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Microsporogenesis and male gametogenesis in *Jatropha curcas* L. (Euphorbiaceae)[1]

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Key words: Euphorbiaceae, *Jatropha curcas*, male gametogenesis, microsporogenesis, Physic nut.

The use of *Jatropha curcas* L. as a source for biodiesel has generated substantial interest in this species (Gübitz et al. 1999). In addition to this potential, *J. curcas* has also been used for insect pest control, fodder, fertilizer, and has the potential to yield new medicines (Duke 1983, Openshaw 2000, Lin et al. 2004). Although recent research has investigated the effective chemical constitutes, their toxicity and pharmacological activities (Naengchomnong et al. 1970, Nath and Dutta 1991, Van et al. 1995, Fagbenro et al. 1998), there have been few studies of its structure and development (Kapil 1994). Apart from Liu et al.'s (2006) study of laticifer anatomy, Liu et al.'s (2007) study of microspore development, Bahadur et al.'s (1998) study of pollen structure, and Puangpaka & Thaya's (2003) investigation of karyology, almost nothing is known about the basic structure and development of the species. This paper offers a partial remedy to this situation by providing an account of anther wall, tapetum, microspore and male gamete development in *J. curcas*.

The Euphorbiaceae s.l. are a diversified family, consisting of approximately 300 genera and 8000 species (Radcliffe-Smith 2001). Their classification has been studied for 150 years by a number of taxonomists. Webster (1975) divided the family into five subfamilies: Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae. Recent molecular studies show that the Euphorbiaceae defined in this sense are not monophyletic, and the Phyllanthoideae and Oldfieldoideae have been raised to family rank (APG 2003). Thus, only three subfamilies (Acalyphoideae, Crotonoideae and Euphorbioideae) remain in the Euphorbiaceae s.s.

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Jatropha L. belongs to the subfamily Crotonoideae and consists of approximately 175 species, with eight species cultivated in China. *Jatropha curcas* is the most widespread of these. It is native to Mexico and Central America, but is cultivated in many other Latin American, Asian and African countries. In China it is cultivated in Guangdong, Guangxi, Yunnan, Sichuan, Guizhou, Taiwan, Fujian and Hainan provinces (Chen and Zheng 1987, Chang 1996).

Male gametogenesis in the Euphorbiaceae is very heterogeneous. Both glandular and amoeboid tapetums have been described from the family, and the pollen grains may be two- or three-celled at shedding (Johri et al. 1992). Neither pollen nor tapetal cell development has been previously described in *Jatropha curcas*.

Materials and Methods. The material of *Jatropha curcas* was collected from a cultivated population in South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China. A voucher specimen is deposited in the South China Botanical Garden Herbarium (IBSC: Liu Huanfang 003).

Anthers prepared for light microscopy were fixed in formalin acetic alcohol (FAA) and stored in 70% ethanol. They were later infiltrated, and embedded in paraffin (Berlyn & Miksche 1976). Serial sections (8 μm thick) were cut on a Leica RM 2016 rotary microtome, stained in Safranin-Fast Green (Johansen 1940), and observed and photographed with an Olympus-AX70 light microscope fitted with an Olympus-DP50 digital camera.

Anthers for light and transmission electron microscopy (TEM) were dissected from fresh flowers, and fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. Air was removed under vacuum. Following fixation the flowers were washed in phosphate buffer, fixed in 1% aqueous osmium tetroxide for two hours at room temperature, washed again in buffer, and dehydrated in an ethanol series. Anthers were embedded in Epon 812 resin. Sections (2 μm) were cut using a LKB-11800 rotary microtome, stained with toluidine blue, and observed and photographed with the Olympus-AX70 light microscope fitted with an Olympus-DP50 digital camera. Ultra-thin sections (80 nm) for TEM were cut using a Leica-Ultracut S ultramicrotome, stained with uranyl acetate/lead citrate (Reynolds 1963) and observed with a JEM-1010 transmission electron microscope at 90 KV (Sajo et al. 2005).

Results. *Jatropha curcas* is monoecious with ten tetrasporangiate stamens in each male flower. Each stamen has four microsporangia arranged in two thecae (Fig. 1A). The young anther wall consists of an epidermis, an endothecium, two or three middle layers and one layer of glandular tapetal cells, each with two or four nuclei. The mature anther wall consists of an epidermis and a highly fibrous, thickened endothecium. Wall development is of the dicotyledonous type (Davis 1966). The cytokinesis following meiosis is simultaneous, producing tetrahedral tetrads. The mature pollen grains are two-celled at anthesis, and the generative cell is spindle shaped.

MICROSPOROGENESIS. The sporogenous cells, produced by the archesporia, are polygonal, larger than the cells of the secondary parietal layer, and have large, obvious nucleoli (Figs. 1B, C). When the anther is 0.5–0.7 mm long, the sporogenous cells give rise to the much larger microspore mother cells, which begin to develop callosic walls (Fig. 1D). The microspore mother cells are in the process of meiosis when the anther is 0.70–0.78 mm long (Fig. 1E). Post-meiotic cytokinesis is of the simultaneous type. Post meiotic tetrads are tetrahedral or irregular tetrahedral, and are surrounded by thick callosic walls (Figs. 1F, 2A).

The development of the microspore mother cells is synchronous within a microsporangium, however in a single anther two or three meiotic stages can be observed at the same time in different microsporangia (Fig. 2B).

MICROGAMETOGENESIS. Free microspores are released into the anther locule by the dissolution of the callose walls when the anther is about 0.83–1.0 mm long. Each microspore is circular, with dense cytoplasm and a prominent and centrally located nucleus (Fig. 2C). The microspores enlarge faster than the anther locule, and become irregularly shaped in the process (Fig. 2D). Later, the anther locule enlarges and the microspore becomes circular again (Fig. 2E, F). By this stage, the intine has developed (Fig. 2E) and a central vacuole develops in each microspore (Fig. 2F). The

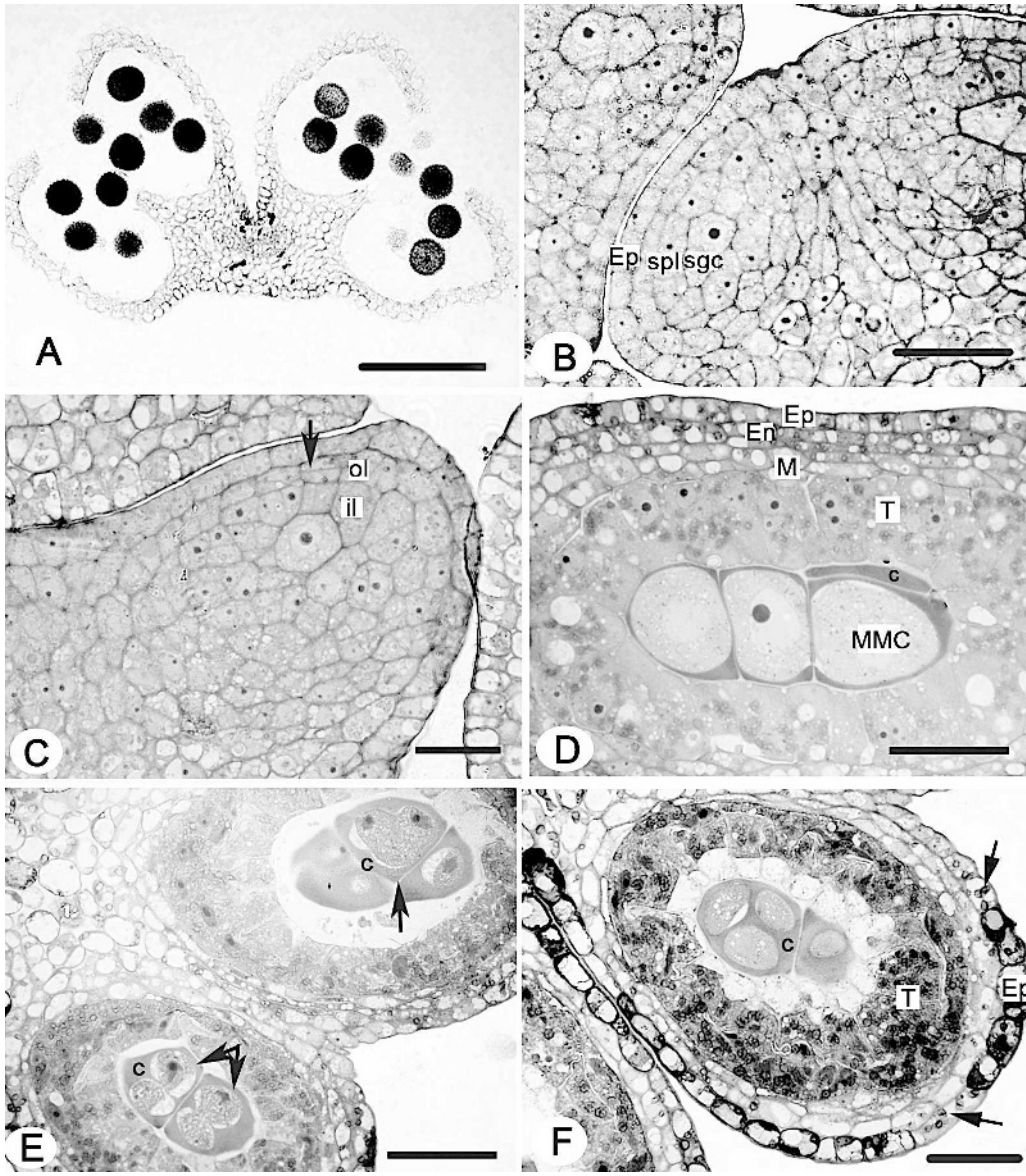

FIG. 1. Light micrographs of anther structure and the early stages of microsporogenesis. A: Four microsporangia at the time of dehiscence. B: Sporogenous cells (sgc) and secondary parietal layers (spl) beneath the protoderm (Ep). C: Cell divisions in the outer secondary parietal layer (ol, arrow) produce the endothecium and middle layer, while the inner layer (il) functions directly as the tapetum. D: Microspore mother cells (MMC) surrounded by callose (c), and an anther wall composed of epidermis (Ep), endothecium (En), middle layers (M), and tapetum (T). E: Microspore mother cells in the process of meiosis (arrows). F: Locule with a tetrahedral tetrad surrounded by callose (c), and tangentially elongated tapetal cells (T). The epidermal cells (Ep) are rich in starch grains (arrows) and fat globules. 1A, scale = 200 μm; 1B, E, F, scale = 30 μm; 1C, scale = 20 μm; 1D, scale = 50 μm.

nucleus now assumes a peripheral position (Fig. 2F).

When the anther is 1.7–2.2 mm long the mitotic division of the microspore nucleus results in the formation of two unequal cells, a large vegetative and a smaller generative cell (Fig. 3A). The generative cell becomes spindle shaped, and is enclosed in the vegetative cell (Fig. 3B). The pollen grains are two-celled at the time of dehiscence, and contain many

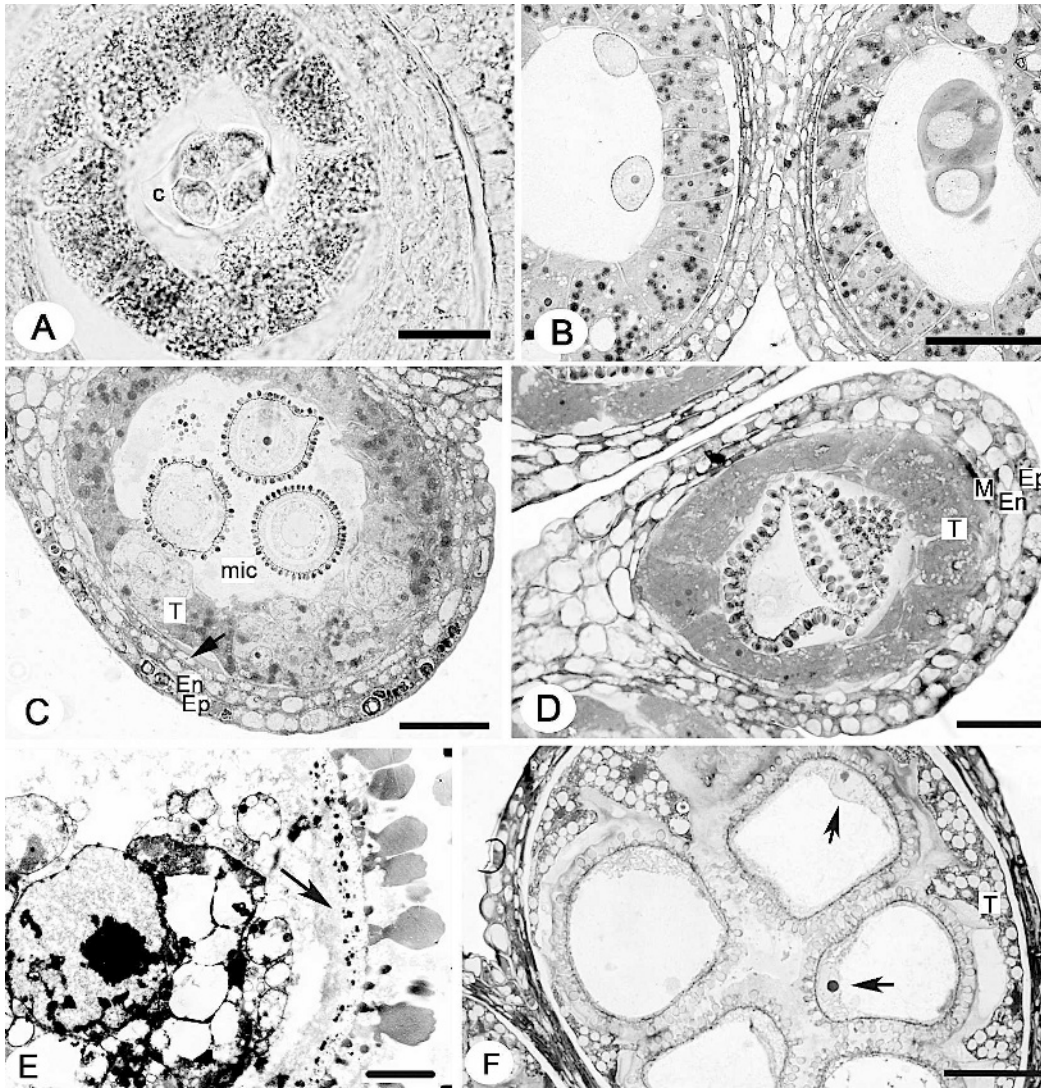

FIG. 2. Tetrad to microspore enlargement. All photographs except E (TEM) are light micrographs. A: An irregular tetrahedral tetrad surrounded by callose (c). B: Two stages of pollen formation in different microsporangia: free microspores in the left, and a tetrad in the right. C: Free microspores with prominent, centrally placed nuclei. The middle layers of the anther wall are flattened (arrow). Ep = epidermis; En = endothecium; T = tapetum. D: Enlarged microspores at the stage where the tapetum (T) is beginning to degenerate. M = middle layer. The radial walls of the tapetal cells have dissolved by this stage. Ep = epidermis; En = endothecium. E: Intine (arrow) present in a free microspore. F: Uninucleate microspore with the nucleus displaced to one side (arrows). The tapetal cells (T) contain numerous vesicles and have partially degenerated at this stage. 2A, scale = 20 μm; 2B, scale = 40 μm; 2C, D, F, scale = 30 μm; 2E, scale = 2 μm.

starch grains in the vegetative cell cytoplasm (Figs. 3B, C). They are inaperturate with an exine layer that is thicker than the intine (Fig. 3C).

FORMATION OF THE ANTHER WALL. The primary parietal layer divides periclinally to produce two secondary parietal layers (Fig. 1B). The outer secondary parietal layer divides again to produce the endothecium and a middle layer, while the inner parietal layer functions directly as the tapetum (Fig. 1C). The anther wall has completed development by the microsporocyte stage and consists of

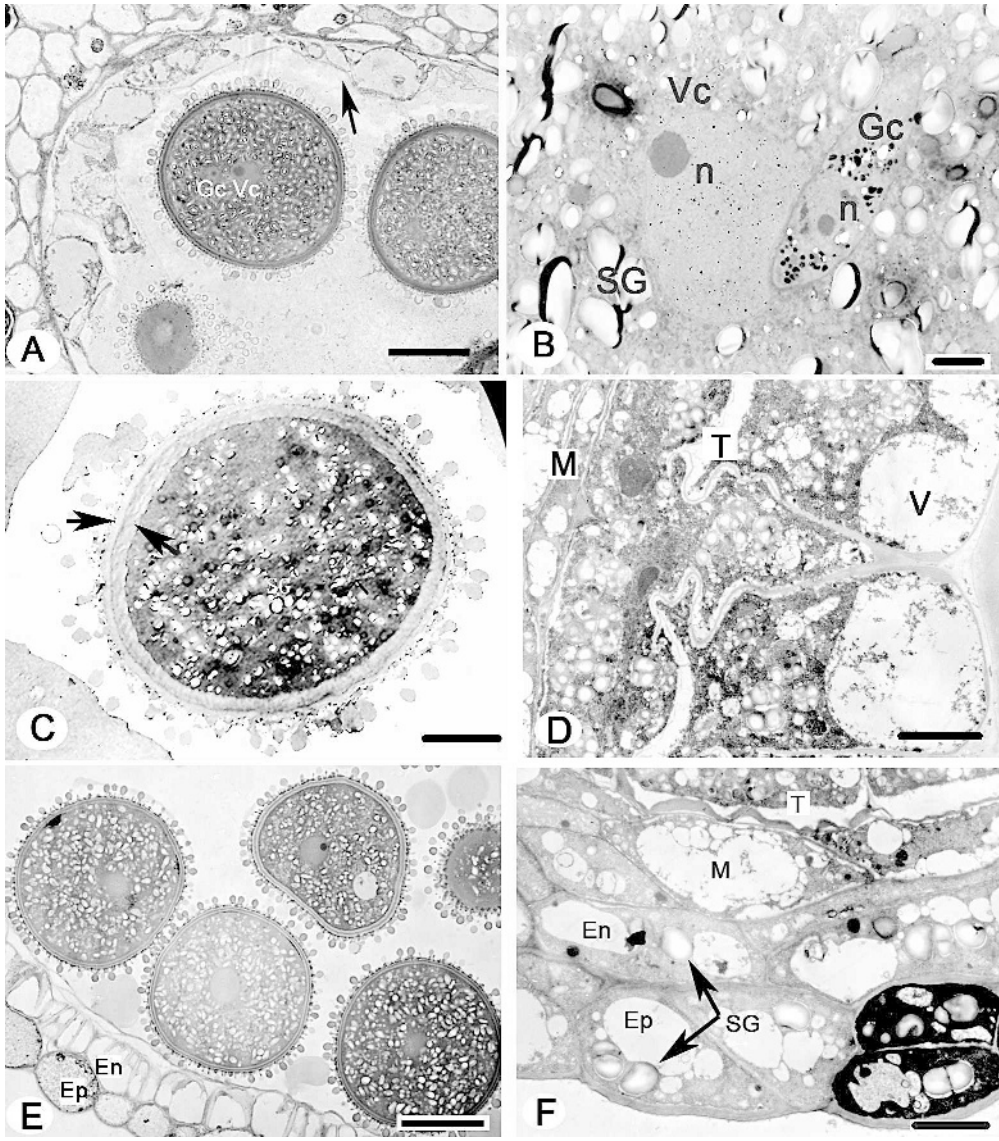

FIG. 3. Microgametogenesis and dissolution of the tapetum. Figs. 3A, E are light micrographs; Figs. 3B, C, D, F are TEM. A: Two-celled pollen grains. All the tapetal cells (arrow) degenerate in their original positions. Gc = generative cell; Vc = vegetative cell. B: Two-celled pollen grain with many starch grains (SG). Gc = generative cell; n = nucleus; Vc = vegetative cell. C: A pollen grain with exine thicker than the intine (arrows) (SG = starch grains). D: Tangentially elongated tapetal cells (T) with folded walls and large vacuoles (V) adjacent to the anther locule. M = middle layers. E: Cross section of mature anther wall, showing epidermis (Ep), highly fibrous thickened endothecium (En). F: Cross section of an anther wall at the stage of tetrad formation, with the innermost of the middle layers (M) degenerating (arrow), and the endothecium (En) tangentially elongated. Ep = epidermis; SG = starch grains; T = tapetum. 3A, E, scale = 30 μm; 3B, scale = 2 μm; 3C, scale = 10 μm; 3D, F, scale = 4 μm.

four layers: an epidermis, endothecium, two or three middle layers and a glandular tapetum (Fig. 1D).

The tapetal cells are initially uninucleate, but become two or four-nucleate after mitotic divisions. They enlarge greatly at the microsporocyte stage, while retaining a dense cytoplasm and small vacuoles (Fig. 1D). During the time of tetrad formation, the tapetal cells have conspicuously folded cell walls, are

elongated tangentially, and form large vacuoles on their adaxial side, i.e., the side towards the microsporangium (Figs. 1F and 3D). They contain numerous small vesicles at the uninulcleate microspore stage, (Fig. 2F), but degenerate gradually at their original sites soon after (Figs. 3A and 3E). As the microspores are released into the locule, the inner periclinal and radial walls of the tapetal cells dissolve and the vacuoles disappear (Figs. 2C and 2D). By the time the pollen grains are mature, the tapetal cells have completely degenerated.

After the tetrads are formed, the innermost of the middle layers begins to degenerate (Fig. 3F). All the middle layers become flattened during the free microspore stage (Figs. 2C and 4A), and have degenerated completely at the mature pollen grain stage (Fig. 3E).

The cells of the endothecium elongate tangentially during the time of tetrad formation (Figs. 1F and 3F). They are vacuolated, and contain few starch grains. The cells then enlarge during the free microspore stage (Figs. 2C and 2D), and develop highly fibrous thickenings with a large central vacuole at the two-celled pollen grain stage (Fig. 3E).

The cells of the epidermal layer undergo divisions in all planes: anticlinal, oblique, and a very few periclinal (Figs. 3F and 4B). The cells are rich in starch grains, and fat globules appear at tetrad formation (Figs. 1F and 3F). At the free microspore stage, they have abundant fat globules (Figs. 2C and 4C). During anther wall maturation, the epidermal cells enlarge greatly (compare Figs. 2C and 3E).

At maturity the anther wall is composed of the epidermis and a highly fibrous, thickened endothecium. At dehiscence, the septa break down and the mature anther becomes bilocular (Fig. 1A).

ABNORMAL PHENOMENA IN MICROSPOROGENESIS AND MALE GAMETOGENESIS. Pollen abortion can cause low fruit set. Unlike other members of the Euphorbiaceae, *Jatropha curcas* has a low ratio of aborted to normal pollen. When it occurs, pollen abortion only takes place in one or two microsporangia per anther. Abortion in all four microsporangia has never been observed. The archesporia and the microspore mother cells of *J. curcas* always develop normally, but occasional irregularities occur in meiosis, tetrad formation and during the free microspore state. In some locules an early degradation of the tapetum causes the microsporocytes to degenerate at the tetrad stage (Fig. 4D). This can result in the formation of a large cavity in the microsporangium (Fig. 4E). In other anthers a few abnormally shaped microspores are found in association with a completely degenerated tapetum, and partially degenerated middle layers (Fig. 4F).

Discussion. On the basis of the formation of the middle layers, Davis (1966) classified the development of anther walls into four types: basic, dicotyledonous, monocotyledonous and reduced. The dicotyledonous type occurs in the majority of dicotyledonous families and one monocotyledonous family, Taccaceae, which is now part of Dioscoreaceae (APG 2003). The monocotyledonous type includes the majority of monocotyledonous families as well as several dicotyledonous families. Only a few families contain species with dicotyledonous and monocotyledonous types of wall formation: Combretaceae, Euphorbiaceae, Sterculiaceae and Thymelaeaceae.

The formation of the anther wall of *Jatropha curcas* conforms to the dicotyledonous type. In Euphorbiaceae, *Euphorbia pulcherima* (Ai et al. 1995) of the Euphorbioideae, and *Manihot esculenta* (Zhang et al. 2003) of the Crotonoideae also have the dicotyledonous type, but *Acalypha* of the Acalyphoideae and *Phyllanthus* of the Phyllanthoideae have the monocotyledonous type (Davis 1966). Bhatnagar and Kapil (1979) found three types of anther wall formation in *Bischofia javonica* (Bischofiaceae of Airy-Shaw (1965); Phyllanthoideae of Webster (1975); now recognized as Phyllanthaceae by the APG (2003)): basic, monocotyledonous and dicotyledonous types. Because of its variability, the type of anther wall development serves a limited purpose in the classification of the Euphorbiaceae.

The arrangement of microspores in a tetrad exhibits one of five patterns: tetrahedral, isobilateral, linear, T-shaped or decussate (Davis 1966). The pattern depends on the shape of microspore mother cells, the position of the meiotic spindles and the resultant division planes (Davis 1966, Blackmore and Crane 1998, Rangaswamy et al. 2001). In the Euphorbiaceae, cytokinesis is simultaneous, and the tetrads are tetrahedral, isobilateral or decussate (Davis 1966, Johri et al. 1992). In

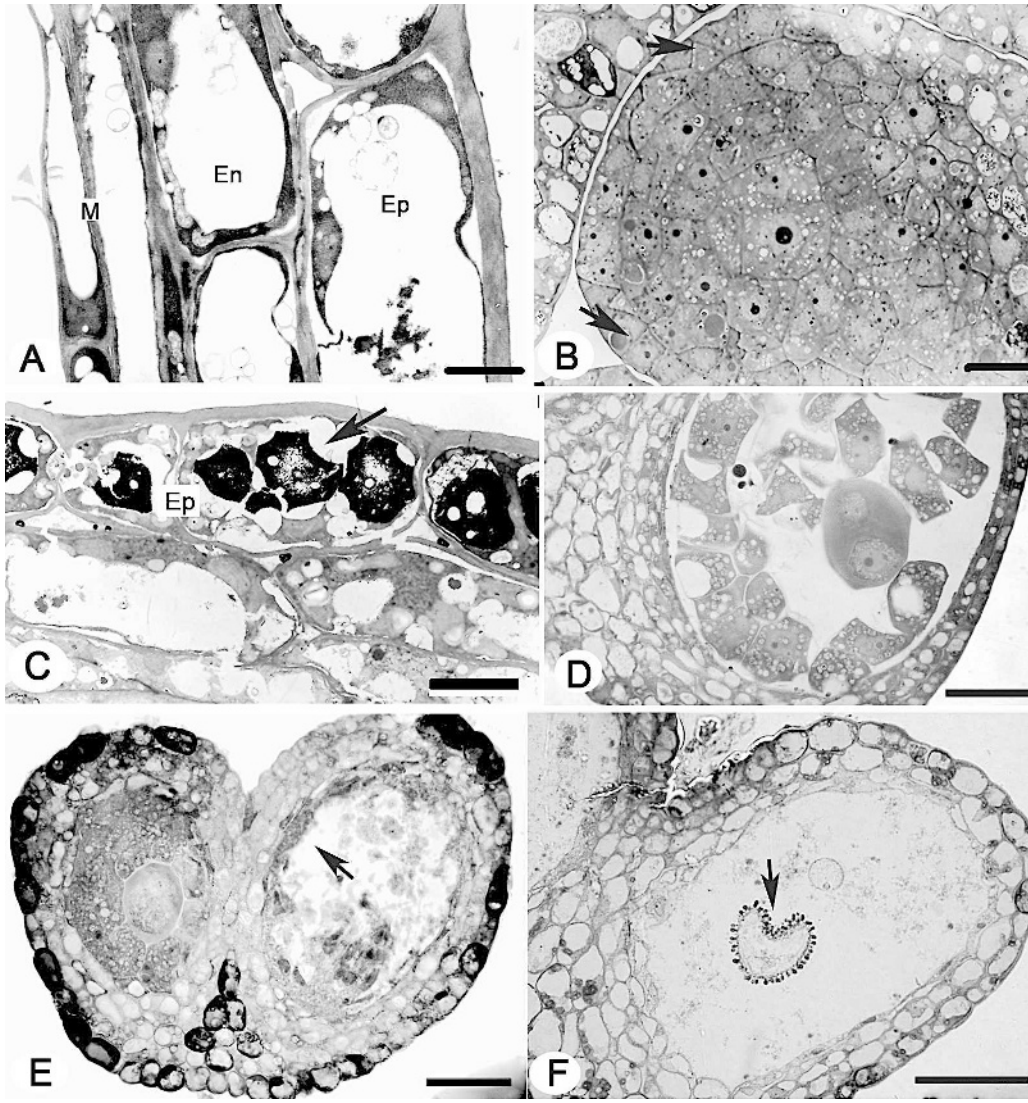

FIG. 4. Anther structure and pollen abortion. Figs. 4A, B, C are TEM; Figs. 4D, E, F are light micrographs. A: Flattened middle layers (M) at the free microspore stage. Ep=epidermis; En=endothecium. B: Protodermal cells with anticlinal and oblique divisions (arrows). C: Fat globules (arrow) in the epidermal cells (Ep) at the free microspore stage. D: Tetrad stage of an abnormal microsporangium with degenerating tapetum and microspores. E: Two thecae; one with a large cavity in the microsporangium because of pollen abortion (arrow). F: A degenerated microsporangium with an abnormal microspore (arrow). 4A, scale = 2 μm; 4B, scale = 10 μm; 4C, scale = 4 μm; 4D, E, scale = 30 μm; 4F, scale = 40 μm.

Euphorbia dulcis Kapil (1961) found all of these types. In *Euphorbia rothiana* (Srivastava 1952), *Euphorbia pulcherima* (Ai et al. 1995) and *Manihot esculenta* (Rao and Sarveswara 1976, Zhang et al. 2003), only tetrahedral tetrads are found. In *Jatropha curcas*, most of the tetrads were tetrahedral, with a few irregular tetrahedral. Irregular tetrahedral tetrads were found in most species from the Asphodelaceae (Penet et al. 2005).

In *Jatropha curcas*, the mature pollen grains are both inaperturate and two-celled. This agrees with the results reported for this species and *Jatropha gossypifolia* by Kajale and Rao (1943). Nowicke (1994) found inaperturate pollen in most Crotonoideae, including *Jatro-*

pha. In most Euphorbiaceae, the pollen grains are generally triporate, and two- or three-celled at anther anthesis (Johri et al. 1992). Nowicke (1994) found that several early branching lineages of subfamily Crotonoideae share inaperturate pollen, an unusual feature among the angiosperms and a strong synapomorphy for most of the subfamily.

In the present study we found that only a few microspores develop abnormally, either at the tetrad stage or in the early stages of microgametophyte development. These results concur with those of Laser (1972), and differ from those of Johri (1984), who described pollen abortion only before the formation of the tetrads in dicotyledons. According to Johri (1984), the degeneration of the tapetum is responsible for the abortion of the pollen. The tapetum is a source not only of nutrients for the microsporocytes, but also of substances that aid pollen development in more specific ways (Pacini et al. 1985, Johri et al. 1992). Malfunctioning tapetal cells have been shown to have an adverse effect on development of the endothecium and on pollen fertility (Warmke and Overman 1972, Nanda and Gupta 1974, Vijayaraghavan and Ratnaparkhi 1979, Hu 1982). In *Jatropha curcas* pollen abortion is correlated with an early degeneration of the tapetum.

In the Euphorbiaceae, pollen abortion occurs in *Manihot esculenta* (Rao and Sarveswara 1976, Zhang et al. 2003), *Euphorbia pulcherima* (Ai et al. 1995), and *Euphorbia dulcis* (Kapil 1961). In all three cases it is correlated with the degeneration of the tapetum. In *Manihot esculenta*, the abortion happens during meiosis or at the free microspore stage, which is similar to the condition in *J. curcas*. In *Euphorbia pulcherima*, abortion occurs between the sporogenous cell and the free microspore stages. In *Euphorbia dulcis*, no fertile pollen grains are produced (Kapil 1961). Some of the microspores degenerate while still within the tetrad, but widespread degeneration of the pollen starts at the uni-nucleate stage, with some grains surviving to the 2-celled condition. The cells that survive to this stage degenerate soon after. The behavior of the tapetum has not yet been reported in this species.

Considering its large size, the Euphorbiaceae are poorly known embryologically (Kapil 1994). Only 16 tribes (about 30 genera), out of 50 tribes recognized by Webster (1987) have been investigated. Further studies of other genera are needed to clarify embryological attributes of the whole family.

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