Ultrastructural study of pollen and anther development in *Luehea divaricata* (Malvaceae, Grewioideae) and its systematic implications: Role of tapetal transfer cells, orbicules and male germ unit

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**Abstract**

Developmental processes of microsporogenesis and microgametogenesis in *Luehea divaricata* (Malvaceae, Grewioideae) were analyzed with transmission electron microscopy. The species studied has perfect flowers. The young anthers are bithecal and tetraropangiate; microspore mother cells undergo simultaneous meiosis, forming tetrads with a tetrahedral arrangement. The development of the anther wall conforms to the basic type and the tapetum is secretory. The results highlight the presence of multinucleate tapetal cells, which acquire ultrastructural features characteristic of transfer cells at the young pollen grain stage, and which are associated with the presence of orbicules. Tapetal transfer cells have not been previously investigated in detail for other species of angiosperms; their function during pollen development is discussed. The present work is the first contribution to the knowledge of ultrastructural pollen development in the genus *Luehea*, as well as in the subfamily Grewioideae.

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**Introduction**

Malvaceae Juss. is a cosmopolitan family comprising 243 genera and probably more than 4300 species (Bayer and Kubitzki, 2003). Morphological and molecular evidence has shown that many of the traditional families of the Malvales are monophyletic (Alverson et al., 1998, 1999; Bayer et al., 1999; Judd and Manchester, 1997). Studies of morphological, molecular, and biogeographical data 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 and Kubitzki, 2003; Bayer et al., 1999). The subfamily Grewioideae Dippel would be the first branching lineage; it comprises 700 species in 25 genera distributed in tropical regions of the New and the Old World (Bayer and Kubitzki, 2003). Within Grewioideae, recent studies based on morphological and molecular phylogenetic evidence have proposed a new subdivision into two tribes, Apeibaeae and Grewieae (Brunken and Muellner, 2012).


*Luehea* Willd. is a small American genus comprising 18 species distributed from Mexico to Uruguay and Argentina (Ferrucci, 2005). This genus includes trees or shrubs, with dense stellate indumentum, with medium to large, pink or white flowers. The genus was traditionally ascribed to the tribe Lueheeae, Tiliaeae (Hutchinson, 1967). However, recent studies based on molecular data have placed *Luehea* in the subfamily Grewioideae (Judd et al., 1999; Bayer and Kubitzki, 2003), and in the tribe Grewieae (Brunken and Muellner, 2012).

Due to the limited embryological data available about the subfamily Grewioideae, the first author is conducting embryological studies in species of four genera present in southern South
America: Corchorus L., Heliocarpus L., Luehea Willd. and Triumfetta L. These studies include the analysis of C. argutus Kuntz, C. hirtus L., H. populifolius Kuntz, L. divaricata Mart., L. candidans Mart., and T. semitriloba Jacq. According to morphological and anatomical characters, L. divaricata presents significant differences from the remaining species studied; for this reason, here the processes concerning the microsporogenesis and the microgametogenesis are analyzed in greater detail.

The aim of this work was to investigate the ultrastructural events of pollen and anther development in Luehea divaricata Mart. Furthermore, the details of pollen and anther ontogeny in this species were compared with known embryological patterns of species from the other subfamilies, in an effort to provide further insight into phylogenetic interpretations within Malvaceae.

Materials and methods

Luehea divaricata. ARGENTINA. Corrientes province: Capital department, 05.III.2009, Lattar & Ferrucci 5 (CTES). The voucher specimen was deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES), Argentina. For transmission electron microscopy (TEM) study, anthers at different stages were pre-fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) at 2 °C for 2 h (only anthers have been used due to the size and consistency of floral buds, which prevent correct fixation) and post-fixed in OsO4 at 2 °C in the same buffer for 3 h. Following dehydration in ethanol series, the material was embedded in Spurr’s resin. Ultrathin sections (750–900 nm) were made on a Reichert-Jung ultramicrotome, stained with uranyl acetate and lead citrate (O’Brien and McCully, 1981), observed and photographed in a Philips EM 301 TEM.

Results

Floral morphology

Luehea divaricata has perfect flowers, calyx and corolla being 5-merous, with petals yellowish to red-purple, a yellow base in pre-anthesis, and reflexed, yellowish-white in post-anthesis. Stamens are numerous, the internal ones connate, filaments glabrous except at base. Anthers have a reniform shape; they are bitemphal and tetrasporangiate. Staminodia externally to the stamens are present.

Stages of anther ontogeny

Stage 1: Microspore mother cell (MMC)

The origin of the anther wall corresponds to the basic type (Davis, 1966), i.e., one parietal layer has a common origin with the endothecium, and the other with the tapetal. The young anther wall consists of an epidermis, an endothecium, one middle layer and a secretory tapetum (Fig. 1). At this stage the microspore mother cells (MMC) show a dense cytoplasm, with abundant mitochondria, endoplasmic reticulum of rough type (ERr), plastids and free ribosomes (Figs. 5 and 6). The tapetal cells are characterized by a dense cytoplasm with mitochondria, plastids and free ribosomes (Fig. 7).

Stage 2: Microspore tetrads

Microspore mother cells undergo simultaneous meiosis, forming tetrads with a tetrahedral arrangement (Fig. 2). At this stage, the middle lamella, primary wall, and callose with a low electron density are observed surrounding the microspores (Fig. 8). The primexine is deposited between the callosic wall and the plasmalemma in mature tetrads. The cytoplasm of the tetrad microspore has a high metabolic activity; it is very dense, with an increase of the ERr, plastids with starch, dictyosomes with numerous vesicles and lipid globules (Fig. 9). The multinucleate tapetal cells present thick and compact cell walls and a dense cytoplasm, with numerous mitochondria, abundant ERr and dic-tysomes; numerous vesicles between the plasmalemma and the tapetal cell wall are also observed (Fig. 10).

Stage 3: Free young microspores

Once the callosic wall disintegrates, microspores become isolated from each other. There is no distinctive difference between the cytoplasm of the microspores at this stage relative to the previous stage. The deposition of the exine wall of the microspores begins at this stage. The exine wall is formed by a basal layer, bacula and tectum. The cytoplasm of microspores shows numerous vacuoles, plastids, ERr, mitochondria and amyloplasts (Fig. 11). Tapetal cells have a dense cytoplasm, with abundant ERr and Golgi vesicles. Their walls have a more loose structure than at the previous stage and exhibit orbicules (Fig. 12).

Stage 4: Young pollen grains

The mitosis in the microspore gives rise to a bicellular pollen grain. The pollen grain exine shows the same characteristics as at the microspore stage, but has a thicker basal layer. Tapetal cells present major changes at this stage (Fig. 3). These cells show a very active cytoplasm with numerous organelles, including mitochondria, lipid globules and abundant ERr with cisternae parallel to the cell wall. The plasmalemma of these cells has numerous invaginations; a new cell wall is observed between the plasmalemma and the original cell wall that keeps the same loose structure as at the previous stage, with orbicules included in it. This new wall layer is composed of a fibrillar matrix with electron-dense inclusions, giving characteristics of transfer cells to the tapetal cells (Figs. 13 and 14).

Stage 5: Mature pollen grains

The cytoplasm of the vegetative cell encloses the generative cell. It is almost fusiform and presents a sinuous wall, transparent to electrons. This cell is observed in intimate contact with lobules of the vegetative nuclei. In the cytoplasm of the generative cell small vesicles and some mitochondria are observed, whereas in the vegetative cell, numerous mitochondria, abundant vesicles and amyloplasts are found (Fig. 15). Both the pollen grain wall and the orbicules have completed their development and possess a lower electron density than at previous stages. The pollen wall consists of a thick fibrillar intine composed of two strata of different electron density, a basal layer, bacula and a discontinuous tectum (Fig. 16). The orbicules are sub-spherical and present a central core transparent to electrons, with an appearance approximately triangular in section. These corpuscles are observed on the tapetal membrane (Figs. 4 and 17). Fibrous thickenings in the endothecium cell wall are observed at this stage (Fig. 4).

Discussion

The ultrastructural study of pollen and anther development in L. divaricata presented interesting characters which permit inferring possible phylogenetic relationships in Grewioideae. The most relevant characters were the tapetum type, the tapetal cells characteristics, the persistent tapetum, the endothecium and the orbicules. Table 1 presents a comparison of these characters to those embryological patterns known for species from the other subfamilies of Malvaceae.

Anther wall development

The development of the anther wall in Luehea divaricata corresponds to the basic type (Davis, 1966), which is shared also by
other species of Grewioideae studied, such as Corchorus argutus, C. hirsutus, Luehea candidans, Helicarpus popayanensis, and Triumfetta semitri folia (Lattar, unpublished) and in Dombeyoideae, Excentrodendron hisien nu (Tang et al., 2006). However, Rao and Rao (1952) and Dnyansagar and 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.

**Secretory tapetum**

The tapetum of L. divaricata is secretory; this character is shared with other members of the subfamily Bombacoideae, such as Adansonia digitata, Bombax malabaricum, Eriodendron anfractuosum, Fuchira aquatica, Ochroma lagopus (Rao, 1954), and Ceiba insignis (Galati and Rosenfeldt, 1998). Moreover, Abutilon pictum has a plasmoidal tapetum (Strittmatter et al., 2000) and Modiolastrum malvifolium has an invasive non-syncytial tapetum (Galati et al., 2007), both species belonging to Malvoideae. On the other hand, Dombeyoideae presents different types: Excentrodendron hisien nu has a secretory tapetum (Tang et al., 2006) and Eriolaena candollei has the plasmoidal type (Tang et al., 2009). The secretory type is considered a primitive state, whereas the plasmoidal tapetum has evolved several times among early branching angiosperms (Furness and Rudall, 2001). In Malvaceae, Eriolaena candollei (Dombeyoideae), which exhibits a plasmoidal tapetum, is considered a bridge to Malvoideae subfamily, which presents species with a plasmoidal or invasive nonsyncytial tapetum (Galati et al., 2007; Strittmatter et al., 2000; Tang et al., 2009). The tapetal cells of the species studied here are multinucleate, a character state present also in two species of Bombacoideae: Bombax malabaricum (Rao, 1954) and Ceiba insignis (Galati and Rosenfeldt, 1998). In the species of Malvoideae studied (Galati et al., 2007; Strittmatter et al., 2000) and in Dombeyoideae (Tang et al., 2006, 2009), the tapetal cells are uninucleate, whereas in Adansonia digitata, Eriodendron anfractuosum, Fuchira aquatica, and Ochroma lagopus (Bombacoideae) these cells are binucleate (Rao, 1954) as well as in C. argutus, C. hirsutus, H. popayanensis and T. semitri folia (Grewioideae) (Lattar, unpublished). The multinucleate tapetal cells observed in L. divaricata would be interpreted as an apomorphy state within Grewioideae; this character state is shared with some species of Bombacoideae, the most derived subfamily within Malvaceae. It should be emphasized that in species of the other subfamilies studied, the tapetal cells are uninucleate or binucleate.

**Ultrastructural features of tapetal transfer cells**

The tapetum plays an essential nutritive role in the formation of the pollen grains (Echlin, 1971). In most of the angiosperms, the tapetal cells reach their maximum development at the tetrad stage, after which a series of degradation changes result in course of their collapse (Raghavan, 1997). In L. divaricata, the tapetum persists to the stage of young pollen grains. Therefore, it appears that here the persistence of the tapetum would be related to important secretory functions involved in pollen development. The ultrastructural features of the tapetal cells of L. divaricata are particular and have previously not been described for other angiosperms. At the microspore tetrad stage, these cells have compact and remarkably thick walls. At the beginning of the free microspore stage, these walls are persistent, and show a lax structure, allowing the extrusion of the orbicules. These corpuscles are fully developed.
and in contact with the locular fluid before the tapetal walls are completely degraded. This is not a common feature, and before it has only been described for *Jacaranda mimosaefolia* D. Don, Bignoniacaeae (Galati and Strittmatter, 1999). These authors mentioned the presence of a loose wall where Ubisch bodies or orbicules can be released, a feature that was also observed in *L. divaricata*. The degradation of the tapetal walls occurs normally during the free microspores stage (Raghavan, 1997). However, in *Jacaranda mimosaefolia* it occurs at the bicellular pollen grain stage (Galati and Strittmatter, 1999), whereas in *L. divaricata* it occurs when the pollen grain is mature. Moreover, these cells develop a new wall between the plasmalemma and the original cell wall at the young pollen grain stage. This new wall has a loose matrix with electron-dense inclusions and numerous invaginations coated by the plasmalemma. As a result of this, the latter considerably increases its contact surface with the anther locule. These features give these cells characteristics of transfer cells, which generally are related to the short-distance transport of solutes (Gunning and Pate, 1969; Jhori, 1984). According to Gunning and Pate (1969), there are cytoplasmic discontinuities between the sporophyte and the developing microspores and, in view of the nutritional interrelationships, it is not surprising to find transfer cells in the tissues involved. So far there is only one record of tapetal cells with characteristics of “transfer cells” for Spermatophyta, described for *Paeonia tenuifolia* L. (Paeoniaceae) by Marquardt et al. (1968). In this species, a new cell wall with invaginations is formed, but the original wall of the tapetal cells is much thinner than that in *L. divaricata*. These authors did not relate the different ontogenetic stages of the tapetal cells to pollen grain development, nor did they mention the presence of orbicules. Numerous mitochondria and a conspicuous endoplasmic reticulum usually accompany the development of invaginations of the transfer cell wall (Gunning and Pate, 1969). Such ultrastructural features can be observed also in the tapetal cells of *L. divaricata*. Studies conducted in wheat xylem transfer cells and in corn endosperm transfer cells using field emission scanning electron microscopy and immunofluorescence confocal microscopy showed that the parallel organization of cellulose microfibrils in flange wall ingrowths was similar to that in secondary walls (Talbot et al., 2007). The new wall in the tapetal cells of *L. divaricata* might be considered a secondary wall, because it is deposited inside original primary walls and it presents a differential electron density. Numerous inclusions are observed inside
the cell wall of tapetal transfer cells of *L. divaricata*, which have the same electron density as the precursors of sporopollenin deposited in the microspores exine and in the developing orbicules. Therefore, these cells might facilitate the release of sporopollenin precursors.

**Development and ultrastructure of the orbicules**

According to Paccini (1990), Paccini and 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 variable size (0.14–20 μm). Orbicules exhibit the same reaction in staining reactions, autofluorescence and resistance to acetylation as pollen exine (Galati, 2003). The beginning of the formation of the orbicules, with the release of a lipid globule or pro-orbicule between the plasmalemma and the tapetal cell wall, was observed in L. divaricata at the tetrad stage. The orbicules of L. divaricata are spherical to sub-spherical, present a central core which is transparent to electrons, are approximately triangular in section, and have a relatively thick wall of sporopollenin. Orbicules with a central core were described for some species of the subfamily Bombaciidae, such as Quararibea asymmetrica and Q. cordata (Nilsson and Robyns, 1974) and Ceiba speciosa (Galati and Rosenfeldt, 1998). Moreover, solid orbicules have been reported for species of the subfamily Malvaceae, such as Abutilon pictum (Strittmatter et al., 2000) and Modiolastrum malviflorum (Galati et al., 2007). The presence of orbicules in the subfamily Grewioidae is reported here for the first time. According to Galati (2003), the development of the orbicules is simultaneous with the formation of the pollen exine. These corpuscles are products of the tapetal cell activity, and the ERr is the organelle involved in this process.

**Endothecium**

The endothecium is one of the layers of the anther wall that undergo most modifications throughout pollen development. In L. divaricata, the presence of fibrous thickenings in the endothecium cell wall is observed at the stage of mature pollen grains, similarly as in Pterospermum and Excentrodendron (Tang et al., 2006). On the other hand, the differentiation of those thickenings occurs at the stage of free microspores in species of Corchorus, Heliocarpus and Triumfetta (Lattar, unpublished) and Excentrodendron hsienmu (Tang et al., 2006). According to the classification of Manning (1996), the fibrous thickenings observed in L. divaricata correspond to the annular type, a character common to other members of Malvaceae.

**Male germ unit (MGU)**

The generative cell of L. divaricata has a sinuous wall that is in close contact with invaginations of the vegetative nucleus. This reminds about the MGU described by Dumas et al. (1985), Hu and Yu (1988), Hu (1990), Rougier et al. (1991), Tian et al. (1998), and Wagner (1990). These authors demonstrated that the cells involved in the MGU can be very different in size, shape, or content of organelles. In the species studied here, both cells exhibit differential ultrastructural features.

**Systematic implications**

The embryological characters analyzed in this study as the persistence of tapetal cells during pollen grain development, and multinucleate tapetal cells that acquire ultrastructural features characteristic of transfer cells support the inclusion of Luehea in the Grewiaceae tribe, within Grewioidae, as they were circumcribed based on morphological and molecular data (Brünken and Muellner, 2012). On the other hand, the embryological characters mentioned above would separate Luehea divaricata from the remaining species studied, present in the Southern Cone of South America, Corchorus argutus, C. hirtus, Helicarpus popayanensis, and
Triumfetta semitri lob a (Lattar, unpublished), genera which belong to the Apeibeae tribe. The secretory tapetum, common to the Grewioidae species studied, supports the basal position of this subfamily within Malvaceae (Bayer and Kubitzki, 2003).

Conclusions

The ultrastructural study of pollen grain and anther development of L. divaricata is the first contribution to these aspects concerning the subfamily Grewioidae. This species presents embryological characters which cannot be recognized in others species of Malvaceae. The secretory and persistent tapetum, with multinucleate cells that acquire ultrastructural features characteristic of transfer cells, would be an adaptive character in L. divaricata, because it would be directly related to the large number of stamens and to pollen production. Under phylogenetic aspects, the tapetal multinucleate cells are interpreted as an apomorphic state within Grewioidae. The presence of orbicules is reported here for the first time for Grewioidae.

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