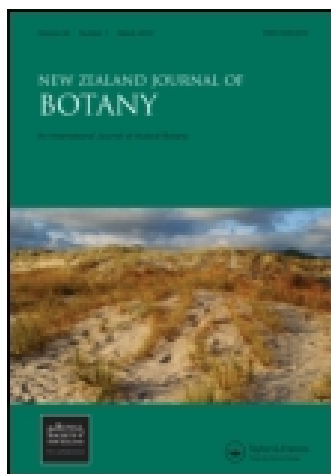


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Comparative study of anther development, microsporogenesis and microgametogenesis in species of *Corchorus*, *Heliocarpus*, *Luehea* and *Triumfetta* Malvaceae: Grewioideae) from South America

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REVIEW ARTICLE

Comparative study of anther development, microsporogenesis and microgametogenesis in species of *Corchorus*, *Heliocarpus*, *Luehea* and *Triumfetta* Malvaceae: Grewioideae from South America

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Anther development, microsporogenesis and microgametogenesis of six species of the genera *Corchorus*, *Heliocarpus*, *Luehea* and *Triumfetta* were analysed. The genera were found to share the following characters: ontogeny of anther wall of basic type; simultaneous microsporogenesis, resulting mainly in tetrahedral tetrads; secretory tapetum and pollen grains shed at the bicellular stage. Moreover, the characters that differentiate them are: presence of uninucleate tapetal cells in *Heliocarpus* and *Triumfetta*; binucleate cells in *Corchorus* and multinucleate cells in *Luehea*; differentiation of the thickenings of the endothecium at free microspores stage in *Corchorus*, *Heliocarpus* and *Triumfetta*, whereas in *Luehea* differentiation occurs at the mature pollen grains stage; late disintegration of sporogenous tissue cell walls in *Luehea*; and the presence of orbicules, absent only in *Corchorus*. This is the first embryological report of the Grewioideae subfamily, contributing to the characterization of the genera studied. The results are discussed in relation to the known data for the family.

Keywords: Apeibae; endothecium; Grewieae; microgametogenesis; microsporogenesis; orbicules; secretory tapetum; systematic

Introduction

Malvaceae Juss. is a cosmopolitan family comprising 243 genera and probably more than 4300 species (Bayer & Kubitzki 2003). Evidence from morphological and molecular studies has shown that many of the traditional families of the Malvales are monophyletic (Judd & Manchester 1997; Alverson et al. 1998, 1999; Bayer et al. 1999). Studies of morphological, molecular and biogeographical data demonstrated that Malvaceae *s.l.* includes nine subfamilies; many tribes and genera of the traditionally circumscribed families are rearranged in the newly proposed system (Bayer et al. 1999; Bayer & Kubitzki 2003). Grewioideae Dippel is considered a basal and monophyletic

subfamily, and together with the sister-group Byttnerioideae, it comprises 25 genera and 700 species, distributed in tropical regions of the New and Old Worlds (Bayer & Kubitzki 2003). Recently, Brunken & Muellner (2012) have proposed a subdivision into two tribes within Grewioideae, Grewieae and Apeibae, based on molecular and morphological data.

Among the scarce embryological studies on Grewioideae are the contributions of Rao & Rao (1952), who studied *Triumfetta rhomboidea* Jacq. and *Corchorus acutangulus* L.; Dnyansagar & Gaoli (1964–1965), who analysed some species of *Corchorus* L. and *Triumfetta* Jacq. and Lattar et al. (2012), who conducted an ultrastructural study

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of anther and pollen development in *Luehea divaricata* Mart. However, there is embryological information about species of most of the other subfamilies of Tilioideae (*Tilia europea* L. and *Tilia plataphyllos* Scop., Chambers & Godwin 1961); Dombeyoideae (*Excentrodendron hsienmu* (W. Y. Chun and F. C. How) H. T. Chang and R. H. Miao, Tang et al. 2006; *Eriolaena candollei* Wall., Tang et al. 2009); Malvoideae (*Abutilon pictum* (Gillies ex Hook. & Arn.) Walp., Strittmatter et al. 2000; *Modiolastrum malviflorum* (Griseb.) K. Schum., Galati et al. 2007; *Ochroma lagopus* Sw., Rao 1954; *Quararibea asymmetrica* Aubl., *Quararibea cordata* Visch., *Quararibea lasiocalyx* Visch., *Quararibea ochrocalyx*, Nilsson & Robyns 1974); and Bombacoideae (*Adansonia digitata* L., *Bombax malabaricum* DC., *Eriodendron anfractuosum* DC., *Pachira aquatica* Aubl., Rao 1954; *Ceiba insignis* (Kunth) P. E. Gibbs & Semir, Galati & Rosenfeldt 1998).

Bayer & Kubitzki (2003) recognized in Malvaceae *s.l.* five pollen types, and they argued that Grewioideae is a stenopalynous subfamily, with most of their species sharing the pollen type *Grewia* with Byttnerioideae. Perveen et al. (2004), in the study of pollen of three subfamilies, among them Grewioideae, recognized three types of pollen: *Mortonioidendron*, *Berrya* and *Corchorus* type, the latter related to the subfamily Grewioideae. Finally, the pollen of southern South American species of Grewioideae was studied in detail by Mambrin et al. (2010). The latter authors found variation in the pollen shape and size, colpus length, endoaperture height and width, and thickness exine, but they emphasized that palynological characters have little taxonomic value at genus or species level; all these species showed tricolporate pollen grains.

During the second half of the nineteenth century and the beginning of the twentieth century, small granular bodies in the anther locule of species of Solanaceae, Boraginaceae and Liliaceae were observed (Rosanoff 1865; Mascré 1922; Schnarf 1923). These are the first references to the presence of orbicules in Angiosperms (Galati et al. 2010). These granules are sometimes in close contact with the pollen grains (von Kosmth

1927; Erdtman et al. 1961), because their development coincides with the process of exine formation during microsporogenesis and microgametogenesis (Vinckier & Smets 2002). Most authors consider that orbicules are exclusive to secretory tapetum (Pacini et al. 1985; Huysmans et al. 1998). However, further studies confirmed their presence in species of Asteraceae (Gotelli et al. 2008) and Malvaceae (Strittmatter et al. 2000; Galati et al. 2007) with plasmodial or invasive non-syncytial tapetum. Finally, the presence of orbicules in Malvaceae has been little explored, with records reported only for the subfamilies Bombacoideae, Tilioideae and Grewioideae (Chambers & Godwin 1961; Nilsson & Robyns 1974; Galati & Rosenfeldt 1998; Lattar et al. 2012).

Here we analyse species of the genera *Corchorus* L., *Heliocarpus* L., *Luehea* Willd. and *Triumfetta* Jacq., present in South America. *Corchorus* is a tropical and subtropical cosmopolitan genus, with c. 30 species comprising shrubs, sub-shrubs and herbs. It is well represented in Africa and Australia, with a smaller number of species in Asia and the Americas (Ferrucci 2005). *Luehea* is a Neotropical genus, with 18 species, including trees and shrubs; it is distributed from Mexico to Uruguay and Argentina (Ferrucci 2005). *Heliocarpus* is represented by tree and shrub species, and is distributed from Mexico to South America (Bayer & Kubitzki 2003). Finally, *Triumfetta* is a pantropical genus, with c. 150 species distributed in the Americas, Asia, Africa and Australia (Meijer 2001; Bayer & Kubitzki 2003). Recent molecular studies have included these genera in the subfamily Grewioideae (Judd et al. 1999; Bayer & Kubitzki 2003), with *Corchorus*, *Heliocarpus* and *Triumfetta* corresponding to the tribe Apeibae and *Luehea* to Grewieae (Brunken & Muellner 2012).

The aim of this paper is to investigate the anther development and the microsporogenesis and microgametogenesis in six species of Grewioideae belonging to the genera *Corchorus*, *Heliocarpus*, *Luehea* and *Triumfetta*, to review embryological characters analysed in relation to the recent Grewioideae tribal classification; and to

compare these data with the information known for other species of Malvaceae.

Materials and methods

The following species were studied: *Corchorus argutus* Kunth, *Corchorus hirtus* L., *Heliocarpus popayanensis* Kunth, *Luehea candicans* Mart., *Luehea divaricata* Mart. and *Triumfetta semitriloba* Jacq. Anthers at different stages of development were fixed with FAA (formalin, acetic acid, ethanol). Transverse serial sections of buds and pre-anthesis flowers at different stages were performed. Permanent preparations were obtained for the ontogeny study. The material was dehydrated in an ethanol series, with a rinse using pre-impregnant Biopur® (Gonzalez & Cristóbal 1997). For infiltration in paraffin, the technique of Johansen (1940) was applied and the material was later embedded in Histoplast® (Biopack, Buenos Aires, Argentina). Sections (10–12 µm) were made with a rotary microtome and stained with astra blue-safranin (Luque et al. 1996) and mounted on slides with synthetic Canada Balsam (Biopur, Buenos Aires, Argentina). The material was observed and photographed with a Leica MZ6 stereomicroscope and a Leica DM LB2 compound microscope (Leica, Wetzlar, Germany), both equipped with a digital camera. Scanning electron microscope micrographs were obtained with a JEOL 5800LV at 10 kV and JEOL 100c. The preserved material was dehydrated and then immersed in CO₂ for critical point drying before coating, and then sputter-coated with gold palladium. To observe orbicules by transmission electron microscopy, the mature anthers were prefixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in OsO₄ at 2 °C in the same buffer for 3 h. Then, the material was dehydrated in an ascending ethanol series and embedded in Spurr's resin. Fine sections were made on a Sorvall ultramicrotome, stained with uranyl acetate and lead citrate (O'Brien & Mc Cully 1981). The photographs were observed using a Philips EM301 microscope. The voucher specimens were deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES), Argentina.

Specimens examined

Corchorus argutus Kunth

Argentina. Prov. Misiones. Dpto. Eldorado, 06. IV.2009, Lattar E. & H. Keller 6 (CTES).

Corchorus hirtus L.

Bolivia. Dpto. Santa Cruz. Prov. Ñuflo de Chaves, 05.VIII.2010, Ferrucci M. S. et al. 3049 (CTES).

Heliocarpus popayanensis Kunth

Argentina. Prov. Misiones. Dpto. Eldorado, 05. VIII.2009, Lattar E. & H. Keller 8 (CTES); Lattar E. & H. Keller 9 (CTES); Dpto. San Ignacio, 11. VI.11, Lattar E. & H. Keller 13 (CTES).

Luehea candicans Mart.

Argentina. Prov. Misiones. Dpto. Montecarlo, 10. III.2010, Keller 8173 (CTES); 14.II.2011, Lattar E. et al. 18 (CTES).

Luehea divaricata Mart.

Argentina. Prov. Corrientes, Dpto. Corrientes, 05. III.2009, Lattar E. & M. S. Ferrucci 5 (CTES). Prov. Misiones, Dpto. Apóstoles, camino a Azara, 13.XII.2011, Miguel et al. 25 (CTES).

Triumfetta semitriloba Jacq.

Argentina. Prov. Corrientes, Dpto. Corrientes, 04. III.09, Lattar E. & J. P. Coulleri 4 (CTES).

Results

Floral morphology

A comparative analysis of flower characters in the species studied is shown in Table 1.

Corchorus argutus

(Fig. 1A)

This species presents perfect flowers, small to medium-sized, yellow, 3–5 mm in length. Calyx is 5-merous, obovate-lanceolate sepals, pubescent in abaxial surface. Corolla is 5-merous, oblong-unguiculated petals. Androecium with numerous (c. 75) free stamens, subcylindrical glabrous filaments, reniform anthers (Fig. 2A). The gynoecium surpasses the androecium, ellipsoid-tetragone pubescent

Table 1 Comparative analysis of flower characters in the species studied.

Characters	<i>Corchorus argutus</i>	<i>Corchorus hirtus</i>	<i>Heliocarpus popayanensis</i>	<i>Luehea candicans</i>	<i>Luehea divaricata</i>	<i>Triumfetta semitriloba</i>
Flower type	Perfect	Perfect	Perfect	Pistillate	Perfect	Perfect
Calix	5-merous	5-merous	4-merous	4-merous	5-merous	5-merous
Sepals	Obovate-lanceolate	Obovate-lanceolate	Oblong	Oblong	Obovate	Obovate
Corolla	5-merous	5-merous	4-merous	Absent	5-merous	5-merous
Petals	Oblong-unguiculatus	Spatulate	Oblong	Absent	Obovate	Obovate
Androecium	c. 75 stamens	20–30 stamens	15 stamens	Absent	Numerous stamens	Numerous stamens
Anthers	Reniform	Reniform	Reniform	Absent	Reniform	Reniform
Staminodes	Absent	Absent	Absent	Absent	Present	Present
						Linear
						Absent

ovary, subcylindrical glabrous style and lobulate-papillae stigma. *Corchorus hirtus* (Fig. 1B) differs from *C. argutus* in flower length (2–4 mm), and in spatulate petals and androecium with a lower number of stamens, c. 20–30 (Fig. 2B).

Luehea candicans

(Fig. 1C)

This species exhibits perfect flowers. Flowers are big, 5–8 cm in length. Calyx is 5-merous, ovate sepals, pubescent in adaxial and abaxial surface. Corolla is 5-merous, white flowers, obovate petals with a basal glandular portion in the inner surface, quadrangular contour, trichomatous nectary and a villose band on the nectary. Presence of smooth coalescent staminodes. Androecium with numerous stamens, cylindrical, glabrous filaments and reniform anthers (Fig. 2D). Gynoecium consists of an ovoid pubescent ovary, pubescent style and capitate stigma.

Luehea divaricata

(Fig. 1D)

This species differs from all the previous ones described in the yellowish to red-purple petals, a yellow base in pre-anthesis and a reflexed, yellowish-white base in post-anthesis. Ovate-lanceolate sepals, suborbicular unguiculatus undulate petals, trichomatous nectary with circular contour on the

inner surface; papillae staminodes and reniform anthers (Fig. 2E). Gynoecium longer than the androecium, ovoid pubescent ovary, pubescent style and capitate stigma.

Heliocarpus popayanensis

(Fig. 1E)

The species has perfect flowers and pistillate flowers. Perfect flowers are small, 1.5–2.5 mm in length. Calyx is 4-merous, oblong sepals, pubescent in abaxial surface. Corolla is 4-merous, oblong petals. Androecium with 15 free stamens, glabrous filaments and reniform anthers (Fig. 2C). Gynoecium consists of a subcylindrical pubescent ovary, glabrous style and bifid stigma.

Triumfetta semitriloba

(Fig. 1F)

It presents perfect flowers. The flowers are of medium length (7–9 mm). Calyx is 5-merous, oblong sepals with subapical apex of 1 mm in length, slightly cucullate in distal quarter, pubescent in adaxial surface. Corolla is 5-merous, obovate-narrow. Androecium with 15–25 stamens, glabrous filaments and linear anthers (Fig. 2F). Androgynophore short, c. 0.4 in length, with five subrectangular contour gland nectaries. Urceolus 5-lobate glabrous. Gynoecium presents ovoid pubescent ovary, glabrous style and bifid stigma.

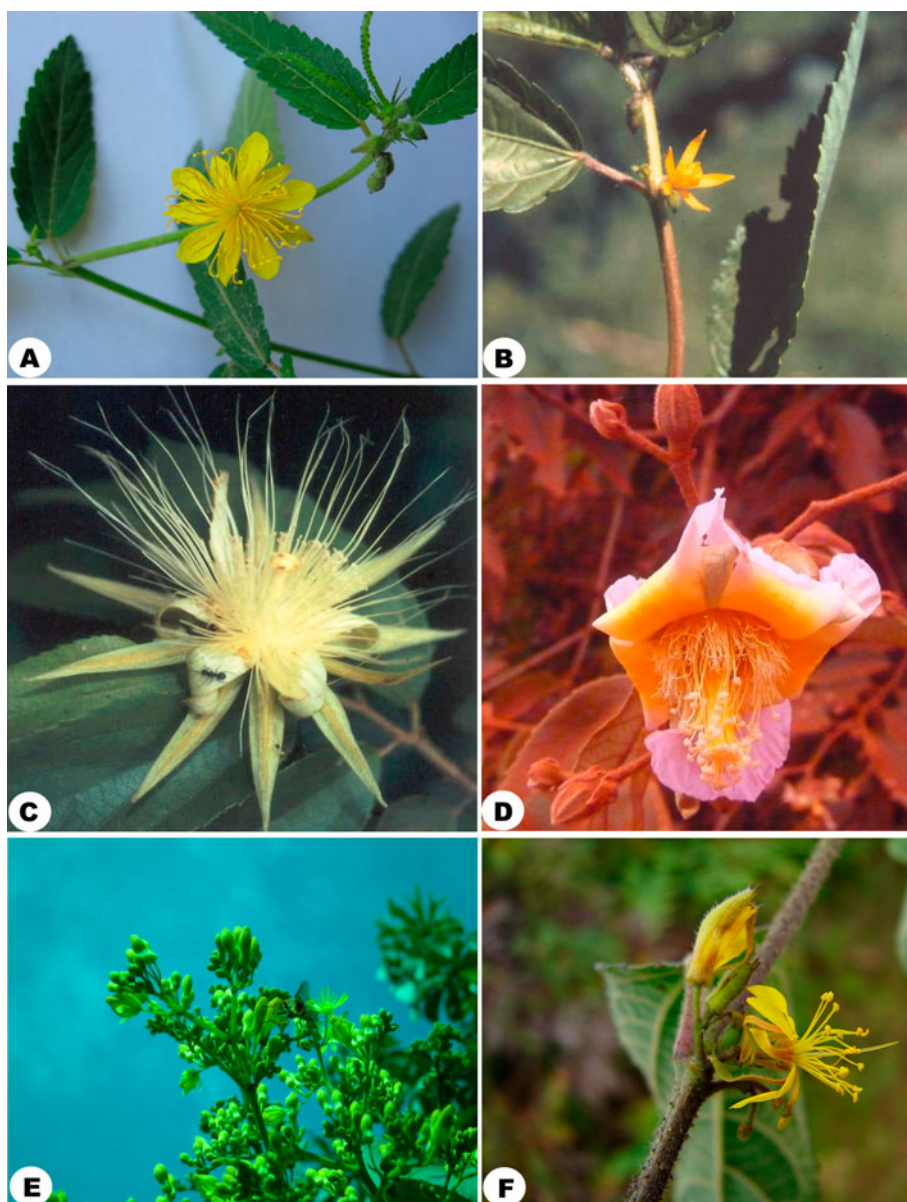


Figure 1 Photographs of the flowers of the species studied: **A**, Detail of the flower of *Corchorus argutus*. **B**, Detail of the flower of *Corchorus hirtus*. **C**, Detail of the flower of *Luehea candicans*. **D**, Detail of the flower of *Luehea divaricata*. **E**, Floriferous branch of *Heliocarpus*. **F**, Detail of the flowers of *Triumfetta semitriloba*.

Microsporangium, microsporogenesis and microgametogenesis development

Microsporangium

Observations with light microscopy. In transverse section, young anther is tetrasporangiate (Fig. 3D,

3E). The anther wall consists of an epidermis, the future endothecium, two middle layers and tapetum (Figs 3C, 5F, 6A). The development of the anther corresponds to the Basic type. The epidermis is the layer that shows smallest change in sporogenesis.



Figure 2 Scanning electron micrographs of mature anthers. **A**, *Corchorus argutus*, dehiscient anther. **B**, *Corchorus hirtus*, dehiscient anther. **C**, *Heliocarpus popayanensis*, dehiscient anther. **D**, *Lueheia candicans*, dehiscient anther. **E**, *Lueheia divaricata*, dehiscient anther showing longitudinal dehiscence and pollen grains. **F**, *Triumfetta semitriloba*, dehiscient anther. Scale bars: A–C, D, F = 50 μm ; E = 20 μm .

At the young pollen grain stage, these cells reach the largest size and exhibit smooth cuticle. Near the stomium, the epidermal cells are papillose in *C. argutus*, *C. hirtus*, *H. popayanensis* and *T. semitriloba*, whereas in *L. candicans* and *L. divaricata* these cells preserve their shape but have abundant tannin content.

The middle layers are ephemeral, because during meiosis they are very compressed and then the microspores are released; their cells are completely degraded in all species.

The endothecium is one of the layers of the anther wall that undergoes most changes throughout development. The endothecium cells increase their size rapidly. In *C. argutus*, *C. hirtus*, *H. popayanensis* and *T. semitriloba* fibrous thickenings

are distinguished in the endothecium cells (Figs 3D, 6D, 6E). Those thickenings develop from the inner tangential and radial walls of the endothecium cells at the free microspore stage, whereas in *L. candicans* and *L. divaricata* they develop at the pollen grain stage (Figs 4G, 5I). Because endothecium cells are specialized, they show the differentiation of the dehiscence zone or stomium. The stomium has small vacuolated parenchymal cells, with thin walls that disintegrate by rupture of the septum wall when the pollen grains are mature, permitting the aperture of the anther.

The tapetum of the studied species is of secretory type (Figs 3D, 4E, 5F, 6B, 6E). During meiosis, the nuclei of the tapetal cells may divide or not; the tapetal cells are binucleate in *C. argutus* and

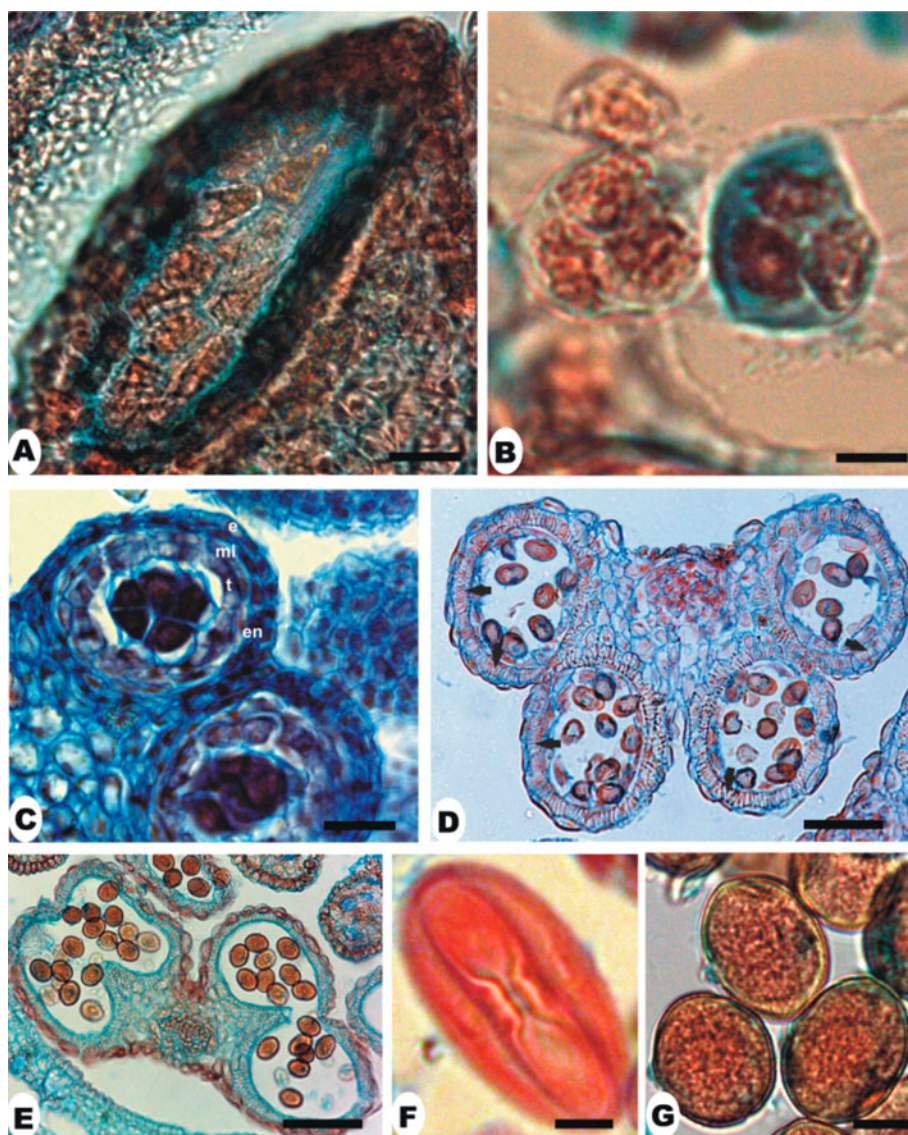


Figure 3 Light micrographs of microsporangium tissues in different stages of development. **A**, *Corchorus hirtus*, cross-section of the young anther, showing wall differentiation and sporogenous cells. **B**, *C. hirtus*, microspore tetrads. **C**, *Corchorus argutus*, microspore tetrads. **D**, *C. argutus*, young free microspores. **E**, *C. hirtus*, young free microspores. **F**, *C. argutus*, detail of mature bicellular pollen grains. **G**, *C. hirtus*, detail of mature bicellular pollen grains. Scale bars: A–C = 50 μ m; D, E = 100 μ m; F, G = 20 μ m.

C. hirtus, multinucleate in *L. candicans* and *L. divaricata*, and 1-nucleate in *H. popayanensis* and *T. semitriloba*.

The tapetal cells are distinguished from the rest of the anther layers by the presence of a dense

cytoplasm and the absence of vacuoles. At the tetrad stage, these cells increase their volume (Figs 3C, 4E, 5F, 6A); and when the microspores are released, degradation starts in *C. argutus*, *C. hirtus*, *H. popayanensis* and *T. semitriloba* (Figs 6B, 6E). However, in

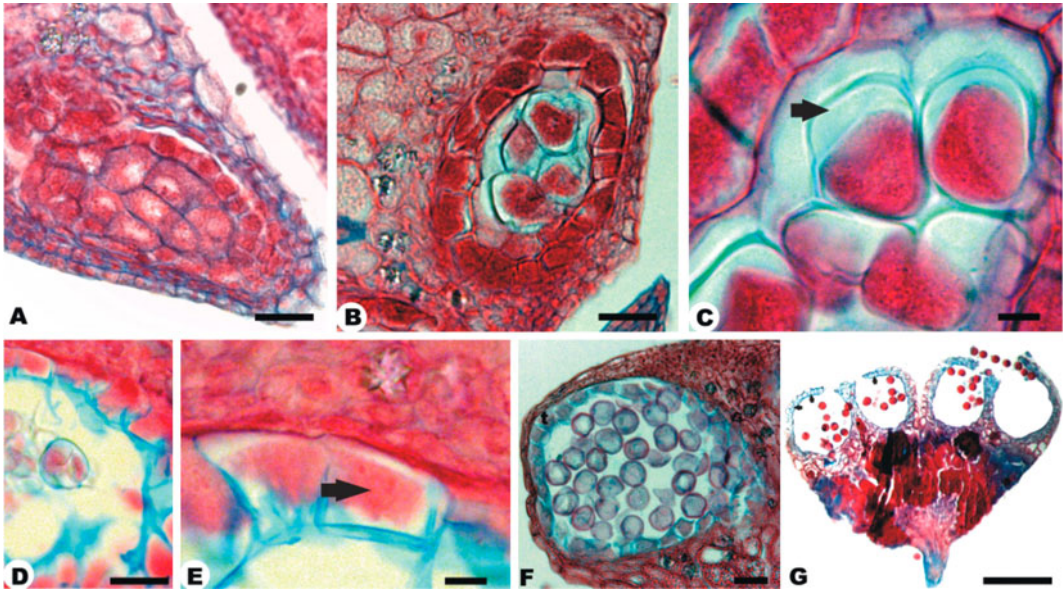


Figure 4 Light micrographs of microsporangium tissues in different stages of development of *Luehea candicans*. **A**, Cross-section of the young anther, showing wall differentiation and sporogenous cells. **B**, General appearance of microspore mother cells. **C**, The primary walls of the microspore mother remain intact, the callose walls undissolved. **D**, Microspore tetrads. **E**, Detail of multinucleate cells of tapetum (arrows). **F**, Detail of young grains of pollen, showing that the tapetum preserves its cells intact (t). **G**, Dehiscent anther with fertile pollen grains, absence of cells of tapetum. Scale bars: A, B, D = 50 μ m; C, E, G = 20 μ m; F = 10 μ m.

L. candicans and *L. divaricata* degradation occurs later, at the mature pollen grain stage (Figs 4G, 5H). In these latter species, the tapetum preserves its cells intact after release of the microspores (Figs 4F, 5G).

Microsporogenesis

The sporogenous tissue does not have intercellular spaces and the cells present a thin wall and dense cytoplasm (Figs 3A, 4A, 5A, 5B). The maturation process, which comprises the preparation for meiosis and differentiation into microspore mother cells, is accompanied by very evident cytological and morphological changes (Figs 4B, 5C). The formation of a callose wall that is deposited between the primary wall and the plasmalemma of the cells is observed. The divisions of the microspore mother cells are simultaneous, resulting mainly in tetrahedral tetrads in all the species analysed (Figs 3B, 3C, 4D, 5E, 6A). Subsequently, the callose wall of the tetrads is dissolved, but the primary walls of the

microspores mother cells remain intact for some time in *Luehea* (Figs 4C, 5D). For this reason, the release of the young microspores into the anther locule in this genus occurs late; however, in other species this wall dissolves rapidly, causing the early release of the microspores. After dissolution of these walls, the deposition of sporopollenin begins on the pollen grain wall. In this stage, the tapetal cells reach maximum physiological activity and the cells of the middle layer are degraded in *Corchorus*, *Heliocarpus* and *Triumfetta*; whereas in *Luehea* the tapetal cells begin to degrade at the pollen grain stage and disappear at the time of release of pollen grains.

Microgametogenesis

After the release of microspores, these incorporate water, increasing their volume, which generates the formation of a large vacuole that is located in parietal position with respect to the nucleus in the species studied. Subsequently, mitotic division of

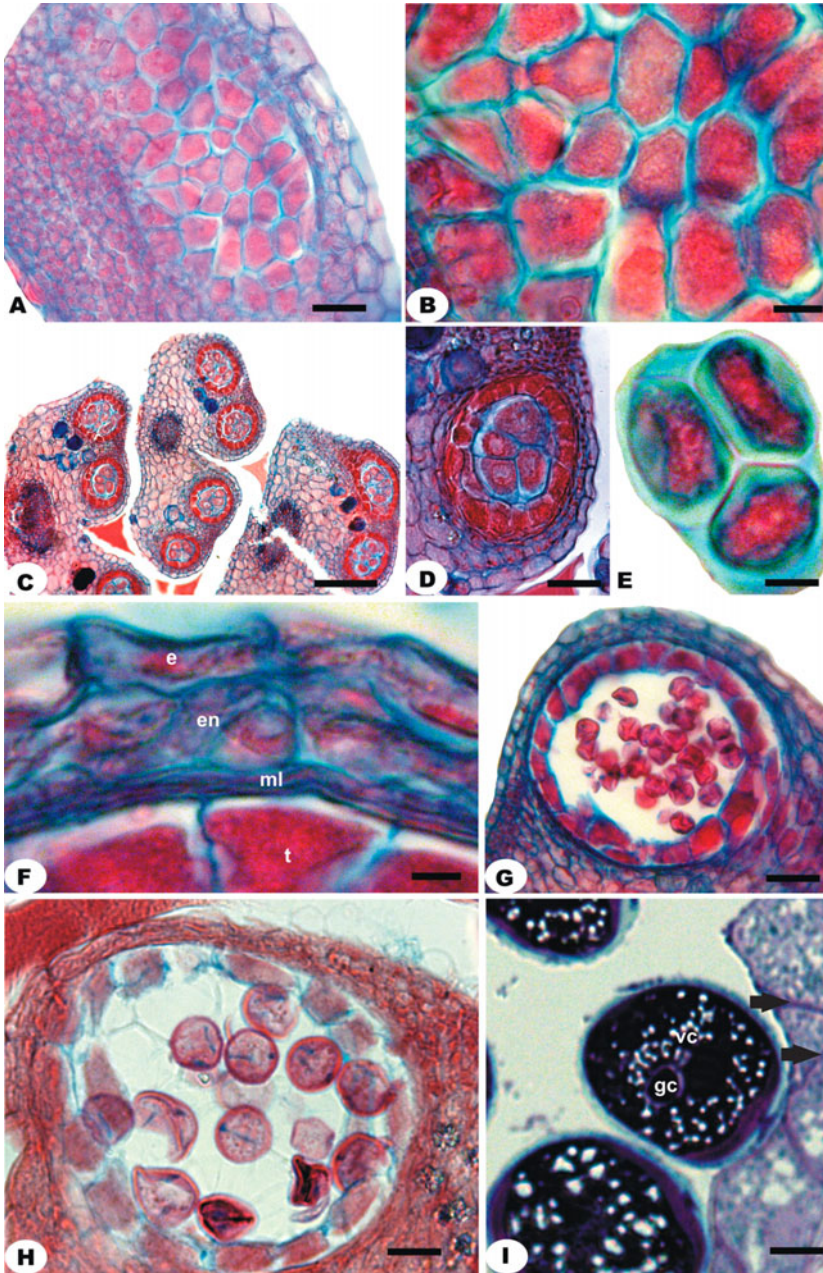


Figure 5 Light micrographs of microsporangium tissues in different stages of development of *Luehea divaricata*. **A**, Cross-section of the young anther, showing wall differentiation and sporogenous cells. **B**, Detail of sporogenous tissue. **C**, General appearance of young anthers. **D**, Detail of a young anther. **E**, Microspore tetrads. **F**, Detail of anther wall: epidermis (e), endothecium (en), middle layers (ml) and secretory tapetum (t). **G**, Detail of young grains of pollen, showing that the tapetum preserves its cells intact (t). **H**, Detail of grains of pollen, the tapetal cells begin to degrade. **I**, Detail of mature bicellular grains of pollen with the vegetative nucleus (vn), and the generative cell (gc). Scale bars: A, D, E, G, H = 50 μ m; B, F, I = 20 μ m; C = 100 μ m.

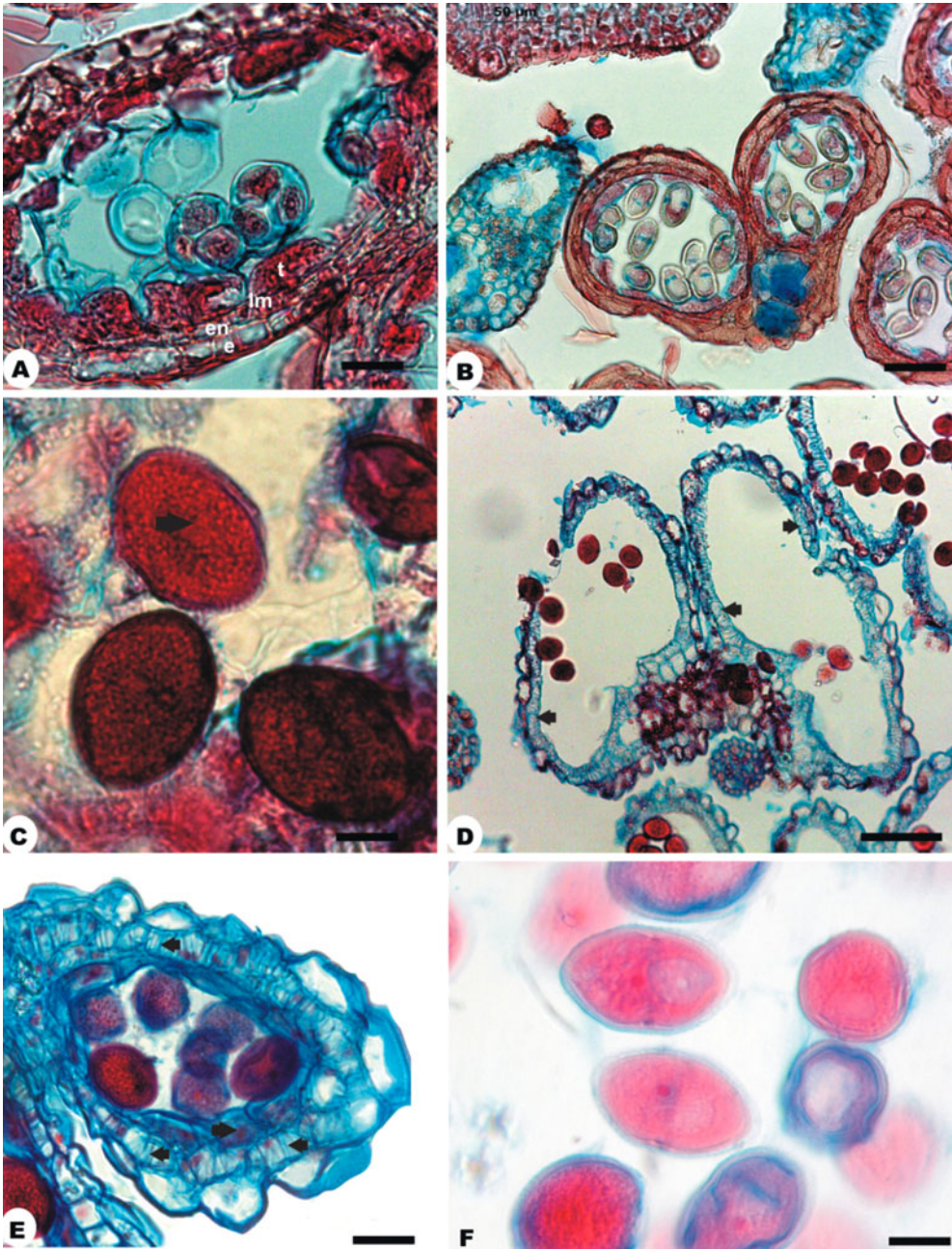


Figure 6 Light micrographs of microsporangium tissues in different stages of development. **A**, *Triumfetta semitriloba*, detail of microspore tetrads. **B**, *T. semitriloba*, detail of young grains of pollen. **C**, *T. semitriloba*, detail of mature bicellular grains of pollen (arrow). **D**, *T. semitriloba*, dehiscent anther. **E**, *Heliocarpus popayanensis*, detail of anther with uninucleate tapetal cells (arrow). **F**, *H. popayanensis*, detail of grains of pollen. Scale bars: A–C, E, F = 50 µm; D = 100 µm.

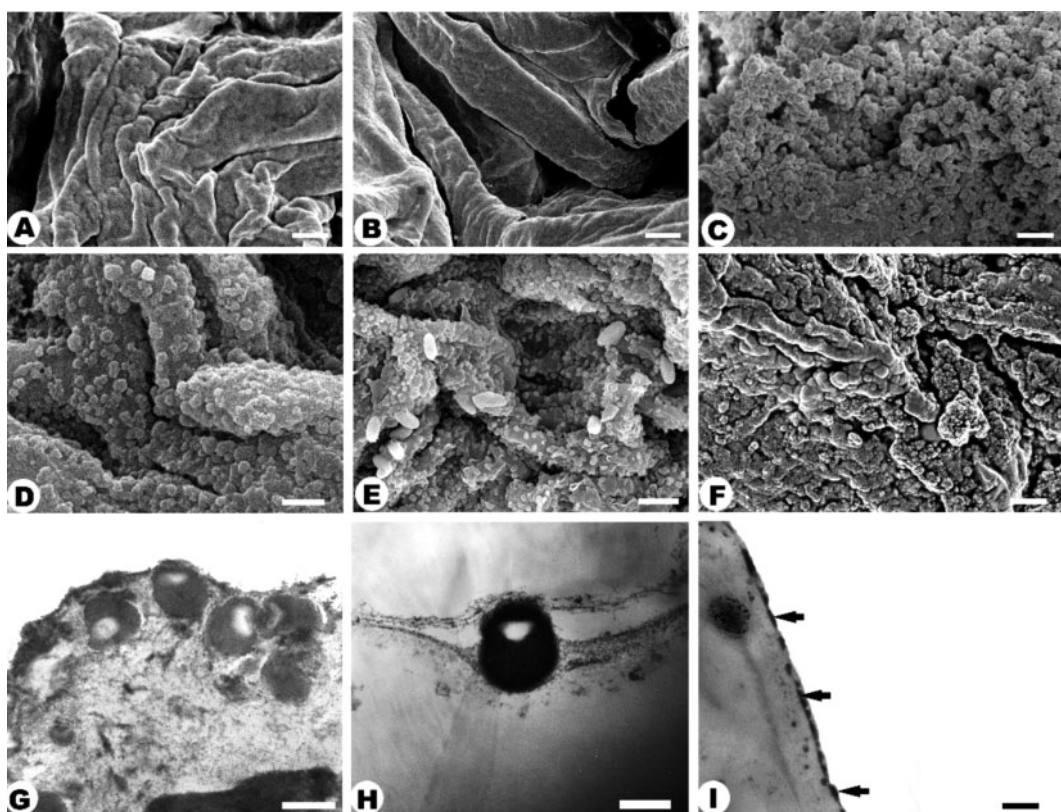


Figure 7 Photomicrographs of scanning and transmission electron microscopy of orbicules. **A**, *Corchorus argutus*, absence of orbicules. **B**, *Corchorus hirtus*, absence of corpuscles. **C**, *Heliocarpus popayanensis*, presence of orbicules. **D**, *Luehea candicans*, presence of orbicules. **E**, *Luehea divaricata*, presence of orbicules. **F**, *Triumfetta semitriloba*, presence of orbicules. **G**, *L. candicans*, orbicules with central core (cc) on the tapetal membrane. **H**, *L. divaricata*, detail of an orbicule with central core (cc). **I**, *T. semitriloba*, detail of orbicules (arrows). Scale bars: A, F = 200 nm; B, C = 300 nm; D, E, H, I = 1 μ m; G = 200 nm.

the nucleus occurs, resulting in a largest volume cell that inherits the big vacuole of microspore, the vegetative cell, and a smaller one, the generative cell (Fig. 5I). During maturation of the pollen grain, the vacuole disappears and a large amount of reserve substances accumulate within the vegetative cell in the form of starch. The pollen grain is released at the bicellular stage in all the species analysed (Figs 3F, 3G, 6C, 6F).

Orbicules

General morphology of the orbicules. Scanning electron microscope observations: *Luehea candicans*, *L. divaricata*, *H. popayanensis* and *T.*

semitriloba possess orbicules distributed over the inner surface of the anther locule, attached to a tapetal membrane. They are spherical to subspherical, with a smooth or irregular surface with small warts (Fig. 7C–7F). Size variation is significant even within the same species (0.1–1.5 μ m). In some cases, two or more orbicules fuse together resulting in aggregates. These aggregates are especially common in *H. popayanensis* and *T. semitriloba*, which result in plate-like structures. Instead, *C. argutus* and *C. hirtus* do not present orbicules (Fig. 7A, 7B).

Transmission electron microscope observations: The orbicules of *L. candicans* and *L. divaricata* are approximately triangular in section;

they have a central core that is transparent to electrons, surrounded by a wall whose electron-density is similar to that of ectexine. Distribution on the tapetal membrane is uniform in both species.

Heliocarpus popayanensis and *T. semitri-loba* do not have a central core, but are solid corpuscles. Distribution on the tapetal membrane is not uniform but forms isolated groups.

According to the differences in the species studied, two types were defined:

Type A. Spherical orbicules, averaging 1–1.5 μm in diameter, smooth surface, with a central core. Solitary or grouped in pairs. Uniform distribution on the tapetal membrane.

Taxa included: *Luehea candicans* and *L. divaricata*.

Subtype A1. Spherical orbicules of 1 μm

Taxon included: *Luehea candicans* (Fig. 7G)

Subtype A2. Spherical orbicules of 1–1.5 μm

Taxon included: *L. divaricata* (Fig. 7H)

Type B. Subspherical orbicules, averaging 0.5–1 μm in diameter, irregular surface with small warts. Distribution on the tapetal membrane is not uniform, but in isolated groups.

Subtype B1. Subspherical orbicules of 0.1–0.5 μm

Taxon included: *Heliocarpus popayanensis* (Fig. 7C)

Subtypes B2. Subspherical orbicules of 0.6–1 μm

Taxon included: *Triumfetta semitri-loba* (Fig. 7I)

Discussion

The embryological characters have been useful in the diagnosis of most of the groups of taxonomic rank (Furness & Rudall 1999; Furness et al. 2002). The study of anther wall structure and pollen development in *C. argutus*, *C. hirtus*, *H. popayanensis*, *L. candicans*, *L. divaricata* and *T. semitri-loba*, provides evidence of embryology characters that allows us to infer possible phylogenetic relationships in Grewioideae. The most relevant characters are determined by the disintegration time of primary cell walls of sporogenous tissue, the number of nuclei of the tapetal cells, the persistence of the secretory tapetum, thickenings of the endothecium and orbicules morphology (Table 2).

Anther wall development in *C. argutus*, *C. hirtus*, *H. popayanensis*, *L. candicans* and *T.*

semitri-loba coincides with the Basic type (Davis 1966), which is shared with *L. divaricata*, Grewioideae (Lattar et al. 2012); *E. hsiennu*, Dombeyoideae (Tang et al. 2006), *O. lagopus* (Malvoideae), *A. digitata* and *B. malabaricum*, Bombacoideae (Rao 1954). However, Rao & Rao (1952) and Dnyansagar & Gaoli (1964–1965) indicated that anther wall development in some species of *Corchorus* and *Triumfetta* corresponds to the dicotyledonous type, as in other species of Malvaceae, such as *A. pictum* and *M. malvifolium*, Malvoideae (Galati unpublished) and *C. insignis*, Bombacoideae (Galati & Rosenfeldt 1998).

In *C. argutus*, *C. hirtus*, *H. popayanensis*, *L. candicans*, *L. divaricata* and *T. semitri-loba* the cytokinesis is of simultaneous type, resulting in tetrads of tetrahedral type, as in *Eriolaena* and *Dombeya* (Tang et al. 2006, 2009). The formation pattern of the microspores in the species studied can be a plesiomorphic character of angiosperms (Nadot et al. 2008), being common in all eudicots (Furness et al. 2002) and can be related to tricolpate pollen apertures and their derivatives, such as tricolporates (Furness & Rudall 1999).

Another important character is the formation time of fibrillar thickenings of endothecium in relation to pollen development of the species analysed. In *C. argutus*, *C. hirtus*, *H. popayanensis* and *T. semitri-loba* the fibrillar thickenings develop at the free microspore stage, as in *E. hsiennu* (Tang et al. 2006). However, in *L. candicans* the fibrillar thickenings complete their development at the immature pollen stage; as well as in *L. divaricata* (Lattar et al. 2012). The latter pattern was also observed in genera belonging to the subfamily Dombeyoideae (Tang et al. 2006), whereas for the remaining species studied in other subfamilies of Malvaceae, the authors did not indicate the development time of these thickenings. According to the classification of Manning (1996), the fibrous thickenings observed in all species studied correspond to the annular type.

The tapetum is a specialized cell layer surrounding the sporogenous tissue. It plays a nutritional role and supplies the pollen grains in the formation of various enzymes and precursors of

Table 2 A comparative analysis of embryological characters in the species studied.

Characters	<i>Corchorus argutus</i>	<i>Corchorus hirtus</i>	<i>Heliocarpus popayanensis</i>	<i>Luehea candicans</i>	<i>Luehea divaricata</i>	<i>Triumfetta semitriloba</i>
Anther wall development	Basic	Basic	Basic	Basic	Basic	Basic
Disintegration of primary tissue walls sporogenous	During meiosis	During meiosis	During meiosis	After meiosis	After meiosis	During meiosis
Tapetum	Secretory	Secretory	Secretory	Secretory	Secretory	Secretory
Tapetum cells	2-nucleate	2-nucleate	1-nucleate	Multinucleate	Multinucleate	1-nucleate
Tapetum persistent	No	No	No	Yes	Yes	No
Endothecium	Free microspores	Free microspores	Free microspores	Mature pollen grains	Mature pollen grains	Free microspores
Orbicules	Absent	Absent	Present	Present	Present	Present
External morphological	Absent	Absent	Subspheroidal with a smooth or irregular surface	Spheroidal	Spheroidal	Subspheroidal with a smooth or irregular surface
Internal morphological	Absent	Absent	Absent central core	Central core	Central core	Absent central core
Reference	Present paper	Present paper	Present paper	Present paper	Lattar et al. 2012	Present paper

the exine (Chapman 1987; Pacini & Franchi 1993; Pacini et al. 1985; Pacini 1990). In angiosperms, this tissue can be of two basic types: amoeboid or secretory, but the presence of an intermediate type called invasive non-syncytial has been mentioned (Furness & Rudall 2001; Galati et al. 2007; Vestraete et al. 2011). All the species studied in this work have a secretory tapetum, which is the most common type in eudicots (Pacini et al. 1985; Furness & Rudall 2001; Furness 2008). This character is shared with other members of subfamily Bombacoideae, such as *A. digitata*, *B. malabaricum*, *E. anfractuosum*, *P. aquatica*, *O. lagopus* (Rao 1954) and *C. insignis* (Galati & Rosenfeldt 1998), as well as *E. hsienmu*, belonging to the subfamily Dombeyoideae. Moreover, *A. pictum* Malvoideae, (Strittmatter et al. 2000) and

E. candollei, Dombeyoideae, (Tang et al. 2009) have plasmodial tapetum, whereas *M. malviflorum*, Malvoideae, has an invasive non-syncytial tapetum (Galati et al. 2007). The secretory tapetum is considered a primitive state, whereas the plasmodial tapetum has evolved several times among early branching angiosperms (Furness & Rudall 2001; Furness 2008). In Malvaceae, *E. candollei* (Dombeyoideae) has plasmodial tapetum; this is considered a bridge to the Malvoideae subfamily, which presents species with a plasmodial or invasive non-syncytial tapetum (Strittmatter et al. 2000; Galati et al. 2007; Tang et al. 2009).

The tapetum cells in *C. argutus* and *C. hirtus* are binucleate, as in *A. digitata*, *E. anfractuosum*, *P. aquatica* and *O. lagopus* (Bombacoideae). This condition of binucleate tapetal cells might be one

of the ways in which total DNA content increases in tapetal tissue (Pandey 1997). By contrast, in *L. candicans*, the tapetum cells are multinucleate, a character shared with *L. divaricata*, Grewioideae (Lattar et al. 2012). This character state was also observed in *B. malabaricum* (Rao 1954) and *C. insignis* (Galati & Rosenfeldt 1998), both species belonging to Bombacoideae. However, in *H. popayanensis* and *T. semitriloba* the tapetal cells are uninucleate, as in other species belonging to Malvoideae and Dombeyoideae (Strittmatter et al. 2000; Tang et al. 2006, 2009; Galati et al. 2007). The multinucleate state in the tapetal cells of *L. candicans* and *L. divaricata* could be interpreted as apomorphic within Grewioideae; this character state is shared with some species of Bombacoideae, one of the derived families within Malvaceae (Lattar et al. 2012). Noticeably, in species of the other subfamilies studied, the tapetal cells are uninucleate or binucleate.

In most angiosperms, the tapetal cells reach maximum development at the tetrad stage; after that, they undergo a series of changes resulting in their degradation (Raghavan 1997). In *C. argutus*, *C. hirtus*, *H. popayanensis* and *T. semitriloba*, the degradation of the tapetal cells occurs at the free microspore stage, whereas in *L. candicans* and *L. divaricata* degradation is manifested at the mature stage of the pollen grain. Lattar et al. (2012) suggested that the persistence of the tapetum in *L. divaricata*, would be associated with the formation of a new wall. This new wall has a loose matrix with electron-dense inclusions and numerous invaginations coated by the plasmalemma, increasing its contact surface with the anther locule. Hence, the tapetal cells of *L. divaricata* acquire features characteristic of transfer cells. The same phenomenon might be happening in *L. candicans*, which presents a persistent tapetum very similar to that of *L. divaricata*; this suggests that, so far, the persistent tapetum would be a character exclusive of the genus *Luehea*.

The study of orbicules is of great interest in Spermatophyta, because they are present in 314 species of 72 families of Angiosperms (Huysmans et al. 1998; Galati et al. 2010); moreover, their function is unknown. According to Pacini (1990),

Pacini & Franchi (1993) and Huysmans et al. (1998), one of the main features of the secretory tapetum is the production of orbicules. The orbicules can be defined as corpuscles of various sizes (0.14–20 µm) that react similarly to pollen exine in staining, autofluorescence and resistance to acetolysis. The orbicules are coating the inner surface of the anther locule, often attached to a peritapetal or tapetal membrane (Galati 2003; Galati et al. 2010). In the subfamily Grewioideae the presence of these corpuscles is recorded for the first time in *L. divaricata* (Lattar et al. 2012). According to the results of this work, within Grewioideae the orbicules of the species studied present differences in size and distribution on the tapetal membrane. The variation in orbicule size within a single species was found in *H. popayanensis* and *T. semitriloba*, whereas in *L. candicans* and *L. divaricata* the size is approximately constant. Variation in orbicule size was observed in species of *Euphorbia* (El-Ghazaly 1989; El-Ghazaly & Chaudhary 1993) as well as in species of the tribe Cinchonoideae, Rubiaceae (Huysmans et al. 1997). In *H. popayanensis* and *T. semitriloba* the orbicules become fused and form small aggregates; this common character is shared with *C. insignis*, Bombacoideae (Galati & Rosenfeldt 1998) and with other species of other Angiosperm families, such as *Eryngium pandanifolium* Cham. & Schltld. (Apiaceae), *Myosotis azorica* L. (Boraginaceae) and *Oziroea acaulis* (Baker) Speta (Asparagaceae) (Galati 2003). The orbicules of the species studied in this work, according to their external and internal morphology, were grouped into two types. Therefore, so far we can define the orbicules of Grewioideae as spherical corpuscles, with smooth surface and with a central core, as occurs in the genus *Luehea*, or with irregular surface with small verrucae without a central core, as in *Heliocarpus* and *Triumfetta*. Orbicules with a central core have been described in *C. insignis* (Galati & Rosenfeldt 1998), *Q. asymmetrica* and *Q. cordata* (Nilsson & Robyns 1974), species belonging to the subfamily Bombacoideae. On the other hand, *Q. ochocalyx*, Bombacoideae (Nilsson & Robyns 1974), *T. europea*, *T. plataphyllos*, Tilioideae (Chambers & Godwin 1961) do not

have a central core. However, in *A. pictum* (Strittmatter et al. 2000) and *M. malvifolium* (Galati et al. 2007), Malvoideae, the orbicules are pyriform without a central core. It would be interesting to extend this type of study to other species to check the variability in shape, size and distribution of the orbicules within Grewioideae.

The characters analysed in this paper, such as characteristics of epidermal cells, number of nuclei in the tapetal cells, persistence of tapetum, moment of development of the fibrillar thickenings of endothecium, and presence or absence of orbicules, support the inclusion of *Luehea* in the Grewieae tribe, and the genera *Corchorus*, *Heliocarpus* and *Triumfetta* in the Apeibeae tribe (Brunken & Muellner 2012). *Heliocarpus popayanensis* and *T. semitriloba* were found to share similar embryological characters, such as epidermal cells morphology, uninucleate tapetal cells and development of fibrillar thickenings at the free microspore stage of endothecium. These characters support the molecular studies and indicate that *Heliocarpus* and *Triumfetta* would be phylogenetically close genera (Alverson et al. 1999; Brunken & Muellner 2012). On the other hand, the characters that support the inclusion of *Luehea* in the tribe Grewieae are: late disintegration of the cell walls of the microspore mother cells, late differentiation of the endothecium thickenings (pollen grain stage), plurinucleate tapetal cells and persistent secretory tapetum.

Concerning the obtained results, additional similar studies in the other subfamilies must be considered to evaluate the phylogenetic relationships among them, as well as intergeneric and/or interspecific relationships within the family Malvaceae. Given the extensive shifts in the classification of the Malvaceae by molecular systematics, the collecting of complementary morphological data remains a priority. Floral development and embryological studies are still lacking for most of the taxa, and are needed for a fuller understanding of the group.

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