Proc. Indian natn. Sci. Acad., **B61**, No. 3, pp. 199-208 (1995)

Structure and Development of Anther, Pollen and Exinal Connections in Jojoba (*Simmondsia chinensis*)

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(Received on 28 January 1994; Accepted on 26 June 1994)

The anther wall comprises epidermis, endothecium, two middle layers and tapetum. The tapetal cells are polarized and become binucleate at meiotic prophase of meiocytes. The primexine is patterned while the microspore tetrads are still invested within the thick callosic walls. The tapetum is of the secretory type and its cells walls are destroyed at the time of dissolution of the callose sheath around the tetrads. During the vacuolization and enlargement of the microspores, the structure of the exine is modified and becomes more complex and massive. Endothecial wall thickenings occur only after pollen engorgement. The inner tangential surface of the tapetum is studied with both single and complex orbicules. The generative cell nucleus is large with an electron opaque nucleolus and is separated from the vegetative cell cytoplasm by a distinct undulating cell wall. The vegetative cell cytoplasm is replete with many starch-containing plastids while the generative cell is bereft of any recognizable plastids. The mature pollen wall is composed of tectum, bacula, foot-layer, endexine and intine. The endexine is lamellated and thickened more at the apertural sites than in the interapertural regions where it is thin and granular. The most striking feature is the presence of exinal connections in many pollen grains that are visible under both light and scanning electron microscope. These thread-like exinal connections are acetolysis resistant and continuous with the ektexine.

Key Words: Simmondsia chinensis, Secretory tapetum, Orbicules, Pollen wall, Exinal connections

Introduction

Simmondsia chinensis (jojoba) is a dioecious shrub, endemic to the Sonoran desert of Mexico and the United States. It is well known as a renewable source of high quality liquid wax that is extracted from the seeds. The seed oil (liquid wax) is unique in the plant kingdom and is a valuable substitute for the oil of endangered sperm whale. The oil has been targeted for several potential uses that include lubricants, cosmetics and pharmaceuticals. Much of the research is confined to the agricultural potential of jojoba and industrial applications of its seed oil but the work on anther and pollen structure has received less attention. The present study analyzes the development and differentiation of pollen grains and reports for the first time the unusual presence of "exinal connections" in this monotypic and esoteric taxon.

Material and Methods

Young male buds and anthers of *Simmondsia chinensis* (Link) Schneider at various stages of development, were fixed in 10% (v/v) aqueous acrolein; dehydrated in methoxyethanol, propanol, butanol series; infiltered

and embedded in pure glycol methacrylate resin. Two micron sections were cut using glass knives and were stained with periodic acid-Schiff's reagent and Coomassie brilliant blue for localization of insoluble polysaccharides and total proteins, respectively (Vijayaraghavan & Shukla 1990).

The tissue, for scanning electron microscopy, was fixed in FAA, dehydrated in an ascending acetone series, critical point dried, coated with gold and observed in a Philips 501 B SEM. Mature pollen grains were acetolyzed in a 9:1 v/v mixture of acetic anhydride : sulphuric acid for 2 or 3 min prior to coating and SEM examination. For transmission electron microscopy, the tissue was fixed in Karnovsky fixative (modified from Karnovsky 1965) prepared in 0.2 M phosphate buffer, at *p*H 7.2, and post-fixed at 4°C in 2% osmium tetraoxide made in the same buffer. The tissue was dehydrated in graded ethanol series and infiltered and embedded in Epon-Araldite Resin mixture. Ultrathin sections cut on a Reichert OM-U2 ultramicrotome, and stained with uranyl acetate and lead citrate were observed on Philips EM-300 operated at 80 KV.

Results

Early in anther ontogeny, each microsporangium differentiates into five distinct wall layers comprising the epidermis, endothecium, two flattened middle layers and tapetum (figure 1A). The sporogenous tissue forms the distinct central mass. The tapetum is of the secretory type and tapetal cells are irregular in shape and much larger than other cells of the anther wall (figure 1A & B). The tapetal cell, per se, has one large convoluted vacuole at one pole and the nucleus at the opposite pole (figure 1A). This arrangement confers polarity to tapetal cells. The meiocytes possess dense cytoplasm, numerous small vacuoles and stain well for proteins (figure 1B). The tapetum stains intensely for cytoplasmic proteins and becomes binucleate around meiotic prophase of meiocytes.

Within a locule, meiosis in meiocytes is normal and synchronous. The protoplasm of each meiocyte is divided into a tetrad of haploid microspores by a simultaneous type of cytokinesis. The microspores are either tetrahedral or decussate in arrangement (figure 1C & D) and remain embedded in an extensive callose sheath (figure 1D). The tapetum, at this stage, shows maximum development, stains intensely for proteins (figure 1C), possesses a few polysaccharide grains and shows extracellular polysaccharidic secretion on its inner tangential walls (figure 1D). The primexine (figure 1D) is laid down around the microspores that are still invested within the spatial confines of the thick callose wall. The inner middle layer (next to the tapetum) becomes compressed, and gradually obliterates (figure 1C). The cytoplasm of the tetrads stains intensely for proteins (figure 1C) and also develops a few perinuclear polysaccharide grains (figure 1D). Concomitant with the dissolution of the callose wall, the tapetal cells disorganize and the haploid microspores are released into the thecal polysaccharidic fluid (figure 1E). A thin exine and incipient apertural sites become visible (figure 1E).

The released microspores round up, enlarge, and their cytoplasm progressively becomes less dense and more vacuolate (figures 1F, 3A). The nucleus initially is centrally located but later migrates to the microspore wall (figure 1F). The exine becomes more complex and massive. The tapetum becomes progressively more disorganized and many tapetal orbicules stud the inner tangential surface facing the locule (figure 3B). The orbicules possess a distinct pro-orbicular core and a marginal electron-dense sporopollenin coat (figure 3B). A few orbicules even become inserted between the tectum perforations and enter the interbacular spaces (figure 3C). Many

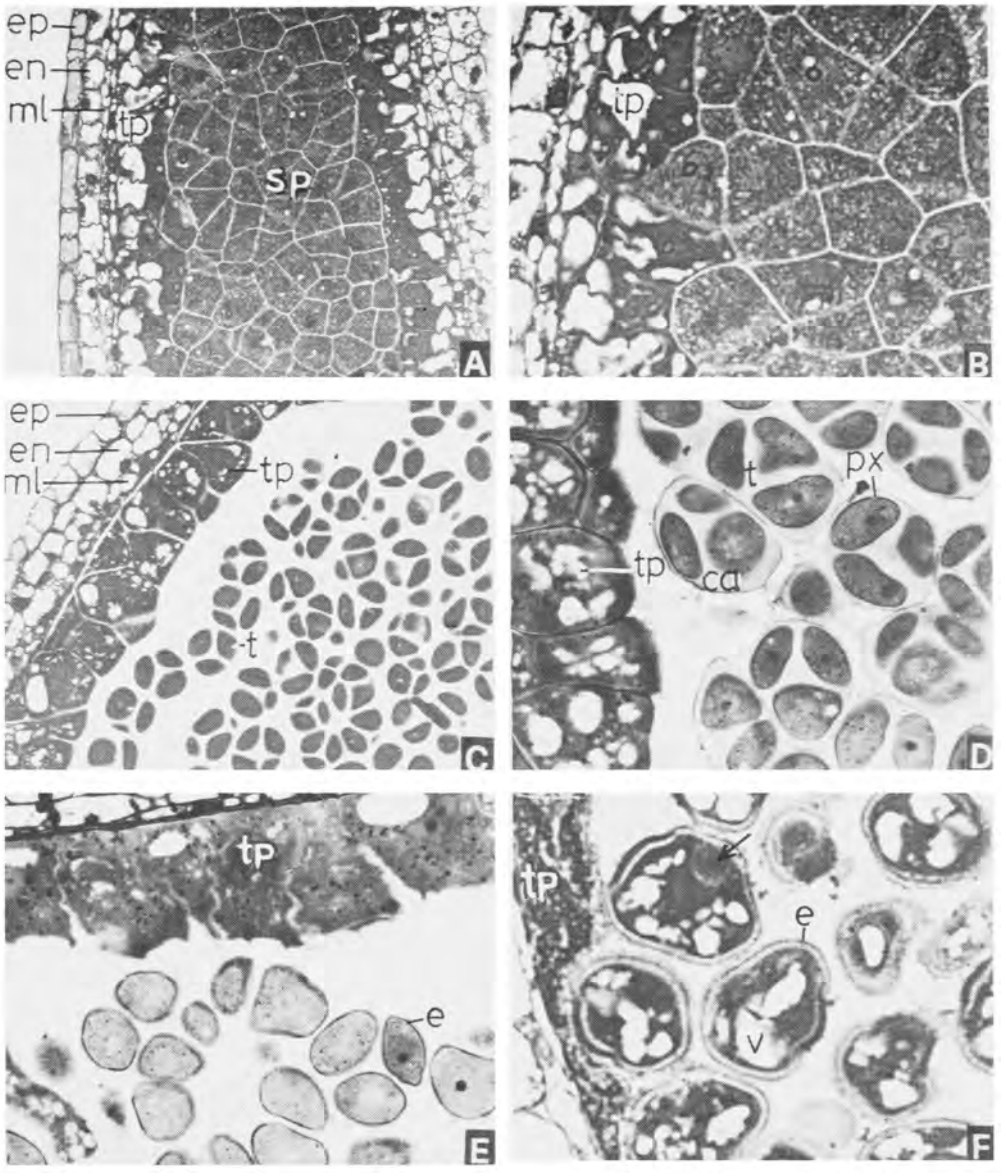

Figure 1 A-F *Simmondsia chinensis*. (**ca**, callose, **e**, exine, **en**, endothecium, **ep**, epidermis, **ml**, middle layer, **px**, primexine, **sp**, sporogenous cell, **t**, tetrad, **tp**, tapetum, **v**, vacuole). **A**, Longisection of young anther stained for proteins showing the wall layers and the central mass of sporogenous tissue. (x 181); **B**, Portion of anther locule to show the large, irregularly shaped, binucleate tapetal cells. The meiocytes stain intensely for total proteins. (× 508; **C**, Anther locule to show microspore tetrads. The crushed inner middle layer is noteworthy. The tapetal cells and the teterads stain intensely for proteins. (× 254); **D**, Microspore tetrads embedded in thick, PAS positive callosic walls. Around the 'tetrads, the original cellulosic mother cell walls persist and each cell of the tetrad reveals PAS positive primexine. The tapetal cells are large and show secretory activity. (× 508); **E**, Portion of anther locule to show the dissolution of callose wall and release of microspores from the tetrad. The tapetum reveals disorganization and its cell walls are destroyed. Early exine deposition starts around each microspore. (× 508); **F**, Microspores at vacuolate stage showing the well differentiated exine. The microspore nucleus migrates towards the periphery (arrow). The tapetum shows degeneration. (× 652).

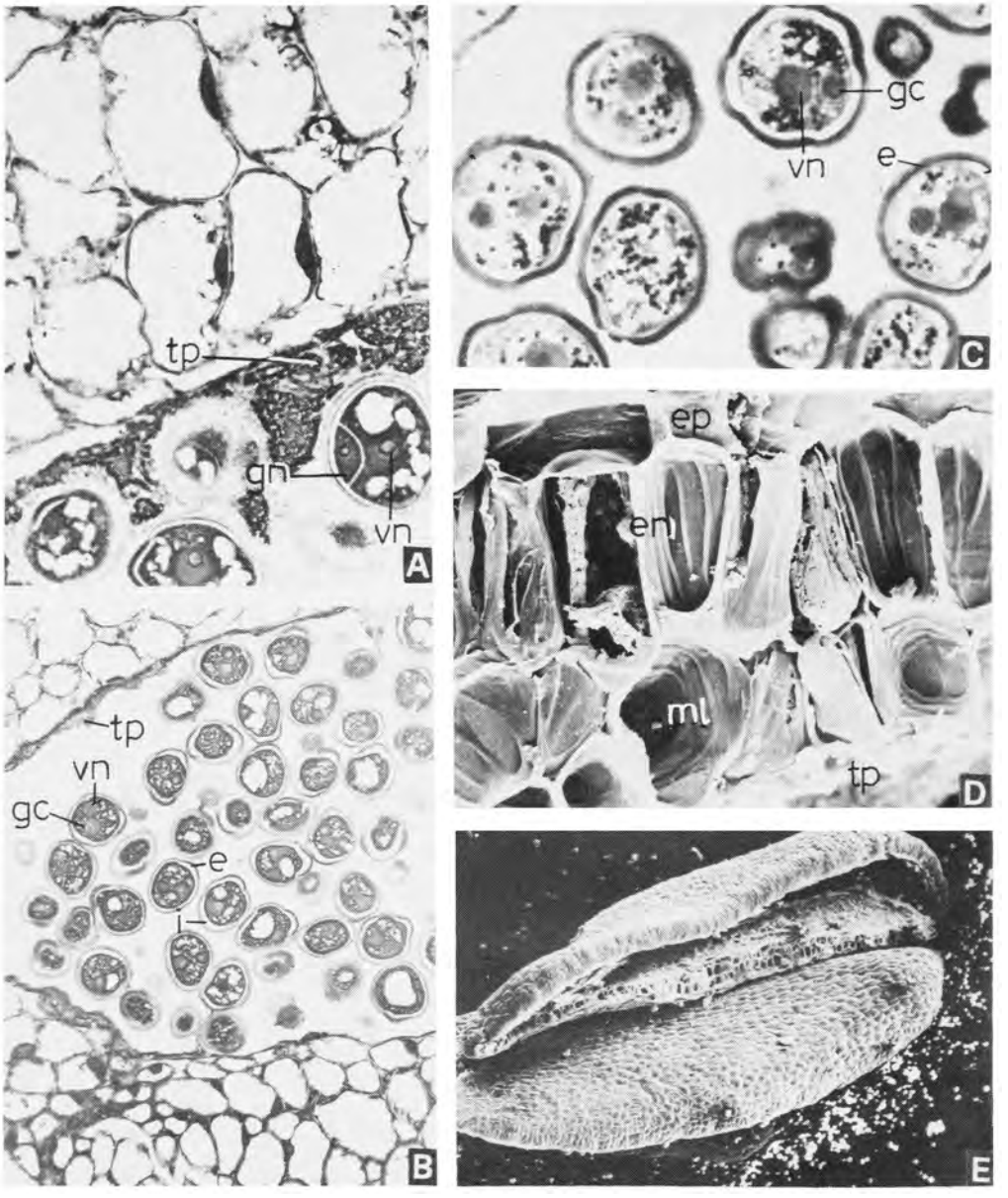

Figure 2 **A-E** (**e**, exine, **en**, endothecium, **ep**, epidermis, **gc**, generative cell, **gn**, generative cell nucleus, **i**, intine, **ml**, middle layer, **tp**, tapetum, **vn**, vegetative cell nucleus). **A**, Portion of anther locule to show the lysed tapetum closely investing the binuclear pollen grains. The newly formed generative nucleus is separated from the vegetative cell cytoplasm by a distinct wall. (× 580); **B**, Mature pollen grains with vegetative cell nucleus and the generative cell enmeshed within the dense, protein-rich cytoplasm. The tapetum is completely lysed. The pollen grain wall comprises the exine and intine. (× 290); **C**, Mature bicellular pollen grains stained with PAS reagent to show the presence of numerous polysaccharide granules. The exine stains intensely. (× 752); **D**, Scanning electron micrograph (SEM) of the anther wall at mature pollen stage to show the intact epidermis, endothecium with well developed, fibrous thickenings and the persistent outer middle layer. The inner middle layer and the tapetum are lysed. (× 696); **E**, SEM of dehiscing anther showing the stomial groove and the release of en masse pollen grains. (× 50)

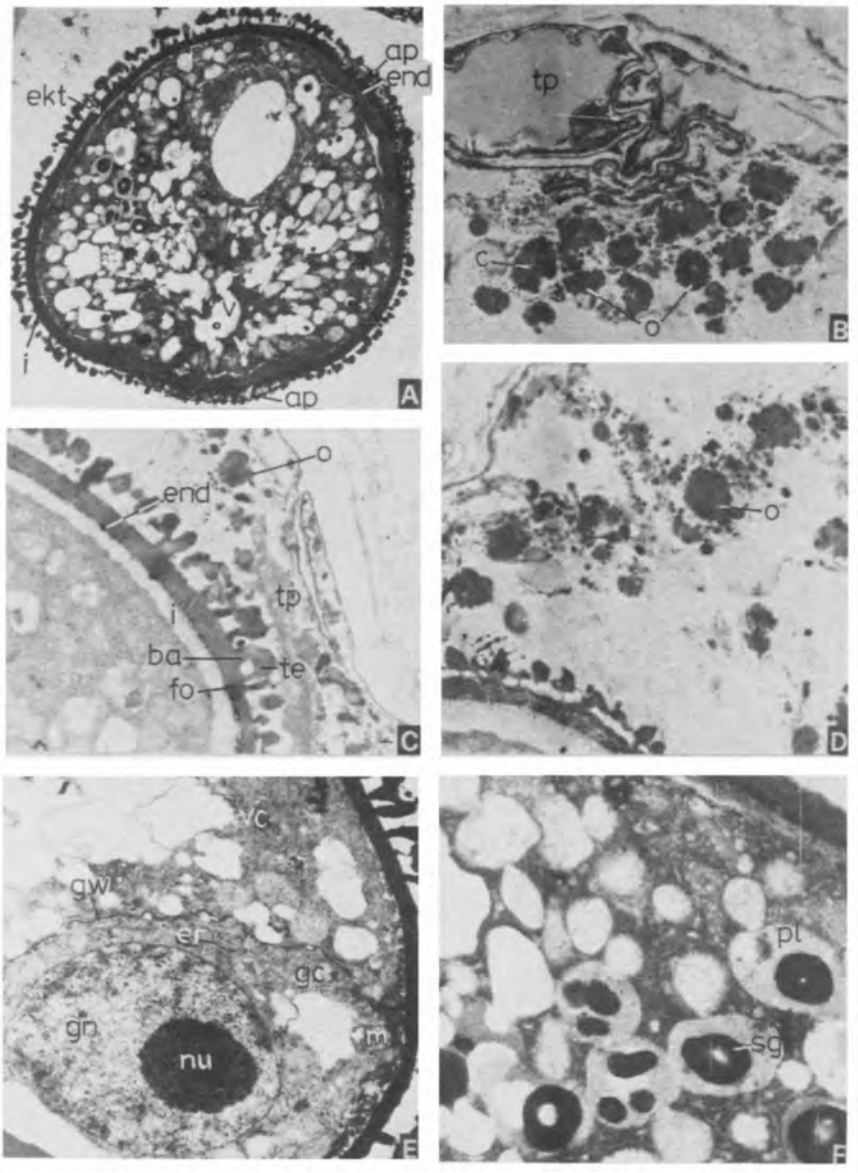

Figure 3 A-F (**ap**, aperture, **ba**, bacula, **c**, core, **ekt**, ektexine, **end**, endexine, **er**, endoplasmic reticulum, **fo**, foot-layer, **gc**, generative cell, **gn**, generative cell nucleus, **gw**, generative cell wall, **i**, intine, **m**, mitochondrion, **nu**, nucleolus, **o**, orbicules, **pl**, plastid, **sg**, starch grain, **te**, tectum, **tp**, tapetum, **v**, vacuole, **vc**, vegetative cell). **A**, Electron micrograph of a vacuolate microspore to show the exine well differentiated into ektexine (tectum, bacula, foot-layer) and endexine. The endexine is lamellate and thick at the aperture regions. (× 3105); **B**, Periphery of the degenerating tapetum showing the orbicules lining the anther locule at vacuolate stage of microspores. The electron-lucent, globular lipid core of the orbicules is noteworthy. (× 8910); **C&D**, Lysed tapetum and orbicules in close proximity with the developing exine. Note the small globules sticking to the orbicules in D. (x 8910) **E**, Electron micrograph of a young bicellular pollen grain showing the generative nucleus separated from the vegetative cell cytoplasm by a distinct, undulating cell wall. (× 5569); **F**, Portion of vegetative cell cytoplasm showing numerous starch containing plastids. (× 7762)

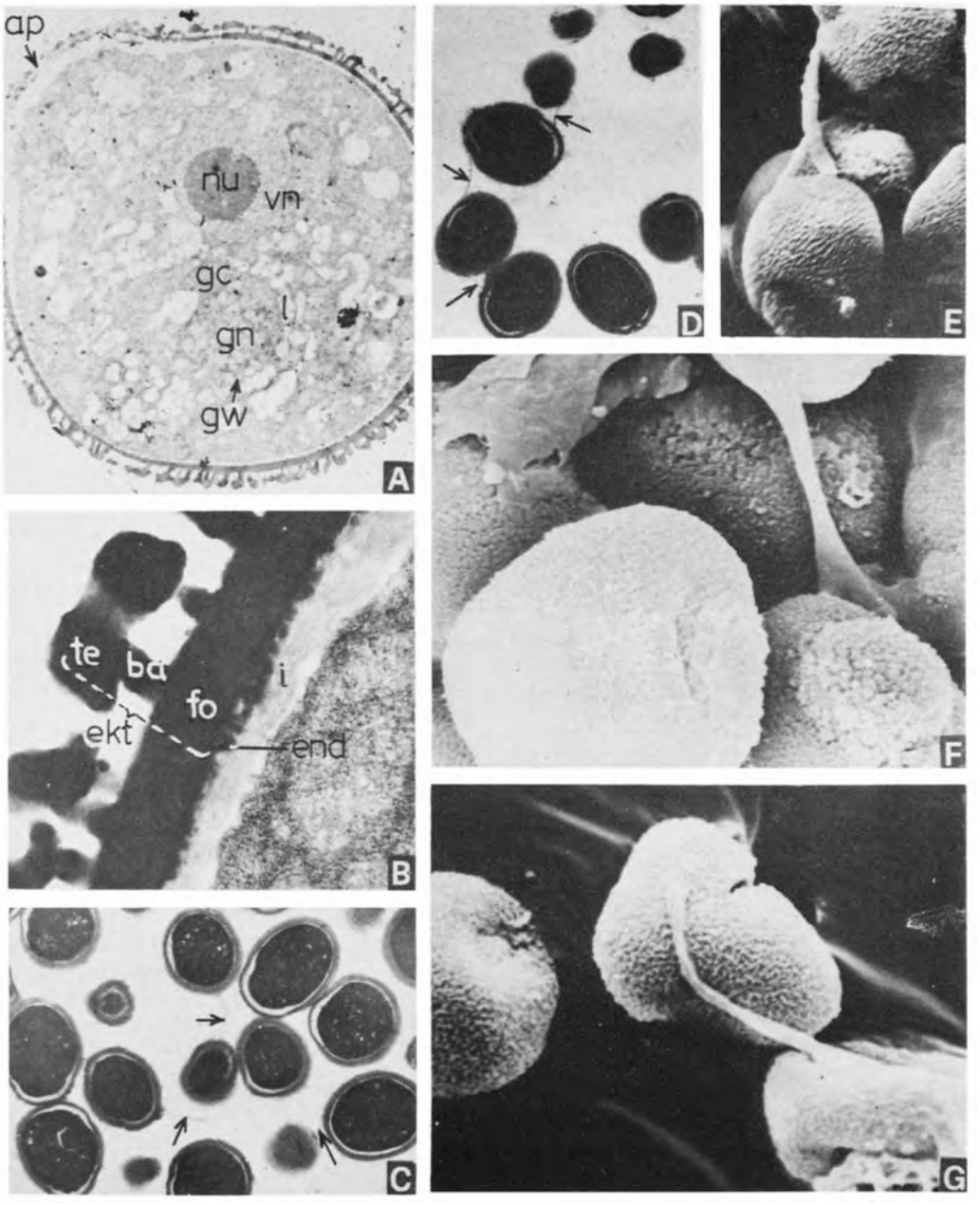

Figure 4 A-G (**ap**, aperture, **ba**, bacula, **ekt**, ektexine, **end**, endexine, **fo**, foot-layer, **gc**, generative cell, **gn**, generative cell nucleus, **gw**, generative cell wall, **i**, intine, **l**, lipid droplet, **nu**, nucleolus, **te**, tectum, **vn**, vegetative cell nucleus). **A**, Electron micrograph of a mature bicelled pollen grain. The generative cell is surrounded by electron-lucent lipid droplets. (× 3897); **B**, Mature pollen grain wall with fully developed fibrillar intine. The exine is well differentiated into ektexine and endexine. The plasmalemma follows the inner convolutions of the intine. (× 19613); **C&D**, Light micrographs of anther locules showing small thread-like structures connecting mature pollen grains (arrows). Note the forked attachment points in **C**. (× 576); **E&F**, Scanning electron micrographs of unacetolysed pollen grains showing continuity of connections and exine. In E the 'Y' shaped attachment point is noteworthy. (E × 1350; F × 2701); **G**, Acetolyzed grains showing continuity of exine and connections. (× 1765)

small globules stick to the orbicules and increase its size (figure 3B & D) and probably represent the second generation of orbicules.

During vacuole fragmentation and cytoplasm densification, the microspore nucleus divides resulting in the formation of a large vegetative cell and a small, lenticular, generative cell adpressed to the intine (figure 2A). The generative nucleus is separated from the vegetative cell cytoplasm by a distinct generative cell wall which shows undulations (figure 3E). The generative cell nucleus is large and contains peripheral zones of condensed chromatin and an electron-opaque nucleolus (figure 3E). The peripheral cytoplasm possesses a few mitochondria, vesicles and thin strands of rough endoplasmic cisternae but does not reveal any recognizable plastids. The vegetative cell cytoplasm, however, is dense with numerous starch containing plastids (figure 3F).

The generative cell soon becomes detached from the intine and migrates to the centre of the pollen grain. A gradual resynthesis of the microspore cytoplasm occurs (figure 2B) followed by a gradual accumulation of polysaccharide granules (figure 2C). The vegetative cell nucleus and generative cell lie enmeshed within the protein-rich cytoplasm (figure 2B). The generative cell is surrounded by electron-lucent lipid droplets (figure 4A). The whole tapetum constitutes a layer of collapsed cells (figure 2B). Even at this stage, there is no evidence of fibrous wall thickenings in the endothecium. The pollen shows no further structural changes except for further engorgement of food reserves (figure 4C & D) but the endothecium develops well-developed thickenings on the inner tangential and radial walls (figure 2D).

The mature pollen grain wall is composed of tectum, bacula, foot-layer, endexine and intine (figure 4B). The tectum, bacula and foot-layer comprise the ektexine. The endexine is very thin and granular (figure 4B) except at the apertures where it is lamellated and thick (figure 3A). The intine is well differentiated, continuous. electron-translucent and fibrillar (figure 4B).

At anther dehiscence, the pollen grains are spherical to subprolate and are shed in large numbers (figure 2E). Both light (figure 4C&D) and scanning electron microscopic (figure 4E-G) studies reveal many small thread-like connections that link pollen grains. The threads are short, thick and have a smooth surface with no evident sculpturing and are extensions of the ektexine (figure 4E-G). In many instances, each end of the thread is observed to be attached to the exine surface of different grains (figure 4E-G). This, however, is not a consistent feature and a few threads with free ends have also been encountered (figure 4C). The threads are firm, non-sticky and acetolysis resistant (figure 4G). There is no obvious pattern of origin of the exinal connections. The threads, in many cases, display forked attachment points (figure 4C&E).

Discussion

In angiosperms, meiosis and subsequent microspore development are accompanied by considerable cytoplasmic changes. In *Simmondsia chinensis,* the primexine (the spatial precursor of the exine) is patterned while the microspores are still invested within the thick callosic wall. In most of the taxa, the pattern elements of the pollen grain wall are laid during the tetrad stage by the synthesis of primexine (Fernandez & Rodriguez Garcia 1988, Perez-Munoz et al. 1993). The callose wall apparently acts as a framework for exine wall deposition because tetrads that are released prematurely from the callose wall have abnormally developed exines and the microspores burst (Worrall et al. 1992). The endexine in *Simmondsia chinensis* reveals lammellations articularly at the aperture sites. In many taxa, the endexine

is reported to grow by the application of platelets of lamellae coated with sporopollenin (Dickinson & Heslop-Harrison 1971, Shoup et al. 1980, Takahashi 1989). The deposition of sporopollenin on the unit membrane is probably the universal method of sporopollenin deposition.

Microspore mitosis is an asymmetric division. The distribution of organelles, during division of the microspore, produces a vegetative cell containing most of the microspore cytoplasm and a generative cell containing very little cytoplasm and organelles. The above observation is well documented in many angiosperms, particularly the lack of plastids in the generative cell cytoplasm (Cresti et al. 1985, Murgia et al. 1986, Hagemann & Schröder 1989, Seth & Vijayaraghavan 1991). Mature generative cells, lacking plastids, are formed either by the unequal distribution of plastids during the first pollen mitosis or by the degeneration/exclusion of plastids during the maturation of the generative cell. In mature generative cells, the absence of plastids indicates a uniparental, purely maternal inheritance of this organelle.

In several taxa characterized by secretory tapeta, the deposition of sporopollenin in the form of übisch bodies/orbicules on the inner faces of tapetal cells has been well established. In *Simmondsia chinensis*, the orbicules during development fuse to form larger agregations, or complex orbicules as also observed in many other taxa (Christensen et al. 1972, Chen et al. 1988). The complex orbicules may be composed only of a few single orbicules as in present work, *Euphorbia palustris* (Hesse 1986) and *Prosopis juliflora* (Vijayaraghavan & Chaudhry 1993) or all the orbicules fuse forming an orbicular wall on the loculus surface as in *Sorghum bicolor* (Christensen et al. 1972).

Pollen aggregation in angiosperms is caused either by the sticky, viscous pollenkitt or by the non-sticky, non-viscous, "exinal connections" and "viscin threads". Pollenkitt is found in all angiosperm families investigated up to now (Hesse 1980, 1984). In contrast, both exinal connections and viscin threads are rarely reported (Skvarla et al. 1978, Cruden & Jensen 1979, Hesse 1984, Patel et al. 1985, Gautam et al. 1991). The occurrence of thread-like exinal connections in *Simmondsia chinensis* is intriguing since the pollen is borne easily by the wind and no insects have been observed on the female flowers which are colourless and without nectaries or scent glands (Gentry 1958). The term "exinal connection" was originally used by Cruden & Jensen (1979) to describe randomly positioned acetolysis resistant threads which connect pollen grains in the Caesalpinioid taxa, *Caesalpinia pulcherrima, Delonix regia* and some species of *Bauhinia*. The mature pollen grains of *Simmondsia chinensis* have been investigated by Nowicke and Skvarla (1984) but they did not report any exinal connections. The thread-like connections in *Simmondsia chinensis* are short, thick and possess a smooth surface. They appear similar to the exinal connections of *Jacqueshuberia* of the Leguminosae, Caesalpinioideae (Patel et al. 1985) in their resistance to acetolysis and continuity with the ektexine. This suggests that they are similar to ektexine in composition (Skvarla et al. 1978).

According to Cruden & Jensen (1979), the exinal connections in Leguminosae increase the efficiency of pollination compared to species which lack them and reflect an adaptation to entomophily. In *Caesalpinia pulcherrima* and *Delonix regia* (Cruden & Jensen 1979), the exinal connections are associated with moth, butterfly and bat pollinators. In any plant with more than one ovule per ovary, small stigma and which is not obligatorily wind pollinated, the cohesion of pollen grains in small clumps may account for the simultaneous transfer of

a large number of pollen grains, and thus permit multiple fertilizations from a single pollination event. The release of en masse pollen grains in *Simmondsia chinensis* and the occurrence of "exinal connections" appears significant in this context and warrants an extensive study and reevaluation of the pollination biology of this genus.

Acknowledgements

One of the authors (BC) gratefully acknowledges the Department of Science and Technology, New Delhi for the award of Young Scientist Project "Reproductive biology of potential petro-crops with special emphasis on susceptibility to insect pests and ovule/seed damage".

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