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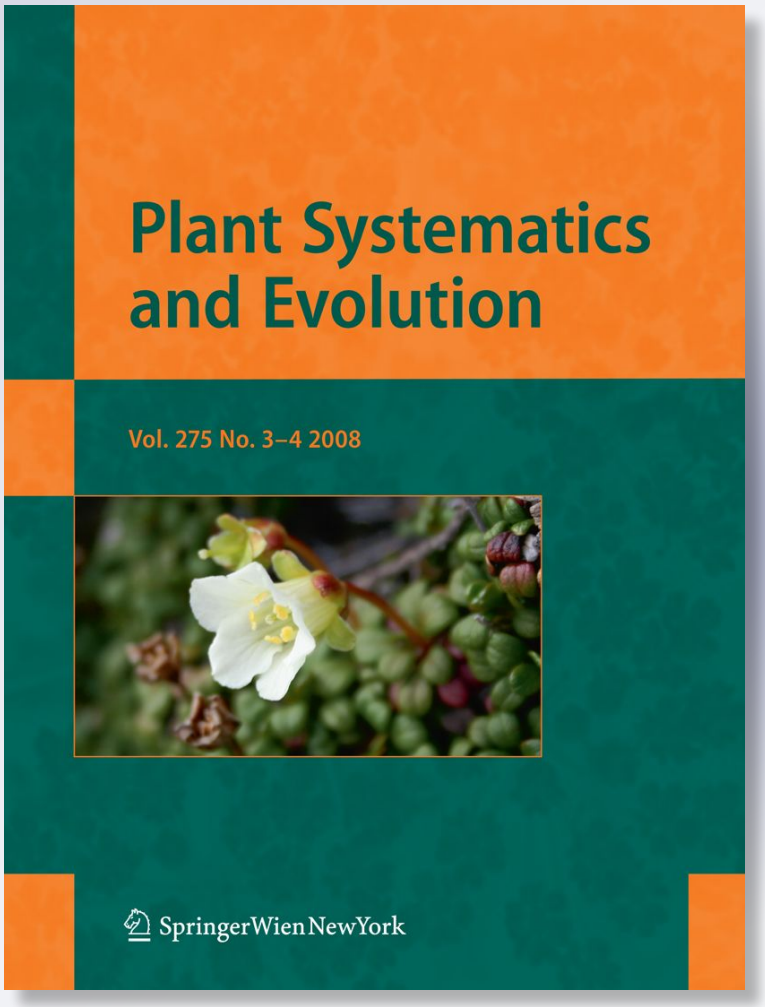

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ORIGINAL ARTICLE

Pollen ontogeny in *Magnolia liliflora* Desr.

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Abstract Pollen ontogeny contributes significantly to the evolutionary analysis and the understanding of the reproductive biology of seed plants. Although much research on basal angiosperms is being carried out there are still many important features about which little is known in these taxa, such as the sporophytic structures related to pollen development and morphology. In this study, pollen development of *Magnolia liliflora* was analyzed by optical microscopy and transmission electron microscopy. The aim of this paper was to supply data that will help characterize basal angiosperms. Microsporogenesis is of the successive type, so that tetrads are decussate or isobilateral. The callosic walls form by the centripetal growth of furrows. The secretory tapetum develops orbicules, which start to form in the microspore tetrad stage. Pollen grains are shed at the bicellular stage. The exine wall has a granular infratectum. Ultrastructural changes observed in the cytoplasm of microspores and tapetal cells are related to the development of the pollen grain wall and orbicules. Centrifugal cell plates are more usual for the successive type of microsporogenesis. The presence of the successive type of microsporogenesis with callosic walls formed by the centripetal growth of furrows could reflect the fact that the successive type in Magnoliaceae is derived from the simultaneous type. The granular infratectum of the ectexine and the presence of orbicules could indicate that this species is one of the most evolved of the genus.

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Introduction

The magnoliid complex comprises most of those lineages typically referred to as "primitive angiosperms" in earlier works (Cronquist 1981, 1988; Takhtajan 1991). This archaic group provides clues to the understanding of the phylogenetic roots of angiosperms. Therefore, some investigators have found the pollen morphology of some integrants of this group of great interest (Xu et al. 2004; Kong 1999; Hesse 2001; Remizowa et al. 2008). In spite of this, there are few studies about pollen ontogeny in basal angiosperms (Gabarayeva 1986, 1991, 1992, 1993, 1995; Gabarayeva and El-Ghazaly 1997; Zavada 1984). Some pollen wall characteristics, such as the absence of an endexine (Lugardon and Le Thomas 1974; Doyle et al. 1975; Walker 1976; Doyle 1977; Le Thomas 1980, 1981; Gabarayeva 1995), the presence of a baculate ectexine (Xu et al. 2004) and the tapetum type in primitive angiosperms (Rowley et al. 1992; Ru-Wen and Zeng-Fang 1995; Furness and Rudall 2001), are discussed in several papers. These facts suggest the importance of studying pollen morphology and ontogeny in other species of this group in order to elucidate angiosperms origin.

Materials and methods

Anthers of *Magnolia liliflora* in different stages of development were collected in the Botanical Garden of the Facultad de Agronomía, Buenos Aires, Argentina. Material was prefixed in phosphate buffer (pH 7.2) containing

1% glutaraldehyde and 4% formaldehyde for 2 h and postfixed in 1.5% OsO_4 at 2°C in the same buffer for 3 h. Then the material was dehydrated in an ascending acetone series and embedded in Spurr's resin. Orbicules and pollen walls were isolated by acetolysis of whole anthers. The acetolysis was carried out following Erdtman's method (Erdtman 1960). Acetolysis-resistant structures were included in agar-agar, dehydrated in an ascending acetone series and embedded in Spurr's resin. Fine sections of both resin-embedded materials were prepared on a Reichert-Jung ultramicrotome, stained with uranyl acetate and lead citrate (O'Brien and McCully 1981), viewed and photographed in a JEOL 100c and a Philips EM 301 transmission electron microscope (TEM). For light microscopy, 1.5-mm thick sections of resin-embedded tissue were prepared and stained with toluidine blue. Callosic walls have been studied using aniline blue at a low concentration (0.1%) which imparts a yellow fluorescence to this material (O'Brien and McCully 1981) when viewed in a fluorescens microscope.

Results

Four stages of pollen development were identified in *M. liliflora*.

Stage 1: Microspore mother cells

The anther is tetrasporangiate and its wall consists of epidermis, endothecium, three middle layers and a secretory type tapetum.

The microspore mother cells are uninucleate with quite a dense cytoplasm and a few small vacuoles. Abundant endoplasmic reticulum of rough type (ERr), free ribosomes and mitochondria are present (Fig. 1a). The callose wall, which is deposited between the plasmalemma and the primary wall, can be clearly seen in more advanced stages.

The cytoplasm of tapetal cells has similar ultrastructural features to the those described for microspore mother cells. Many free ribosomes can be identified, along with endoplasmic reticulum of rough type, mitochondria with a well-developed cresta and dictyosomes (Fig. 1b).

Stage 2: Tetrad formation

The middle lamella of the archesporic tissue cells has disintegrated but the primary walls are still present. The last ones are more electron-dense than the callose wall (Fig. 2a).

Cytokinesis is successive resulting in isobilateral and decussate microspore tetrads (Fig. 3b, c). The callosic walls form by the centripetal growth of furrows (Fig. 3a).

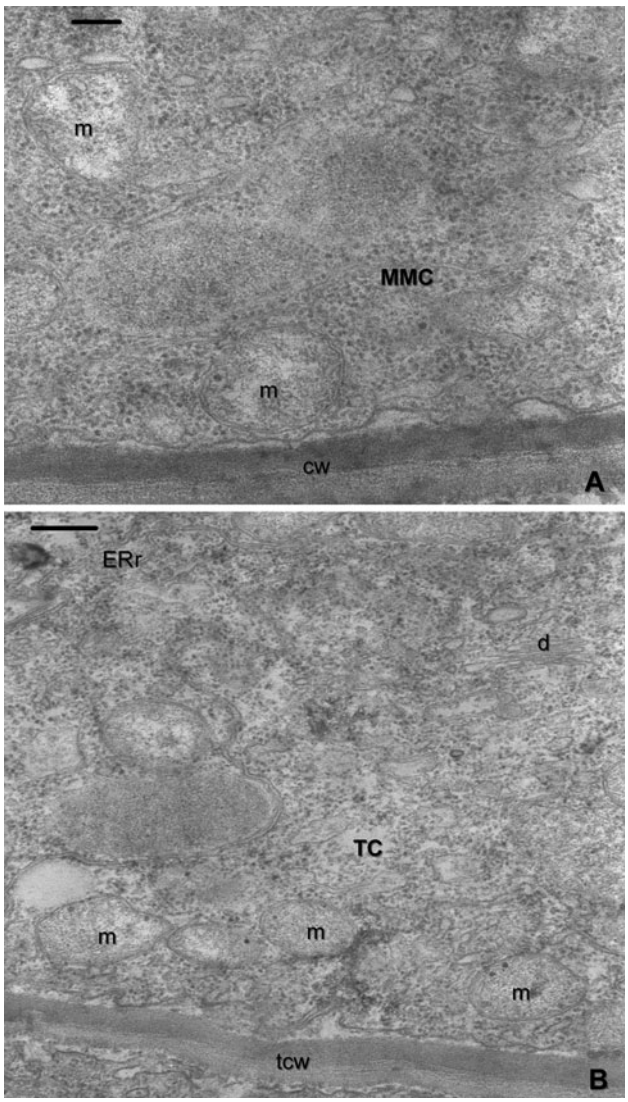

Fig. 1 TEM images of the microspore mother cell stage. **a** Detail of a microspore mother cell. **b** Detail of a tapetal cell (*cw* cell wall of microspore mother cell, *d* dictyosome, *ERr* endoplasmic reticulum of rough type, *m* mitochondrion, *MMC* microspore mother cell, *TC* tapetal cell, *tcw* tapetal cell wall). *Scale bars* **a** 0.2 μm, **b** 0.5 μm)

A primexine fibrillar matrix is observed between the callosic wall and the plasmalemma of the tetrad microspores (Fig. 2a). On it, the protectum can be seen as a more electron-dense layer (Fig. 2b).

The microspore cytoplasm is highly active with many mitochondria, ERr, dictyosomes, protoplastids and small lipid globules (Fig. 2a, b).

Tapetal cell walls are still present but with a conserved lax structure as in the previous stage. Some electron-dense spherical vesicles (pro-orbicules) are released into the space between the plasmalemma and the tapetal cell wall (Fig. 2c). The mitochondria are very evident; they are more numerous and have a well-developed cresta. The tapetal

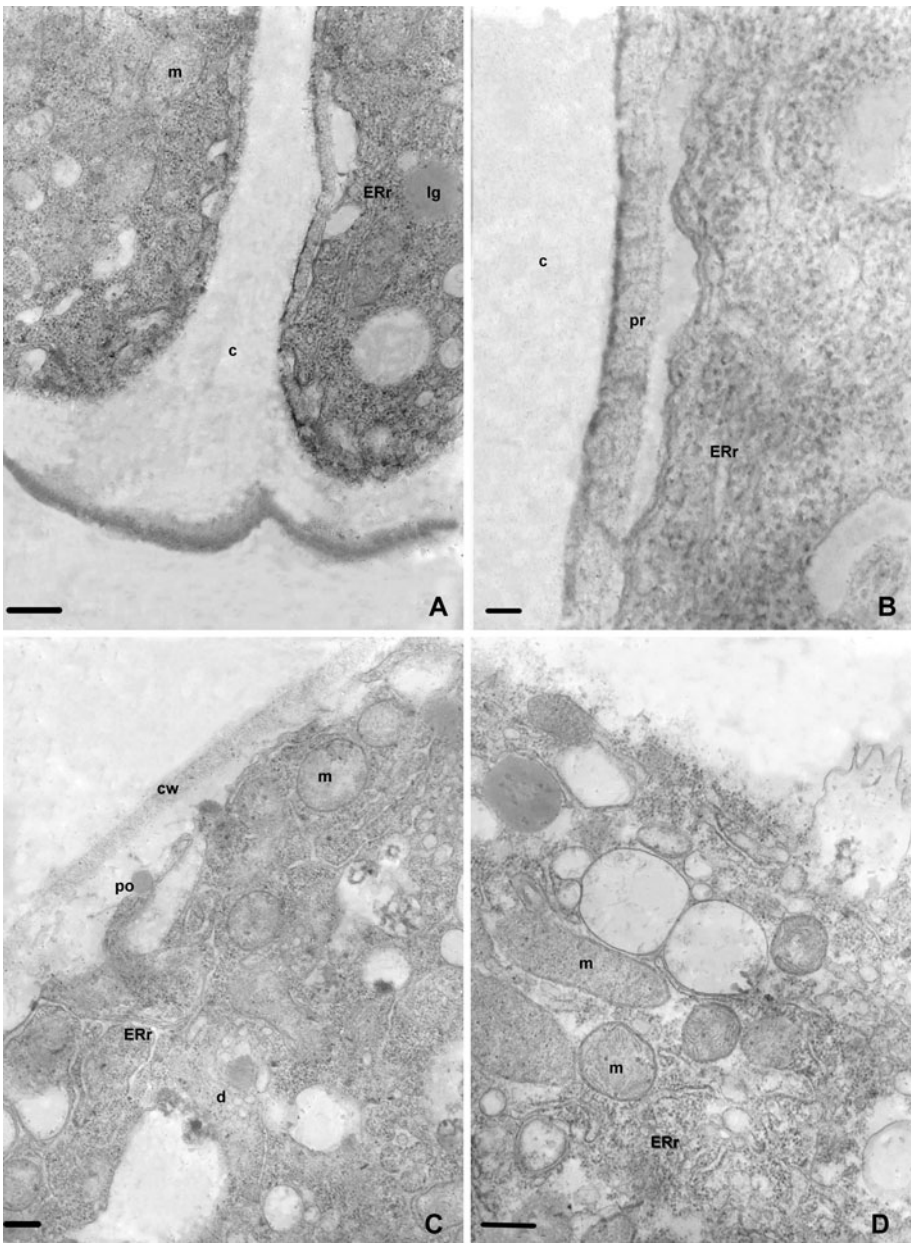

Fig. 2 TEM images of the tetrad stage. **a** Detail of two microspores of the tetrad. **b** Detail of the primexine with the protectum more electron-dense. **c**, **d** Detail of the tapetal cell cytoplasm (*c* callose, *cw* cell wall, *d* dictyosome, *ERr* endoplasmic reticulum of rough type, *lg* lipidic globule, *m* mitochondrion, *po* pro-orbicule, *pr* primexine). *Scale bars* 0.5 μm)

cell cytoplasm shows a great amount of ERr, free ribosomes and plastids (Fig. 2c, d).

Stage 3: Free microspores

The anther wall shows the same layers as in the previous stages: epidermis, endothecium, three middle layers and a secretory type tapetum (Fig. 3d).

Once the callose wall dissolves, microspores are freed (Fig. 3d, e). An electron-dense substance is deposited over the microspore surface forming a thick exine wall (Fig. 4a). Different electron-dense layers can be distinguished: the ectexine, with a thick discontinuous tectum, a granular infratectum and a thinner foot layer; the endexine is still under development. A thick layer (the membranous granular layer, MGL), with moderate electron density and many granular inclusions can be observed between the plasmalemma and the future endexine (Fig. 4a).

Microspores contain numerous mitochondria, abundant ERr, dictyosomes and lipid globules (Fig. 4a, c).

Tapetal cells walls are almost completely degraded and the cytoplasm is dense and shows mitochondria and ERr (Fig. 4a). Orbicules are abundant on the inner tangential surface of the tapetal cells. They have a central core with

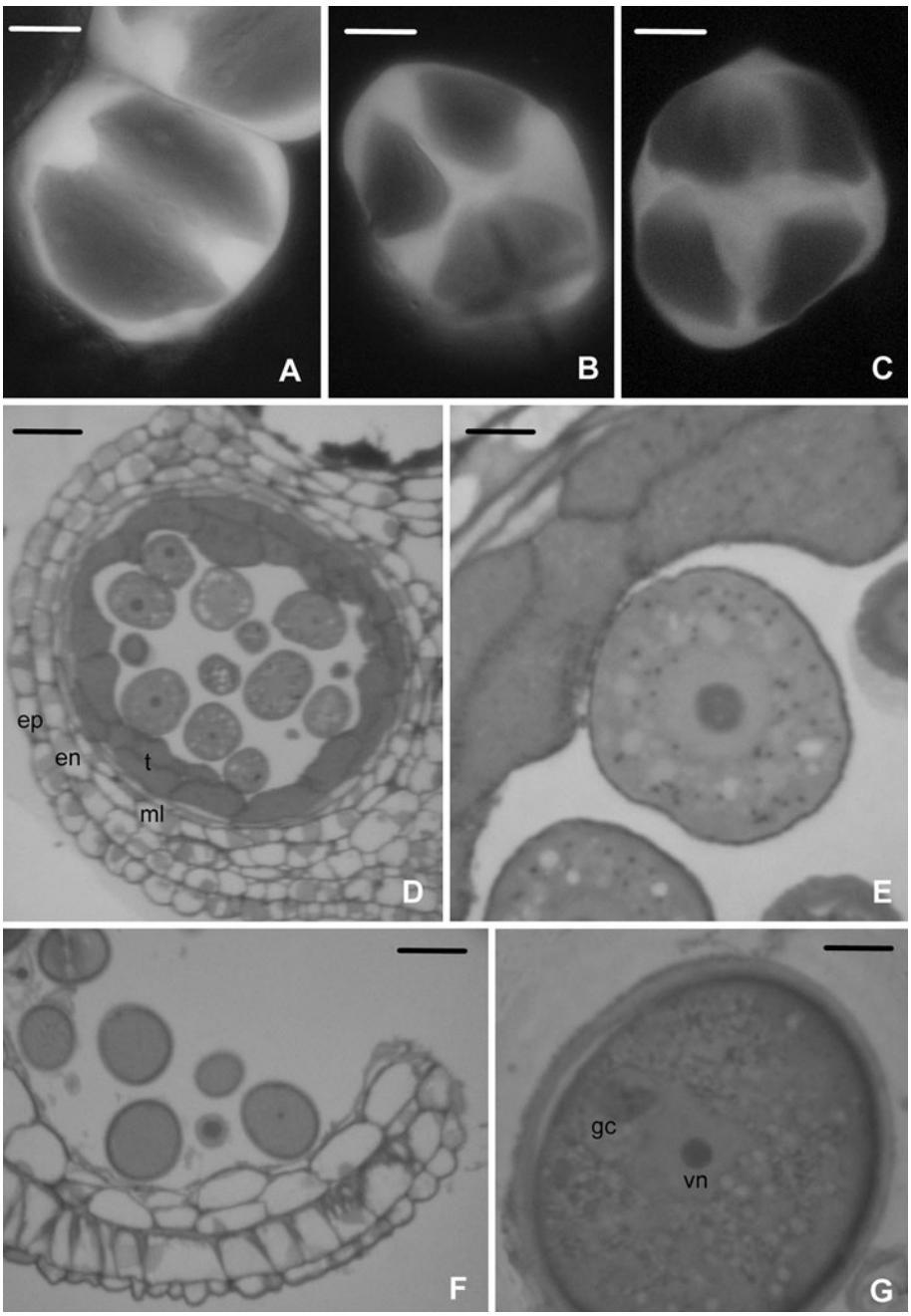

Fig. 3 Microsporogenesis and microgametogenesis. **a–c** Fluorescence microscope images of microspore tetrad formation: **a** centripetal growth of the callosic wall after of the first meiotic division; **b** decussate tetrad; **c** isobilateral tetrad. **d–g** Light microscope images (**d**, **e** free microspore stage; **f**, **g** mature pollen grain stage: **d** detail of an anther locule (*en* endothecium, *ep* epidermis, *ml* middle layers, *t* tapetum); **e** detail of one microspore and tapetal cells; **f** anther wall and pollen grains; **g** detail of pollen grain (*gc* generative cell, *vn* vegetative nuclei). *Scale bars* **a–c**, **e**, **g** 20 μm; **d** 80 μm; **f** 100 μm

moderate electron density and a thin wall with the same electron density as the exine (Fig. 4a).

Stage 4: Mature pollen grain

The endothecium cells develop fibrillar thickenings. One or two the middle layers are observed in this stage and their cells show a higher volume than in the younger stages (Fig. 3f).

The generative cell formed by a mitotic division of the microspore is enclosed by the vegetative cell cytoplasm (Fig. 3g). The generative cell has a very thin wall transparent to the electrons and a reduced cytoplasm with scarce organelles, some ERr, mitochondria and dictyosomes. This cell is surrounded by numerous vesicles with an electron-transparent content. The vegetative cell cytoplasm is dense and shows elaioplasts, lipid globules and mitochondria (Fig. 5a, b).

The pollen wall is fully developed. The endexine is very thin and lamellate. Below it, a thick MGL is very evident. The intine is the innermost pollen wall layer. Two layers can be seen within it. The outer layer is thicker, more electron-dense and has spherical to subspherical inclusions (Fig. 4b). Neither the MGL nor the intine are resistant to acetolysis (Fig. 4c).

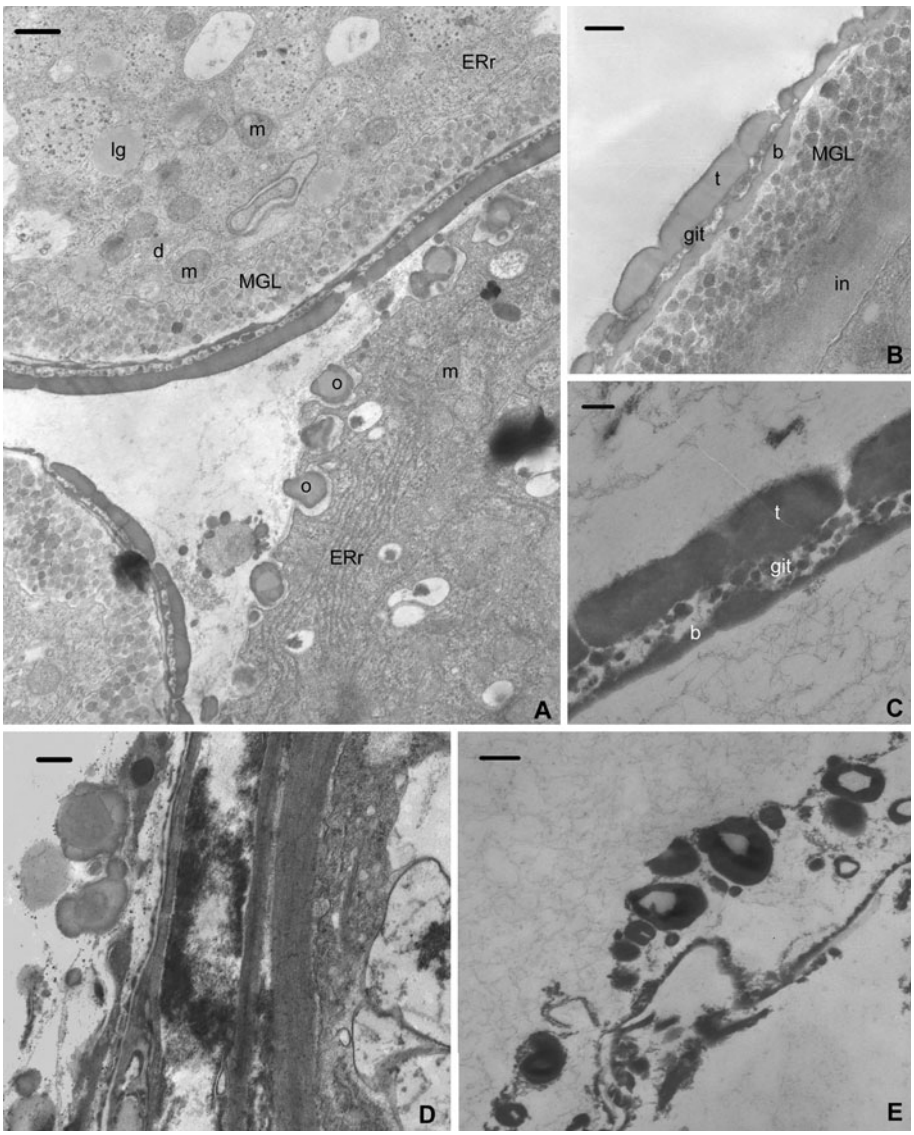

Fig. 4 TEM images of free microspore and mature pollen grain stages. **a** Free microspore stage. Detail of a portion of a tapetal cell with orbicules in exocytosis and a microspore (*d* dictyosome, *ERr* endoplasmic reticulum of rough type, *lg* lipidic globule, *m* mitochondrion, *MGL* membranous granular layer, *o* orbicule). **b** Detail of mature pollen wall (*b* basal layer, *git* granular infratectum, *in* intine, *MGL* membranous granular layer, *t* tectum). **c** Detail of acetolyzed mature pollen wall (*b* basal layer, *git* granular infratectum, *t* tectum). **d** Orbicules in mature anther. **e** Detail of orbicules after acetolysis. *Scale bars* **a** 1 μm; **b**, **d**, **e** 0.5 μm; **c** 0.2 μm

Only rests of tapetal cells cytoplasm can be observed. The orbicules are spherical in shape and show an electron-dense central core and a wall with an irregular surface (Fig. 4d). These structures are resistant to acetolysis, but after this treatment the central core loses its electron density (Fig. 4e).

Discussion

The microsporogenesis in *M. liliflora* is of the successive type and the tetrads are isobilateral and decussate. However, the callosic wall form by the centripetal growth of furrows as is usual in the simultaneous type (Raghavan 1997). There is uncertainty about the type of microsporogenesis in the genus *Magnolia*. According to Kapil and Bhandari (1964) the microsporogenesis in *M. stellata* and *M. obovata* is of the simultaneous type and the cytokinesis takes place by furrowing. Wang et al. (2005) have described the cytokinesis during meiosis of the microspore mother cell in *M. biloba* as a modified simultaneous type. In *M. soulangeana*, Dinis and Mesquita (1993) observed actin filaments at the level of cell division planes constituting cleavage furrows following both meiosis I and II. The successive type of microsporogenesis is common in many basal angiosperms (Galati 1985; Taylor et al. 2008). However, simultaneous cytokinesis is apparently plesiomorphic within the order Piperales, where successive microsporogenesis was found only in species of *Aristolochia* (González et al. 2001). The presence of the successive type of microsporogenesis with callosic walls form by the centripetal growth of furrows could reflect the fact that the successive type in Magnoliaceae is derived from the simultaneous type. This is in accordance with the conclusions of Furness (2008) for the family Berberidaceae.

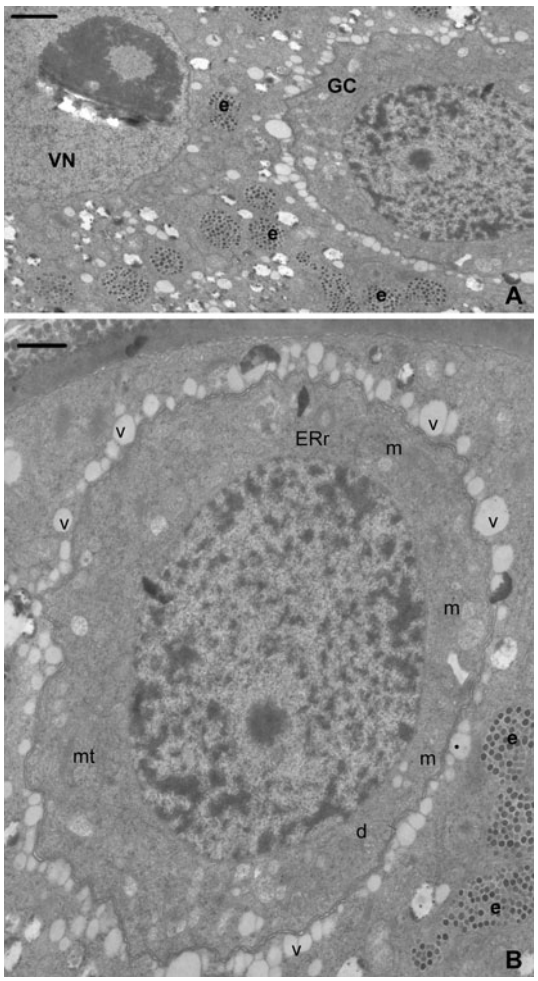

Fig. 5 TEM images of mature pollen grain stage. **a** Detail of vegetative nuclei and generative cell (*e* elaiosome, *GC* generative cell, *VN* vegetative nuclei). **b** Detail of generative cell (*d* dictyosome, *e* elaiosome, *ERr* endoplasmic reticulum of rough type, *m* mitochondrion, *mt* microtubule, *v* vesicle). *Scale bars* **a** 2 μm; **b** 1 μm

The tapetum in *M. liliflora* is of the secretory type. The majority of basal angiosperms share a secretory tapetum with their anthophyte ancestors. The invasive tapetum type has not been reported in Magnoliaceae until now. According to Furness and Rudall (2001), the invasive tapetum has evolved at least four times independently in primitive angiosperms: in Nymphaeaceae, Annonaceae, Winteraceae and Monimiaceae. Gabarayeva and El-Ghazaly (1997) have described the tapetum of *Nymphaea mexicana* as invading the loculus at least twice and then retreating to the initial parietal position. Taylor et al. (2008) consider that the tapetum of *Cabomba caroliniana* is of the plasmodial type. However, the figures illustrate the contrary. We consider that the tapetum type in the Nymphaeaceae family requires new investigations.

Orbicules are present in the *M. liliflora* anthers. They have an electron-dense central core and a wall with an irregular surface. Orbicules formation starts during the tetrad stage and they are more evident in the free microspore stage. In this last stage the orbicules are very conspicuous. The presence of orbicules has been reported in some species of basal angiosperms (Zavada 1984; Rowley et al. 1992; Gabarayeva 1995; Taylor et al. 2008). The function of orbicules is still controversial. There are many possible functions, but none of them has been tested (Huysmans et al. 1998). One of the first functions attributed to the orbicules is the idea that these corpuscles might contribute to the development of the exine (Maheshwari 1950). In the majority of the studied species, the orbicules are attached to a tapetal or peritapetal membrane (Galati 2003; Galati et al. 2007) and they are not observed free in the anther locule. Therefore, this function has been rejected (Galati 2003). According to Galati et al. (2010) there are coincidences between orbicule morphology and pollination syndrome, so they support the theory proposed by Heslop-Harrison (1968a, b), who suggested that orbicules could be related to pollen dispersal by a nonwettable surface from which pollen can be easily freed. According to Pacini and Franchi (1993), since orbicules and pollen exine are composed of sporopollenin, they repel each other. This would allow the expulsion of pollen grains. Galati et al. (2010) add that the different orbicule morphologies would facilitate each pollination mode. In *M. liliflora* the effective pollinator is unknown, but according to its orbicule morphology (spherical to subspherical with an irregular surface) this species could be psichophilous.

The pollen wall of *M. liliflora* has a noncontinuous tectum and a granular infratectum with incipient low collumellae. The majority of the species of *Magnolia* studied have infratectal columellae (Xu et al. 2004). Only *M. denudata* has spherical granules mixed up with the small intraexinous spaces of several incipient columellae, sometimes in columella. This species belongs to the same subgenus (Yulania) as *M. liliflora,* but a different section. However, molecular phylogeny places the two species in the same node (Azuma et al. 1999). According to Xu et al. (2004), the development of columella supports the idea of considering the pollen of *Magnolia* as primitive. Phylogenetic analyses indicate that the granular structure was derived from a columellar structure within Magnoliales and Laurales, which is an important synapomorphy of a major subgroup (Doyle 2009). Therefore, according to the ultrastructure of the exine, *M. liliflora* could be a more evolved species of the genus.

A thin lamellate endexine is seen under the basal layer in *M. liliflora*. The hypothesis that the endexine is absent in primitive angiosperms is dominant. The absence of this layer in some species of this group has been reported by several authors (Lugardon and Le Thomas 1974; Doyle et al. 1975; Walker 1976; Doyle 1977; Le Thomas 1980,

1981; Gabarayeva 1995). However, detailed ontogenetic studies of the pollen of species of Magnoliaceae and Annonaceae have shown the presence of tangential lamellae (Gabarayeva 1986, 1991, 1992, 1993, 1995). According to Gabarayeva (1995), we can trace the evolutionary continuity of the endexine from fossil extant gymnosperms and primitive angiosperms to advanced angiosperms. Gabarayeva considers that the ectexine and the endexine in gymnosperms are homologous with the ectexine and the endexine in angiosperms.

Magnolia liliflora has a thick MGL and a thin intine. The presence of a MGL has been reported in *M. grandiflora* by El-Ghazaly and Huysmans (2001). According to these authors this layer is granular and shows a clearly distinct development, both in timing and mode of formation, from the endexine as well as the intine, and its composition is also characteristic because MGL is resistant to acetolysis. Other authors interpret this layer as part of the intine (Gabarayeva 1986, 1991; Xu and Kirchoff 2008). In *M. liliflora*, the MGL is not resistant to acetolysis, so we consider this layer as part of the intine in agreement with the latter authors.

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