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Embryological studies in *Lotus glaber* (Fabaceae)

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The development of the female gametophyte and the pollen grain, the ultrastructure of the mature megagametophyte and the endothelial cells in *Lotus glaber* were examined. The anther wall development follows the dicotyledonous type. The tapetum is secretory, the microspore tetrads are tetrahedral and pollen grains are shed at a bicellular stage. The mature ovule is anatropous, crassinucellar and bitegmic; integuments form a zig-zag micropyle. A T-shaped megaspore tetrad is formed and the lowest megaspore is functional and produces a 7-celled embryo-sac corresponding to the *Polygonum* type. The synergids show ultrastructural differences, involving dictyosomes and ERr; these differences suggest a functional differentiation, probably related to the reception of the pollen tube.

Key words: embryology, endothelium, *Lotus*, megagametophyte, ultrastructure

Introduction

The genus *Lotus* comprises many annual and perennial species distributed all over the world. In Argentina two originally European species of this genus are found, *Lotus glaber* and *L. corniculatus*. They are important pasture legumes adapted to different climatic and soil conditions. Their seeds are morphologically similar, however, they present differences in their aril region and in their chemical composition (Dizeo de Strittmatter *et al*. 1985, Kade *et al*. 1997).

Lotus is embryologically unknown and the literature on this subject in the Fabaceae family in general is meagre (Cooper 1933, Rembert 1971, Aziz *et al*. 1972, Desphande & Bhasin 1974, Biddle 1979, George *et al*. 1979, Bharathi & Murty 1984, Kennell & Horner 1985, Prakash 1987, Cameron & Prakash 1990, 1994, Ashrafunnisa & Pullaiah 1997, Schutte 1997).

There are few ultrastructural studies on the megagametophytes of angiosperms (Johri *et al*. 1992) and this is the first ultrastructural description of the embryo-sac in the Fabaceae.

The purpose of this work was to investigate in detail the female and male gametophyte development of *L. glaber* and provide information on the ultrastructural organization of the embryo-sac and the endothelium cells.

Material and methods

Flowers of *L. glaber* were collected during 1995–1996 from plants grown from seeds that were provided by AgroVerónica, Verónica,

Buenos Aires Province and sown on 17 May at experimental fields of the Faculty of Agronomy, Buenos Aires, Argentina, (34°35´S, 58°29´W) (BAA 25.497).

The embryo-sac and pollen development were studied with light microscopy and the mature embryo-sac with transmission electron microscopy.

Light microscopy (LM)

Ovules and anthers in different stages of development were fixed in FAA, dehydrated, embedded in paraffin wax, serially sectioned (10 μm thick) with a Minot microtome and stained with a safranin-fast green combination (D'Ambrogio 1986). Material was observed and drawn with a Wild M20 microscope. The photomicrographs were made with a Zeiss microscope.

Transmission electron microscopy (TEM)

Mature ovules were pre-fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 24 hrs and then post-fixed in OsO_4 at 2 °C in the same buffer for 3 hrs; they were dehydrated in an ethanol series and embedded in Spurr's resin. Ultrathin sections, 750–900 Å thick, were obtained with a Sorval ultramicrotome using glass knives. Thin sections were stained with uranyl acetate and lead citrate (O'Brien & McCully 1981), and observed and photographed with a Jeol JeM-1200 ExII microscope.

Results

Microsporangium

The anther contains four sporangia. The anther wall consists of epidermis, endothecium, one middle layer and a secretory tapetum (Fig. 1A–D). The microsporangial wall follows the dicotyledonous type of ontogeny (Davis 1966). The outer secondary parietal layer gives rise to two layers, the outermost forming the endothecium and the innermost the middle layer. The inner layer directly functions as tapetum (Fig. 1A). The tapetal cells are uninucleate and have a dense cytoplasm (Fig. 1B–E).

During the maturation of anther, the endothecial cells acquire thickenings as fibrous bands in the radial walls (Fig. 1F–H). These thickenings are star-shaped in the inner tangential wall (Fig. 1I).

Microsporogenesis and microgametogenesis

The microspore mother cells are uninucleate and poorly vacuolate (Fig. 1A). When they enter the phase of meiotic division, they become enclosed in a thick callose wall (Fig. 1B and C). Cytoplasmic connections cannot be observed.

Cytokinesis is simultaneous and the arrangement of microspores is tetrahedral (Fig. 1C and D).

Once released from the tetrad, the microspores enlarge. A central vacuole appears in them, pushing the nucleus towards the periphery (Fig. 1E and F). As a result, the mitotic division of the microspore is unequal. Therefore, a small generative cell and a large vegetative cell appear (Fig. 1G). The first one gets separated from the wall of the pollen grain, and comes to lie in the cytoplasm of the vegetative cell (Fig. 1H). In the last stage of development, the cytoplasm of the vegetative cell is filled with starch grains and the generative cell acquires a fusiform shape. Pollen grains are two-celled at the time of shedding.

Ovule

The mature ovule is anatropous, crassinucellar, and bitegmic with a zig-zag micropyle (Fig. 2D). The ovule originates as a small protuberance. The ovule primordium is bizonate and it is initiated by periclinal divisions in the second cell layer of the placenta. One of the cells of the subdermal layer develops directly into the archesporial cell, which divides into a primary parietal cell and a megaspore mother cell (MMC). Then, the primary parietal cell undergoes one or two periclinal divisions (Fig. 2A).

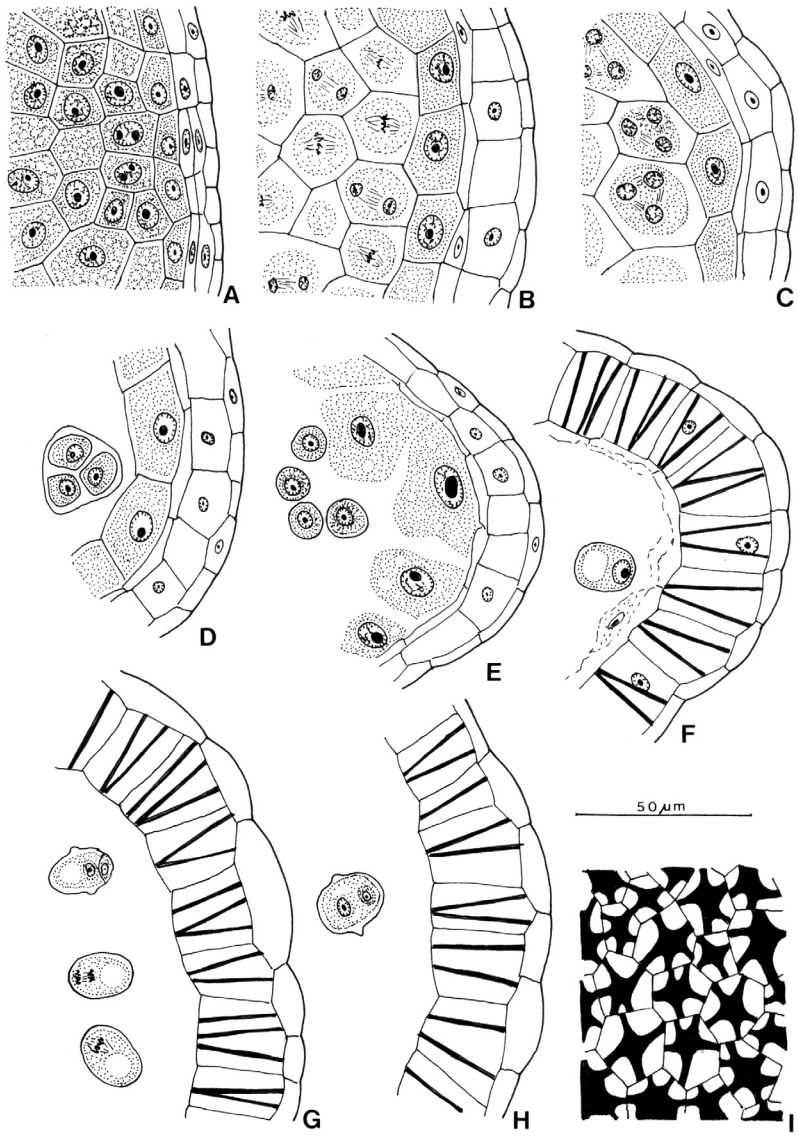

Fig. 1. *Lotus glaber*, anther in transverse section. — **A**: Young anther, wall formation. — **B** and **C**: Wall with all strata formed and meiosis in microspore mother cells. — **D**: Microspore tetrad. — **E**: Young free microspores. — **F**–**H**: Mature anther wall and mature microspore. — **G**: Mature anther wall and young bicellular pollen grains. — **H**: Mature anther wall and mature bicellular pollen grain. — **I**. Superficial view of inner tangential wall of endothecial cells.

The ovule primordium starts bending at an early stage. The initiation of the integuments takes place when the ovule shows a nearly 180° curvature (Fig. 2A).

The integuments are of dermal origin and two cells thick (Fig. 2A). On the opposite side of the funiculus, the outer integument (OI) grows faster (Fig. 2B), resulting in the exostome becoming eccentric with respect to the endostome; the two integuments thus constitute the zig-zag micropyle (Fig. 2C and D). When the embryo-sac starts its development, the ovule is completely inverted, so the nucellus and integuments lie alongside the funiculus (Fig. 2D).

Simultaneously with the development of the embryo-sac, a structure consisting of nucellar tissue resistant to the absorbing activity of the embryo-sac is observed. This nucellar structure looks like a pedestal for the megagametophyte, having the antipodals at its apex (Fig. 2D).

An endothelium is originated from the inner layer of the inner integument. The cells of this layer become radially stretched and they contain prominent nuclei and dense cytoplasm (Fig. 2D).

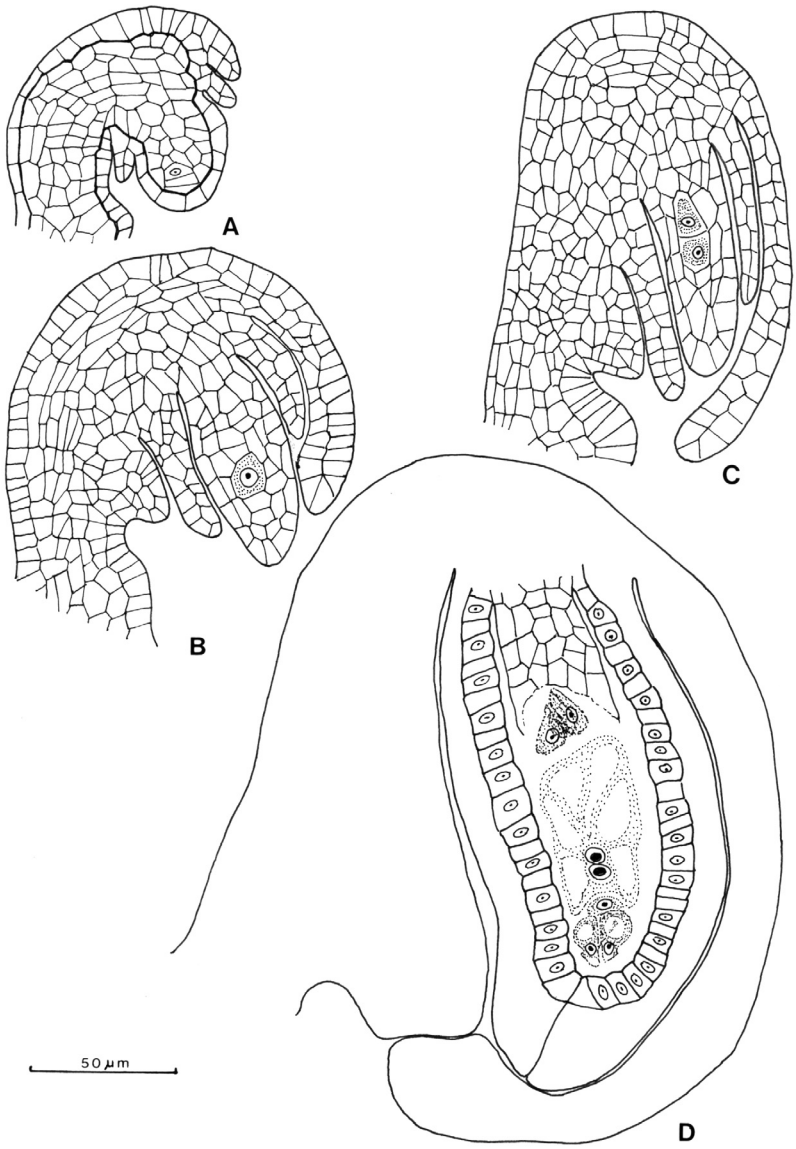

Fig. 2. *Lotus glaber*, longitudinal sections of developing ovules, showing initiation of integuments and anatropous curvature. Both integuments are of dermal origin. — **A**: Ovule primordium showing archesporial cell division. — **B**: Ovule primordium with MMC. — **C**: Ovule showing a dyad of megaspores. — **D**: Mature ovule with zig-zag micropyle, showing endothelium and "podium".

Megasporogenesis and female gametophyte

The megaspore mother cell (MMC) divides meiotically and undergoes two successive divisions resulting in a T-shaped tetrad (Fig. 3A–D). The three micropylar megaspores degenerate, and the chalazal one develops into the megagametophyte. Three successive mitotic karyokinesis give rise to an eight-nucleate embryo-sac (Fig. 3E–G). One central vacuole is formed and four nuclei are positioned in the micropylar end of the cytoplasm, and the other four nuclei in the chalazal end (Fig. 3G). After the eight-nucleate stage, the coenocytic megagametophyte becomes partly cellular. This process is simultaneous at the micropylar and chalazal ends.

The embryo-sac consists of seven cells: the egg cell, two synergids, the central cell and three antipodal cells (Fig. 3H). Such an embryo-sac represents the *Polygonum* type. The micropylar part of the egg cell is filled by a large vacuole and the chalazal end is filled with cytoplasm containing the egg nucleus. The chalazal part of

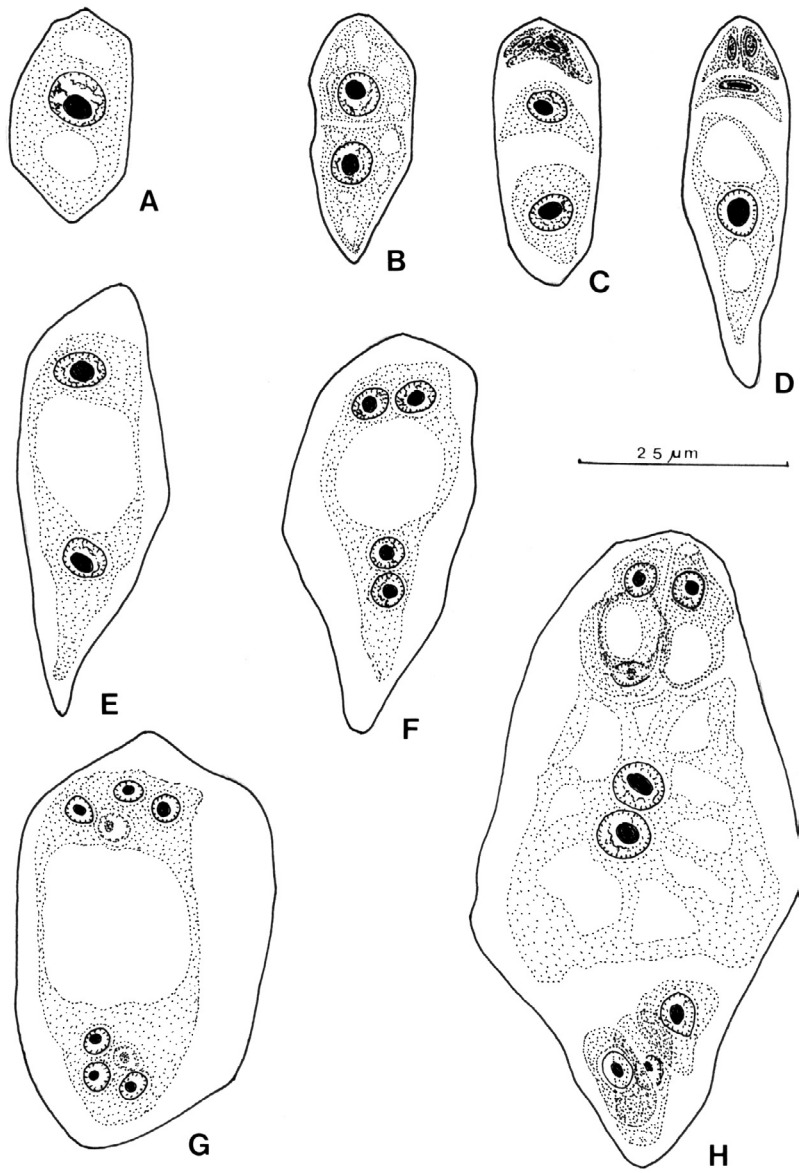

Fig. 3. *Lotus glaber.* — **A**: Megaspore mother cell. — **B**: Meiosis I in megaspore mother cell. — **C** and **D**: T-shaped tetrad of megaspores. — **E**: Two-nucleate embryo-sac. — **F**: Four-nucleate embryo-sac. — **G**: Eight-nucleate embryo-sac. — **H**: 7-celled embryo-sac (micropylar end up, chalazal end down).

the synergids is occupied by one large vacuole and the nuclei are in the micropylar region; they are hooked. The antipodals are the smallest cells of the embryo-sac (Fig. 3H).

Ultrastructure of endothelial cells

The endothelial cells are separated from the embryo-sac by a cuticle (Fig. 4A–C).

The cell walls of the endothelium are irregular in thickness (Fig. 4A), with the adjacent cell walls to the embryo-sac being the thickest (Fig. 4B–C). Many primary pit-fields with numerous plasmodesmata are observed in the radial cell walls (Fig. 4F). The endothelial cell walls show ingrowths or small projections (Fig. 4D).

The nuclei are conspicuous (Fig. 4A). The cytoplasm has abundant dictyosomes and free ribosomes, many mitochondria, endoplasmic reticulum of rough type (ERr) and plastids (Fig. 4B, C, E, F).

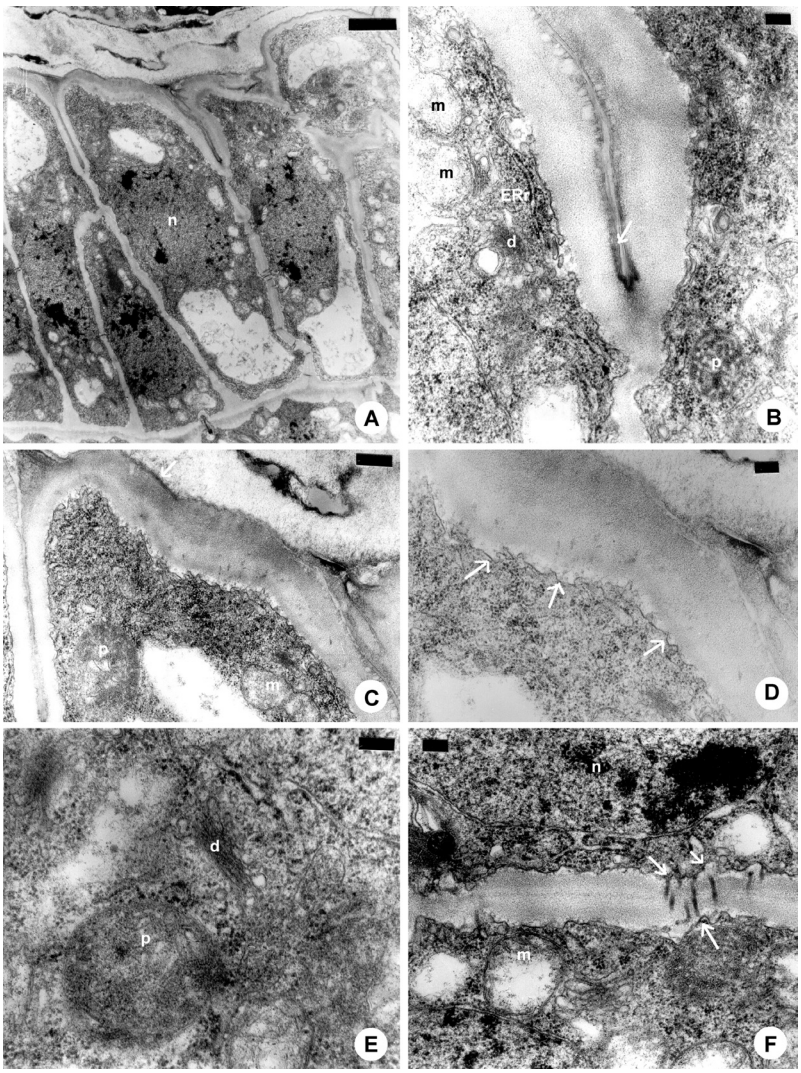

Fig. 4. *Lotus glaber*, ultrastructure of endothelial cells. — **A**: General aspect. — **B** and **C**: Details of wall and cuticle (arrow). — **D**: Detail of transfer cell (arrows). — **E**: Detail of cytoplasm. — **F**: Plasmodesmata (arrows). ERr = endoplasmic reticulum of rough type, m = mitochondria, p = plastid, n = nucleus, d = dictyosome. Scale bars: in **A** = 2 µm; in **B** and **D**–**F** = 200 nm; in **C** = 500 nm.

Ultrastructure of mature megagametophyte

Synergids

The micropylar end of each synergid is occupied by its nucleus and a large vacuole is found at the chalazal end. The synergids wall is thickened at the micropylar pole, developing a small filiform apparatus (fa) that exhibits a relatively homogeneous structure, low electron density and an irregular surface (Fig. 5A). This wall thins gradually towards the basal part up to the chalazal region of the synergids, where in some regions it stretches considerably or disappears, leaving the plasma membranes of the synergids and the central cell in close contact.

The synergids show some ultrastructural differences (Fig. 5B). The cytoplasm of both synergids has abundant mitochondria and free ribosomes. One of them has abundant dictyosomes with numerous associated vesicles, a well-developed ERr and some lipidic globules (Fig. 5C). The other synergid shows a more electrondense cytoplasm; it has an ERr with very dilated cisternae, lipidic globules and few dictyosomes (Fig. 5D).

Egg cell

The micropylar part of the egg cell contains a vacuole and the large nucleus is situated at the

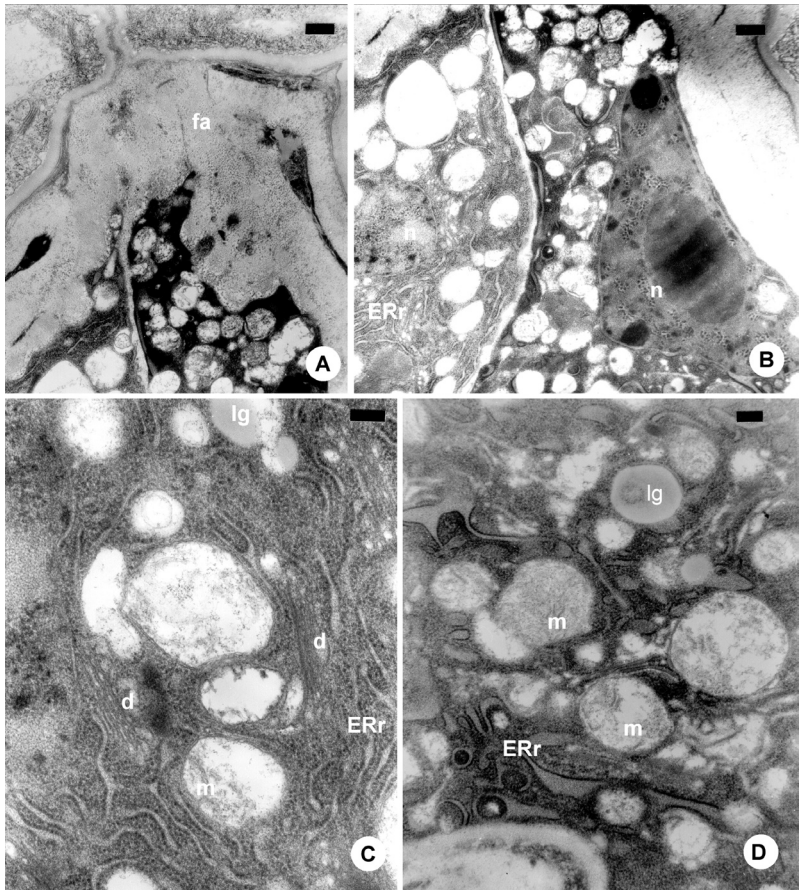

Fig. 5. *Lotus glaber*, ultrastructure of synergids. — **A**: Filiform apparatus (fa). — **B**: Cytoplasm of both synergids. — **C**: Detail of cytoplasm of synergid 1. — **D**: Detail of cytoplasm of synergid 2. ERr = endoplasmic reticulum of rough type, m = mitochondria, n = nucleus, d = dictyosome, lg = lipid globule. Scale bars: in **A** and **B** = 500 nm; in **C** and **D** = 200 nm.

chalazal end (Fig. 6A). The egg cell is partially surrounded by a cell wall. This wall thins in some regions of contact with the central cell, so that the plasma membranes of both cells are in close contact (Fig. 6A).

The cytoplasm shows abundant dictyosomes, mitochondria, scarce endoplasmic reticulum of rough type (ERr) and many free ribosomes (Fig. 6A).

Central cell

The central cell is highly vacuolated and its cytoplasm is confined to a thin layer along the embryo-sac wall. It has two polar nuclei with conspicuous nucleoli. These have many little nucleolar vacuoles (Fig. 6B).

The cell wall is absent at the micropylar end in those regions where the neighbouring egg-apparatus also lacks a cell wall (Fig. 6A). The cell wall against the nucellus shows small projections increasing the cell surface (Fig. 6C). The cytoplasm has numerous mitochondria, abundant ERr, dictyosomes, lipidic globules and plastids (Fig. 6B–D).

Discussion

Development of anther and male gametophyte in Fabaceae have been studied only in the genera *Alysicarpus* (Raju & Deshpande 1977, Seshavatharam 1982, Ashrafunnisa & Pullaiah 1997), *Desmodium* (Pantulu 1942), and *Lespedeza* (Hanson 1953, Hanson & Cope 1955). Until now all the members studied are uniform in showing a simultaneous cytokinesis of pollen mother cells, glandular anther tapetum and two-celled pollen grains at anthesis.

In *L. glaber* the anther wall is four-layered including epidermis, in accordance with the

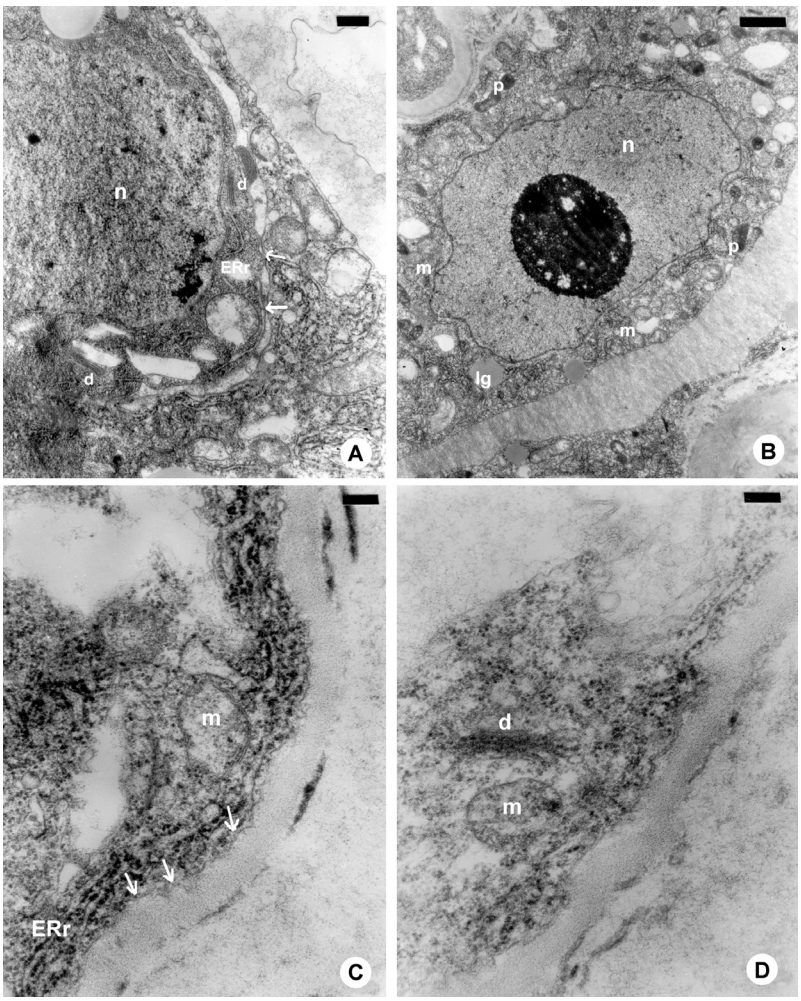

Fig. 6. *Lotus glaber,* ultrastructure of egg and central cells. — **A**: Cytoplasm and nucleus (n) of egg cell, detail of thinning of cell wall (arrows) between egg cell (ec) and central cell (cc). Note the presence of dictyosomes (d) and endoplasmic reticulum of rough type (ERr). — **B**: Cytoplasm and nucleus (n) of central cell. lg = lipid globules, p = plastids, m = mitochondria. — **C**: Detail of cell wall adjacent to nucellus showing small projections (arrows). ERr = endoplasmic reticulum of rough type, m = mitochondria, v = vacuole. — **D**: Detail of cytoplasm of central cell with dictyosome (d) and a mitochondrion (m); v = vacuole. Scale bars: in **A** = 500 nm; in **B** = 2 µm; in **C** and **D** = 200 nm.

majority of the investigated species of this family. A five-layered anther wall has been reported only in two species, *Alysicarpus longifolius* (Raju & Deshpande 1977) and *Desmodium gangeticum* (Pantulu 1942). According to Ashrafunnisa and Pullaiah (1997), however, these reports are erroneous.

Lotus glaber has a bizonate ovular primordium, a feature previously not reported for any species of Faboideae.

Both linear and T-shaped megaspore tetrads are found within Fabaceae (Johri *et al*. 1992). In *L. glaber,* only T-shaped megaspore tetrads were observed.

The development of the embryo-sac in *L. glaber* follows the *Polygonum* type. *Allium* type of development has reported in *Laburnum anagyroides* and *Pongamia*, and *Oenothera* type in *Vigna unguiculata* (Johri *et al*. 1992).

Considerable variation exists in the formation of the megagametophyte in Papilionoideae, and according to the types described by Cameron and Prakash (1994), *L. glaber* corresponds to their type 1, i.e. *Polygonum* type with normal antipodals.

The partial or total disappearance of the egg and synergid cell walls facing the chalaza in the mature megagametophyte is rather common (Willemse & Van Wen 1984, Jane 1992, Wang & Wang 1992, Pandey 1997, Liu *et al*.1998).

The ultrastructure of the synergids of *L. glaber* shows differences that involve the presence of more dictyosomes, ERr with no dilated cisternae and a cytoplasm less electrondense

in one of them. Jane (1992) discovered a slight ultrastructural difference between each synergid of *Arundo formosana* in that one had a denser cytoplasm, and Amela *et al*. (2003) described important differences in *Passiflora caerulea* that involve the filiform apparatus, the nucleus and the ERr. Jane (1992) observed the ERr as dilated and scattered vesicles in the synergids cytoplasm of *Arundo formosana*. This last characteristic was observed in only one of the synergids in *L. glaber.*

The differences between synergids probably indicate which one will receive the pollen tube.

In *L. glaber*, a true endothelium is originated from the inner layer of the inner integument. The same has been observed in *Jasione montana* (Erdelska 1975) and *Bellis perennis* (Engell & Petersen 1977). Similar structures were described for *Zornia diphylla* and *Vigna catjang* by Johri *et al*. (1992). In the first species, the nucellar cells in contact with the inner integument elongate and acquire an endothelium-like appearance. In the second species, the organization of a tapetum-like layer, successively formed by the nucellus, inner integument and endosperm, was observed. However, according to Johri *et al*. (1992) these observations require confirmation.

Ultrastructural studies of the endothelium are too few to allow reliable generalizations. However, the ultrastructural characteristics of this tissue described for *Helianthus annuus* (Newcom 1973a, 1973b) are similar to the ones observed in *L. glaber*. The presence of abundant dictyosomes and mitochondria in the endothelial cells of *L. glaber* suggests that the most important function of this tissue is to supply nutrition to the embryo-sac. According to Newcomb (1973a), the tapetum cells' location and the occurrence of many organelles associated with synthesis suggest that this tissue may be involved in the metabolism of the solutes before these enter the embryo-sac. Moreover, wall ingrowths in the endothelial cells increase the cell surface for the absorption of metabolites from adjacent tissues, inferred as a transfer cell function (Gunning & Pate 1969).

In *L. glaber,* a nucellar structure that looks like a pedestal for the megagametophyte was observed. This structure may be considered a "podium" or a "postament" (Bouman 1984). It is similar to the hypostase that consists of nucellar tissue resistant to the absorbing activity of the embryo-sac.

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