ACTA BIOLOGICA CRACOVIENSIA Series Botanica 45/2: 63–73, 2003

STUDY OF OVULE AND MEGAGAMETOPHYTE DEVELOPMENT IN FOUR SPECIES OF SUBTRIBE PHASEOLINAE (LEGUMINOSAE)

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Ovule development, megasporogenesis and megagametogenesis were studied in *Macroptilium bracteatum*, *Phaseolus augusti*, *P. vulgaris* var. *aborigineus* and *Vigna adenantha* to elucidate their taxonomic position. The ovule is anacampylotropous, bitegmic and crassinucellate. Megaspore tetrads are linear in *M. bracteatum* and T-shaped in the other three species. In all species the chalazal megaspore produces an embryo sac of Polygonum type. The results are discussed in relation to the current taxonomic classification. Our findings support the position of *Macroptilium* as a separate genus and the inclusion of *V. adenantha* in the genus *Phaseolus*. This is the first report of the use of embryological characters to solve intergeneric relationships of the *Phaseolus-Vigna-Macroptilium* neotropical complex, pointing out the method's usefulness.

Key words: Phaseolinae, ovule, megaspore tetrad, embryo sac.

INTRODUCTION

The Phaseolinae subtribe belongs to the subfamily Papilionoideae. Several species of Phaseolinae show palynological and morphological characters that make its taxonomic position uncertain. Several phytochemical and morphological studies have attempted to improve the generic delimitation (Marèchal et al., 1978; Zallocchi, 1992; Di Stilio, 1994; Drewes, 1995; Palacios and Hoc, 2001).

Embryological data have been of great importance in addressing taxonomic problems (Prakash, 1987). Previous embryological investigations of subtribes are few (Johri et al., 1992) and are restricted to a few species of *Phaseolus* (Brown, 1917; Weinstein, 1926; Desphande and Bhasin, 1974; George et al., 1979) and *Vigna unguiculata* (Ojega and Samyaolu, 1970).

This paper reports the ontogeny of the ovule and embryo sac in *Macroptilium bracteatum*, *Phaseolus augusti*, *P. vulgaris* var. *aborigineus* and *Vigna adenantha*. The aim of this work is to give an account of ovule structure and embryo sac development in order to provide new information for better delimitation of taxa within the Phaseolinae subtribe.

MATERIALS AND METHODS

The material was collected in natural populations of Argentina:

1. *Macroptilium bracteatum* (Nees et Mart.) Maréchal et Baudet in Prov. de Entre Ríos, Dpto. Concordia, Parque Rivadavia. Coll. A. Faigón, 2000; s/n (BAFC),
2. *Phaseolus augusti* Harms. in Prov. de Salta, Dpto. Rosario de Lerma, Quebrada del Toro, 1 km W. of Campo Quijano. Coll. P. Hoc, 1995; 355 (BAFC),
3. *P. vulgaris*. var. *aborigineus* (Burkart) Baudet. in Prov. de Salta, Dpto. Rosario de Lerma, Quebrada del Toro, 1 km W. of Campo Quijano. Coll. P. Hoc, 1995; 346 (BACF),
4. *Vigna adenantha* (G. Meyer) Maréchal, Mascherpa et Stainier in Ciudad Autonoma de Buenos Aires, Ciudad Universitaria. Coll. P. Hoc, 2001; 372 (BACF).

Flowers in different stages of development were fixed in FAA and embedded in paraffin. Sections (7–10 μm thick) were cut and stained with safranin combined with fast green (D'Ambrogio, 1986) and

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PL ISSN 0001-5296

TABLE 1. Embryological features of Phaseolinae

Taxon/Author	Ovule	Endothelium	Archesporial tissue	MMC position	Tetrad type	Embryo sac development
Dolichos labab Rembert, 1969; 1972	–	–	One subhypodermal cell	–	Linear/ T-shaped	–––––
Macroptilium bracteatum	Anacampylotropous, crassinucellate, with epistase	Yes	One hypodermal cell	Deep	Linear	Polygonum
Phasealus augusti	Anacampylotropous, crassinucellate	Yes	One hypodermal cell	Shallow	T-shaped	Polygonum
P. vulgaris Brown, 1917; Weinstein, 1926	Anacampylotropous, crassinucellate	Yes	One hypodermal cell	Shallow	Linear	Polygonum
P. vulgaris var. *aborigineus*	Anacampylotropous, crassinucellate	Yes	One hypodermal cell	Shallow	T-shaped	Polygonum
Strophostyles helvola Rembert, 1969; 1972	–	–	One hypodermal cell/multicellular	Deep	Linear	–––––
Vigna aconitifolia (ex *Phaseolus aconitifolius*) Deshpande and Bhasin, 1974	Anacampylotropous, crassinucellate	Yes	One hypodermal cell	Deep	Linear	Polygonum
V. adenantha	Anacampylotropous, crassinucellate	No	One hypodermal cell	Shallow	T-shaped	Polygonum
V. radiata (ex *P. aureus*) George et al., 1979	Campylotropous, crassinucellate	No	Multicellular	Shallow	Linear	Polygonum
V. unguiculata Ojega and Samyaolu 1970	–	–	–	–	Linear	Oenothera

observed with a Wild M20 microscope. The photomicrographs were taken with a Nikon Labphot AFX-II microscope. For SEM studies the material was dehydrated in an acetone series (70%, 80%, 90%, 100%), critical-point dried with liquid CO_2, and sputtercoated with gold-palladium for 3 min (O'Brien and McCully, 1981).

RESULTS

The descriptions are common for all the studied species. The distinctive features are specifically mentioned.

STYLE AND STIGMA

In the flower bud of the investigated species, the style is hollow along most of its length, and the canal is coated by an undifferentiated layer of epidermis (Fig. 1a). In mature flowers the epidermis of the style canal and the subjacent layers of cells become transmitting tissue (Fig. 1d). At the tip of the style, a solid region with a core of transmitting tissue connects the stigma surface to the canal. The stigma surface is composed of a layer of one-celled hairs with a thick cuticle (Fig. 1a,c). At anthesis the cuticle breaks. This rupture releases the stigmatic exudate beneath and allows pollen germination (Fig. 1b).

OVULE

In the four analyzed species the mature ovules are anacampylotropous, bitegmic and crassinucellate (Figs. 1e,f, 2b,f, 3e, 4i, 6e). The ovule originates as a small protuberance from the marginal placenta (Fig. 3a). The ovule primordium is bizonate in longitudinal section. It is straight at the beginning but starts

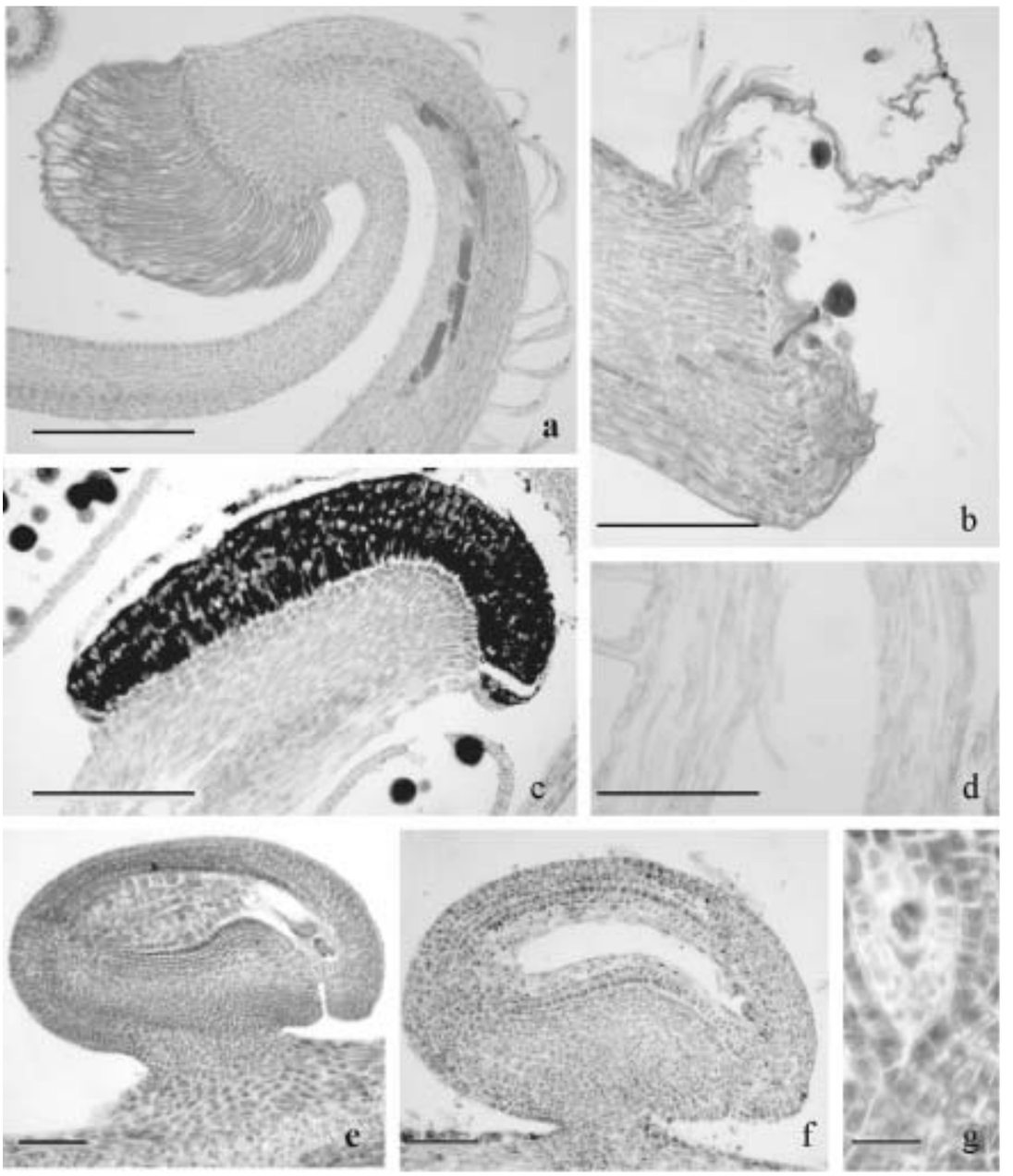

Fig. 1. *Vigna adenantha* (**a–b**). Longitudinal section of stigma and style at bud stage (**a**), and stigma with detached cuticle and pollen grain germinating (**b**). *Phaseolus augusti* (**c–d**). Longitudinal section of stigma at anthesis and solid portion of style (**c**), and stylar channel revested by transmission tissue (**d**). *P. vulgaris* var. *aborigineus*, ovule at mature female gametophyte stage (**e**). *V. adenantha*, ovule with female gametophyte (**f**). *Macroptilium bracteatum*, detail of epistase tissue (**g**). Bars in a–f = 50 µm, in g = 20 µm.

bending at megaspore mother cell (MMC) stage (Figs. 2a, 3b,c).

Both integuments differentiate simultaneously at the sporogenous cell stage (Figs. 2a,d,c, 3b,c, 6a). The inner integument is of dermal origin (Figs. 3b,c, 6a) and is 2 cells thick, except at the micropylar end on the funicular side where it is 3–4 cells thick. The outer integument is of hypodermal origin (Figs. 3b,c,

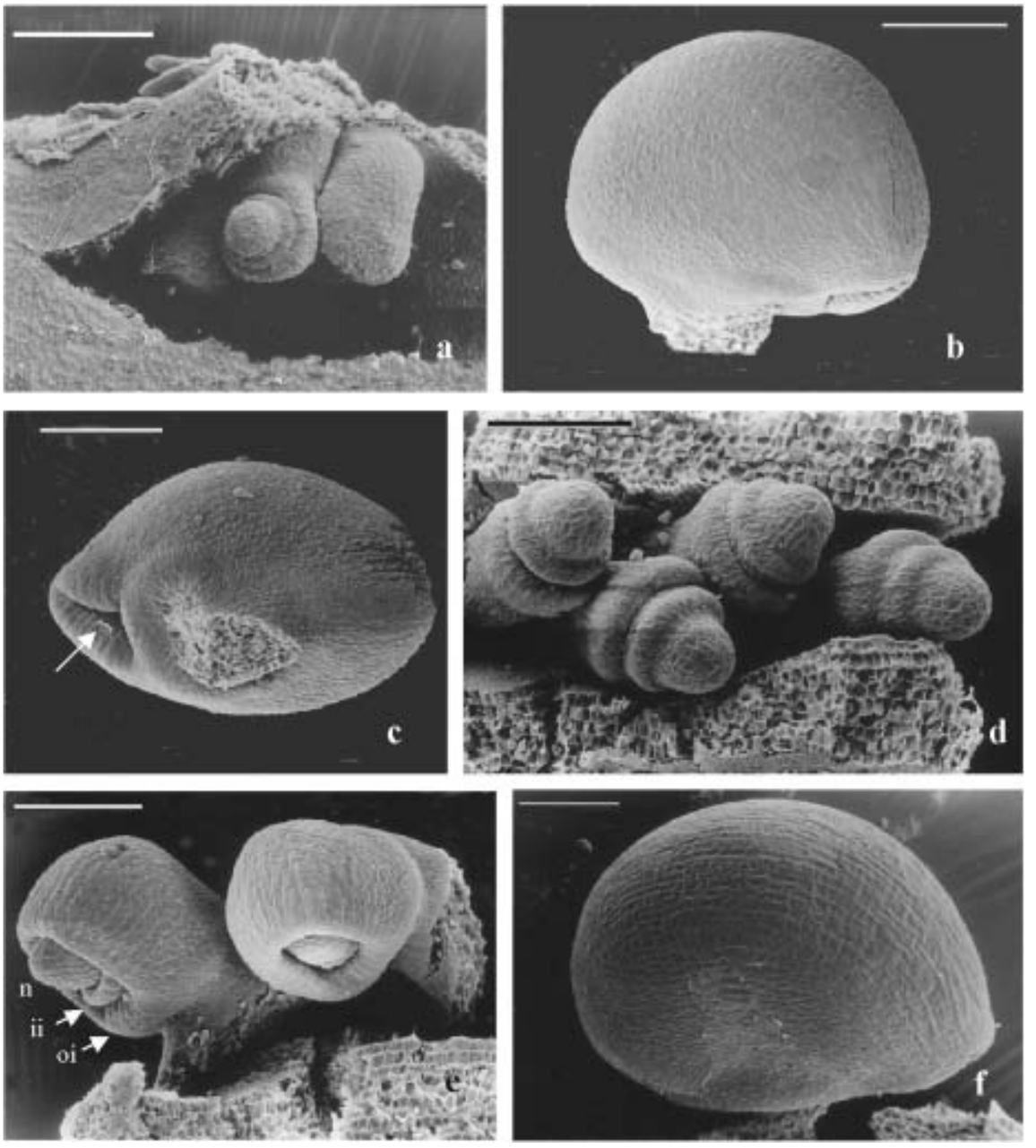

Fig. 2. SEM of critical-point dried material. *Macroptilium bracteatum* (**a–c**). Ovule primordia (**a**), mature ovule in lateral view (**b**), mature ovule showing micropyle (arrow) (**c**). *Vigna adenantha* (**d–f**). Ovule primordia (**d**), young ovule (**e**), mature ovule (**f**). n – nucellus; ii – inner integument; oi – outer integument. Bar = 0.1 µm.

6a) and multilayered (4–7 cells thick in *P. augusti*, *P. vulgaris* var. *aborigineus* and *V. adenantha*, and 10–12 cells thick in *M. bracteatum*).

The outer integument develops more rapidly than the inner integument (Fig. 2e), so at the dyad stage it encloses the inner integument and reaches the nucellar end (Fig. 3d).

In the mature ovule, in the tip of the outer integument opposite the raphe, repeated divisions form a massive structure covering the inner integu-

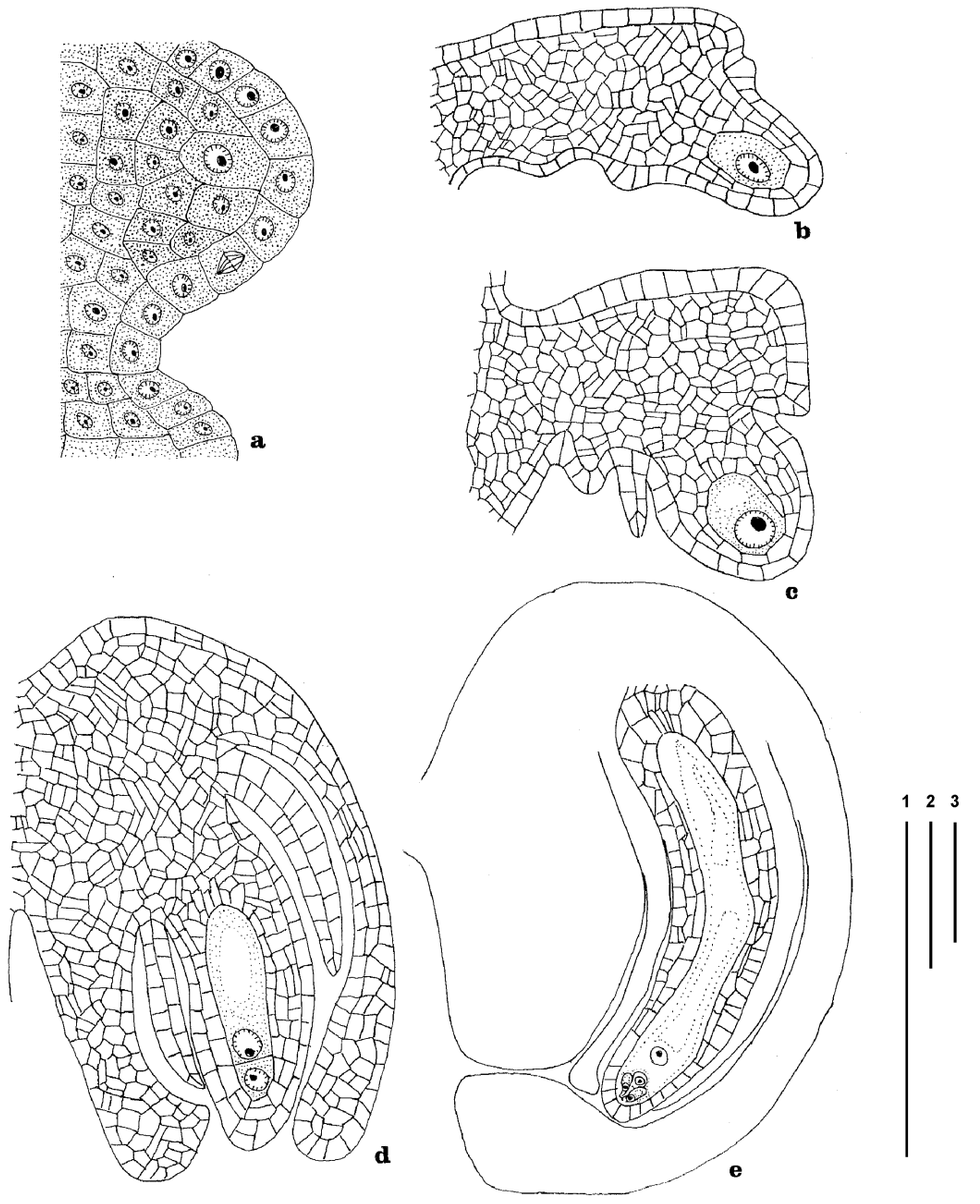

Fig. 3. *Vigna adenantha.* **(a–d)** Longitudinal section of developing ovules showing initiation of integuments and anatropous curvature, (**e**) Mature anacampylotropous ovule. Bar 1 = 50 µm, for (a); Bar 2 = 50 µm, for (b–d); Bar 3 = 25 µm, for (e).

ment and the micropylar end of the nucellus (Figs. 2c, 3e; 6e). The inner integument opposite the raphe never reaches the micropylar end of the nucellus. In consequence, the micropyle is composed of an exostome formed by the outer integument and by an endostomatic channel delimited by the inner integument at the raphe side and by the internal face of the outer integument at the opposite side (Figs. 3e, 4g, 6e).

In *Phaseolus augusti, P. vulgaris* var. *aborigineus* and *M. bracteatum*, the inner epiderm of the inner integument forms an endothelium or integumentary tapetum. The cells of this tissue have prominent nuclei and dense cytoplasm (Figs. 1e, 6g). During its development the embryo sac increases in size at the expense of nucellar tissue; hence in *P. augusti, P. vulgaris* var. *aborigineus* and *V. adenantha* the mature embryo

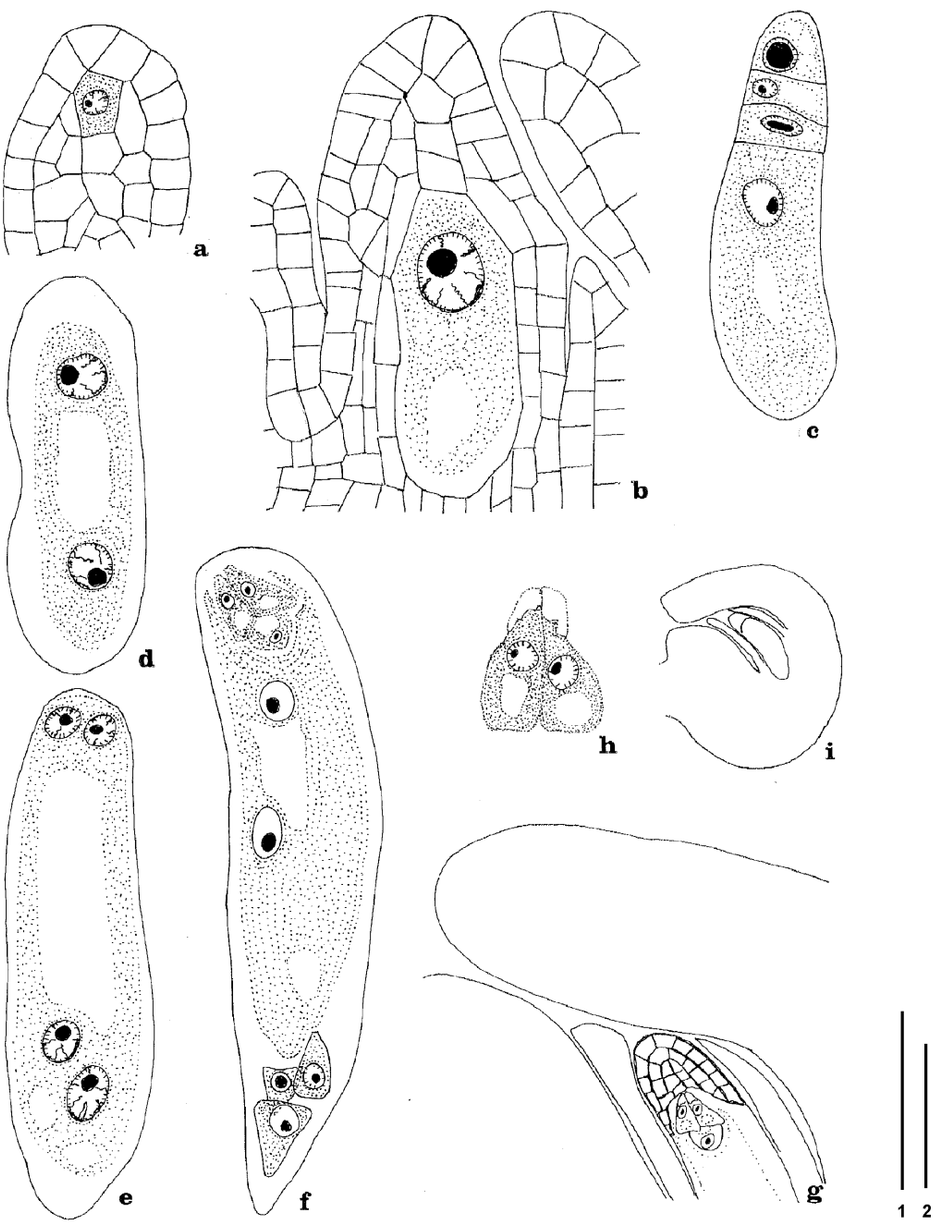

Fig. 4. *Macroptilium bracteatum*. (**a–c**) Megasporogenesis. Hypodermal archesporial cell (**a**), megaspore mother cell (MMC) situated deep within ovule (crassinucellate) (**b**), linear megaspore tetrad (**c**), (**d–f**) Megagametogenesis. 2-nucleate megagametophyte (**d**), 4-nucleate megagametophyte (**e**), Young 7-cellular megagametophyte (**f**), detail of epistase (**g**), detail of mature synergids (**h**), ovule at stage of mature gametophyte (**i**). Bar 1 = 50 µm, for (g); Bar 2 = 25 µm, for (a–f) and (h).

sac is surrounded by a single-layered nucellar epidermis at the micropylar end (Fig. 6f).

In *M. bracteatum* the 3 to 4 layers of nucellar tissue at the micropylar end form an epistase. The cells of the epistase are poor in cytoplasmic content and have thick walls (Figs. 1g, 4g).

MEGASPOROGENESIS AND MEGAGAMETOGENESIS

One of the cells of the hypodermal layers develops directly into the archesporial cell (Figs. 3a, 4a), which divides into a primary parietal cell and an MMC (Fig. 3b). In *M. bracteatum* only, the primary

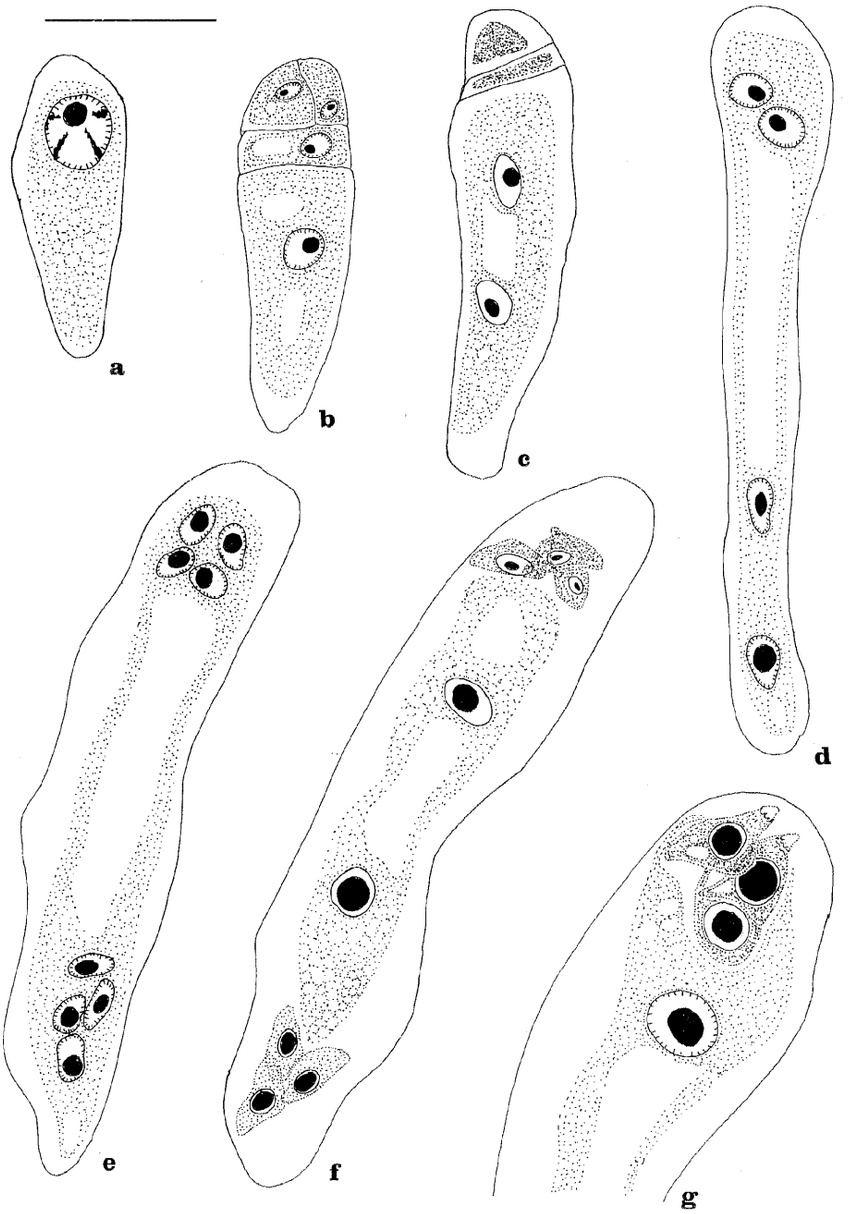

Fig. 5. *Phaseolus augusti*. (**a**) MMC, (**b**) T-shaped megaspore tetrad, (**c**) 2-nucleate megagametophyte and abortive megaspores, (**d**) 4-nucleate megagametophyte, (**e**) 8-nucleate megagametophyte, (**f**) Young 7-cellular megagametophyte, (**g**) Micropylar end of mature megagametophyte. Bar = 25 µm.

parietal cell undergoes further periclinal divisions to form the parietal tissue. This situates the MMC deep within the nucellus (crassinucellate) (Fig. 4b).

The MMC (Figs. 4b, 5a, 6b, 7a) has a microvacuolated cytoplasm at the chalazal end and a prominent nucleus at the micropylar end. The MMC divides meiotically (Fig. 7b), resulting in a linear tetrad in *M. bracteatum* (Fig. 4c) and in a T-shaped tetrad in the other three species studied (Figs. 5b, 6c, 7c). The three micropylar megaspores degenerate, and the chalazal megaspore develops into the megagametophyte (Figs. 5c, 6d). Three successive mitotic divisions give rise to the 8-nucleate female gametophyte (Figs. 4d–e, 5c–e). A central vacuole is

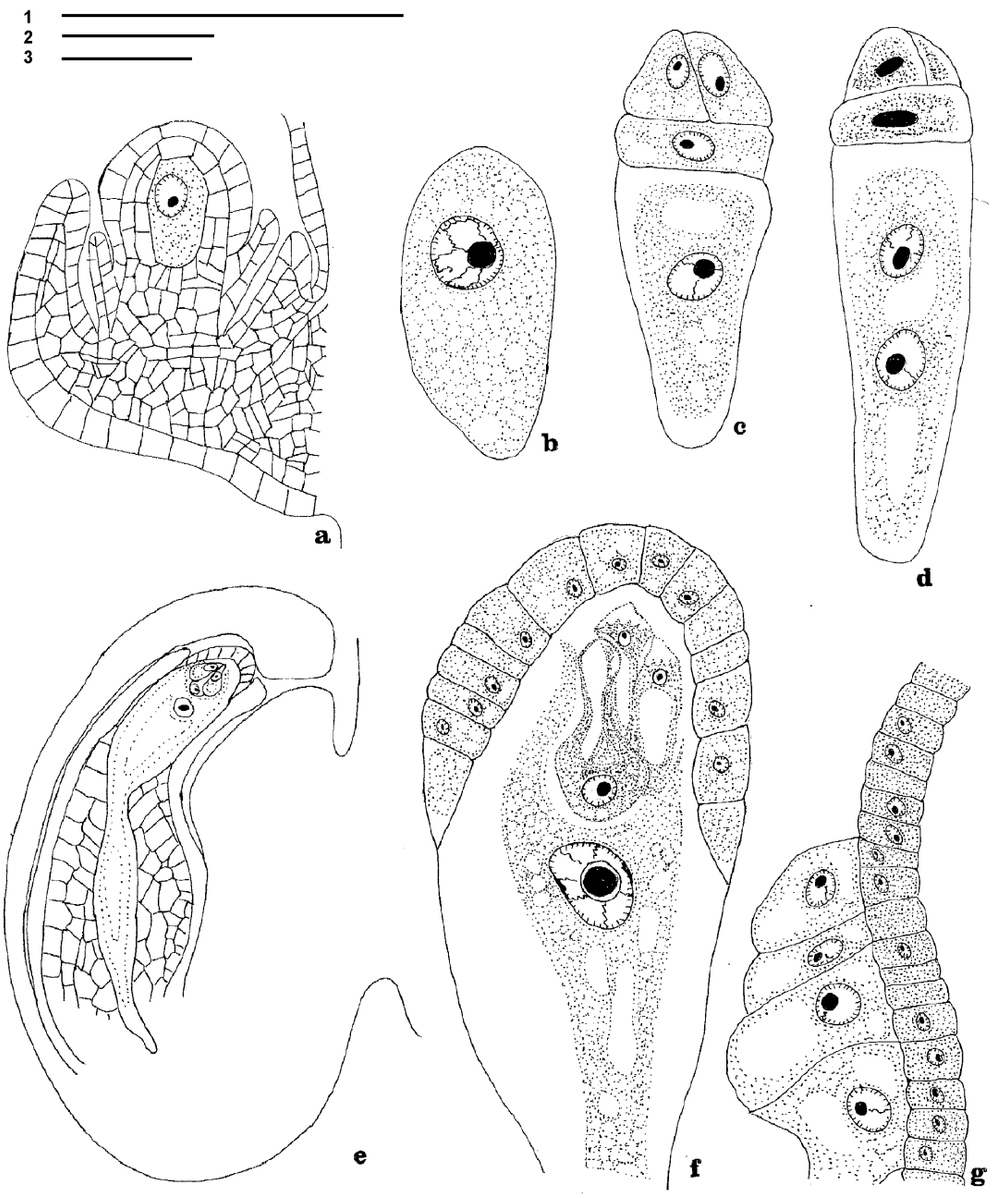

Fig. 6. *Phaseolus vulgaris* var. *aborigineus*. (**a**) Young ovule, (**b**) MMC, (**c**) T-shaped megaspore tetrad, (**d**) 2-nucleate megagametophyte and abortive megaspores, (**e**) Ovule at mature female gametophyte stage, (**f**) Micropylar end of mature megagametophyte and nucellar epidermis, (**g**) Detail of endothelium and some vacuolate nucellar cells. Bar 1 = 50 µm, for (a); Bar 2 = 25 µm, for (e); Bar 3 = 25 µm, for (b–d) and (e–f).

formed between the four nuclei at the micropylar end and the four at the chalazal end. After the 8-nucleate stage, the coenocytic megagametophyte becomes cellular. This process is simultaneous in the micropylar and chalazal parts. The embryo sac consists of seven cells: the egg cell, two synergids, the central cell and three antipodal cells (Figs. 4f,h, 5f,g, 6f, 7d–e). Megagametogenesis follows the Polygonum type.

The central cell is highly vacuolate and contains two polar nuclei which fuse and form a diploid central nucleus before fertilization (Figs.

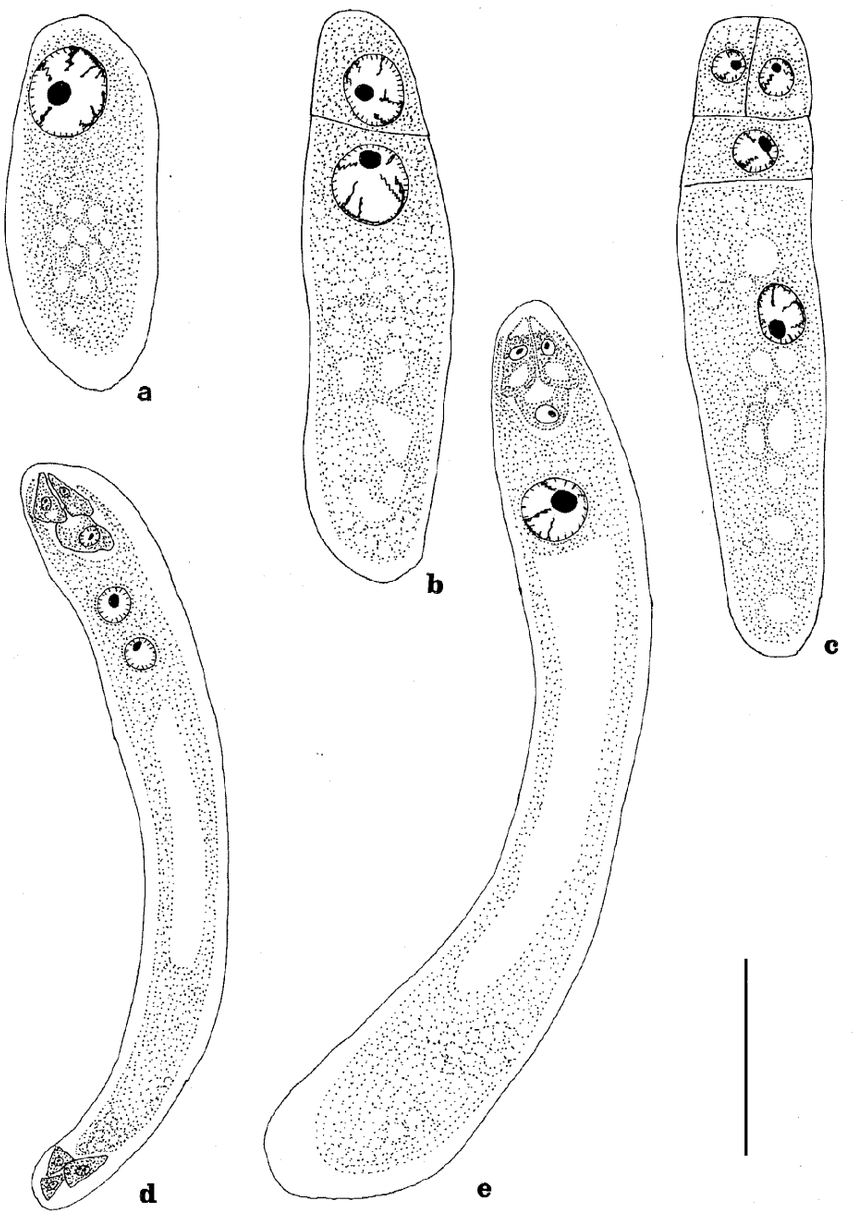

Fig. 7. *Vigna adenantha.* (**a**) MMC, (**b**) Dyad, (**c**) T-shaped megaspore tetrad, (**d**) Young 7-cellular megagametophyte, (**e**) Mature megagametophyte. Bar = 25 µm.

5f,g, 7d,e). The micropylar end of the egg cell is filled by a large vacuole and the chalazal end is filled with cytoplasm containing the egg nucleus. In contrast, the chalazal end of the synergids is occupied by one large vacuole and their nuclei are in the micropylar region. At the micropylar pole, the synergid wall is strongly thickened and develops the filiform apparatus. The filiform apparatus consists of a mass of pectic wall projections extending deep into the cytoplasm (Figs. 4,; 5g, 6f, 7e).

The antipodals are slightly vacuolate; they are ephemerals. They degenerate during maturation of the megagametophyte. When the egg apparatus is totally differentiated, the antipodal cells have disappeared (Figs. 4f, 5f, 7e).

DISCUSSION

The morphology and development of the ovule reaffirms earlier observations on the subfamily (Prakash, 1987; Jorhi et al., 1992) except in the structure of the micropyle. Previous studies describe a zigzag micropyle with an exostome, mesostome and endostome constituted by both integuments. A true endostome is not formed in the four species studied here. The inner integument at the opposite funicular side never reaches the micropylar end of the nucellus, and an endostomatic channel is formed by the inner integument at the funicular side and by the internal face of the outer integument at the opposite funicular side.

The occurrence of an integumentary tapetum or endothelium is not constant throughout the subfamily or even throughout species of the same genus. It is present in *Paseolus aconitifolius* (Deshpande and Bhasin, 1974) and in *P. vulgaris* (Weinstein, 1926) but absent in *P. aureus* (George et al., 1979). In *Vigna adenantha* this tissue does not differentiate, but it was observed in both *Phaseolus* species studied in this work and in *Macroptilium bracteatum*. The functions ascribed to the endothelium are to supply nutrition to the megagametophyte and to act as a barrier tissue to resist the aggressive action of the growing female gametophyte (Bouman, 1984).

The ovule of *M. bracteatum* shows a zone of thick-walled cells above the megagametophyte. These cells form an epistase tissue which plugs the micropyle. This is the first report of this tissue in Leguminosae. This structure has been observed only in Zingiberaceae, Nymphaceae, and a few members of Myrtales (Bouman, 1984). In *M. bracteatum, P. augusti, P. vulgaris* var. *aborigineus* and *V. adenantha*, a large cytoplasmic concentration at the chalazal pole of the MMC was seen. This polarity may be related to selection of the functional megaspore.

Phaseolus augusti, P. vulgaris var. *aborigineus* and *V. adenantha* show T-shaped megaspore tetrads, while in *M. bracteatum* a linear tetrad is formed. Brown (1917) and Weinstein (1926) reported a triad of megaspores in *P. vulgaris*. The triads may have been T-shaped tetrads observed from the other longitudinal plane.

Female gametophyte development belongs to the Polygonum type, which seems to be a common feature in the Leguminosae, except for members of the tribe Miribelieae which exhibit unique patterns (Cameron and Prakash, 1994).

To delimit *Vigna* and *Phaseolus*, Verdocurt (1970) and Marèchal et al. (1978) decided to include only about 50 Andean species in the latter genus, so they transferred many species from *Phaseolus* to *Vigna*. As a consequence, *Vigna* increased its heterogeneity. The current assignment of *V. adenantha* to the genus *Vigna* is controversial. *V. adenantha* shares many morphological features with *Phaseolus* which are absent in the remaining species of *Vigna*: epigial cotyledons, the apical position of the stigma, the rim aril not raised, and tricolporate pollen grains with reticulate exine (Marèchal et al., 1978; Di Stilio, 1994; Palacios and Hoc, 2001).

Phenetic analysis based on phytochemical characters reveals that *V. adenantha* has an affinity with *Phaseolus*, while *V. luteola* and *V. peduncularis* have an affinity with some members of *Macroptilium* (Zallochi, 1992).

According to our study, *V. adenantha*, *P. augusti* and *P. vulgaris* var. *aborigineus* have several embryological characters in common which they do not share with *M. bracteatum*: a semioval mature ovule in longitudinal section, the shallow position of the MMC in the nucellus, the lack of an epistase, an outer integument 4–7 cells thick, and an exclusively T-shaped megaspore tetrad. Embryological features of *M. bracteatum*, *P. augusti*, *P. vulgaris* var. *aborigineus* and *V. adenantha* and literature data for Phaseolinae are summarized in Table 1.

Rembert (1969, 1972) considers that megaspore tetrad patterns are a legitimate tool to pursue relationships within groups of angiosperms. *V. adenantha* shows a different megaspore tetrad type, opposite to the other *Vigna* species studied so far (Tab. 1). The similar arrangement of megaspores in *V. adenantha* and *Phaseolus* species observed in this research and the other shared embryological characters must be taken in account to verify the taxonomic position of *V. adenantha*.

This is the first report of the use of embryological characters as a promising tool for solving intergeneric relationships of the *Phaseolus-Vigna-Macroptilium* neotropical complex.

ACKNOWLEDGEMENTS

We wish to thank Dr. Maria T. Amela Garca for a critical reading of the manuscript.

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