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Anther structure and pollen development in *Melicoccus lepidopetalus* (Sapindaceae): An evolutionary approach to dioecy in the family

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ABSTRACT

Anther and pollen development in staminate and pistillate flowers of dioecious *Melicoccus lepidopetalus* (Sapindaceae) were examined by light and electron microscopy. Young anthers are similar in both types of flowers; they consist of epidermis, endothecium, two to four middle layers and a secretory tapetum. The microspore tetrads are tetrahedral. The mature anther in staminate flowers presents compressed epidermal cells and endothecium cells with fibrillar thickenings. A single locule is formed in the theca by dissolution of the septum and pollen grains are shed at two-celled stage. The mature anthers of pistillate flowers differ anatomically from those of staminate flowers. The epidermis is not compressed, the endothecium does not develop fibrillar thickenings, middle layers and tapetum are generally persisting, and the stomium is nonfunctional. Microspore degeneration begins after meiosis of microspore mother cells. At anthesis, uninucleate microspores and pollen grains with vegetative and generative nuclei with no cytokinesis are observed. Some pollen walls display an abnormal exine deposition, whereas others show a well formed exine, although both are devoid of intine. These results suggest that in the evolution towards unisexuality, the developmental differences of anther wall tissues and pollen grains between pistillate and staminate flowers might become more pronounced in a derived condition, such as dioecy.

Introduction

The family Sapindaceae s.str. comprises about 140 genera and 1800 species (Ferrucci, pers. comm.), mainly distributed in tropical and subtropical regions. The species possess climber, tree or shrub habit, and are mostly monoecious, rarely dioecious or polygamous. Radlkofer (1931–1934) reported dioecy in 28 genera from 10 tribes of the family. Both genera of the American tribe *Melicocceae*, *Melicoccus* P. Br. (Radlkofer, 1931–1934) and *Talisia* Aubl. (Ferrucci, 1991), present dioecy. The genus *Melicoccus* consists of 10 tree species, distributed throughout Central and South America, and only *M. lepidopetalus* Radlk. occurs naturally in a larger area comprising parts of Argentina, Bolivia (Santa Cruz), Brazil (Mato Grosso do Sul) and Paraguay (Ferrucci, 1991; Acevedo-Rodríguez, 2003).

Although Sapindaceae is a diverse family with a wide distribution, works on general embryological descriptions have focused on a limited number of species. Most of the available works include Asian species of *Filicium* Thwaites ex Benth. & Hook. f. (Gulati and Mathur, 1977), *Allophylus* L. (Mathur and Gulati, 1980, 1989), *Lepidopetalum* Bl. (Mathur and Gulati, 1981), *Xerospermum* Bl., *Nephelium* L., *Pometia* J.R. Forst. & G. Forst. (Ha et al., 1988), as well as *Cardiospermum halicacabum* L., which is widely distributed (Kadry, 1946; Nair and Joseph, 1960). Moreover, in all these studies, light microscopy was the only technique used to examine anther structure and male gametophyte development.

Sterility mutations that give rise to specialized unisexual flowers are the starting point for the evolution of separate sexes from combined sexes in flowers (Bawa, 1980; Freeman et al., 1997). A common feature in both monoecious and dioecious species of Sapindaceae is the presence of staminate flowers with rudimentary gynoecium and perfect flowers that are functionally pistillate, with nonfunctional stamens, although also perfect flowers exceptionally occur in *Dodonaea* Mill. (Ferrucci, 2005). Thus, these flower morphs are unisexual by abortion of the nonfunctional reproductive organs of the opposite sex, which are recognized in the literature as type I (Ainsworth, 2000; Mitchell and Diggle, 2005), as opposed to unisexual flowers from inception, referred to as type II. Although the stamens of pistillate flowers appear to be always indehiscent, interestingly the mechanisms that could lead to androecial termination development are diverse and may occur at different stages (Ainsworth, 2000), representing several distinct developmental transitions in the evolution from perfect to unisexual flowers (Mitchell and Diggle, 2005). Anatomical examinations in these types of flowers are scarce in the family. However, a recent paper has revealed ultrastructural details on anther development,

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and identified cytological events in the tapetum layer, related to male sterility in functionally pistillate flowers of *Cardiospermum grandiflorum* Sw. and *Urvillea chacoensis* Hunz., both monoecious species with climbing habit (Solís et al., 2010).

Melicoccus lepidopetalus has been studied in terms of taxonomy and pollen morphology, and it has been included in a phylogenetic analysis of Sapindaceae (Muller and Leenhouts, 1976; Acevedo-Rodríguez, 2003; Buerki et al., 2009). However, anther and pollen development in this genus has not been investigated.

The aim of this paper was to provide a comparative description of pollen and anther development in the two flower morphs of *Melicoccus lepidopetalus*, using light and transmission electron microscopy. Features accompanying development in anthers and pollen of pistillate flowers were compared with those of previously studied species to identify possible differences in general mechanisms involved in preventing the production of functional pollen grains.

Material and methods

Light microscopy

Flowers of both types at different developmental stages were fixed in formalin–alcohol–acetic acid 1:1:3 (FAA). Voucher specimens were deposited at the Instituto de Botánica del Nordeste herbarium (CTES), Argentina.

Transverse serial sections of floral buds and flowers at anthesis were made by conventional methods. The material was dehydrated in a series of graded ethanol solutions with a rinsing pre-impregnant of tertiary butyl alcohol (González and Cristóbal, 1997). For infiltration in paraffin, the technique of Johansen (1940) was applied, and the material was later embedded in 'Histoplast®' (Fisher Scientific, Hampton, USA). Sections (12 μm thick) were cut with a rotary microtome, stained with Astra blue-safranin (Luque et al., 1996), and mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). Slides were examined using a Leica DM LB2 (Leica, Wetzlar, Germany) binocular microscope fitted with a digital camera.

Scanning electron microscopy (SEM)

Stamens for scanning electron microscopy were dehydrated by transfer through an acetone series and critical-point dried; the specimens were then sputter coated with gold-palladium. SEM micrographs were obtained with a scanning electron microscope JEOL 5800 LV at 20 kV (JEOL USA, Peabody, MA, USA).

Transmission electron microscopy (TEM)

For transmission electron microscopy examination, fresh anthers at different developmental stages were fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in 1.5% OsO_4 at 2 °C in the same buffer for 3 h. The material was dehydrated in an ascending acetone series and embedded in Spurr resin. Ultrathin sections (750–900 nm) were made on a Sorval ultramicrotome and stained with uranyl acetate and lead citrate (OïBrien and McCully, 1981). The sections were examined with a JEOL 100C TEM (JOEL USA, Inc., Peabody, MA).

Material examined

Melicoccus lepidopetalus Radlk.: ARGENTINA. Province of Corrientes: Capital. Corrientes, 8-2008, *Zini & Ferrucci 1*; *Idem*, *Zini & Ferrucci 2*. Province of Corrientes. Departament Capital, 9-1978. *Martínez Crovetto 11313*. Province of Chaco. Departament 1° de Mayo, Colonia Benítez, 10-1951, *Schulz 8119*. Province of Corrientes. Departament Capital, 10-1983. *Ferrucci 196*.

Results

Floral morphology

Flowers of *M. lepidopetalus* are actinomorphic, 5 mm in length; staminate flowers have eight stamens, exserted, 4 mm in length, with dehiscent anthers (Fig. 1A), and a gynoecium reduced to a pistillode, whereas pistillate flowers have smaller stamens, 2 mm in length, with indehiscent anthers (Fig. 1B), and a fully developed pistil.

Anther development

Anthers of both staminate and pistillate flowers are bithecal and tetrasporangiate. At anthesis, SEM observations show that the epidermal cells of staminate flower anthers are dehydrated and show a cuticle slightly striated, whereas in pistillate flowers, those cells are turgescent and smooth (Fig. 1C and D). Unlike in staminate flowers, pollen grains of pistillate flowers are dehydrated (Fig. 1E and F). Both types of flowers share the anatomy of the filament; in transverse sections, two or three layers of subepidermal tanniferous cells are observed, and a single periphloematic vascular bundle supplies each stamen.

Archesporial isodiametric cells differentiate in young anthers, dividing periclinally to form outer primary parietal cells and inner sporogenous cells. The latter undergo periclinal divisions to form secondary sporogenous cells, which finally differentiate into microspore mother cells. Primary parietal cells undergo periclinal divisions, resulting in two layers of secondary parietal cells. The outer one divides periclinally, forming two cell layers, namely the endothecium and the upper middle layer, whereas the inner one forms the lower middle layer and the tapetum. The two middle layers may further divide periclinally.

At microspore mother cells stage the anther wall consists of an epidermis, an endothecium with thin-walled cells and conspicuous nuclei, two-four middle layers and a secretory tapetum.

For a clear interpretation, the results are organized by ontogenetic stages. Four stages were identified: microspore mother cell, tetrad, free microspore and mature pollen grain stages.

Microspore mother cell stage

Staminate flowers

Sporogenous tissue differentiates into microspore mother cells (MMC), which are surrounded by callose deposited between the plasmalemma and the MMC wall (Fig. 2A). The cytoplasm of the MMC presents numerous mitochondria, rough endoplasmic reticulum (ERr) with expanded cisternae, free ribosomes and dictyosomes (Fig. 3A and B).

The tapetum is well differentiated and can be clearly distinguished from the other anther wall layers by the denser cytoplasm of its cells (Fig. 2A) and the abundant mitochondria, ERr and dictyosomes with numerous vesicles (Fig. 3C). At this development stage, most tapetal cells suffer nuclear divisions, giving rise to two-nucleate cells. Cytoplasmic connections between these cells were not observed.

Pistillate flowers

Microspore mother cells contain a conspicuous nucleus (Fig. 4A); the cytoplasm of these cells has some mitochondria and abundant free ribosomes (Fig. 4B). The tapetum cells present a similar ultrastructure but with less ERr than staminate flowers (Fig. 4B).

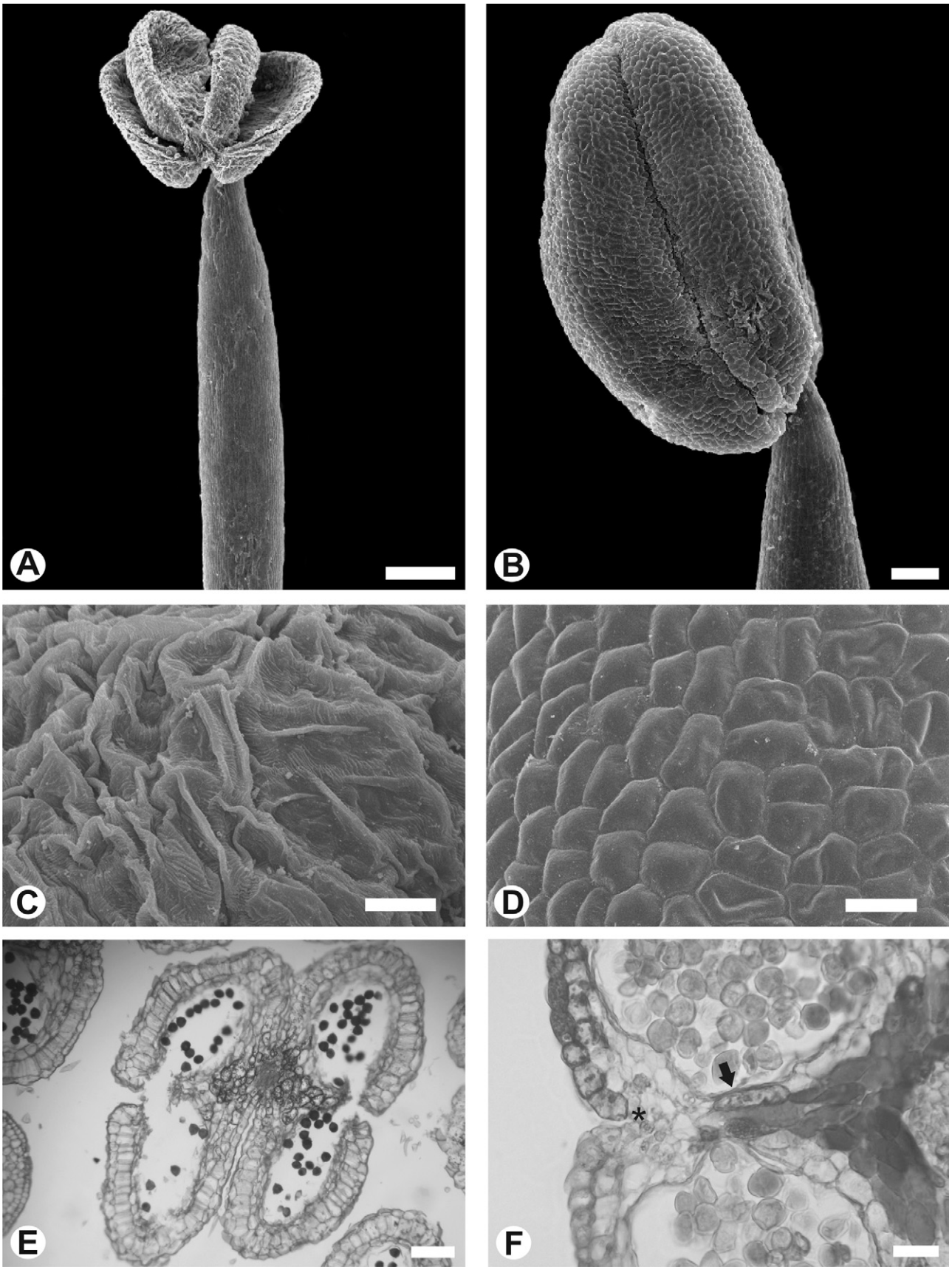

Fig. 1. Comparison of the mature anther between both flower morphs in *M. lepidopetalus*. (A–D) SEM micrographs. (A) Dehiscent anther of staminate flower, (B) indehiscent anther of pistillate flower. (C) Dehydrated anther epidermis of staminate flower. (D) Anther epidermis of pistillate flower. (E–F) Light micrographs of transversal sections of mature anthers. (E) Detail of staminate flower anther. (F) Detail of pistillate flower anther, with unbroken septum (arrow) and nonfunctional stomium (asterisk). Scale bars: A, 300 μm; B, 100 μm; C–D, 20 μm; E, 150 μm; F, 25 μm.

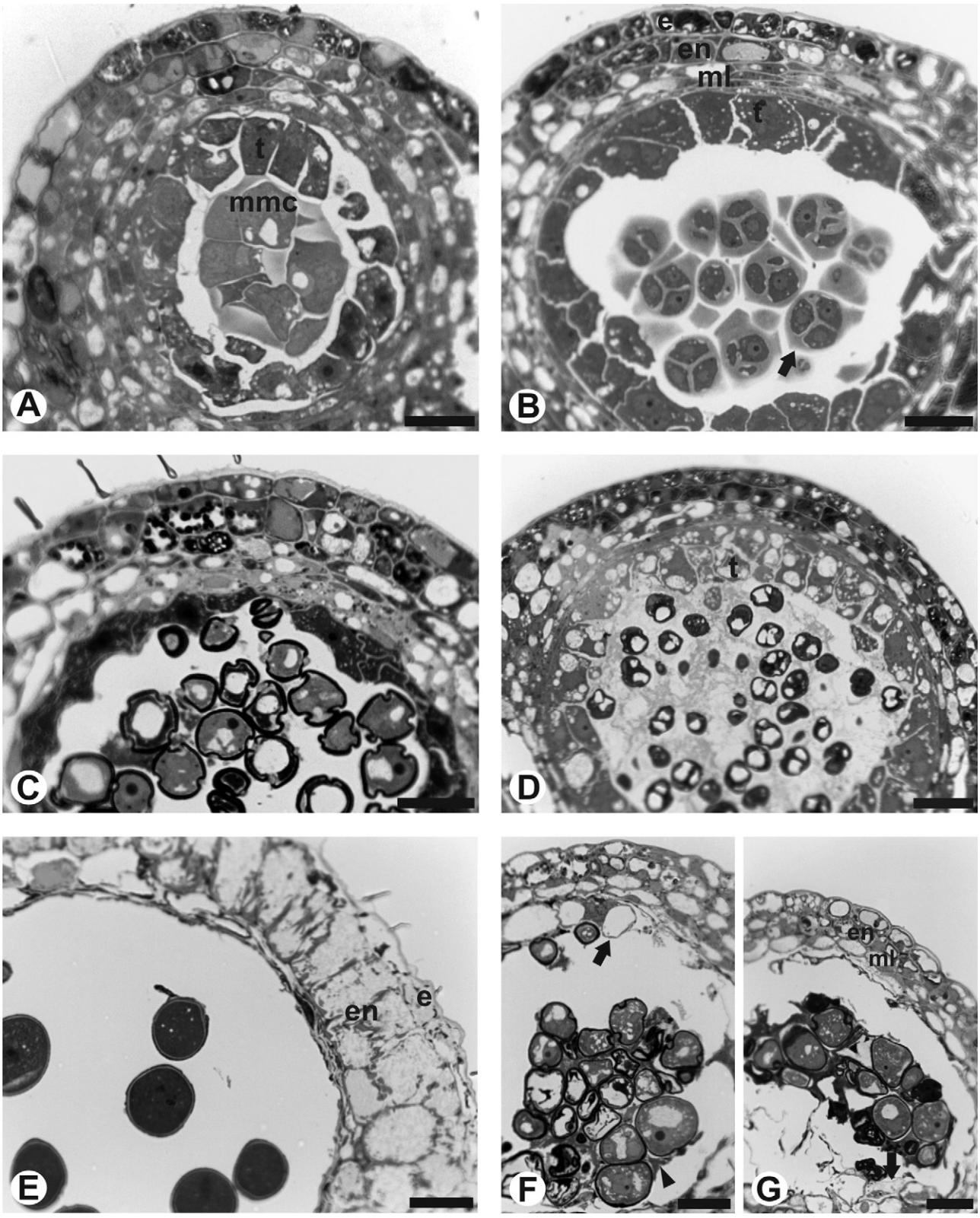

Fig. 2. Light micrographs at different developmental stages of anthers in *M. lepidopetalus*. (A) Cross section in pistillate flower anthers showing microspore mother cells surrounded by a thick callose layer and a well differentiated tapetum. (B) Tetrads of staminate flower anthers with tetrahedral configuration and surrounding callose walls (arrow), and detail of anther wall layers: epidermis, endothecium cells, middle layers and tapetum. (C) Free microspore stage in staminate flowers showing tapetum degeneration and vacuolated microspores. (D) Young free microspores of pistillate flowers, with tapetum highly vacuolated. (E) Detail of mature anther locule of a staminate flower showing epidermis, endothecium with secondary wall thickenings, and bicellular pollen grains. (F–G) Mature anther locule of a pistillate flower showing aborted microspores and cytoplasmic debris released from dead tapetum (arrowhead), also collapsed tapetal cells, underdeveloped endothecium, middle layers, and tapetal cells (arrows) can be distinguished. Scale bars (A–G) 25 μm. Abbreviations: e: epidermis, en: endothecium, ml: middle layers, mmc: microspore mother cell, t: tapetum.

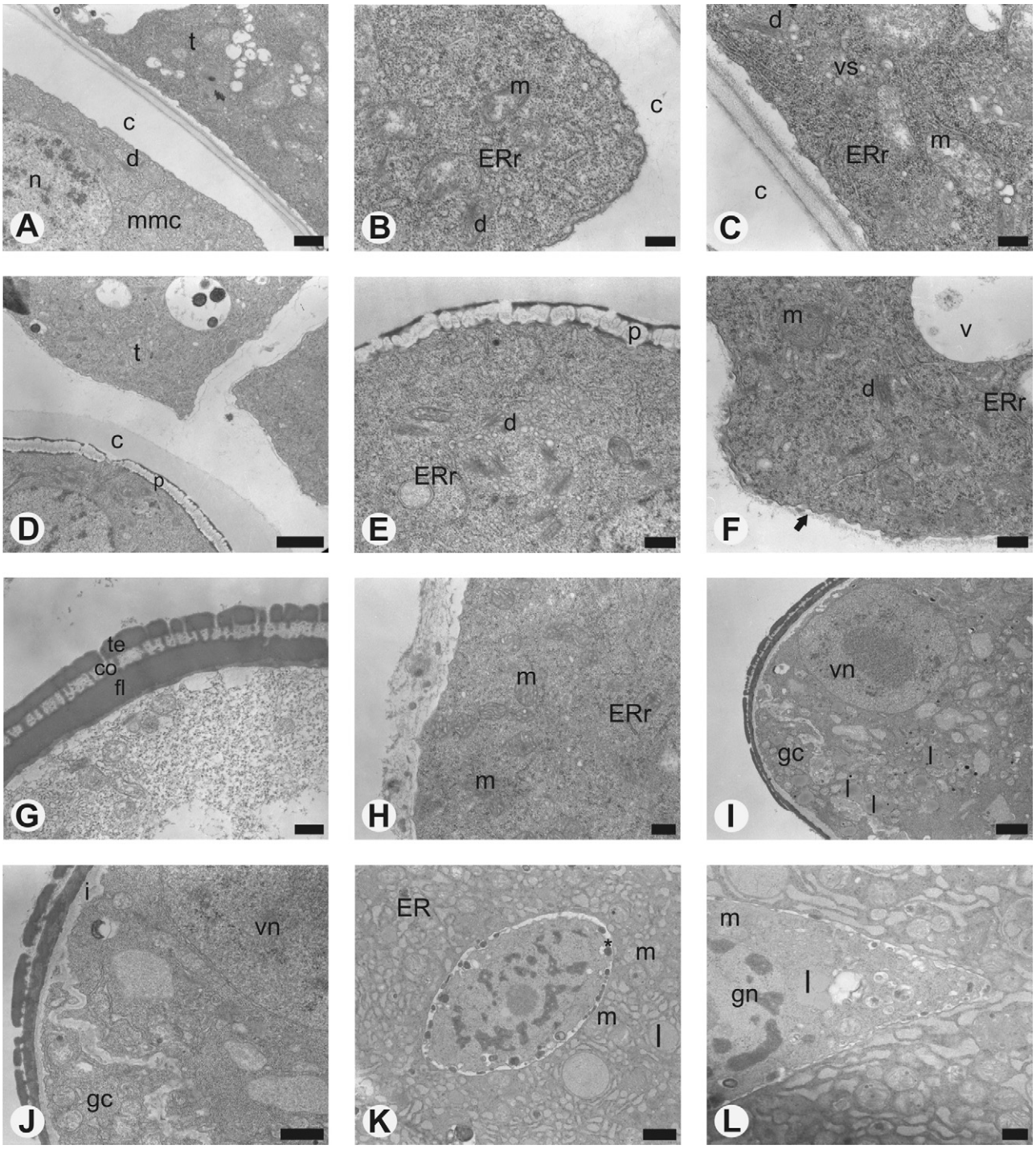

Fig. 3. Transmission electron micrographs at different developmental stages of tapetum and pollen grains in staminate flowers. (A) Microspore mother cell stage showing portions of tapetal cells and microspore mother cells. (B) Detail of a microspore mother cell similar in structure to the tapetum and surrounded by a thick callose wall. (C) Detail of a tapetal cell containing abundant endoplasmic reticulum of rough type, mitochondria and ribosomes. (D) Tetrad stage showing a portion of a microspore of the tetrad and tapetum. (E) Detail of the microspore wall at tetrad stage; the primexine has thickened below the callose and electron-dense procolumellae and protectum have formed. The cytoplasm shows numerous dictyosomes and endoplasmic reticulum. (F) Portion of tapetal cell, the lax structure of the cell wall and slightly undulated plasmalemma are evident (arrow), the cytoplasm contains several vacuoles. (G) Detail of a free microspore showing the complete ectexine wall layers: a discontinuous tectum, columellae, and a foot layer. (H) Detail of tapetal cell showing the cell wall with a lax structure. Note that the tapetal cytoplasm remains dense and with numerous mitochondria. (I) Mature pollen grain containing lipid globules. (J) Detail of pollen wall with well formed intine, the cytoplasm with generative cell in parietal position. (K) The cytoplasm of the vegetative cell contains abundant endoplasmic reticulum with dilated cisternae. The generative cell shows electron-dense corpuscles included in the cell wall (asterisk). (L) Detail of a generative cell showing cytoplasm with lipid globules and mitochondria. Scale bars: D, I 2 μm, (A, G, J, K) 1 μm, (E, F, H, L) 500 nm, and B, C 400 nm. Abbreviations: c: callose, co: columellae, d: dictyosome, ERr: endoplasmic reticulum of rough type, fl: foot layer, gc: generative cell, i: intine, l: lipid globules, mmc: microspore mother cell, m: mitochondria, n: nucleus, p: primexine, r: ribosomes, t: tapetum, te: tectum, v: vacuole, vs: vesicles.

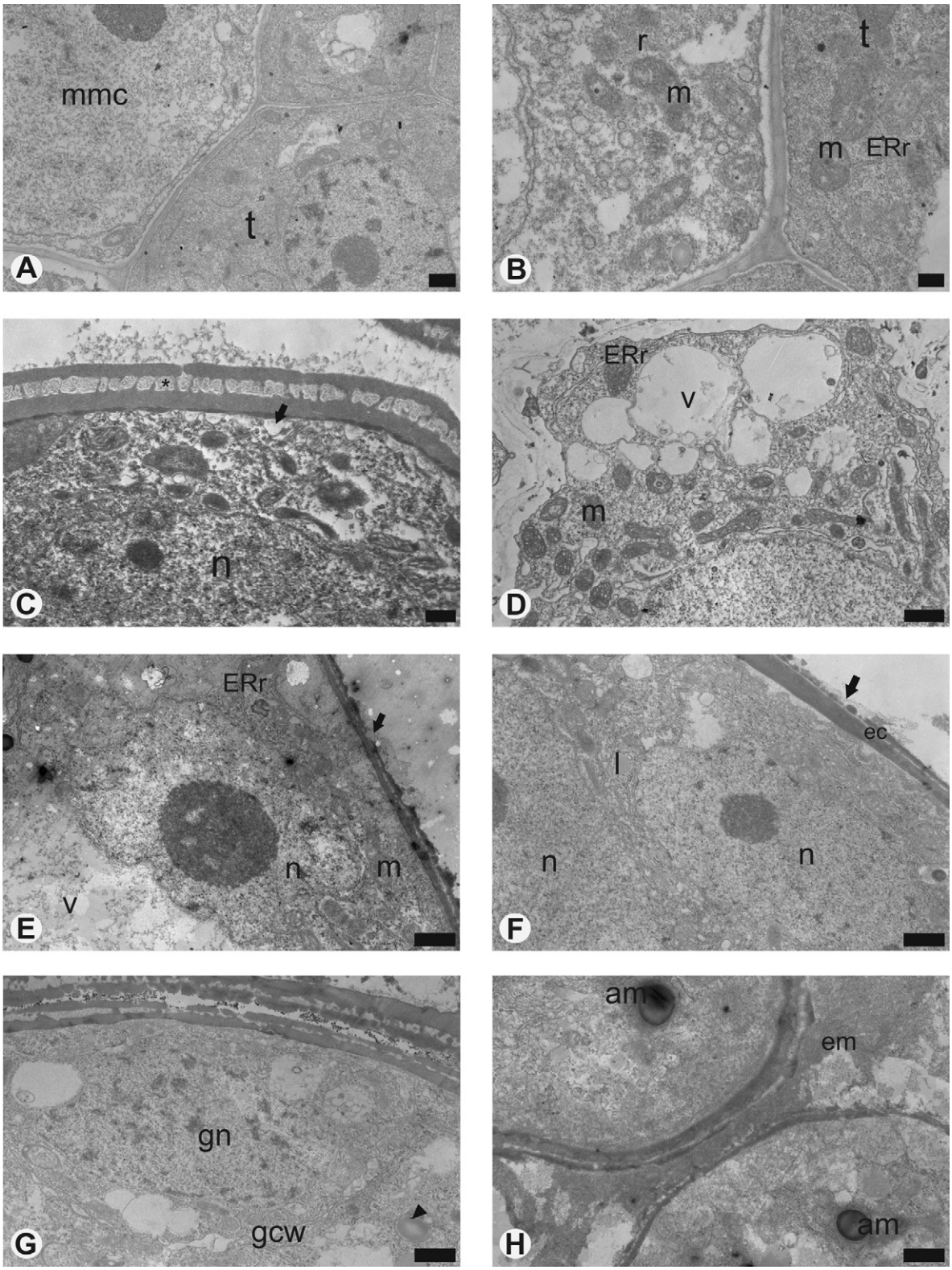

Fig. 4. Transmission electron micrographs at different developmental stages of pollen grains and tapetum in pistillate flowers. (A) Aspect of microspore mother cell and tapetum. (B) Detail of a microspore mother cell containing abundant endoplasmic reticulum with dilated cisternae, mitochondria, free ribosomes, and tapetal cell with some mitochondria and less extensive endoplasmic reticulum. (C) Detail of uninucleate microspore showing remnants of primexine (asterisk), a normal exine structure, blebbing of plasma membrane (arrow) and the nucleus. (D) Detail of tapetal cell highly vacuolated at the free microspore stage, with numerous mitochondria and elongated cisternae. (E) Detail of a vacuolated microspore at the time of flower anthesis, showing the nuclei, scarce mitochondria and endoplasmic reticulum. (F) Pollen grain cytoplasm, with the generative cell showing an irregular cell wall, though with anomalous pattern of tectum and collumellae deposition (arrow). (G) Detail of a microspore with vacuoles, endoplasmic reticulum with elongated cisternae and an elaioplast (arrowhead). (H) Detail of two microspores with amyloplasts and showing anomalous pattern of exine. Electron-dense material are observed surrounding microspores. Scale bars: B, D 500 nm, A, C, E, F, G, H 1 μm. Abbreviations: am: amyloplast, em: electron-dense material, ERr: endoplasmic reticulum of rough type, ec: ectexine, gc: generative cell, gcw: generative cell wall, mmc: microspore mother cell, m: mitochondria, n: nucleus, t: tapetum, v: vacuole.

Tetrad stage

Staminate and pistillate flowers

At this stage, both types of flowers have the same ultrastructural characteristics. Therefore, only one description is given.

Microsporogenesis is simultaneous, resulting in microspore tetrads with tetrahedral arrangement. The callose wall that surrounds the entire tetrad is thick, whereas thinner callose extends between the individual microspores (Fig. 2B). At this stage, the fibrillar matrix of primexine starts to develop (Fig. 3D and E). The future basal layer, probacula and protectum can be seen on the fibrillar matrix as more electron-dense zones. The cytoplasm of microspores shows some mitochondria, abundant dictyosomes and ERr (Fig. 3E).

The tapetum reaches maximal development (Fig. 2B); the cells have abundant mitochondria, ERr, and dictyosomes, and some vacuoles. Tapetal cell walls are still present but show a lax structure (Fig. 3F).

Free microspores

Staminate flowers

Once the callosic wall disintegrates, microspores release from the tetrads into the locule. They develop a large central vacuole displacing the nucleus to a parietal position (Fig. 2C). The exine of microspores is well development, and the beginning of intine formation can be observed. The microspore cytoplasm has ERr and mitochondria (Fig. 3G).

Tapetal cells exhibit a dense cytoplasm with abundant mitochondria and are devoid of large vacuoles (Figs. 2C and 3H), whereas the middle layers are still visible (Fig. 2C). Tapetal cell walls present a laxer structure than at the previous stage, with some electron-dense inclusions (Fig. 3H). By the end of the young free microspore stage, the tapetum is mostly degraded and endothecium cells begin to elongate radially.

Pistillate flowers

At this stage, a general disorganization, darkening and shrinkage of the microspore cytoplasm are observed, although exine development is apparently normal, with a tectum, collumellae, and a foot layer, as in staminate flowers (Fig. 4C).

Tapetal cells are less dense and with larger vacuoles than those of the staminate flowers (Figs. 2D and 4D). They have small amount of ERr and numerous mitochondria with normal structure (Fig. 4D).

Mature pollen grains

Staminate flowers

The mature anther wall consists of dehydrated epidermal cells and the endothecium, which reaches its maximum radial expansion and develops thickenings on inner tangential and radial walls. The middle layers and the tapetum are entirely degenerated at this stage (Fig. 2E).

The nucleus of the microspore divides by mitosis, giving rise to the generative and vegetative cells. Young pollen grains have the generative cell in parietal position and with a sinuous cell wall (Fig. 3I–L). In mature pollen grains, the generative cell is enclosed by the vegetative cell. The latter possesses a cytoplasm with lipid globules, mitochondria and abundant ERr with expanded cisternae (Fig. 3I–K). The generative cell has a prominent nucleus and a cytoplasm with few organelles, only some mitochondria and lipid globules are evident. Conspicuous electron-dense corpuscles are observed near the plasmalemma and within the generative cell wall (Fig. 3K).

At this stage, the parenchyma septum that separates both pollen sacs of an anther theca breaks down and the anther dehisces through longitudinal slits (Fig. 1E). Pollen grains are shed at two-celled stage, they are tricolporate, prolate to prolate-spheroidal and the exine is tectate striate.

Pistillate flowers

The mature anther wall consists of an epidermis with turgescent cells, and the endothecium with cells that do not increase in volume and fail to produce fibrous thickening. The middle layers remain vacuolated; some tapetal cells are disintegrated, whereas others persist until anther maturity (Fig. 2F and G).

The parenchymal septum does not disintegrate, the stomium remains closed, and therefore the resulting anthers are indehiscent (Fig. 1F).

At anthesis, simultaneously in a same locule, microspores at different stages of maturity and exine development are observed (Fig. 4E–H). The cytoplasm of these microspores has some visible organelles as endoplasmic reticulum and mitochondria, and presents a large vacuole typical of young microspores (Fig. 4E). Some of them have vegetative and generative nuclei, but the generative cell wall is not observed (Fig. 4F). Only some microspores reach the young pollen grain stage with the generative cell in parietal position (Fig. 4G). The cytoplasm of these pollen grains accumulates very few lipid globules, which are not observed in mature pollen grains. On the other hand, numerous microspores show abnormal shapes or are completely collapsed (Fig. 2F and G). Some pollen walls display an exine with irregular depositions of collumellae and tectum (Fig. 4E, F and H). Others have a well formed exine but always lack intine (Fig. 4F). Many cellular remains and an electron-dense substance is observed between the pollen grains in the loculus (Fig. 4H).

Discussion

The comparison of staminate and pistillate flowers in which pollen grains abort and the anthers do not dehisce showed differences between anther and pollen development in *Melicoccus lepidopetalus*. The evolutionary significance of nonfunctional stamens and pollen in pistillate flowers remains a controversial issue. In a study of reproductive biology of the monoecious Sapindaceae species *Cupania guatemalensis* Radlk., Bawa (1977) hypothesizes that the floral morphs of this species would represent a transitional stage in the evolution of dioecy, and that the permanence of the androecium may represent an adaptive advantage, increasing the attractiveness to pollinators. Mayer and Charlesworth (1991) agree with Bawa (1977) in the attractive role in pollination, and suggest the retention of stamens is also due to genetic correlations between androecium and gynoecium that delay the suppression of one or the other in functionally staminate or pistillate flowers. By contrast, Cane (1993) proposes that an androecium of pistillate flowers, such as those of the andromonoecious *Xerospermum* (Sapindaceae), would serve neither as a pollinator reward nor as a functional male gametophyte; thus, the stamens may become vestigial or lost in the evolutionary future.

Anther and pollen development

Anther ontogeny and development reveal that the pattern of secondary parietal layer divisions corresponds to the basic type (Davis, 1966). This feature agrees with that reported in *Cardiospermum halicacabum* (Ha et al., 1988), *C. grandiflorum* Sw., and *Urvillea chacoensis* Hunz. (Solís et al., 2010). However, in *Allophylus alnifolius* Radlk. ex Engl. (Mathur and Gulati, 1980), *Lepidopetalum jackianum* Radlk. (Mathur and Gulati, 1981) and *A. zeylanicus* L. (Mathur and Gulati, 1989), anther *wall development* follows the *dicotyledonous type.*

The secretory tapetum presents one to two nucleate cells, a character also observed in *Filicium decipiens* (Wight & Arn.) Thwaites ex Hook. f. (Gulati and Mathur, 1977) and *A. alnifolius* (Mathur and Gulati, 1980). However, in *C. halicacabum* the tapetal cells are uninucleate (Nair and Joseph, 1960; Solís et al., 2010), in *A. zeylanicus* they are three-nucleate (Mathur and Gulati, 1989); in *L. jackianum* they are multinucleate followed by fusion of nuclei to form a polyploid nucleus (Mathur and Gulati, 1981). Since this character is variable within Sapindaceae, it might be of systematic value in the family. Although the number of nuclei may vary among species, Furness and Rudall (2001) consider that this character may not be reliable.

Tetrads with tetrahedral arrangement occur as a result of simultaneous divisions of the MMC in *M. lepidopetalus*. This character is also variable among different species of Sapindaceae. Indeed, in *C. halicacabum* (Nair and Joseph, 1960) and *Xerospermum intermedium* Radlk. (Ha et al., 1988) the tetrads are tetrahedral or tetragonal, in *A. alnifolius* (Mathur and Gulati, 1980) tetrads are isobilateral, decussate and less frequently arranged linearly, formed by a failure in the separation of one or two MMCs, and in *A. zeylanicus* the tetrads are tetrahedral and isobilateral (Mathur and Gulati, 1989).

During anther development, concurrently with degradation of tapetum and middle layers, endothecium cells enlarge radially and develop fibrous thickenings before dehiscence. Manning (1996) recognized generally U-shaped and base plate patterns of endothecial wall thickenings for Sapindaceae. The U-shaped pattern is consistent with that observed in staminate flowers of *M. lepidopetalus*. In this genus, pollen grains are shed at the two-celled stage, which is consistent with previous studies on other genera (Nair and Joseph, 1960; Mathur and Gulati, 1981; Ha et al., 1988; Solís et al., 2010). In *Allophylus zeylanicus* and in *A. alnifolius* pollen grains were also recorded to be tricellular (Mathur and Gulati, 1980, 1989).

Structural evidences of male sterility

The phenotypic manifestations of male sterility are very diverse, ranging from the complete absence of male organs, the failure to develop normal sporogenous tissue, the abortion of pollen in some stage of their development, the absence of stamen dehiscence, and through the inability of mature pollen to germinate on a compatible stigma (Budar and Pelletier, 2001). In *M. lepidopetalus*, anther wall development in both floral morphs follows the same pattern at early stages. However, at advanced stages, the endothecium, tapetum and microspores of pistillate flowers show some anomalies in their development that result in nonfunctional pollen grains.

In mature anthers of pistillate flowers, the endothecium shape is not modified, and the cells do not show secondary wall thickenings. These features differ from those observed in *C. grandiflorum* and *U. chacoensis*, in which the cells are radially elongated and develop fibrillar thickenings, although less visible in pistillate flowers than in staminate ones, which are absent in endothecium cells along the stomium margins (Solís et al., 2010).

Failure in anther dehiscence of pistillate flowers is a character shared by all species of Sapindaceae studied so far. Goldberg et al. (1993) mentioned that one of the essential events for anther dehiscence involves the sequential lysis of septum, connective and stomium cells, and that failure in anther dehiscence is associated with a defect in the programmed degradation and breakage of these anther wall tissues. Keijzer (1987) suggests that in a normal pattern of dehiscence, both the swelling of epidermis and endothecium cells, and rigidity of endothecial wall generates an inwardly directed force in the anther wall that causes the rupture of the weakened stomium, whereas the stomium remains closed in the less expanding epidermal and endothecial cells in deviant anthers. In anthers of pistillate flowers, intersporangial septum and stomium cells that remain intact may be directly associated with a failure of programmed cell death.

Laser and Lersten (1972), who presented the earliest review of the microsporogenesis in species with male sterility, noted that the abortion of pollen grains may occur at almost any stage of development, mostly at post-meiotic stages. The results of the present study show that the development of pollen grains in pistillate flowers of *M. lepidopetalus* after meiosis is arrested between the unicellular microspore stage and the mitotic division of the nucleus, since cytokenesis is generally absent. This feature distinguishes *M. lepidopetalus* from *C. grandiflorum* and *U. chacoensis*, in which cytokinesis always occurs (Solís et al., 2010), and from *C. halicacabum*, in which abortion occurs earlier, at tetrad stage (Nair and Joseph, 1960).

The abnormal tapetum development and irregular exine formation are determining factors in causing male sterility (Radice et al., 2008). In many male-sterile lines, irregular exine formation was attributed to premature degeneration of tapetal cells at the first meiotic division of microspores mother cells (Dundas et al., 1981), or at tetrad stage (Kapoor et al., 2002) or soon after the release of the microspores (Loukides et al., 1995; Ku et al., 2003). Taking in account that the exine pattern is under the control of the sporophytic genome and the precursors of the pollen wall are released mainly by the tapetum at the vacuolated microspore stage (Heslop-Harrison, 1972), problems in tapetum behavior and programmed death of *M. lepidopetalus* pistillate flowers probably have led to abnormal pollen grain exine structures and pollen abortion. These disturbances are reflected in the rapid vacuolation of tapetal cells at vacuolated microspore stage, and the permanence of some of these cells at the final step of anther development. The cytological events accompanying male sterility in pistillate flowers of *C. grandiflorum* and *U. chacoensis* are also those concerning the persistence of tapetum in fully mature anthers (Solís et al., 2010). Regarding the origin of protein components of the intine, these are synthesized by the vegetative cell; therefore, it is controlled by gametophytic gene expression (Shivanna, 2003). For this reason, the absence of intine in pollen grains of pistillate flowers may be attributed to a developmental consequence of a defective gametophytic tissue.

The ultrastructural analysis of pollen grains revealed that pistillate flowers of *M. lepidopetalus* are distinguished by lack of an intine and the presence of an irregular exine, compared with *C. grandiflorum* and *U. chacoensis* (Solís et al., 2010) which always undergo normal pollen wall development. These features, plus the absence of generative and vegetative cell differentiation, would indicate an earlier detention of pollen grain development than in the previously mentioned species. All the above observations on microsporogenesis in pistillate flowers suggest that this floral morph would have no fertile pollen grains; further *in vitro* and *in vivo* tests for pollen viability confirmed these results in *M. lepidopetalus* (Zini et al., unpubl. data) and were in agreement with previous studies on monoecious *C. halicacabum* (Nair and Joseph, 1960; Das et al., 1997).

An evolutionary approach to dioecy in Sapindaceae

Within Sapindaceae monoecy is common and dioecy is comparatively less frequent. In a monograph of the tribe *Melicocceae* Acevedo-Rodríguez (2003) found that species of *Talisia* are sequentially monoecious (i.e., changing sex during their lifetime), whereas he points out that *Melicoccus* is always dioecious. In dioecious plants, the susceptibility to produce flowers of the opposite sex, under extreme environmental conditions, is in accordance with the predictions for species involved via the evolutionary pathway from monoecious ancestors (Freeman et al., 1997). Our more than 30 years of observations on some reproductive individuals of *M. lepidopetalus*, cultivated in Corrientes, reveal that they are

not sequentially monoecious. Thus, this species appears to have a well established dioecy, being firmly dimorphic and having no sexual lability.According to our observations, we conclude that loss of male fertility in pistillate flowers would be more pronounced in the dioecious species *M. lepidopetalus* than in previously studied monoecious species (Solís et al., 2010). The most common evolutionary pathway in dioecious species is hermaphroditism, resulting first in monoecy and then to dioecy (Bawa, 1980; Freeman et al., 1997). Thus, an origin via monoecy involves the origin of unisexual flowers prior to separation of sexes (Mitchell and Diggle, 2005). This suggests that in the evolution towards unisexuality, differences in development between both types of flowers, pistillate and staminate, might become more pronounced in a derived condition, such as dioecy.

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