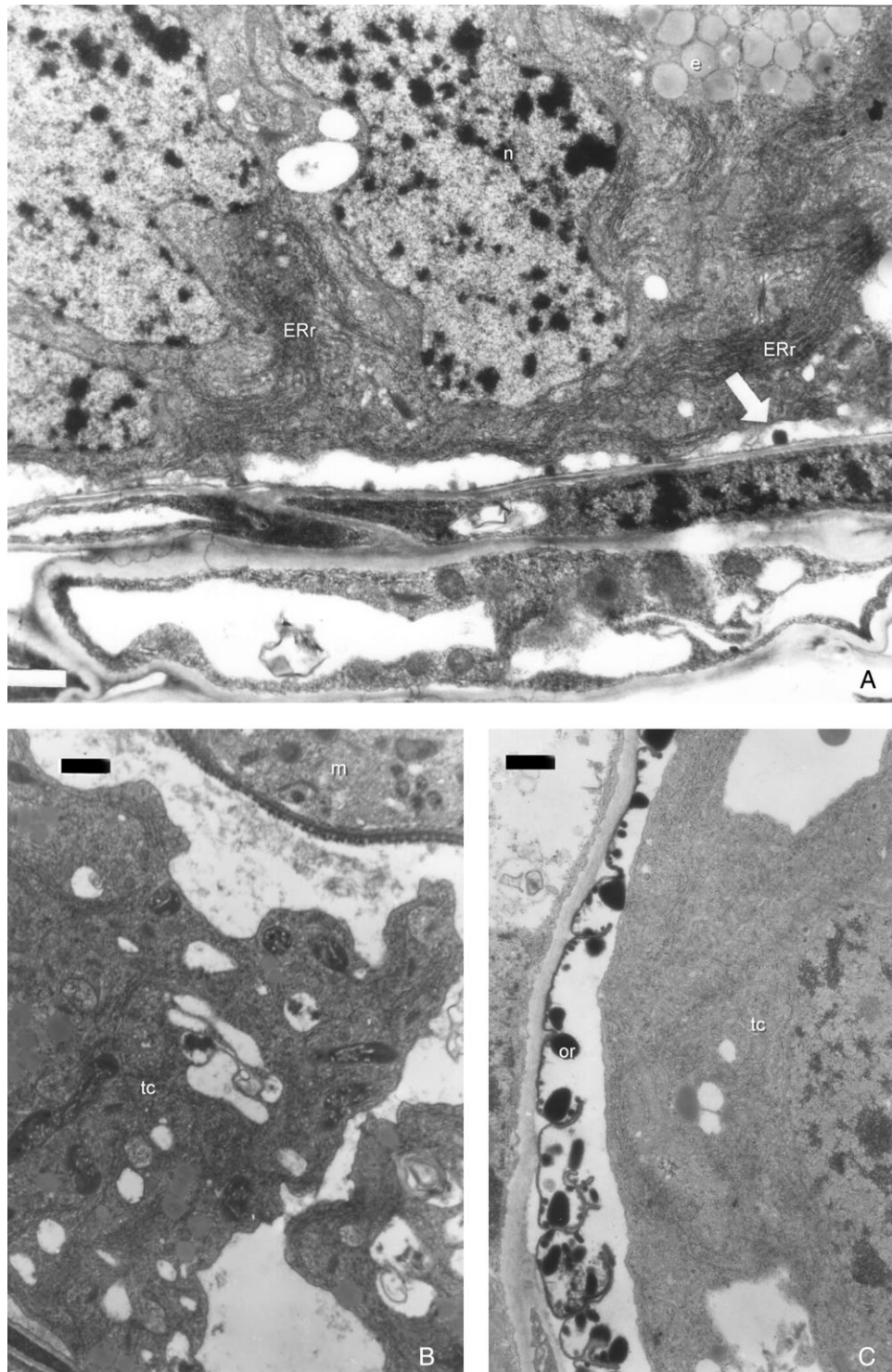


FIG. 5. Observations made under a TEM. (A, B) Free, young microspore stage. (A) Detail of a three-nuclei (n) tapetal cell with elaioplasts (e), abundant ERr and pro-Ubisch (arrow) developing in towards the middle layer, young endothecium. (B) Detail of the radial and tangential inner wall of two tapetal cells (tc) and part of a microspore (m). (C) Microspore with exine wall formed, the peritapetal membrane folded with orbicules (or), and part of a tapetal cell (tc). Scale bars = 1  $\mu$ m.

appear in species characterized by a plasmodial tapetum. However, there are three reports on the presence of corpuscles with electron density and structure similar to orbicules (although not confirmed as such) in plasmodial

tapeta: *Gentiana acaulis* (Lombardo and Carraro, 1976), *Butomus umbellatus* (Fernando and Cass, 1994) and *Tradescantia virginiana* (Tiwari and Gunning, 1986; Raghavan, 1997). These reports do not indicate anything

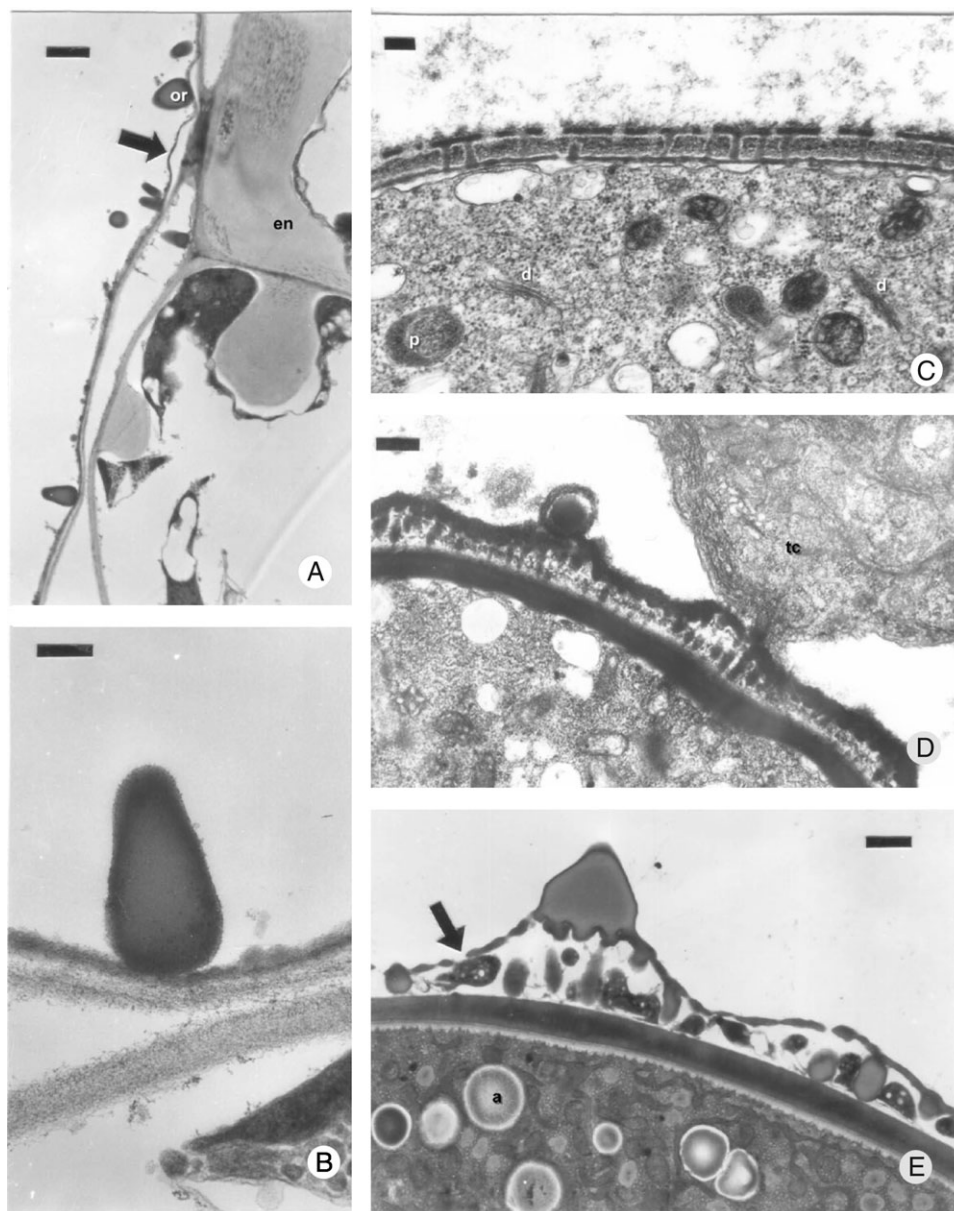


FIG. 6. Observations made under a TEM. (A) Peritapetal membrane (arrow) with orbicules (or), and part of an endothecium cell (en). (B) Detail of an orbicule. (C–E) Sequence of pollen grain wall development. (C) Free, young microspore stage: plastid (p), dictyosomes (d); sporopollenin precursors laying over probacules and protectum. (D) Free microspore stage: exine wall not yet compressed; tc, tapetal cell. (E) Mature pollen grain stage: exine wall compressed and filled with tapetal derivates between columellae (arrow); a, amiloplasts. Scale bars: A, E = 1  $\mu$ m; B, C = 200 nm; D = 500 nm.

about their resistance to acetolysis or about being related to a peritapetal membrane.

Heslop-Harrison (1969) noted that there is a peritapetal membrane resistant to acetolysis in certain Compositae possessing a plasmodial tapetum. He reported that it is formed during exine deposition, but made no reference to the presence of orbicules.

In previous research on *Abutilon pictum* (Malvaceae; Strittmatter *et al.*, 2000), corpuscles attached to a peritapetal membrane that surrounds the plasmodial tapetum have been observed. These corpuscles have the same reaction to

colourants, the same electron density, autofluorescence and resistance to acetolysis as orbicules. This was the first account of the presence of orbicules on a peritapetal membrane (Strittmatter *et al.*, 2000). Although *M. malvifolium* presents an invasive, non-syncytial tapetum, it shows similar characteristics to those cited for *A. pictum*.

Thus, according to the observations made in this study and in the previous report on *A. pictum*, it can be concluded that the presence of orbicules is not a stable criterion to differentiate between a secretory and plasmodial, or invasive, non-syncytial tapetum.



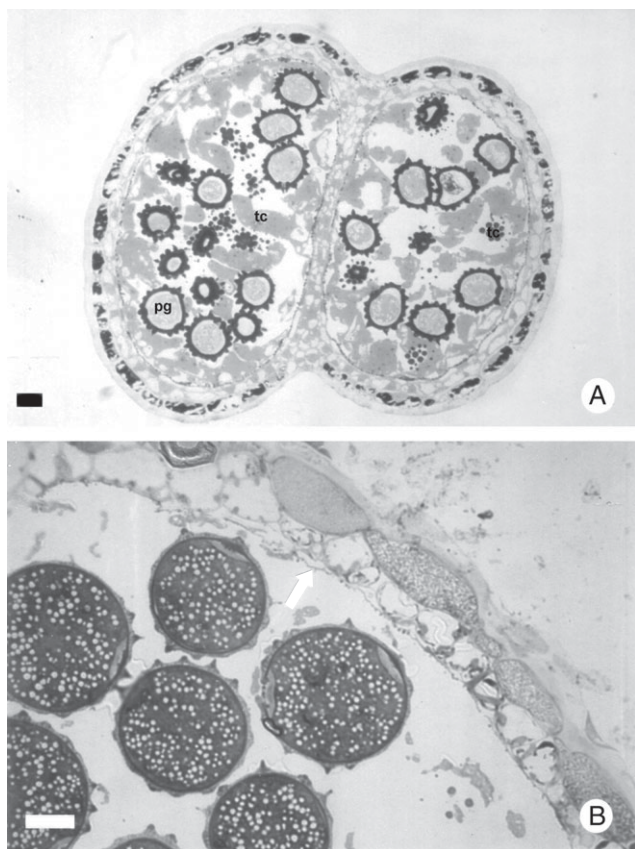


FIG. 7. Anther transversal section observed under a light microscope. (A) Young pollen grain (pg) stage: tapetal cells (tc) invading the locule. (B) Mature pollen grain stage. Remains of the tapetum, anther wall: epidermis, mature endothecium and peritapetal membrane with orbicules (arrow). Scale bars = 20  $\mu$ m.

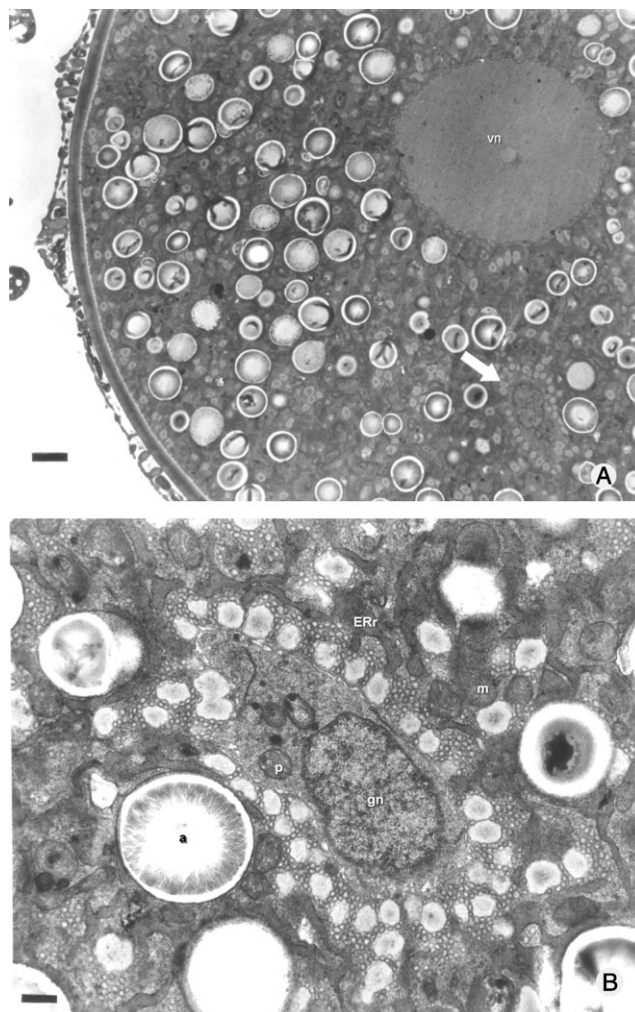


FIG. 8. Mature pollen grain observed under a TEM. (A) General aspect of the pollen grain: generative cell (arrow) surrounded by the vegetative cell; vn, vegetative nucleus. (B) Detail of the generative cell, with a conspicuous nucleus (gn), reduced cytoplasm with some plastids (p), thin, electron-transparent irregular wall. The vegetative cell cytoplasm presents abundant thickened cisterns of the ERr, mitochondria (m) and amiloplast (a). Scale bars: A = 2  $\mu$ m; B = 500 nm.

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