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Ovule and megagametophyte development in selected species of Apeibeae and Grewieae (Malvaceae–Grewioideae) from South America and its systematic implications

Elsa Lattar[A,B,D], *Beatriz Gloria Galati*[C] *and María Silvia Ferrucci*[A,B]

[A]Instituto de Botánica del Nordeste (IBONE-UNNE-CONICET), C.C. 209, W3400CBL, Corrientes, Argentina.
[B]Cátedra de Morfología de Plantas Vasculares, Facultad de Ciencias Agrarias (FCA-UNNE), W3400CBL, Corrientes, Argentina.
[C]Cátedra de Botánica General, Facultad de Agronomía, Universidad de Buenos Aires (FAUBA), C1417DSE, Ciudad Autónoma de Buenos Aires, Argentina.
[D]Corresponding author. Email: elsilattar@gmail.com

Abstract. This is the first embryological report of the Grewioideae subfamily, which is meant to contribute to the characterisation of the genera *Corchorus*, *Luehea* and *Triumfetta*. Ovule and female gametophyte development in *C. argutus*, *L. divaricata* and *T. semitriloba* was analysed. The ovules of all species are anatropous, bitegmic and of crassinucellate mixed type. The micropyle of the studied species is formed by the outer integument (exostome). The ovule of *L. divaricata* differs from those of the other two species because the chalazal tissue expands forming a cap, which gives rise to a wing in the seed. All species present one hypostase. The megaspore mother cell gives rise to a linear megaspore triad in *C. argutus* and *L. divaricata*, whereas in *T. semitriloba*, triads and diads can be observed in the same ovule. The chalazal megaspore develops a seven-celled and eight-nucleate female gametophyte corresponding to the Polygonum type. The synergids of *L. divaricata* have hooks and a conspicuous filiform apparatus. The antipodal cells in *C. argutus* are persistent, whereas in the other species, they are small and ephemeral. The embryological characters are compared with those of other taxa within the family and the megagametophyte formation in these species is discussed.

Additional keywords: callose, *Corchorus*, female gametophyte, hypostase, *Luehea*, *Triumfetta*.

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Introduction

Malvaceae Juss. is a cosmopolitan family that comprises 243 genera and 4300 species (Bayer and Kubitzki 2003). Morphological, molecular and biogeographical studies have 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 and Kubitzki 2003). Grewioideae Dippel is considered a basal and monophyletic subfamily that comprises 25 genera and 700 species distributed in tropical regions of the New and the Old Worlds (Bayer and Kubitzki 2003). Brunken and Muellner (2012) proposed the subdivision of Grewioideae into two tribes, Grewieae and Apeibeae, on the basis of molecular and morphological data.

Studies on the development of the megasporogenesis and megagametogenesis in the subfamily Grewioideae and the family Malvaceae are scarce, despite the high number of species of this family (Johri *et al*. 1992). The first contributions in the family Malvaceae described the type of ovule as anatropous, hemi-anatropous or campylotropous. According to the nucellus development, the ovule is crassinucellate type (Rao and Rao 1952; Davis 1966; Johri *et al*. 1992). The micropyle is composed of the outer integument in *Corchorus acutangulus* L. and *Triumfetta rhomboidea* Jacq. (Rao and Rao 1952), only by the inner integument in some species of *Entelea* R.Br. (Johri *et al*. 1992) or by both integuments in species of *Muntingia* L. and *Tilia* L. (Johri *et al*. 1992). The development of female gametophyte was described as Polygonum type (Davis 1966; Johri *et al*.1992) and the megaspore mother cell undergoes meiotic division, giving rise to linear or occasionally T-shaped megaspore tetrads (Banerji 1941; Venkata Rao 1953, 1954; Tang and Pan 1994).

The aim of the present work was to characterise the ontogenetic sequences of the ovule and female gamethophyte in representatives of Grewioideae (*Corchorus argutus* Kunth, *Triumfetta semitriloba* Jacq. (Grewieae) and *Luehea divaricata* Mart. (Apeibeae). The study intends to extend the embryological knowledge to the subfamily and to provide data of taxonomic value for the studied genera.

Materials and methods

Floral buds and open flowers were fixed with formalin–acetic acid–alcohol (FAA). For anatomical analyses, permanent slides were prepared by processing the fixed material. Samples of gynoecia were dehydrated with histological dehydrating BIOPUR ® SRL (Gonzalez and Cristóbal 1997) and infiltrated in paraffin Histoplast ® (Biopack, Buenos Aires, Argentina) according to Johansen (1940). The material was sectioned transversely and longitudinally (10–12-µm thickness) with a rotary microtome (Microm, Walldorf, Germany). Sections were stained in a safranin–astra blue combination (Luque *et al.* 1996) and mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). The serial sections were examined under a Leica DMLB2 (Leica, Wetzlar, Germany) light microscope equipped with a digital camera (Canon Power Shot S 50 AIAF, Canon, Tokyo, Japan).

Callosic walls were 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 Zeiss (Germany) Axioplan epifluorescens microscope.

Herbarium material of *C. argutus*, *L. divaricata* and *T. semitriloba* are deposited at the Instituto de Botánica del Nordeste herbarium (CTES), Corrientes, Argentina.

Examined material

Corchorus argutus. ARGENTINA. Prov. Misiones. Dpto Eldorado, 06 Apr. 2009, *Lattar E. & H. Keller 6* (CTES).

Luehea divaricata. ARGENTINA. Prov. Corrientes. Dpto Corrientes, 05 Mar. 2009, *Lattar E. & M. S. Ferrucci 5* (CTES). Prov. Misiones. Dpto. Apóstoles, camino a Azara, 13 Dec. 2011, *Miguel et al. 25* (CTES).

Triumfetta semitriloba. ARGENTINA. Prov. Corrientes, Dpto Corrientes. 04 Mar. 2009, *Lattar E. 4* (CTES).

Results

Ontogeny and general characteristics of the ovule

The mature ovule is anatropous and bitegmic. The inner integument is shorter and does not delimit an endostome (Fig. 1*a–c*). This character is less notable in *L. divaricata*, where the inner integument grows up to the proximity of the exostome (Fig. 1*b*) and, in turn, in *C. argutus* the inner integument is shorter than in *T. semitriloba* (Fig. 1*a, c*). In all the species, the micropyle is formed only by the outer integument (exostome) (Fig. 1*a–c*).

Ovule primordia are initiated by periclinal divisions in the second layer of the placenta. Both integuments have dermal origin (Fig. 1*d*). The inner integument is initiated first as a ring of cells at the base of the nucellus, and does not completely cover the nucellus in the mature ovule. Immediately after that, the outer integument is initiated and grows faster than the inner integument. The nucellus is reduced and differentiates into a primary archesporial cell, which is conspicuous by its larger size (Fig. 1*c*). The archesporial cell usually undergoes mitotic division, resulting in a bottom cell that generally acts as the megaspore mother cell and a top cell that divides repeatedly, giving rise to several nucellar cell layers located in subdermal position (Fig. 1*d*). Moreover, the nucellar epidermal cells divide periclinally to form two to five cell layers, giving rise to a nucellar cap (Fig. 1*c*). Nucellar cap cells of *T. semitriloba* and *C. argutus* accumulate abundant starch grains (Fig. 1*a, c*).

The ovule of *L. divaricata* differs from that of the other species because the chalazal tissue expands forming a cap, which gives rise to a wing in the seed (Fig. 1*b*). Moreover, the ovule of all species has an hypostase. In *L. divaricata*, the hypostase cells present thickened primary walls with callose that stain more intensely with astra-blue, separating from the rest of the nucellus cells (Fig. 1*b*). In contrast, in the remaining two species, the cells of this tissue are separated only when are stained with aniline blue and observed with an epifluorescens microscope because their walls contain callose (Fig. 2*b, f*). *Triumfetta semitriloba* presents a hairy obturator (Fig. 1*a*).

Megasporogenesis

The megaspore mother cell divides meiotically, giving rise to a triad of megaspores in *C. argutus* and *L. divaricata* (Fig. 2*a, b*). In *T. semitriloba*, the archesporic cell can be divided, giving rise to two megaspore mother cells (Fig. 2*c, d*). Generally, one of them is divided meiotically, originating a triad of megaspores, and the other results in a diad (Fig. 2*e*). Only in *T. semitriloba*, callosic walls are formed between the megaspores (Fig. 2*f*), whereas in the other species, callose was not identified during the megasporogenesis (Fig. 2*b*). In all species, the two micropylar megaspores degenerate and the chalazal one develops into the megagametophyte.

Megagametogenesis

The functional chalazal megaspore is characterised by its larger size, a denser cytoplasm and a larger nucleus with conspicuous nucleolus. This cell undergoes three successive mitotic karyokineses (Fig. 3*a*) and the result is an eight-nucleate embryo sac. After this stage, the coenocytic megagametophyte becomes cellular. This process occurs simultaneously at the micropylar and chalazal poles. The female gametophyte has an egg cell, two synergid cells, a central cell with two polar nuclei and three antipodal cells (Figs 3*b–d*, 4*a–d*).

The nucleus of the synergids is usually polarised towards the micropylar end and the vacuole is polarised towards the chalazal end (Figs 3*b–d*, 4*a–d*). In *L. divaricata*, the synergids have hooks and a filiform apparatus well developed (Figs 3*b*, 4*b*). The egg cell is vacuolated and its nucleus occupies a chalazal position (Fig. 3*b–d*). The central cell is highly vacuolated, with two polar nuclei in all the studied species (Fig. 3*b–d*). The antipodal cells have dense cytoplasm. In *C. argutus*, these cells persist in the mature megagametophyte (Figs 3*c*, 4*d*), whereas in the other species, they are smaller and ephemeral (Fig. 3*b, d*).

Discussion

The most useful characters to describe the studied species are the megaspore tetrad arrangement, the presence or absence of callose in the megagametogenesis, the hypostase, the type of megagametophyte development and the number and persistence of the antipodal cells. These characters are summarised and compared with those known for other species of Malvaceae in Table 1.

Ovule morphology

Embryological studies addressing the structure of the ovule and megagametophyte development in the subfamily Grewioideae are scarce. Rao and Rao (1952) described the structure of ovule

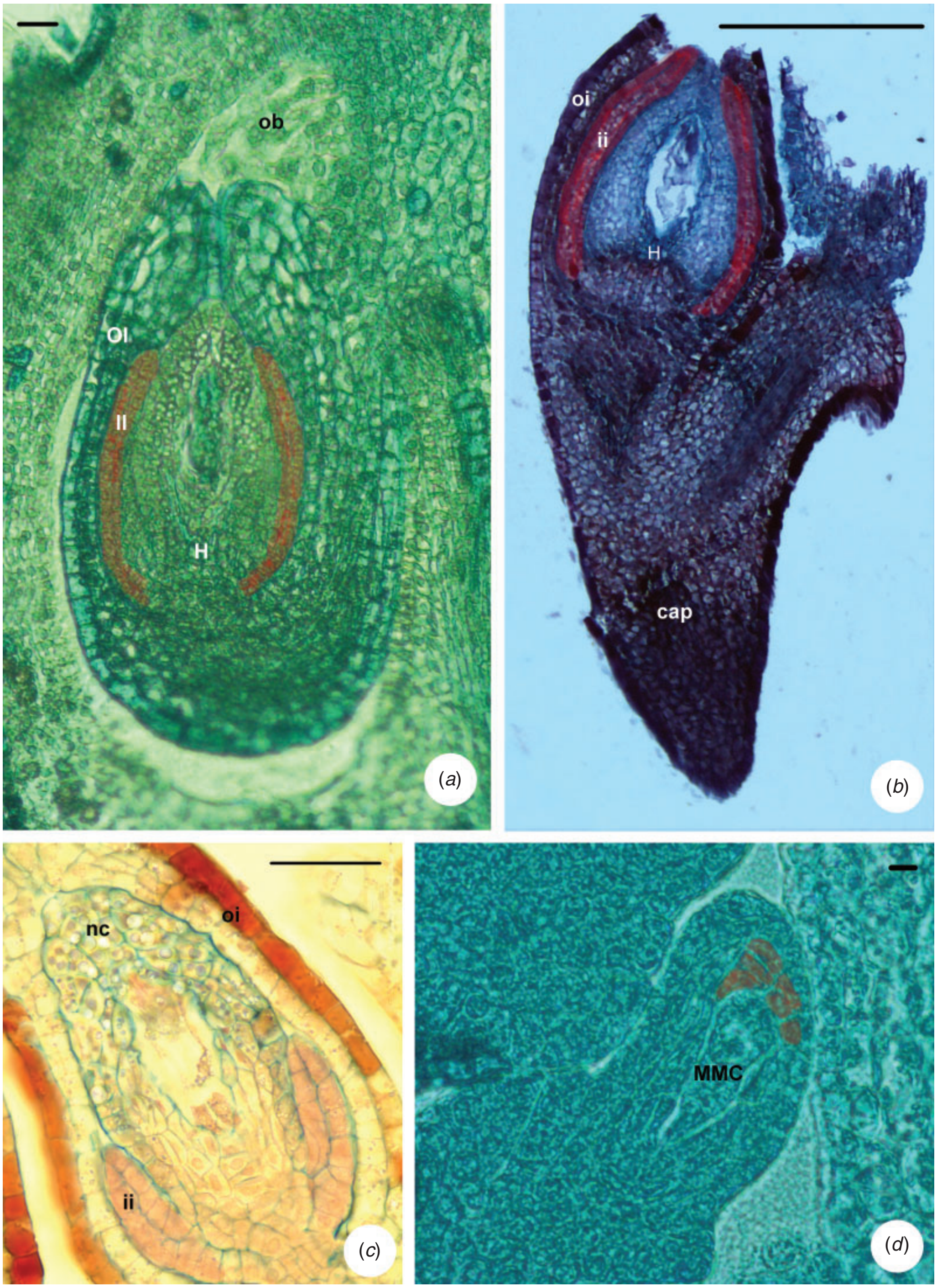

Fig. 1. Ovule morphology observed with brightfield microscope. (*a–c*) General aspect of mature ovules with the inner integument highlighted in red. (*a*) *Triumfetta semitriloba*. (*b*) *Luehea divaricata* showing the vascular bundle and the cap. (*c*) *Corchorus argutus.* (*d*) Details of ovule primordium of *C. argutus*, showing the nucellar cell layers resulting from mitotic division of the archesporial cell (red colour). ob, hairy obturator; oi, outer integument; ii, inner integument; H, hypostase; nc, nucelar cap; and MMC, megaspore mother cell. Scale bars = 20 µm (*a*), 200 µm (*b*), 50 µm (*c*) and 5 µm (*d*).

as anatropous, bitegmic and crassinucellate in *C. acutangulus* and *T. rhomboidea*, which is in agreement with the characteristics observed in the studied species here. These characters are shared with *Eriodendron anfractuosum* DC., *Pachira aquatica* Aubl., *Adansonia digitata* L., *Ochroma lagopus* Sw. (Venkata Rao 1954) and *Ceiba insignis* (Kunth) Gibbs and Semir (Galati and Rosenfeldt 1997) belonging to Bombacoideae and *Burretiodendron kudiifolium* Y.C.Hsu et R.Zhuge (Tang 1998) and *Eriolaena candollei* Wallich (Tang *et al*. 2009), both species included in Dombeyoideae.

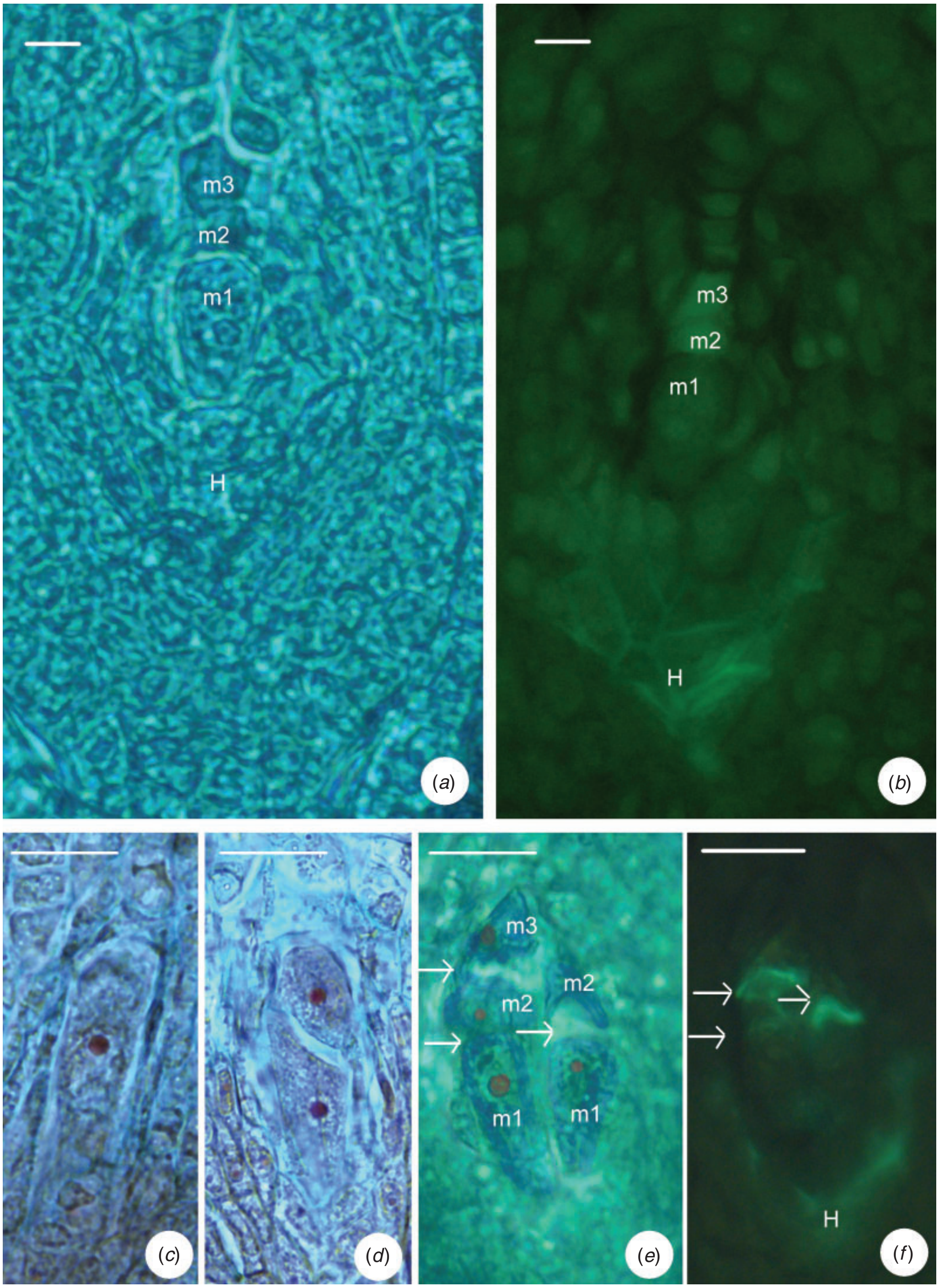

Fig. 2. Megasporogenesis. (*a*, *b*) *Corchorus argutus.* (*a*) Detail of triad of megaspores (m1, m2, m3) observed with brightfield microscope. (*b*) Detail of megaspore triad (m1, m2, m3) and hypostase observed with epifluorescens microscope. (*c–f*) *Triumfetta semitriloba*. (*c*, *d*) Brightfield microscope. (*c*) Detail of the archesporial cell. (*d*) Division of the archesporial cell. (*e*) Detail of megaspore triad (m1, m2, m3) and megaspore dyad (m1, m2) in the same ovule. (*f*) Detail of callosic walls between the megaspore triad and the megaspore dyad (arrows) and the hypostase. H, hypostase. Scale bars = 5 µm (*a–f*).

Triumfetta semitriloba has a hairy obturator. This character has been described previously for *T. rhomboidea* (Rao and Rao 1952).

In the studied species, the nucellar epidermis divides periclinally and gives rise to a nucellar cap. The presence of a nucellar cap was mentioned previously by Donato (1991) and Galati and Rosenfeldt (1997) for *Ceiba insignis*. Because the nucellar layers above the megaspore mother cell (MMC) have different origin (daughter cells of MMC and of epidermal cells), the ovule can be considered crassinucellate mixed.

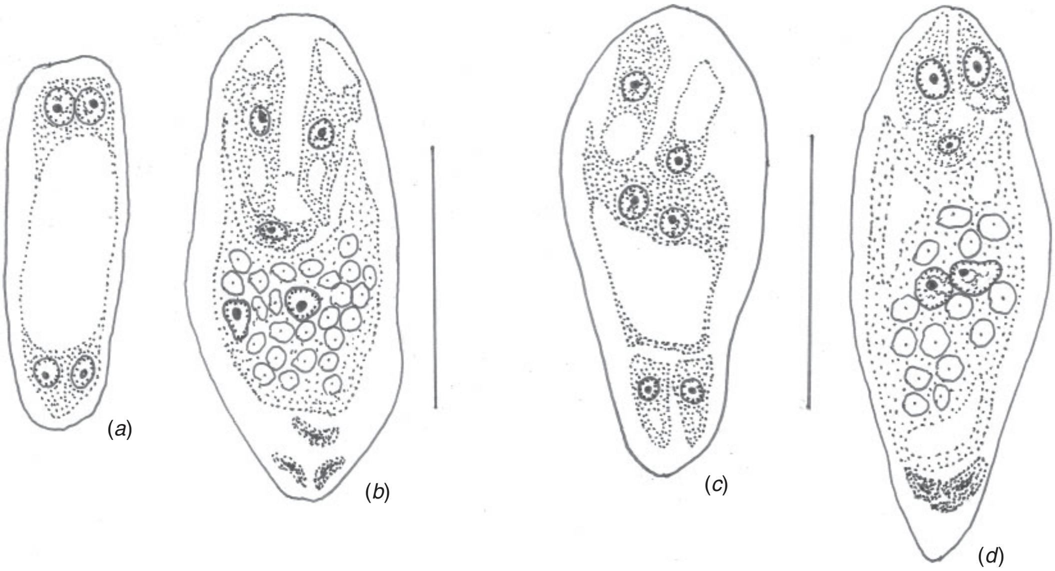

Fig. 3. Megagametophyte. (*a*, *b*) *Luehea divaricata.* (*a*) Tetranucleate embryo sac. (*b*) Mature megagametophyte with abundant starch grains in the central cell. (*c*) *Corchorus argutus*: mature megagametophyte, showing the egg apparatus observed in side face. (*d*) *Triumfetta semitriloba*: mature megagametophyte with abundant starch grains in the central cell. Scale bars = 25 µm (*a*, *b*) and 50 µm (*c*, *d*).

Davis (1966) and Johri *et al*. (1992) suggested that the ovules in Malvaceae can be anatropous or hemianatropous. However, Bayer and Kubitzki (2003) indicated that the ovule in some species of Grewioideae is anatropous, whereas in Malvoideae it is campylotropous; however, these authors did not indicate the species or genera that possess each type of ovule.

Integument

The number of ovule integuments is a very important embryological feature (Sporne 1969; Philipson 1974; Dahlgren 1975, 1980). The ovules generally have one (unitegmic) or two (bitegmic) integuments. The bitegmic ovule is the most common and primitive condition (Johri *et al*. 1992). Several authors, such as Sporne (1969) and Johri *et al*. (1992), have indicated that the evolutionary changeover from bitegmic to unitegmic must have taken place many times.

According to Reinheimer and Kellogg (2009) and Endress (2011), the inner integument of angiosperms is probably homologous to the single integument in their gymnospermous ancestor. However, the outer integument may have been derived from the wall of a cupule and a reduction of ovule number to one per cupule in angiosperm ancestors.

Johri *et al*. (1992) suggested the following three different pathways to unitegmisation: (1) the elimination of one of the two integuments by retardation or complete suppression of its development; (2) the fusion of integumentary primordial when the two integuments are initiated in the same fashion; or (3) integument shifting process, involving the fusion of primordial initials common structure or a shifting of the inner integument or an arrested growth of the inner integument. In *C. argutus*, *L. divaricata* and *T. semitriloba*, the ovule is bitegmic, but the micropyle is formed exclusively by the outer integument, whereas the inner integument arrests its growth. This character matches with previous descriptions of other species of *Corchorus* and *Triumfetta* by Rao and Rao (1952), whereas in *Entelea* R.Br., also belonging to Grewioideae subfamily, the micropyle is formed only by the inner integument (Johri *et al*. 1992), and in *Tilia* L., belonging to Tilioideae subfamily, it is formed by both integuments. However, in some species of *Burretiodendron* Rehder, belonging to Dombeyoideae, the ovule is unitegmic (Davis 1966; Tang 1998). *Ceiba insignis* (Bombacoideae) has a zig-zag micropyle, because the outer integument develops earlier and grows faster than the inner integument (Galati and Rosenfeldt 1997). In the present paper, we consider that the inner integument of the species analysed presents different levels of delayed growth. Studies of ovule ontogeny in more species could provide phylogenetic clues to understand unitegmic evolution within the subfamily. This would be a very interesting character where the plesiomorphic state could be the two integuments.

Hypostase

According to Davis (1966) and Johri *et al*. (1992), the presence of hypostase in Malvaceae is an infrequent character. The hypostase was present in all studied species. These nucellus cells were intimately connected with the embryo sac in each species studied here. According to Venkata Rao (1963), the hypostase acts as a connecting link between the vascular strand and the embryo sac, and helps in the transportation of nutrients. The hypostase in *L. divaricata* is much more noticeable; the cells have thickened walls and dense cytoplasm. These cells could acquire ultrastructural features of transfer cells. Several studies have described transfer cells in ovular tissues as integuments or nucellar cells that surround the female gametophyte (Gunning and Pate 1969; Sumner and van Caeseele 1990; Galati *et al*. 2005).

The cells of the hypostase have thickened primary walls that differ from the rest of the nucellus tissue in *L. divaricata*. In all the species studied, this tissue has callosic walls. It has long been known that callose is hygroscopic in nature and has

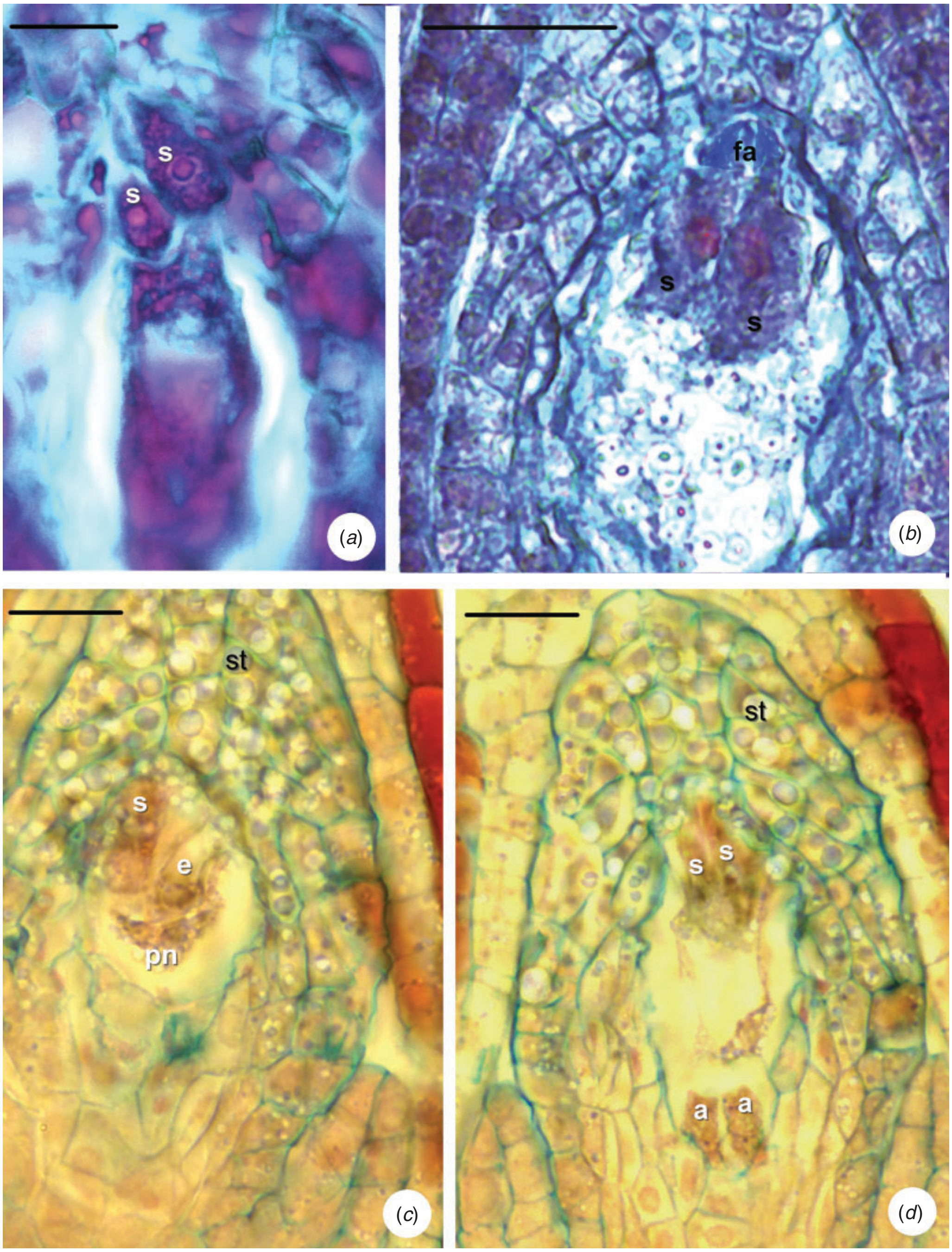

Fig. 4. Mature megagametophyte observed with brightfield microscope. (*a*) *Triumfetta semitriloba*. (*b*) *Luehea divaricata*. (*c*, *d*) *Corchorus argutus*. s, synergids; fa, filiform apparatus; e, egg cell; pn, polar nuclei; a, antipodals cells; and st, starch grains. Scale bars = 25 µm (*a*), 50 µm (*b*) and 12.5 µm (*c*, *d*).

a pronounced ability to absorb water (Bhandari 1984). According to Galati and Rosenfeldt (1997), the hypostase callosic walls may help in the hydric regulation of the embryo sac.

Megasporogenesis

One MMC per ovule was observed in *C. argutus* and *L. divaricata*, whereas in *T. semitriloba*, two MMCs are present in the same ovule. Four to five MMCs have been described for *T. rhomboidea* (Rao and Rao 1952).

Linear or occasionally T-shaped megaspore tetrads are characteristic in species of Malvaceae (Banerji 1941; Venkata Rao 1953; Venkata Rao 1954; Tang and Pan 1994). However, *C. argutus* and *L. divaricata* possess linear triads of megaspores, whereas *T. semitriloba* develops triads and diads in the same ovule.

In most of the species investigated, the localisation of the first callose in the megaspore mother cell wall agrees with the localisation of the active megaspore in the tetrad (Rodkiewicz

1970; Rodkiewicz and Bednara 1976). Callose in the megaspore mother cell wall has not been observed in any of the species studied here. This polysaccharide is present in the transverse walls of the megaspore triad only in *T. semitriloba*, and is absent from the remaining species. The presence of callose in the megasporogenesis process was studied only in *Ceiba insignis* (Bombacoideae) and this character agrees with the observation in *T. semitriloba* in the present paper.

Table 1. A comparative analysis of embryological characters in the species studied
Some species of Malvaceae subfamilies included here are from previous studies

Subfamily	Species	Arrangement of megaspores	Hypostase	Typeof megagametophyte development	Apomixis	Number of antipodal cells	Persistent or ephemeral of antipodal cell	Reference
Grewioideae	*Corchorus argutus*	Linear triad without callose	Present with callosic walls	Polygonum	Absent	3	Persistent	Present paper
Grewioideae	*Luehea divaricata*	Linear triad without callose	Present with callosic walls	Polygonum	Absent	3	Ephemeral	Present paper
Grewioideae	*Triumfetta semitriloba*	Linear triad and diad with callosic walls	Present with callosic walls	Polygonum	Absent	3	Ephemeral	Present paper
Grewioideae	*C. acutangulus*	Linear tetrad	Unconfirmed	Polygonum	Absent	3	Ephemeral	Rao and Rao (1952)
Grewioideae	*T. rhomboidea*	Linear tetrad	Unconfirmed	Polygonum	Absent	3	Ephemeral	Rao and Rao (1952)
Dombeyoideae	*Burretiodendron kydiifolium*	Linear or tetrad	Absent	Polygonum	Absent	3	Ephemeral	Tang (1998)
Dombeyoideae	*Eriolaena candollei*	Linear tetrad	Absent	Polygonum	Absent	3	Ephemeral	Tang *et al.* (2009)
Bombacoideae	*Adansonia digitata*	Linear tetrad	Absent	Polygonum	Absent	3	Ephemeral	Venkata Rao (1954)
Bombacoideae	*Bombax malabaricum*	Linear tetrad	Absent	Polygonum	Absent	3	Ephemeral	Banerji (1941)
Bombacoideae	*Ceiba insignis*	Linear tetrad with callosic walls	Present with callosic walls	Polygonum	Absent	3	Ephemeral	Galati and Rosenfeldt (1997)
Bombacoideae	*Eriodendron anfractuosum*	Linear tetrad	Absent	Polygonum	Absent	3	Ephemeral	Venkata Rao (1954)
Bombacoideae	*Erioteheca pubescens*	Linear tetrad	Absent	Unconfirmed	Present	3	Ephemeral	Mendes-Rodrigues *et al.* (2005)
Bombacoideae	*Pachira aquatica*	Linear tetrad	Absent	Polygonum	Absent	2 or 3	Ephemeral	Venkata Rao (1954)

Female gametophyte development

The development of the female gametophyte in the species studied here coincides with the Polygonum type, which is seven-celled and eight-nucleate type and has developed from the chalazal megaspore of the tetrad. This character agrees with the observations made by Rao and Rao (1952) in *C. acutangulus* and *T. rhomboidea*, and in *Adansonia digitata*, *Burretiodendron kudiifolium*, *Ceiba insignis*, *Eriolaena candollei*, *Eriodendron anfractuosum*, *Ochroma lagopus* and *Pachira aquatica* (Venkata Rao 1954; Galati and Rosenfeldt 1997; Tang 1998; Tang *et al*. 2009).

The antipodal cells show much variation in number and size. The usual position of the antipodal cells is in the chalazal pole of the embryo sac. The antipodal cells of *L. divaricata* and *T. semitriloba* are ephemeral. This character coincides with the observations reported by Rao and Rao (1952) in *C. acutangulus* and *T. rhomboidea*, as well as with observations in *A. digitata*, *B. kundiifolium*, *C. insignis*, *E. candollei*, *E. anfractuosum*, *O. lagopus* and *P. aquatica* (Venkata Rao 1954; Galati and Rosenfeldt 1997; Tang 1998; Tang *et al*. 2009). *Corchorus argutus* has persistent antipodal cells. These cells may be metabolically very active in the transport of nutrients to the embryo sac (Johri *et al*. 1992). In *C. argutus*, the antipodal cells are conspicuous and present in the mature female gametophyte, suggesting their possible nutritive function during embryo development.

The species analysed here can be distinguished by the synergid morphology. In *T. semitriloba* and *L. divaricata*, the synergids are more developed than in *C. argutus* and in *L. divaricata*, they are characterised by the presence of hooks. Rao and Rao (1952), Davis (1966) and Johri *et al*. (1992) suggested that the presence of a well developed filiform apparatus is not common in Malvaceae. In the present work, a conspicuous filiform apparatus was observed in *L. divaricata*. The function of this apparatus and its development has been described in only few species (Van der Pluijm 1964; Mogensen 1972; Johri *et al*. 1992). The morphology of the synergids of *A. digitata*, *B. kundiifolium*, *C. insignis*, *E. candollei*, *E. anfractuosum*, *O. lagopus*, *P. aquatica* and *T. rhomboidea* was not described in detail by the authors (Rao and Rao 1952; Venkata Rao 1954; Galati and Rosenfeldt 1997; Tang 1998; Tang *et al*. 2009). According to the present results, the morphology of the synergids might have taxonomic value.

Conclusions

The present study conducted on ovule ontogeny, megasporogenesis, megagametogenesis and female gametophyte in *Corchorus argutus*, *Triumfetta semitriloba* and *Luehea divaricata* is the first report for these native South

American species. The interesting embryological characters such as the ovule type, the reduction of integument, the megaspore tetrad arrangement, the presence or absence of callose in the megagametogenesis, the hypostase, the type of megagametophyte development, the number, morphology and persistence of the antipodal cells, and the morphology of the synergids would be of importance for phylogenetic studies. It would be interesting to extend this type of study to other species of Malvaceae, so as to elucidate the affinity of the genera and subfamilies within the family.

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