Taiwania 68(1): 51–74, **2023**
DOI: 10.6165/tai.2023.68.51

Development of seed and fruit in *Bischofia javanica* Blume (Phyllanthaceae)

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(Manuscript received 23 May 2022; Accepted 16 January 2023; Online published January 28 2023)

ABSTRACT: Taxonomic affinity of genus *Bischofia* needs to be reaffirmed in view of the splitting of the erstwhile Euphorbiaceae into six distinct families. In addition, the role of the reproductive features in reported invasive nature of the tree species *Bischofia javanica* also requires to be investigated. To answer these questions, reproduction and fruit/ seed development have been investigated in, *Bischofia javanica* Blume (Phyllanthaceae). All stages of flowering and fruit/ seed development were collected, fixed in formalin-acetic-alcohol, sectioned with rotary microtome and stained with safranin-astra blue for study with light microscope. The ovule is anatropous, bitegmic and crassinucellate. Distinct obturator and hypostase are present. Embryo sac development is of Polygonum type. Pollination is anemophilous. Development of endosperm is of nuclear type. The embryogeny conforms to the Capsella variation of the onagrad type. At the organized embryo sac stage, the outer as well as the inner integument consist of three or four layers of cells. Seed is exotegmic. As the pericarp matures, the inner subdermal cells become sclerosed, acquire prismatic crystals and constitute the stony endocarp. Cells of the middle zone enlarge considerably and several trichosclereids are also differentiated. The exocarp is smooth and tanniniferous. Fruit is a 3-loculed, loculicidal capsule or schizocarp. Based on the embryological features, placement of *Bischofia* in the family Phyllanthaceae is justified. Profuse flowering, anemophily, high rate of fruit/seed set, extended seed viability and ability of seedling to grow well in shade or light are among reproductive features that can aid invasive behaviour of the species.

KEY WORDS: Bischofieae, fruit, integuments, invasive species, ovule, Phyllantheae, seed.

INTRODUCTION

Bischofia javanica Blume (Phyllanthaceae), commonly known as bishop tree, is widespread from Indo-Malayan to pacific region. It is grown as an ornamental or timber tree in many countries within and outside its natural range, and is even regarded as an invasive species in many locations (Morton, 1984; Yamashita *et al*., 2003).

Bischofia javanica is a dioecious species and sex changes have been often reported (Yamashita and Abe, 2002). The classification and affinities of *B. javanica* have been debatable. It has been placed either in tribe Phyllantheae or in independent tribe Bischofieae within sub-family Phyllanthoideae of Euphorbiaceae (Hutchinson 1969, Webster, 1987). Airy Shaw (1966) assigned the same genus to independent family Bischofiaceae. Many taxonomists include it in a separate family Phyllanthaceae (Bingtao, 1994; Savolainen, 2000; Hoffmann *et al*., 2006; APG IV, 2016).

The taxonomic significance of some embryological features of *B. javanica* were discussed by Bhatnagar and Kapil (1973). A detailed study of anther development was also carried out (Bhatnagar and Kapil, 1979). The study showed that similarities in embryological characteristics of *B. javanica* and other Euphorbiaceae affirm its placement in the same family. Kapil and Bhatnagar (1994) discussed the similarities and differences among five subfamilies of Euphorbiaceae as circumscribed by Webster (1975). Of these, *Bischofia* bears close embryological resemblance with the Phyllanthoideae. APG IV (2016) recognized three biovulate families (Picrodendraceae, Putranjiviaceae and Phyllanthaceae) and two uniovulate families (Euphorbiaceae and Pandaceae) from Euphorbiaceae *sensu lato*. Phyllanthaceae is the largest of the biovulate lineage. According to the Ajaib and Khan (2012) the genus *Bischofia* has been placed in family Phyllanthaceae, which is split apart from Euphorbiaceae as it shows various growth forms.

Owing to its multipurpose uses, researchers are looking at the secondary metabolite profiles, economic and pharmaceutical uses. The plant produces red dye from bark, wine from fruits and oil from edible seeds (Christopherson, 1935, Mahanta and Tiwari, 2005). Leaf extract has been shown to possess antidiabetic chemical constituents (Mai, 2017). Antimicrobial properties of leaves and their importance in curing various fungal diseases have recently been identified (Sarmah *et al.,* 2020). According to Das *et al.* (2012), the leaves contain vitamin C and bark is rich in tannin. Nutritional profiling of the *Bischofia* seeds has also been carried out by Indra *et al*. (2013). It has been found that seeds have high content of carbohydrate, minerals, and proteins. Also, the edible oil extracted from the seeds contain essential omega 3 fatty acids.

The tree has been planted in many areas as it shows rapid growth and dense dark green glossy foliage (Streets, 1962; Morton, 1984). However, in the past few years there is an increase in concern of the spread of this tree as it is turning out to be an invasive species (Yashimata and Abe, 2002; Itou *et al*., 2015; Gilman *et al.*, 2018).

Emphasis on physiology and ecological attributes of the species and their relevance to invasion has gained momentum lately (Naoko *et al*., 2003). However, there hasn't been any recent conclusive study on morphological and embryological aspects, especially the study of ovules and seeds. Importance of embryological traits vis-à-vis functional biology are also gaining significance as these give some idea about invasiveness of species. Besides, detailed investigation and comparison of plant morphology, anatomy and embryology have acquired an important place in systematics.

The embryological features in Euphorbiaceae are highly conservative and have been used in delimitation of taxa and understanding the taxonomic relationships. Division in Euphorbiaceae is primarily based on the number of ovules. Tokuoka and Tobe (1995) suggested five characters of seed development that can be taken into consideration for comparison between and with the sub-families of Euphorbiaceae. These are (i) presence or absence of vascular bundles in inner integument, (ii) thickness of inner integument, (iii) whether ovules and seeds are pachychalazal, (iv) whether seeds are arillate, and (v) whether the exotegmen is fibrous. In view of the importance of these attributes, the present study was undertaken to investigate the reproductive features, including seed and fruit development in *B. javanica,* and evaluate its relationship with Euphorbiaceae and Phyllanthaceae. The study established that the reproductive traits of this tree species play a significant role in its invasive behaviour in some native as well as introduced locations.

MATERIALS AND METHODS

The plants were observed in the field at the study sites and morphological characters were noted. Buds, flowers, and fruits were collected from Forest Research Institute and Motharanwala swamp, subtropical locations at foot of the Himalaya, Dehradun (Uttarakhand), India and Acharya Jagdish Chandra Bose Indian Botanical Garden, tropical location on bank of the river Ganga, Sibpur (West Bengal), India. Progressive stages of flowering on male and female trees were observed from March to June, and fruit development through monsoon period till October. Developmental stages progressed in a synchronous manner in both male and female trees. Therefore, to complete all life cycle stages, collections were made over many years from a few tagged trees at each site. Voucher specimens have been deposited at the Delhi University Herbarium (DUH). The material was fixed in FAA (1:1:18) and dehydrated through graded tertiary butyl alcohol series and embedded in paraffin wax (congealing temperature 58-60°C). Sections were cut between 5–15 µm thickness with the microtome (Leica) and stained with safranin-astra blue combination. Crystals of calcium oxlate were observed with the Pizzolato method.

RESULTS

External Morphology

Bischofia javanica is a tall, dioecious tree with spreading branches. Its leaves are spirally arranged and palmately three- or five foliolate. The leaflets are ovate, acuminate, crenate-serrate with terminal leaflets having longer petioles. Stipules are caducous.

Male flowers are small, bracteate, pentamerous, arranged in axillary and pendant thyrses. The pistillate flowers are also arranged in axillary thyrses. In pistillate flowers, calyx consists of five cuneate, acuminate sepals and corolla is absent. Staminodes are represented by a minute outgrowth in the axil of each sepal. Gynoecium is tricarpellary, syncarpous, trilocular (rarely tetralocular) with two pendulous ovules in each locule. Style is short. Stigma is linear or reflexed. The fruit is a 3-loculed, loculicidal capsule or schizocarp with a smooth surface, but vertical lines of dispersal are present in each compartment of stony endocarp. Mature seed is estrophiolate, trigonous and has fluted surface. Six seeds, two in each compartment, are compactly arranged in the capsule.

Megasporangium

The young ovular primordium is distinguishable into three zones: (a) dermatogen, whose cells divide anticlinally; (b) subdermatogen whose cells initially divide anticlinally, but later divide periclinally and form two layers; and (c) inner core of cells arranged in vertical rows formed by anticlinal and some periclinal divisions (Fig. 1A–D). The primordium begins to curve even before initiation of integuments (Fig. 2A–D) and the ovule subsequently becomes anatropous at anthesis (Fig. 2E–J). An instance of reverse curvature of the ovule has been noticed (Fig. 2K). A subsessile and orthotropous ovule was also observed (Fig. 2L).

The ovule is bitegmic and crassinucellate. One of the two ovules in each locule arises at the junction of the carpellary wall and central axis whereas the other originates slightly higher on the placental axis. Both the ovules shift upwards because of elongation of the ovarian axis below the place of ovule attachment.

Integuments

The inner integument arises as an annular swelling around the ovular primordium. This is accompanied or closely followed by the initiation of outer integument along the transfunicular and lateral sides (Fig.1B). The patterns of initial divisions of dermal cells which give rise to the outer and the inner integuments are similar. An epidermal cell enlarges slightly and undergoes periclinal divisions giving rise to outer and inner derivatives, Ia and Ib (Fig. 3 A–D). This is succeeded (Fig. 3B–E) or sometimes preceded, by oblique vertical divisions in the epidermal cells flanking it on upper and lower sides, each

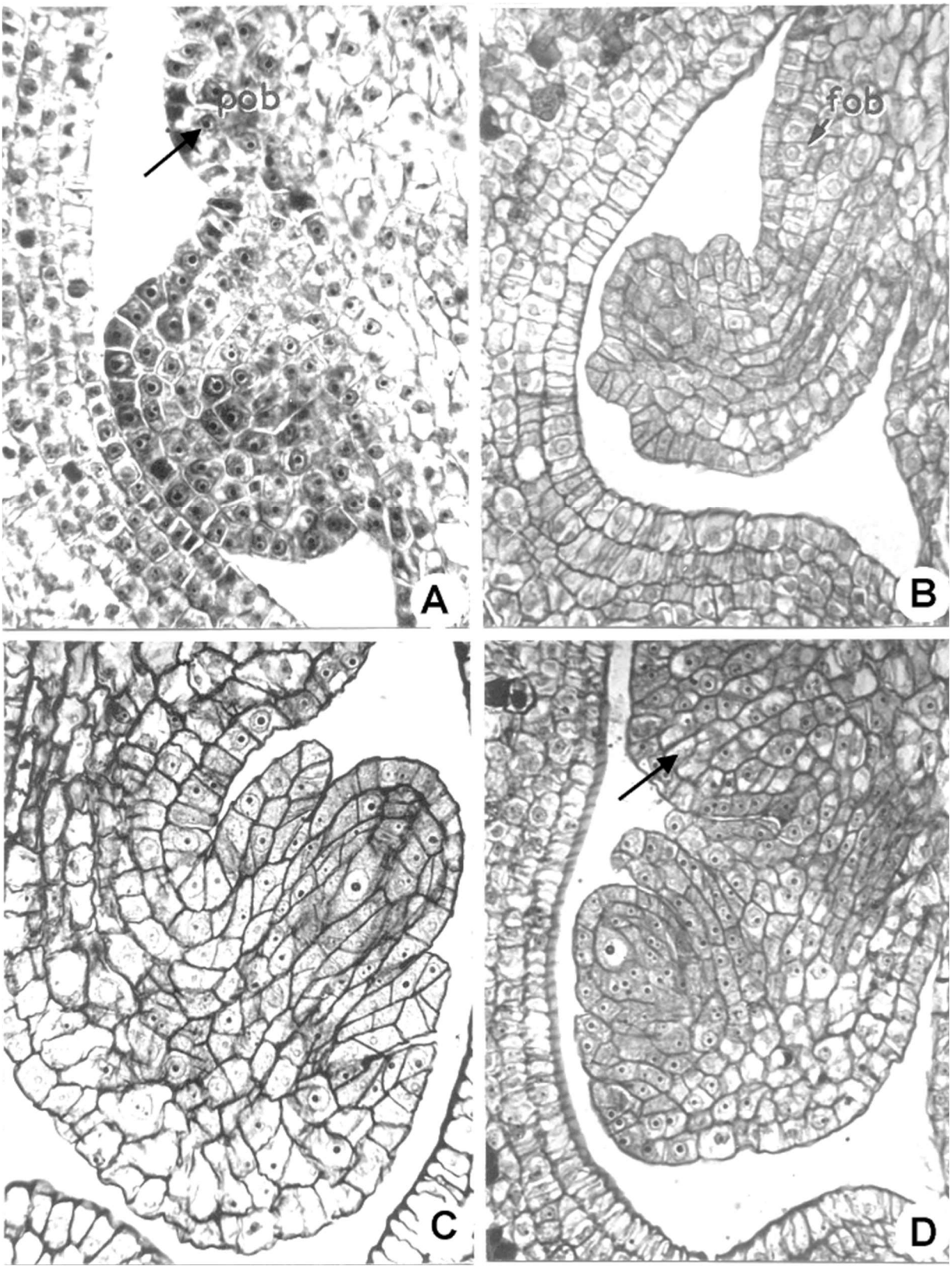

Fig 1. ***Bischofia javanica***. Megasporangium and obturator. **A**. Longisection through ovular primordium showing histogenic differentiation; initials of placental obturator (pob) are also seen (x730). **B**. Young ovule in longisection; both integuments are initiated simultaneously. Some cells of funiculus have enlarged (fob) (x360). **C**. Longisection of ovule at megasporocyte stage; outer integument is not distinguishable toward funiculus (x480). **D**. Ovule with persistent archesporial cell; outer integument can be seen toward funiculus (x345).

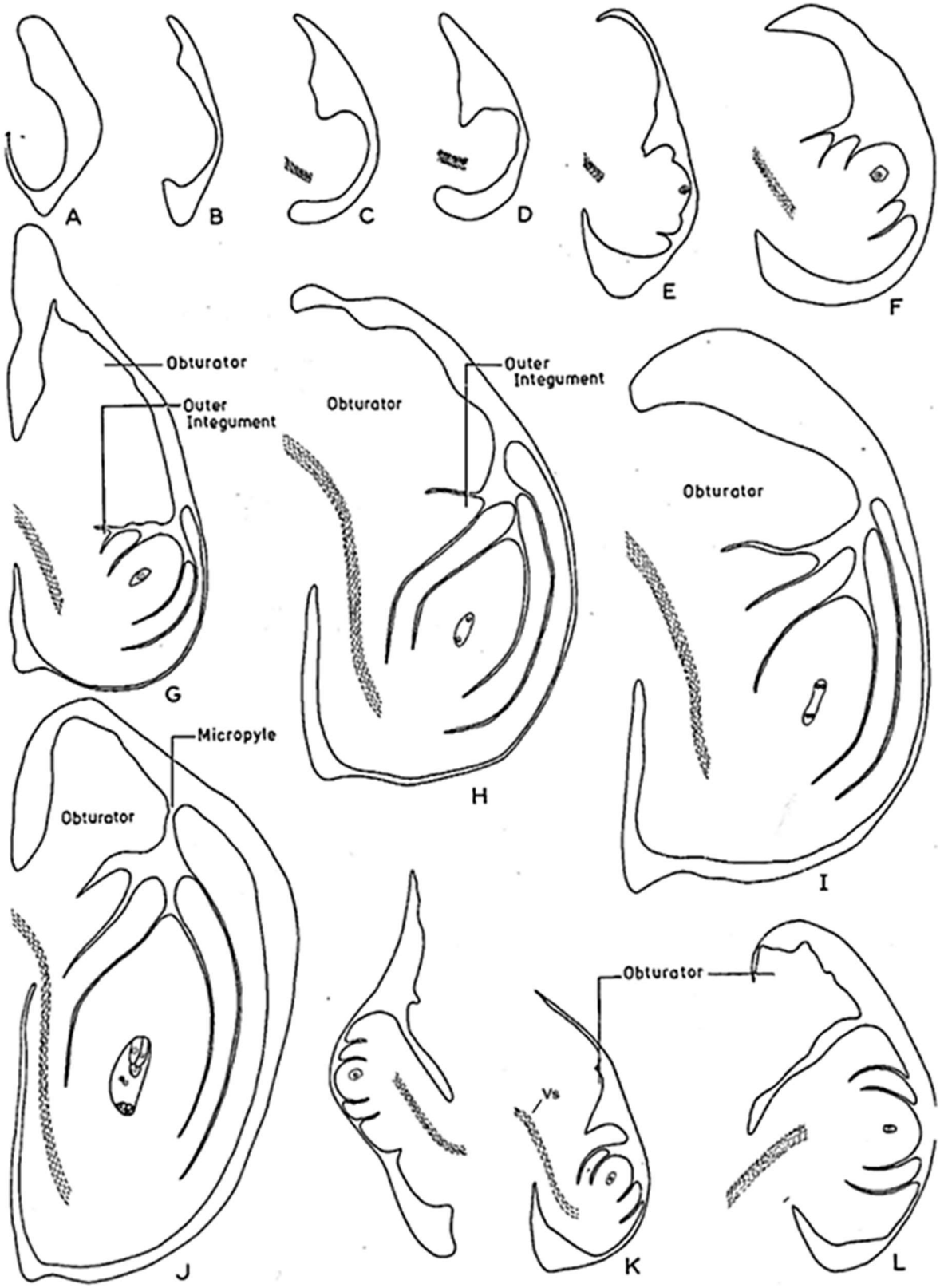

Fig. 2. ***Bischofia javanica***. Megasporangium. **A**–**J**. Stages leading to anatropy of ovule; development of obturator from placentum is also shown (x130). **K**. Longisection of ovary with one ovule having reverse curvature (Vs- vascular starnd) (x130). **L**. Abnormal subsessile, orthotropous ovule along with placental obturator (x130).

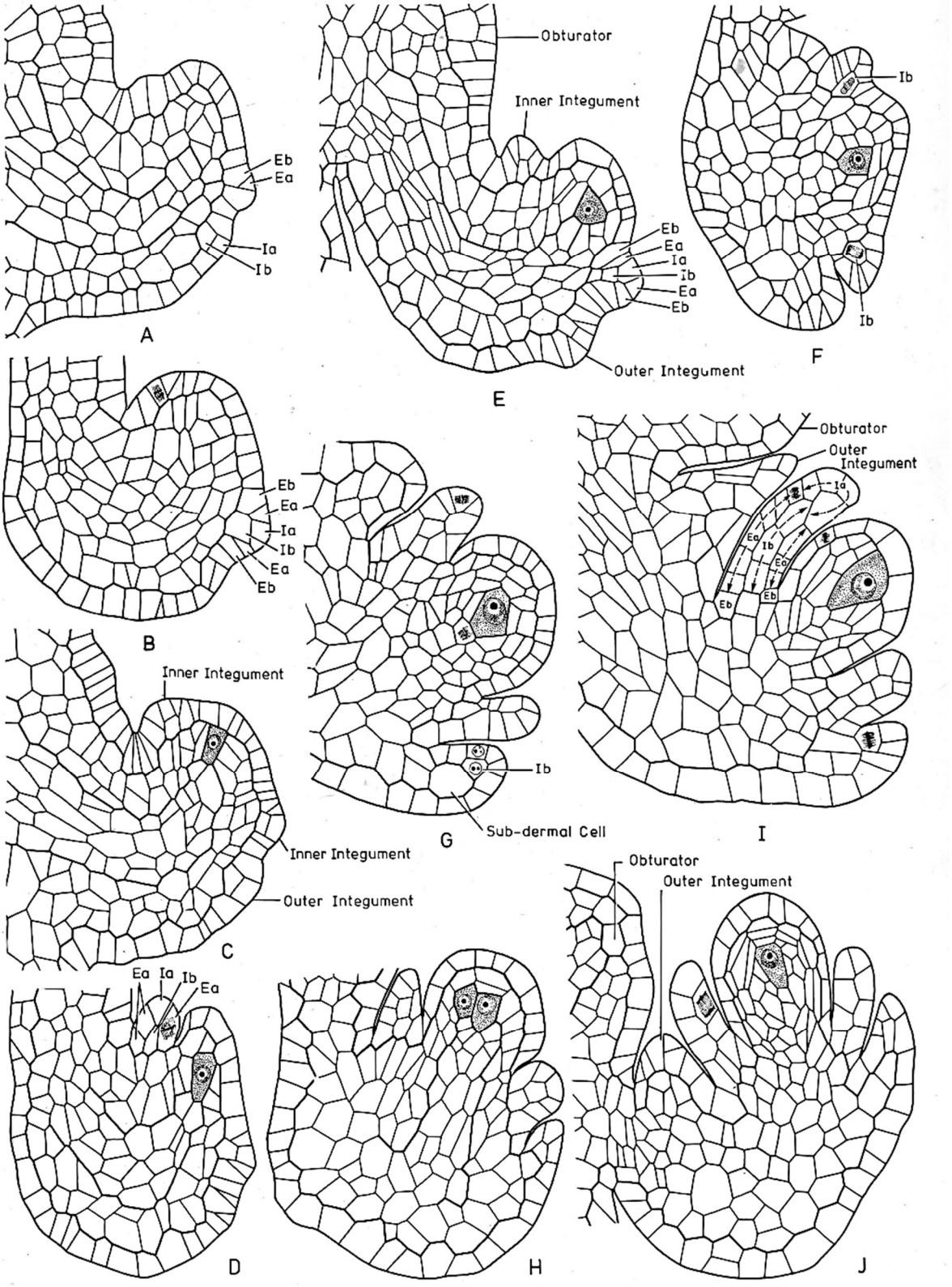

Fig 3: ***Bischofia javanica***. Integument initiation. **A**–**E**. Longisections of ovules at early stages of development. Initials of inner and outer integuments have divided to form cells Ea and Eb and Ia and Ib (x 340). **F**. Cells Ib of inner integument divide periclinally (x 340). **G**. Derivatives of Ia divide anticlinally; in outer integument one subdermal cell has become prominent (x340). **H**. Young ovule with two sporogenous cells in longisection (x 340). **I**, **J**. Ovules in longisection showing initiation of outer integument towards raphe; inner integument is two-layered in J (x340).

resulting in cells Ea and Eb. The cell Ib undergoes periclinal divisions (Fig. 3F–I), whereas Ia and Ea and their daughter cells divide anticlinally. Further growth of the integument is brought about by division and enlargement of Ia, Ea, Eb and their derivatives (Fig. 3E-J). The pattern of cell division in outer integument is identical during early growth (Fig. 3C, E), but the cell Ib remains undivided (Fig. 3G) or divides only sporadically (Fig. 1C, D). Instead, the subjacent subdermal cells multiply and add to the rapid growth of the outer integument (Fig. 3H–J). Toward the raphe the outer integument is represented by a relatively small, belatedly-formed projection produced by the activity of dermal as well as subdermal cells (Figs. 1D; 3I, J; 4A–D).

Occasionally, two adjascent dermal cells undergo oblique division and their subsequent anticlinal divisions give rise to a two-layered inner (Fig. 3J) or outer integument. Less often the cell Ib or its derivatives undergo anticlinal divisions and constitute a four-layered inner integument.

Inner Integument- The initial growth of the inner integument is mainly due to: (a) periclinal divisions of derivatives of the cell Ib and (b) anticlinal divisions in derivatives of the cells Ia and Ea. Subsequently, cells of the middle layer of the integument divide periclinally so that the inner integument becomes four or five cells thick, especially toward the raphe (Fig. 4D). At the two-nucleate stage of embryo sac, inner epidermal cells along the micropyle divide periclinally, resulting in radial rows of dermal cells in this zone. The outer epidermis of inner integument differentiates into a layer of densely cytoplasmic cells whereas the inner epidermis becomes tanniniferous (Fig. 4C, D).

Outer Integument- Subdermal cells divide rapidly and form the core of the basal part of outer integument (Fig. 4 A–D). During later development the cell Ia undergoes oblique divisions, and the resulting cells divide anticlinally. Because of this activity a two-cell-thick flange is produced at the apical rim of outer integument (Fig. 4B) which grows rapidly and envelopes the inner integument. The cells of the outer epidermis are filled with tannin (Fig. 4C).

Nucellus

The archesporial cell (or cells) is differentiated in hypodermal position (Fig. 3C). It divides to form the outer primary parietal cell and inner primary sporogenous cell. The former undergoes repeated periclinal divisions and gives rise to an axial row (or rows, if archesporium is multicellular) of cells (Fig. 5A). These cells are richly cytoplasmic and constitute a micropylar axial meristem, whose cells divide periclinally as well as anticlinally. The peripheral derivatives resulting from such anticlinal divisions join the vacuolate nucellar cells rank by rank and, like them, divide periclinally at a comparatively slower rate than the axial cells. Some rows of cells below the sporogenous tissue also become meristematic and constitute a chalazal axial meristem. Their divisions contribute to the growth of nucellar region underneath the sporogenous cells (Fig. 4A, B).

In some abnormal ovules the primary parietal cell is not delimited. The nucellar cells surrounding the archesporial cell divide periclinally and the chalazal axial meristem becomes more active. The persistent archesporial cell, therefore, comes to lie near the tip of massive nucellus (Fig. 5B).

During the development of embryo sac, the extent of micropylar axial meristem remains constant because of the differentiation of laterally delimited cells. However, the cells of chalazal axial meristem multiply and form several vertical rows of cells extending between the embryo sac and the chalaza (Fig. 5C). A few dermal cells at the nucellar apex also divide periclinally and form a conical nucellar cap (Fig. 5D).

Obturator

It is initiated by cytokinetic activity of subdermal cells of the placentum (Fig. 1A, D), and forms a canopy over the ovule. In the ovules which are partly attached to the carpellary wall some subdermal cells of the funiculus divide and form the obturator (Fig. 1B, C). At anthesis the obturator forms a hood over the exostome and some of its elongated, glandular dermal cells approach the endostome. In the carpellary locules in which an ovule fails to curve and become anatropous, obturator is formed in the normal placental position (Fig. 2L); but in those having ovules curved in the reverse direction, obturator-like projections occur on the placentum as well as on the funiculus (Fig. 2K). The obturator is fully developed at the mature embryo sac stage. Soon after fertilization it degenerates.

Megasporogenesis and Female Gametophyte

The primary sporogenous cell is covered by 10-15 layers of parietal tissue. It enlarges and functions as megaspore mother cell. During first meiotic division the spindle is oriented parallel to the long axis of the megasporocyte (Fig. 6A). Meiosis II occurs synchronously in the dyad (Fig. 6C) and a linear tetrad of megaspores is produced (Fig. 6E). Sometimes, the meiotic spindle in the micropylar dyad cell is perpendicular to that of the chalazal cell (Fig. 6D), resulting in a T-shaped tetrad (Fig. 6F). Less often, the micropylar dyad cell divides horizontally and the chalazal at right angles to it so that the daughter megaspores are disposed in an inverted T-shaped manner (Fig. 6H, I). Triads, formed by division of the chalazal dyad cell and degeneration of the micropylar one are also observed (Fig. 6B, G; 7A).

The chalazal cell of the dyad is generally larger than the micropylar. Meiosis II in the former is mostly unequal so that the chalazal most megaspore in a tetrad is the

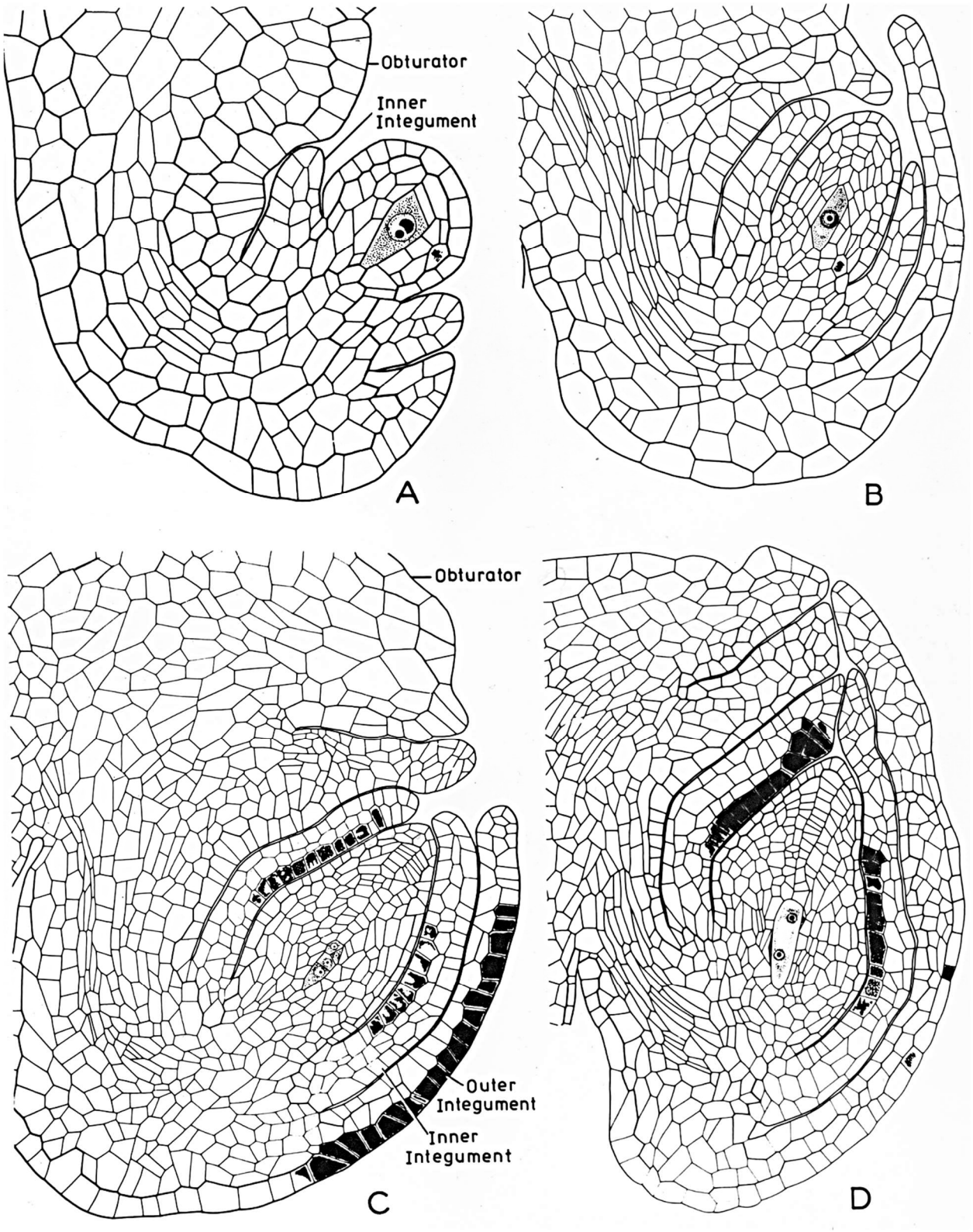

Fig. 4. ***Bischofia javanica***. Integuments. **A**, **B**. Longisections of ovules at megasporocyte stage showing cell division pattern in integuments (A. x665; B. x475). **C**. At megaspore tetrad stage tannin (dark due to staining) is deposited in some cells of integuments (x320). **D**. Ovule with two-nucleate gametophyte; inner integument is four-layered toward funiculus. Some distal cells of inner epidermis of inner integument have divided (x370).

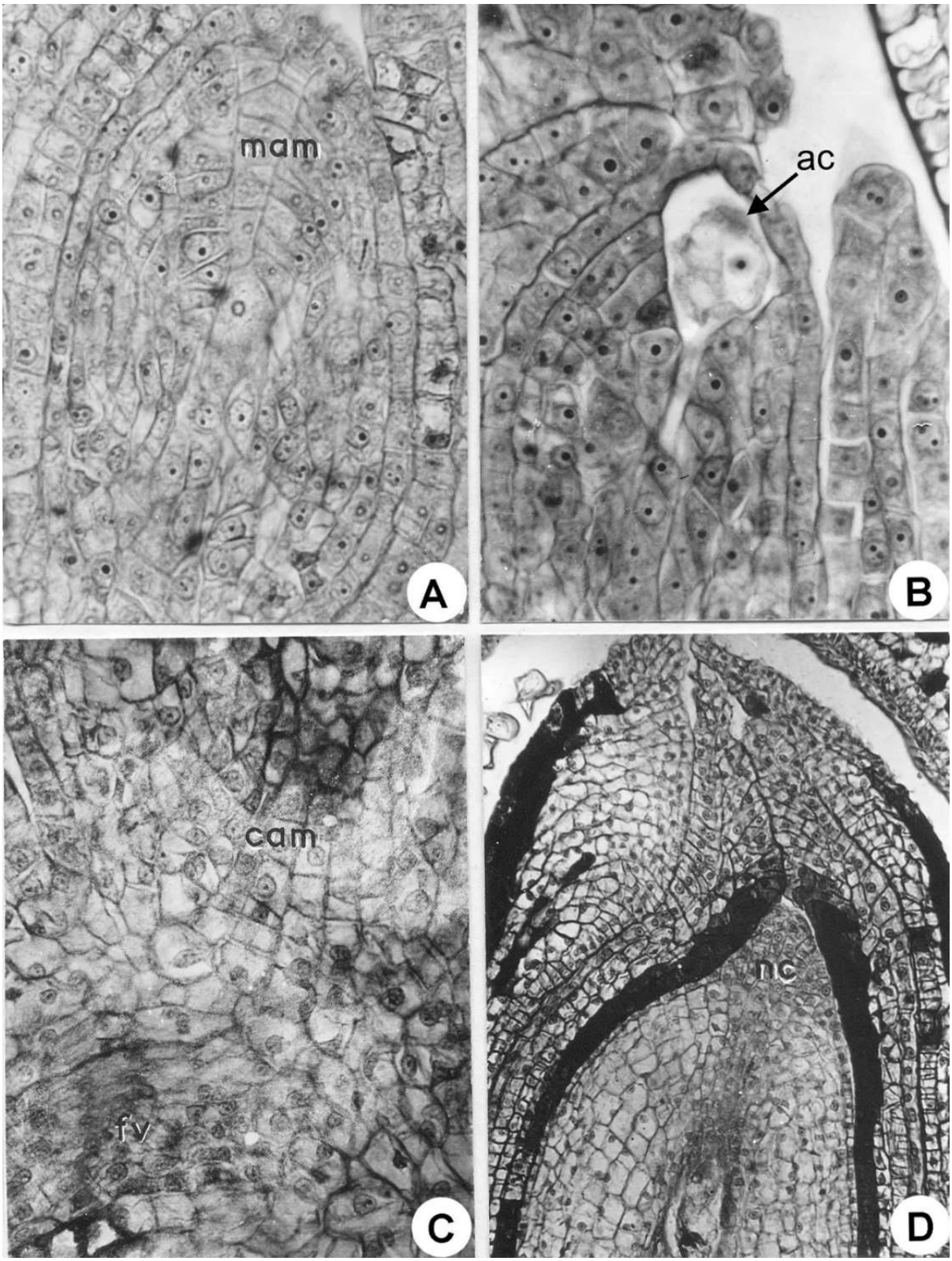

Fig. 5. ***Bischofia javanica***. Nucellus. **A**. Longisection through nucellus to show position of micropylar axial meristem (mam) (x695). **B**. In some abnormal ovules the nucellar cells surrounding the archesporial cell divide and become more active. Note young ovule with a large, persistent archesporial cell (ac); nucellus is massive (x725). **C**. Ovule at mature embryo sac stage; cells of chalazal axial meristem (cam) approach funicular vasculature (fv) (x590). **D**. Micropylar part of ovule in longisection showing nucellar cap (nc) and micropylar axial meristem (x195).

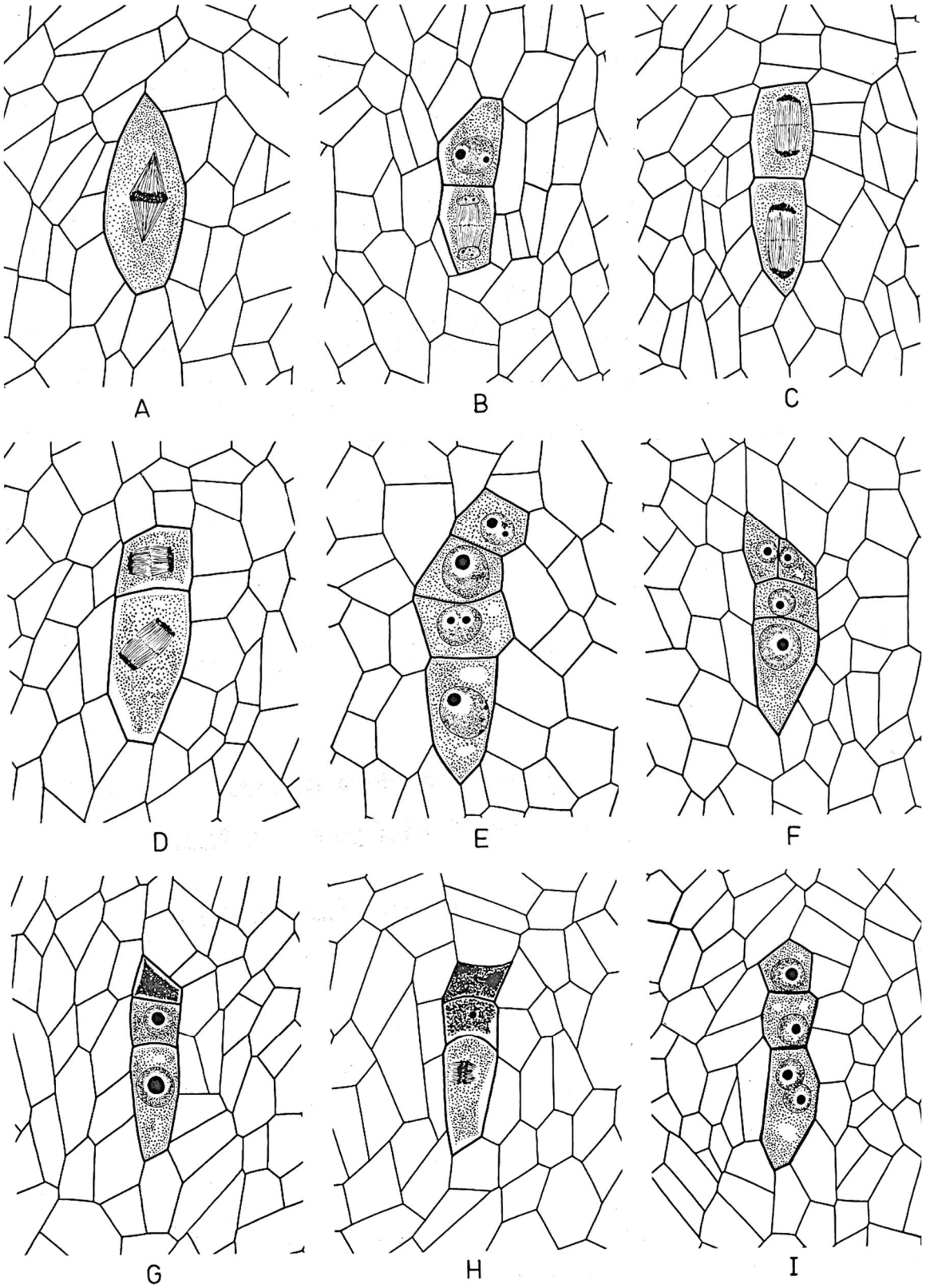

Fig. 6. ***Bischofia javanica***. Megasporogenesis**. A.** Megasporocyte in division. (x750). **B.** Chalazal cell of dyed undergoing division (x760). **C, D.** Both dyed cells dividing synchronously; in C both spindles are oriented lengthwise whereas in D they are almost at right angles (x750). **E, F.** Linear and T-shaped tetrads respectively (x750). **G.** Triad: micropylar dyed cell has degenerated (x750); **H.** Micropylar megaspores have degenerated whereas chalazal dyad cell is in division (x750). **I.** Tetrad with inverted T-shaped orientation (x750).

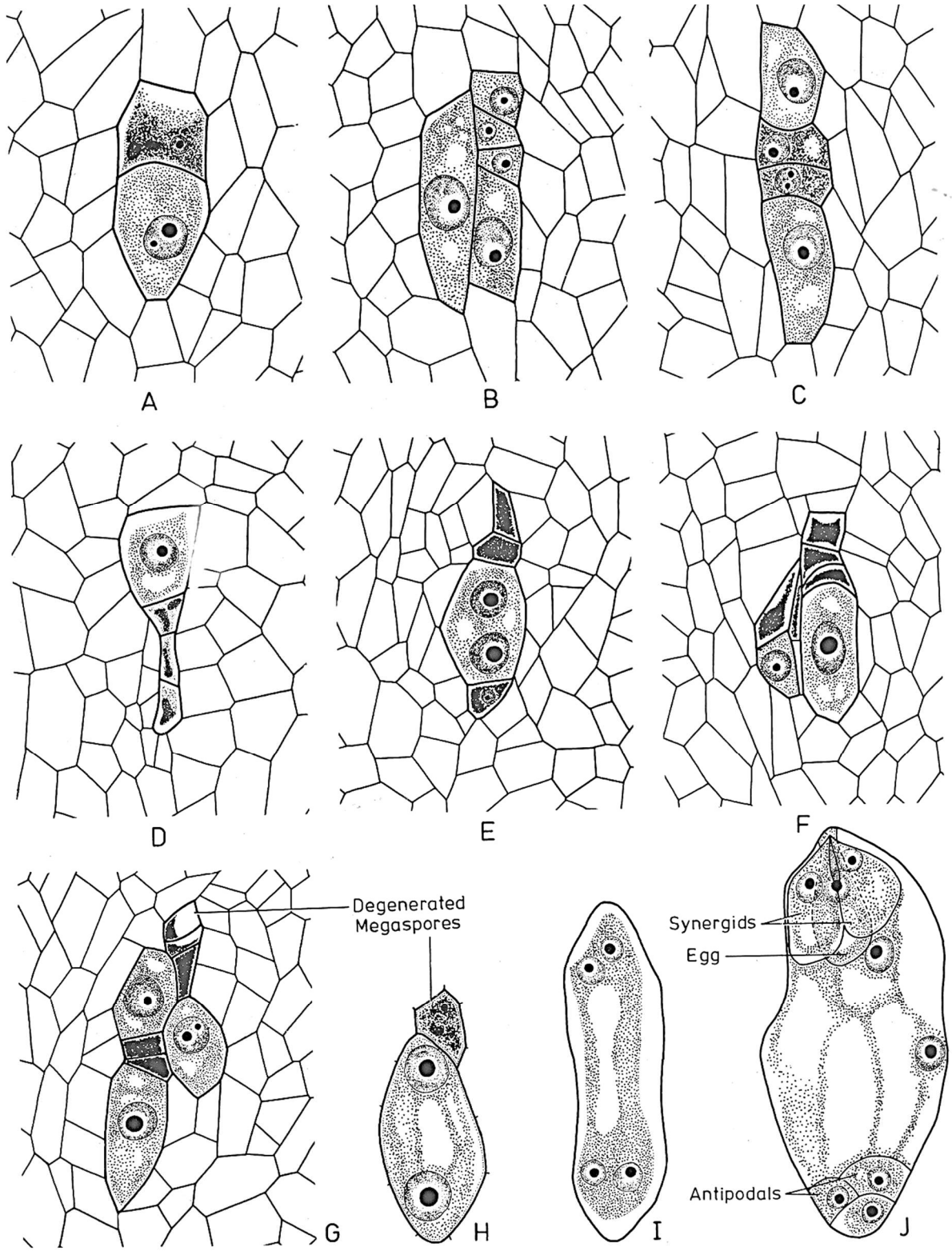

Fig. 7. ***Bischofia javanica***. Megasporogenesis and Megagametogenesis. **A**. Dyad with degenerated micropylar cell. **B**. Coexisting megasporocyte and megaspore tetrad with enlarged chalazal megaspore. **C**. Tetrad; chalazal and micropylar megaspores have enlarged. **D**. Linear tetrad with functional micropylar megaspore. **E**. Two-nucleate gametophyte developed from sub-chalazal megaspore. **F**. Tetrad with chalazal functional megaspore, accompanied by a dyad with degenerated micropylar cell. **G**. Two megaspore tetrads – one shows enlarged micropylar as well as chalazal megaspores whereas in other chalazal is functional. **H**, **I**. Two- and four-nucleate embryo sacs, respectively. **J**. Organized embryo sac (Figs. A-J; all magnifications: x740).

largest and functional (Fig. 7B, F) while the outer megaspores degenerate (seen as dark). Rarely any one of the other megaspores may function to form the embryo sac (Fig. 7D, E). Tetrads with more than one enlarged megaspore were also observed (Fig. 7C, G). Two to four sporogenous cells co-exist in some ovules. (Fig. 7F, G).

The functional megaspore undergoes three mitotic divisions forming, successively, two-, four- and eight-nucleate embryo sacs (Fig. 7H, I). The organized embryo sac (Fig. 7J) is of the Polygonum type. It contains two synergids and an egg at the micropylar pole, three antipodal cells at the chalazal end and two polar nuclei in the central cell. The synergids are usually pear-shaped, with an inconspicuous hook and prominent, Petunia type (Van Went and Linskens, 1967) filiform apparatus. The apical half of each synergid is vacuolate, whereas the basal is richly cytoplasmic and contains the nucleus. The central cell has large vacuoles and prominent cytoplasmic strands. Antipodal cells are oriented in a T-shaped (8A) or triangular fashion (Fig. 8 C).

An embryo sac with the antipodal cells simulating the egg apparatus was also observed (Fig. 8B). Often one of the synergids is much smaller and seems to be in a degenerated condition (Fig. 8C). In one of the embryo sacs observed, the egg was seen in lateral position (Fig. 8A).

Fertilization

Pollen dispersal occurs through anemophily. Pollen tube penetrates the tip of the stigmatic papillae (Fig. 9A). A short canal, lined by glandular cells akin to those of the stigma, leads down to the proximity of the ovarian chamber (Fig. 9B). Close to the locules, the stylar canal divides into three channels which terminate between the two obturators of each locule.

Several changes take place in the embryo sac prior to fertilization. Most prominent is the extension of egg beyond the synergids and shifting of its nucleus to the apical part (Fig. 8D). Cells of the egg apparatus have thick walls along two-third of the basal boundary, but the apical surfaces, facing the central cell, are virtually hyaline. The polar nuclei fuse near the egg apparatus, and the antipodal cells degenerate before fertilization (Fig. 8D). An embryo sac with one of the synergids in a degenerated state and the other healthier was also observed (Fig. 8E). The discharge of pollen tube contents and fusion of gametes could not be observed because of the degenerated state of the synergid and consequent darkening of the cytoplasm. Subsequently, the second synergid also degenerates (Fig. 8F).

Endosperm

The primary endosperm nucleus divides earlier than the zygote (Fig. 8F). Rapid free-nuclear divisions result in the formation of a large coenocyte (Fig. 8G, H). The karyokinetic activity becomes asynchronous after four or five divisions and is more intense at the densely cytoplasmic chalazal part of embryo sac (Fig. 8H). Wall formation begins after more than 100 nuclei have been formed. At first a peripheral layer of cells is organized, which then cuts off cells centripetally to fill the embryo sac cavity. Wall formation is more rapid in the micropylar part of the embryo sac (Fig. 8I). Eventually the entire endosperm becomes cellular.

Embryogeny

The resting zygote undergoes a distinct reduction in volume (Fig. 10A, B). First division is transverse and results in two unequal superposed cells *ca* and *cb* (Fig. 10 B, C). The hemispherical apical cell has dense cytoplasm whereas the elongated or bulbous basal cell contains large vacuoles.

The second cell generation precedes in the basal cell, which divides transversely to form a smaller, deeply cytoplasmic cell *m* and a larger, vacuolate cell *ci* (Fig. 10D). The cell *ca* divides vertically (Fig. 10 E, F) to give rise to two juxtaposed cells. The four-celled proembryo is, thus inverted T-shaped.

At the third cell generation the sequence of divisions is discordant in different cells of the proembryo. Cell *ci* segments transversely to form two unequal cells *n* and *n'* (Fig. 10G); *m* divides vertically to produce a tier of two cells (Fig. 10H, I); and *ca* undergoes vertical divisions perpendicular to the previous wall to form four circumaxial cells in tier *q* (10I, J).

The cells *n* and *n'* may remain undivided (Figs. 10P-R; 11A), or both of these may divide transversely (Fig. 10H). In some proembryos *ci* is segmented vertically (Fig. 10M, N), and occasionally the resulting cells divide again (Fig. 10 O) to form a tier of four cells.

Tier *m* remains in a two-celled condition until histogenic differentiation begins in the proembryo. Subsequently, these cells undergo vertical divisions and form two horizontal row of four cells each (Fig. 11A)

Next divisions in each cell of the quadrant are asynchronous. Figure 10K shows a proembryo in which one of the cells has completed cytokinesis whereas others are still undivided. Another proembryo in which two of the cells of the quadrant have undergone segmentation, one is in division and the fourth is undivided is seen in Figure 10L. Eventually, these transverse divisions result in the formation of an octant consisting of two superposed tiers *l* and *l'* of four cells each (Fig. 10 M-O). Variations in the topography of the octant are quite frequent. In two of the proembryos, the walls segmenting the quadrant cells are oblique (Fig. 10S). In another instance, two tiers of cells are formed, each with four cells in a linear plane (Fig. 10T).

Histogenic differentiation begins by tangential divisions of the tier *l* and *l'*. Proembryos in which *l* and *l'* divide transversely (Fig. 10R) or vertically (Fig. 10U) before the onset of tangential divisions are occasionally seen. Tangential divisions in *l* demarcates an outer layer of dermatogen which gives rise to epidermal initials (Figs. 10V, W; 11A). The inner derivatives divide tangentially

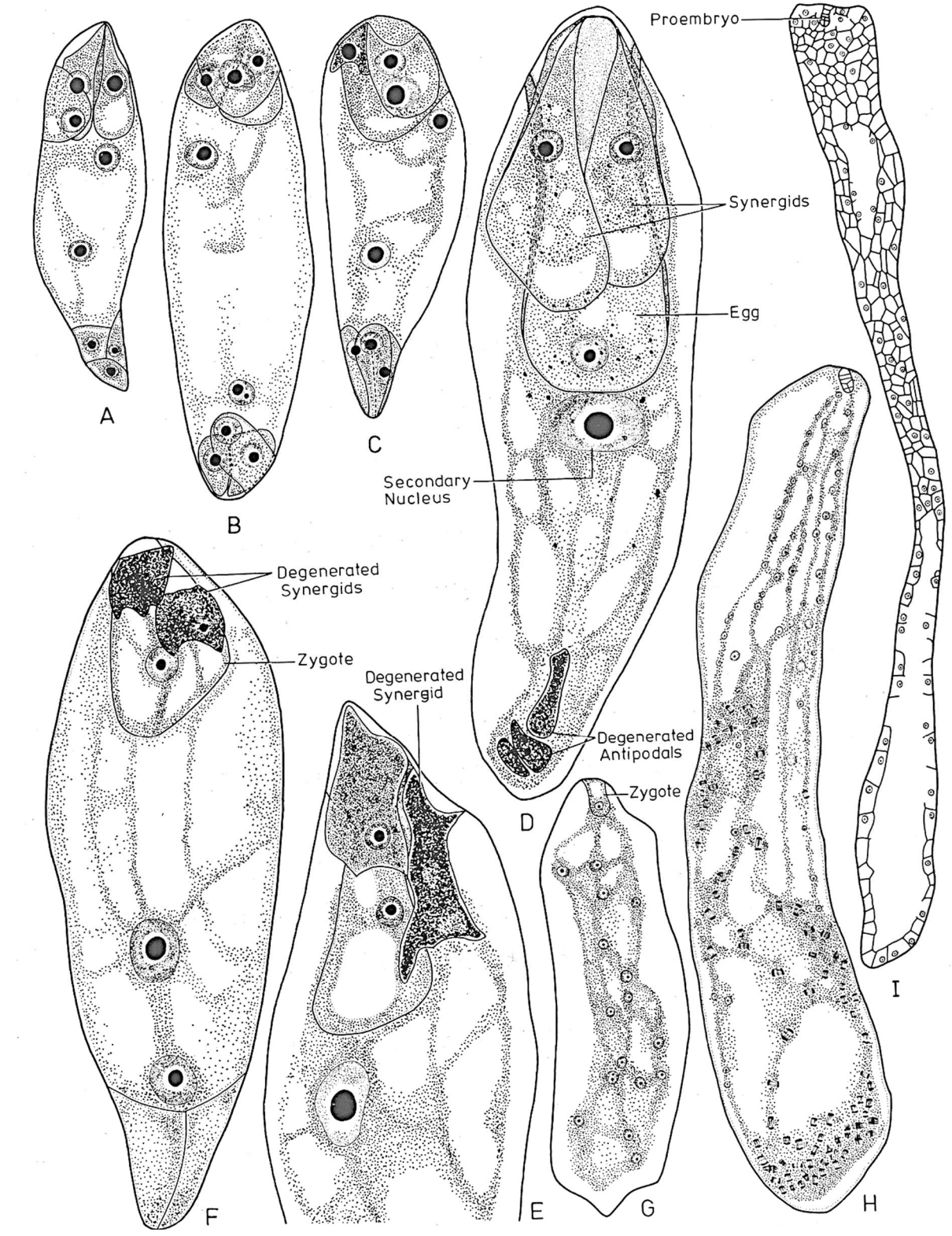

Fig. 8. ***Bischofia javanica***. Embryo sac and endosperm. **A.** Embryo sac with egg in lateral position. **B.** Same, antipodals simulate egg apparatus. **C.** Mature embryo sac; one of the synergids is in degenerated condition. **D.** Embryo sac prior to fertilization; egg has extended beyond synergids and antipodals have degenerated. **E.** Micropylar part of embryo sac; one synergid has degenerated, whereas other has a healthy nucleus. **F.** Two-nucleate endosperm; zygote is vacuolate and synergids and antipodals have degenerated. **G-H**. Free nuclear endosperm at zygote and octant stages of embryo, respectively; intense karyokinesis is seen at chalazal pole in H. **I.** Cell wall formation at octant stage of proembryo (Magnifications: A-F, x750; H,I, x58).

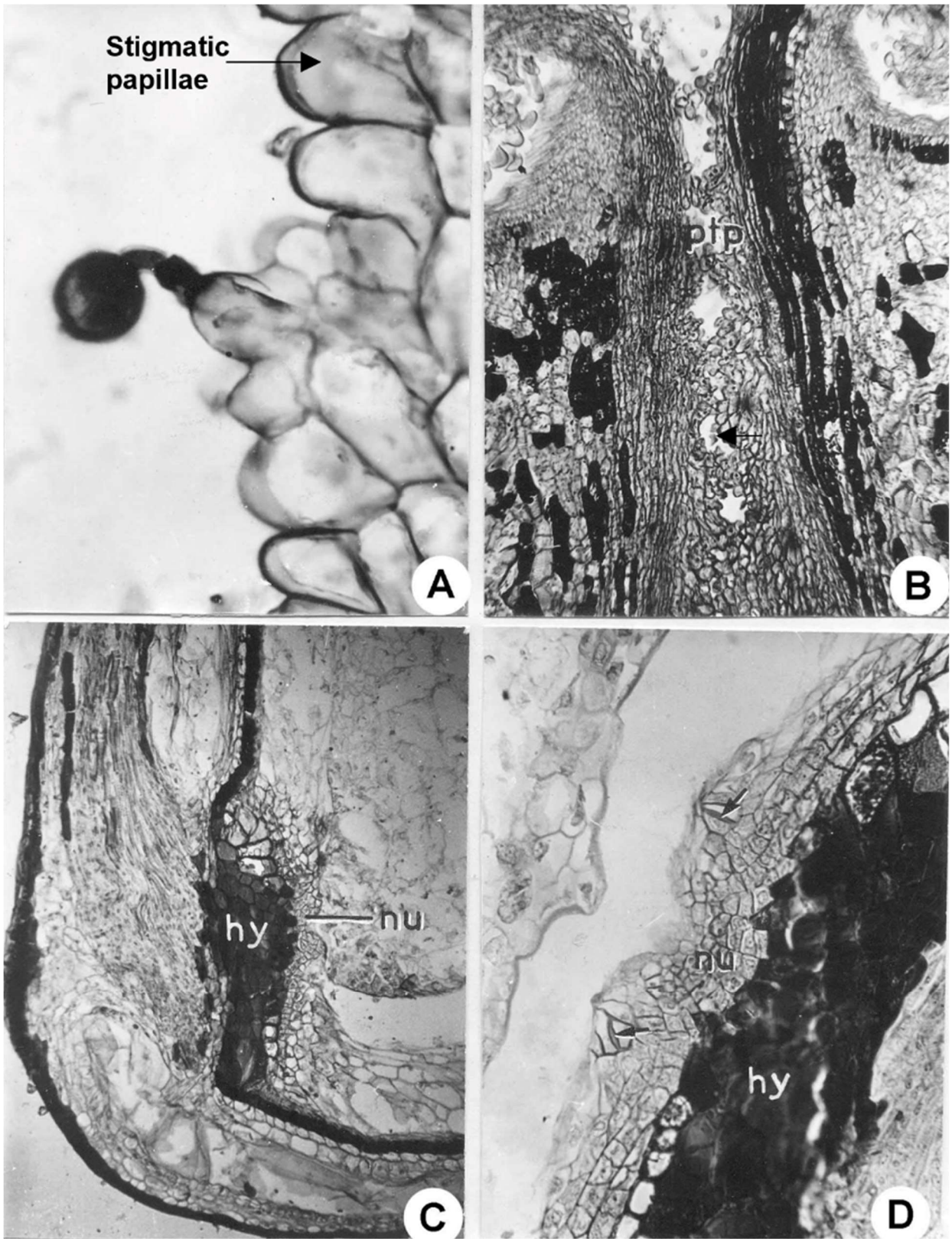

Fig. 9. ***Bischofia javanica***. Fertilization and hypostase. **A**. Germinating pollen grain on stigmatic papillae (x780). **B**. Pollen tube passage (ptp) lined by glandular stigmatoid cells (arrows) (x110). **C**. Chalazal portion of longisection of young seed showing persistent nucellar cells (nu) and tannin-filled hypostase (hy). (x70). **D**. Persistent nucellar cells enlarged to show their arrangement in vertical files; peripheral cells have casparian-like thickenings (arrows). Disalignment of endosperm from nucellus is due to artifact (x220).

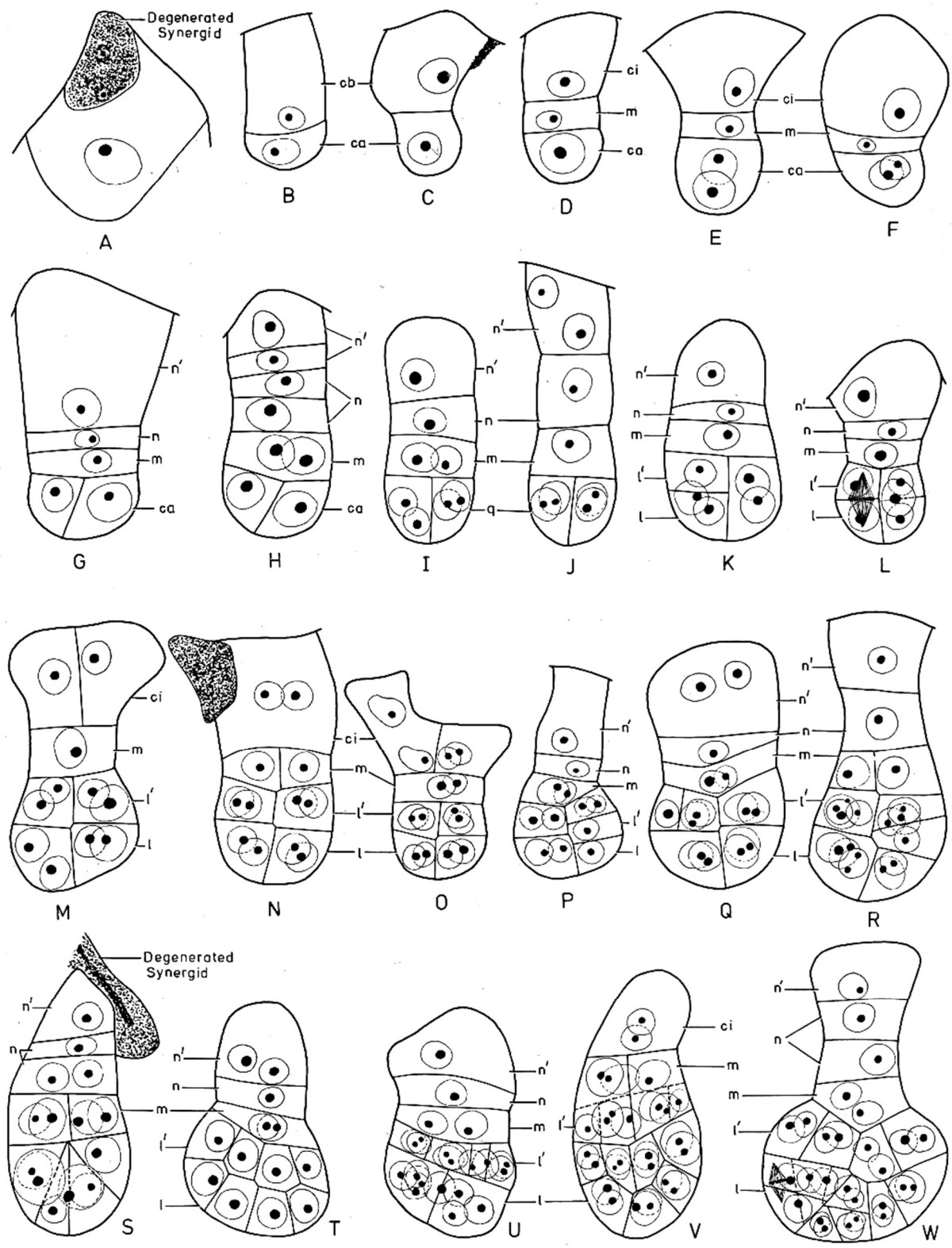

Fig. 10. ***Bischofia javanica***. Early embryogeny. **A.** Zygote in longisection; a degenerated synergid is also seen (x664). **B, C.** Two-celled proembryos (x664). **D.** Three-celled proembryos (x664). **E, F.** Four-celled proembryos (x664). **G, H.** Proembryos; cell *ci* has divided to form a short suspensor. In H, *m* has also divided (x664). **I, J.** Proembryos at quadrant stage (x664). **K, L.** Asynchronous divisions in quadrant cells (x664). **M-O.** Proembryos at octant stage; cells *m* and *ci* have divided vertically (x664). **P-R.** Proembryos showing variation in divisions of octant cells (x664). **S, T.** Proembryos showing abnormal divisions in derivatives of *ca* (x664). **U-W.** Early histogenic differentiation in proembryos. (x664).

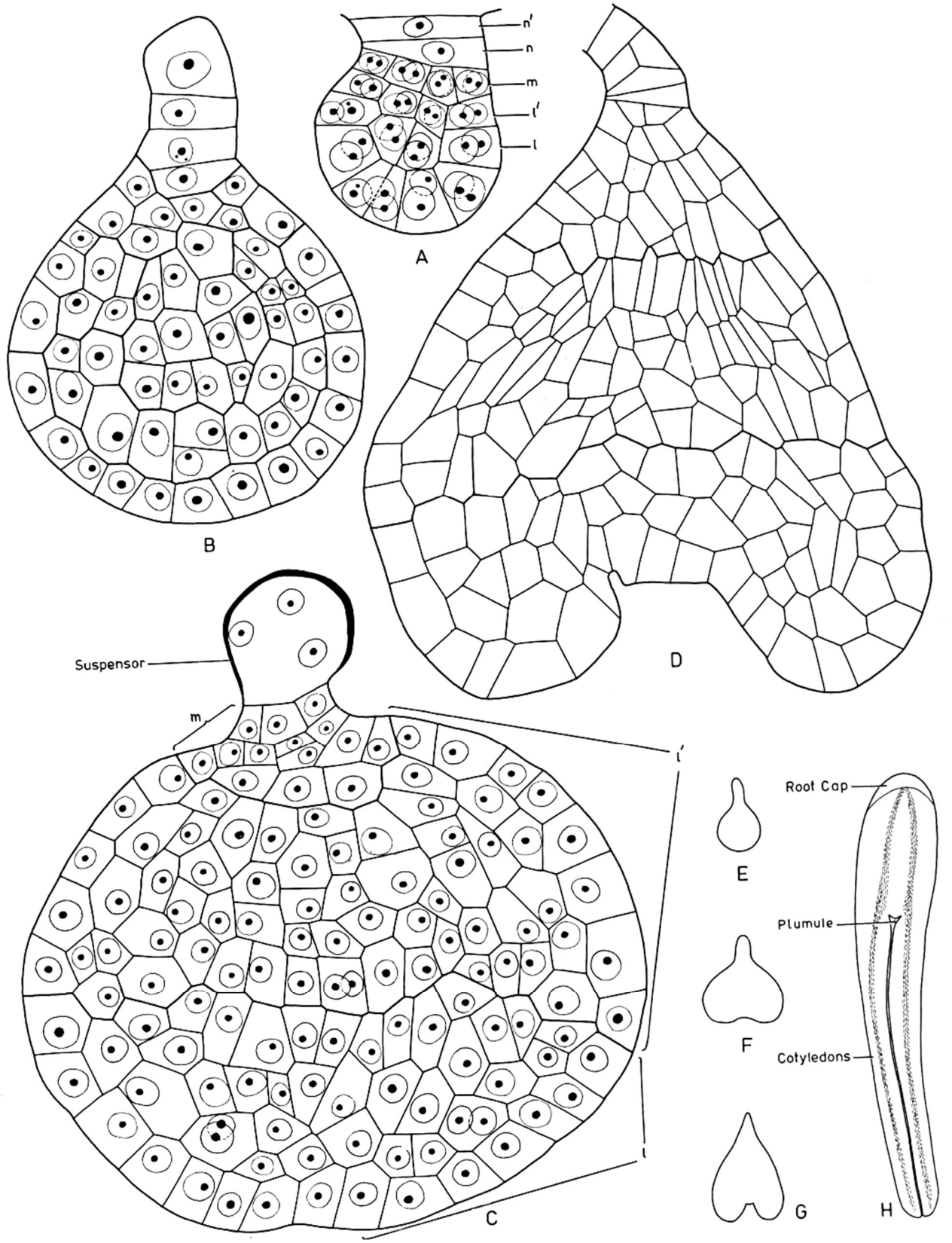

Fig. 11. ***Bischofia javanica***. Late embryogeny. **A**–**C**, Longisections of different stages of globular proembryos; suspensor is thick walled and coenocytic in C. (x 640). **D**. Embryo with two cotyledonary primordia (x640). **E**–**G**. Outlie drawings of whole mounts of globular, heart shaped, torpedo-shaped embryos, respectively (x90). H. Longisection of mature dicotyledonous embryo (x30).

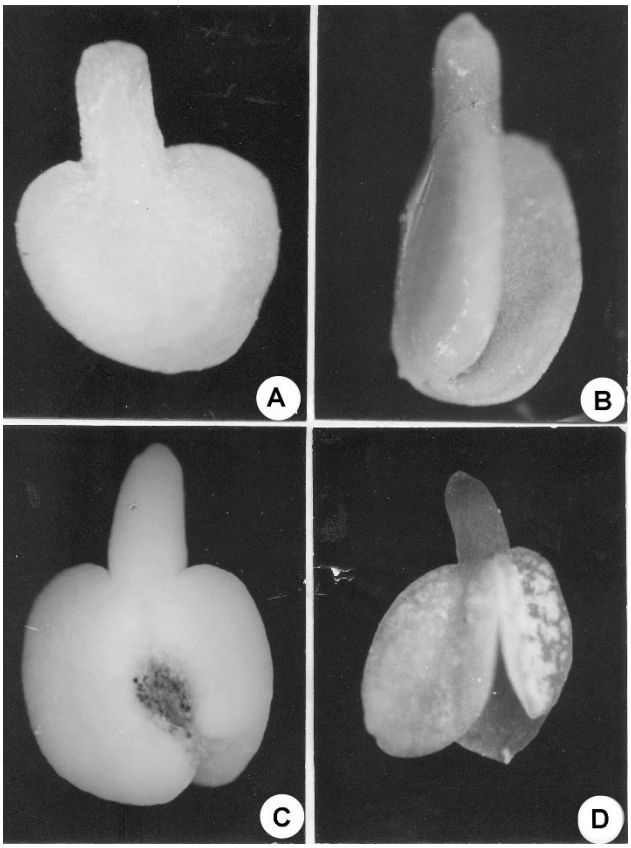

Fig. 12. ***Bischofia javanica***. Embryo. **A**. Mature embryo with broad leafy cotyledons (x42). **B**. Embryo with folded cotyledons (x42). **C**. Same with lobed cotyledons (x42). **D**. Tricotyledonous embryo (x42).

again and the daughter cells toward the exterior differentiate as cotyledonary initials (Fig. 11D), and the interior ones constitute the precursors of stem apex. Likewise, the outer derivatives of tier *l'* divide by anticlinal walls to form the dermatogen, whereas, the inner divide periclinally as well as anticlinally to produce the initials of periblem and plerome of the stem and hypocotyl (Fig. 11A-D).

The tier *m* divides comparatively slowly to engender the initials of central cylinder of root. The cell *n* contributes to the initials of root cap, and *n'* forms a short suspensor consisting of vesicular, occasionally multinucleate cells. In the proembryos in which *ci* does not divide, it forms a thick-walled multinucleate suspensor cell (Fig. 11C).

This pattern of development conforms to the Capsella variation of the onagrad type of embryogeny (Johansen, 1950). Late globular, heart shaped, torpedo-shaped and dicotyledonous embryos are depicted in Figure 11E-H. The mature embryo is straight or slightly curved and is spread along the entire length of the seed. It has broad leafy cotyledons (Fig. 12A) and well-developed root cap. Excepting the procambial and meristematic cells of the shoot and root apices, other cells of the embryo contain compound starch grains, aleurone bodies and crystals of calcium oxalate.

In ca. 300 seeds dissected, some embryos with folded (Fig. 12B), lobed (Fig. 12C) and unequal cotyledons were observed. Eight tricotyledonous embryos (Fig. 12D) were also encountered.

There is a solid core of provascular tissue in the radicle. In the hypocotyl this tissue forms a continuous ring surrounding a medulla of isodiametric cells (Fig. 13A). In dicotylous embryos, below the cotyledonary region four provascular strands are present (Fig. 13B)., two being median cotyledonary and two intercotyledonary. Near the shoot apex the two strands divide further, and each cotyledon receives one trace (Fig. 13C, D) which branches repeatedly.

In the hypocotyl of tricotylous embryos six provascular strands are seen. Three are median cotyledonary (Fig. 14A), and in alternating positions two strands are intercotyledonary. Each cotyledon receives two traces which diverge and ramify (Fig. 14B), and a median vascular bundle continues in each cotyledon (Fig. 14C, D).

Seed and Seed Coat

At the organized embryo sac stage, the outer integument consists of three or four layers of cells (Fig. 15A), except at the base where it is four- or five-layered. Cells of its outer epidermis are tanniniferous, whereas others are vacuolate. Inner integument is three-or four-layered along much of its length, and its outer epidermal cells are tangentially elongated and richly cytoplasmic. The cells in between the outer and inner epidermis are columnar and vacuolate whereas those of inner epidermis are large, isodiametric, and tannin-filled.

After fertilization the inner epidermal cells at the distal part of outer integument divide repeatedly and form 8- to 10-cell-thick rim over the micropyle. Cells of the middle layer of inner integument also divide rapidly, rendering this integument four- to six-layered. The outer epidermal cells enlarge further.

At the globular stage of proembryo, the outer integument is represented by the cutinized, much-compressed, tanniniferous outer epidermis, and some cells of inner epidermis (Fig. 15B). Of the inner integument, cells of middle layers begin to degenerate and those of outer epidermis enlarge enormously, so much so that their thin walls become almost imperceptible.

The seed coat is formed by both the integuments. Outer integument persists as a brown, membranous covering. Middle cells of the inner integument degenerate, tannin-filled cells of its inner epidermis persist, and the outer epidermis differentiates, as a sclerotic layer (Fig. 15C, D). The exotegmen consists of ribbon-like thickenings with simple pits (Fig. 15 C). Near the chalaza and micropyle, however, radially elongated sclereids are present (Fig. 15 D).

Hypostase- After fertilization, the cells of chalazal axial meristem multiply and enlarge, and those toward the

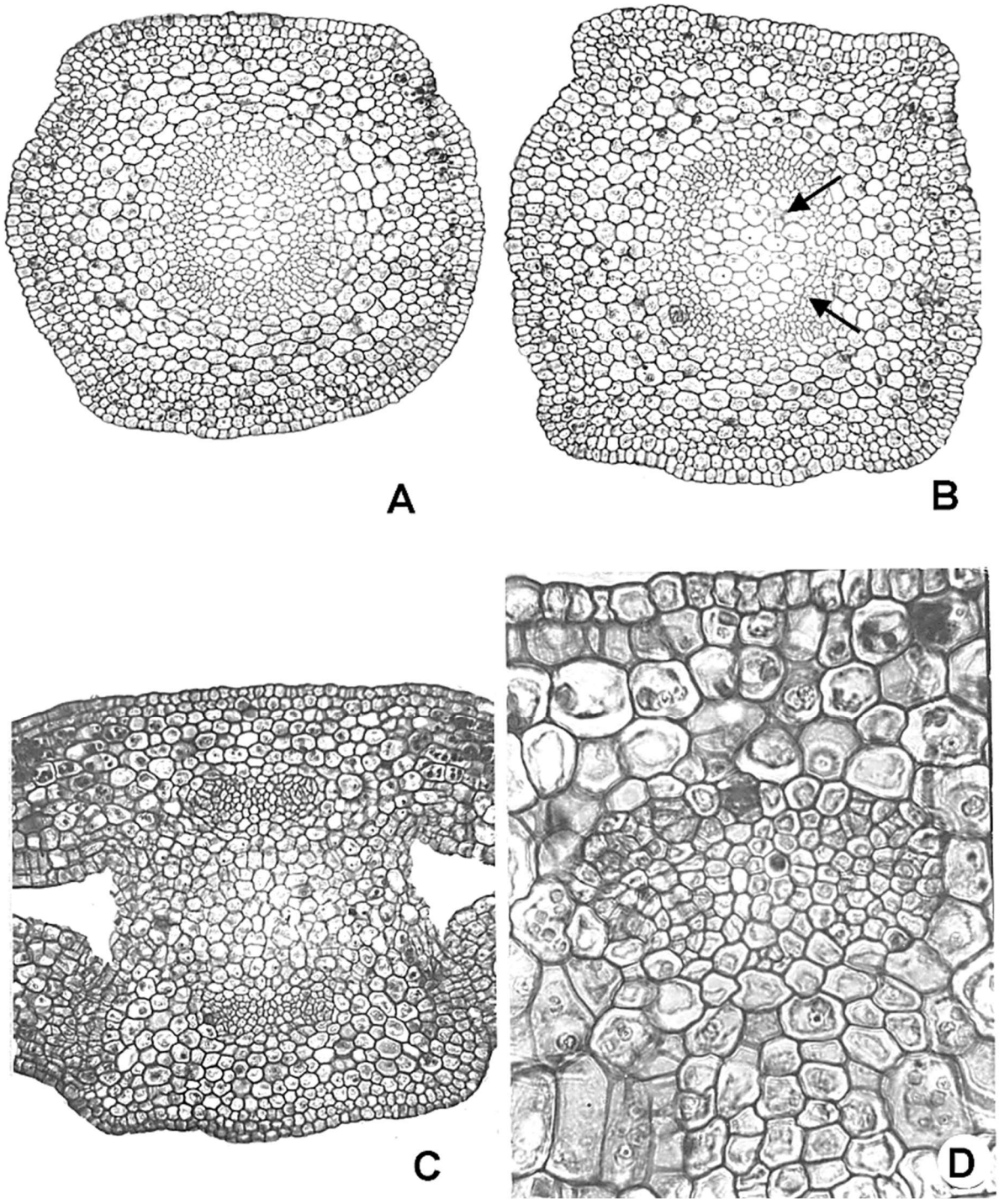

Fig. 13. ***Bischofia javanica***. Anatomy of dicotylous embryo. **A.** Transection of embryo at basal part of hypocotyl; a ring of provascular tissue is seen (x176). **B.** Same, in region of hypocotyl close to cotyledons; four (arrows) provascular strands (x170). **C.** Transection of embryo near shoot apex showing two pairs of cotyledonary strands (x160). **D.** Part of a cotyledon showing a pair of strands; aleurone and starch grains in embryonal cells are also visible (x515).

base become filled with tannin. These cells constitute the hypostase (Fig. 9C). Rest of the cells surround the chalazal extremity of developing endosperm. Some nucellar cells surrounding the axial tissue develop casparian-like thickenings (Fig. 9D). Cells of the micropylar axial tissue become thick-walled and store starch grains and lipid droplets. Rest of the nucellar cells surrounding the embryo sac are soon resorbed. The epidermal cells and a few layers of nucellus at the chalaza persist in the mature seed.

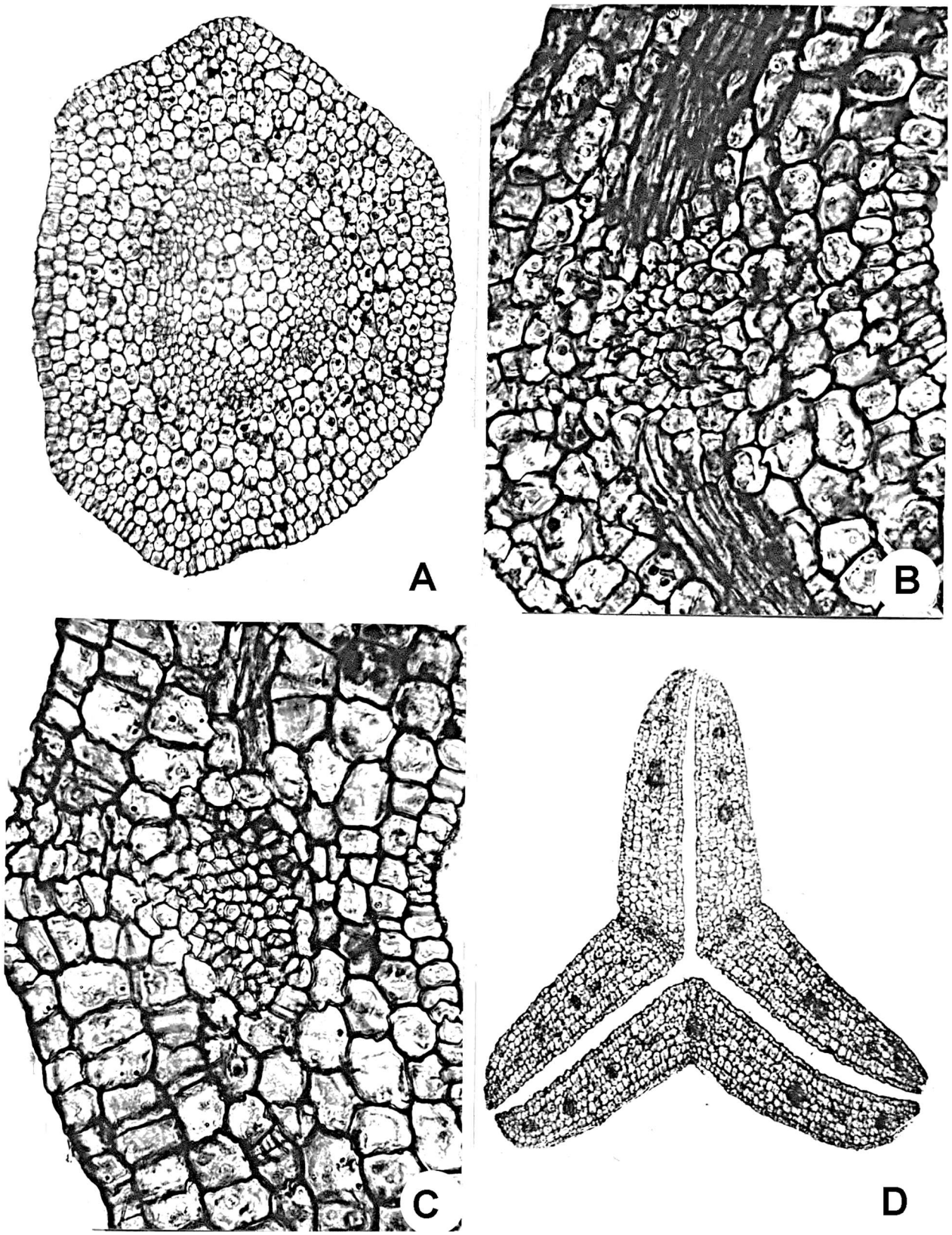

Fig. 14. ***Bischofia javanica***. Anatomy of tricotylous embryo. **A.** Transection through hypocotyl near cotyledonary region showing three pairs of cotyledonary pro-vascular strands (x170). **B.** Two strands diverging laterally near basal region of cotyledon (x390). **C.** Continuation of one strand at a little higher level of cotyledon (x400). **D.** Cross section of embryo at cotyledonary level (x68).

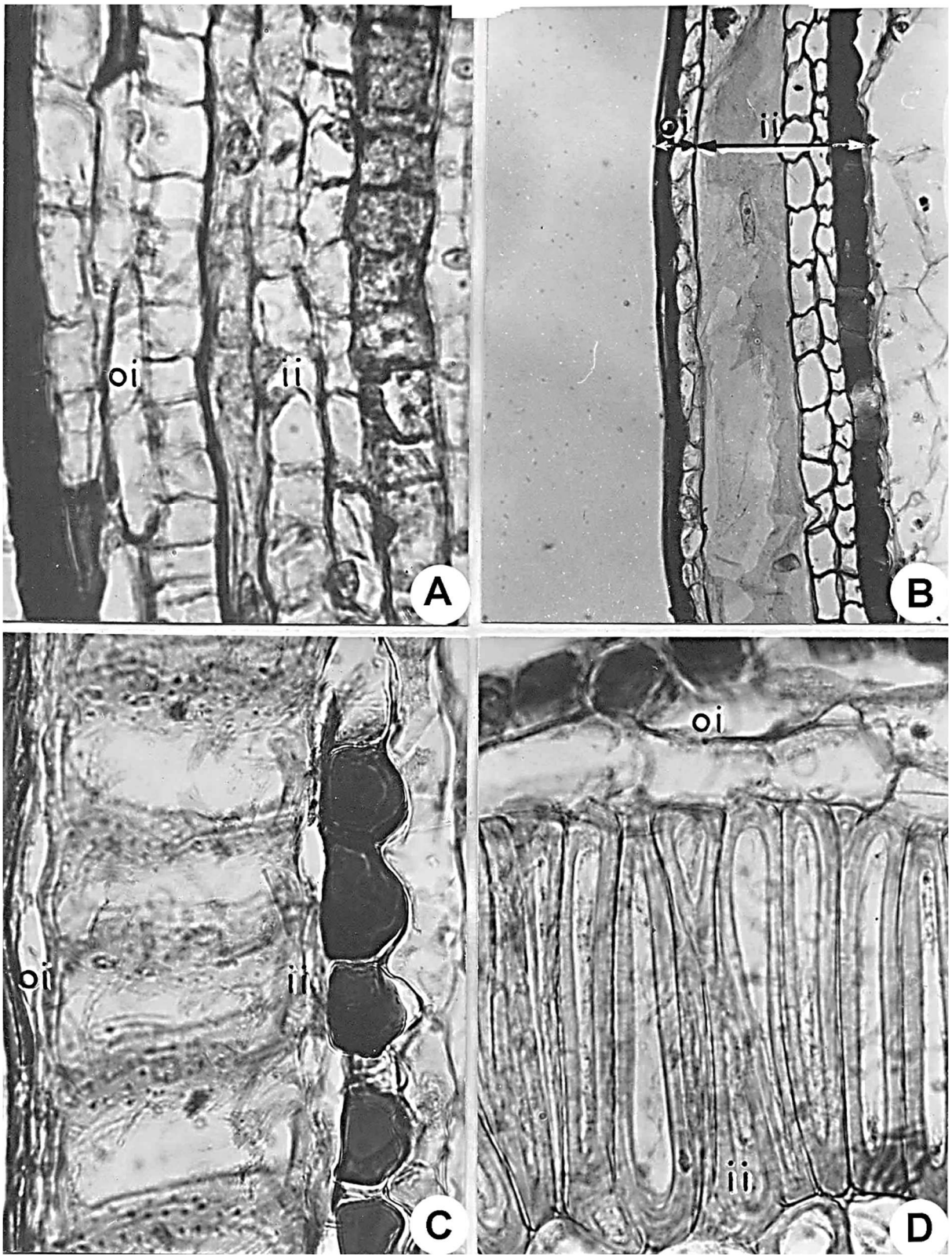

Fig. 15. ***Bischofia javanica***. Seed coat. **A**. Portion of longisection of outer (oi) and inner (ii) integuments at mature embryo sack stage; cells of outer epidermis of inner integument are tangentially elongated (x695). **B**. Same, at globular stage of embryo; cells of outer epidermis of inner integument have enlarged (x235). **C**. Portion of seed coat from broad middle portion of seed; outer integument is compressed, whereas outer epidermis of inner integument develops ribbon like thickenings (x415). **D**. In the micropylar portion exotegmen has macrosclereids (x480).

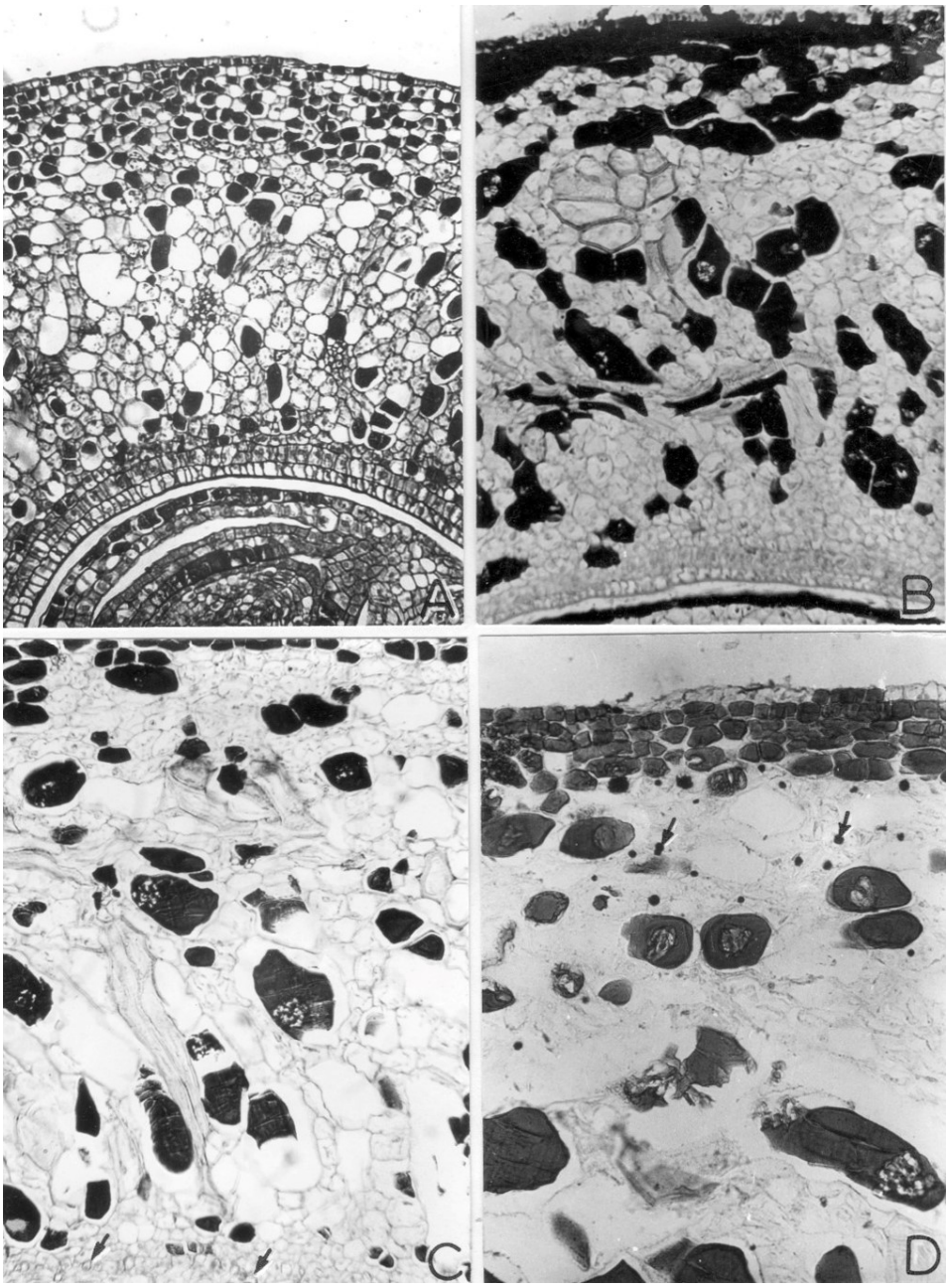

Fig. 16. ***Bischofia javanica***. Ovary wall and pericarp. **A**. Portion of longisection of ovary at megasporocyte stage to show histogenic composition of ovary wall (x300). **B**. At organized embryo sack stage groups of brachysclereids differentiate in outer part of ovary wall (x115). **C**. Pericarp at globular stage of proembryo; mesocarp has trichosclereids and cells of endocarp have crystals (arrows) (x145). **D**. Outer part of ovary wall showing distribution of crystals of calcium oxalates (arrows) silvered by Pizzolato method (x180).

Pericarp

At megasporocyte stage the ovary wall consists of 20-25 layers of parenchymatous cells (Fig. 16 A). Majority of cells of the outer epidermis and two or three layers below it are tanniniferous. Tannin-containing cells are also scattered in the rest of the ovary wall. Cells of the inner epidermis and hypodermis elongate in a radial plane.

At the mature embryo sac stage, the ovary wall becomes 35–40 cells wide (Fig. 16 B). Inner hypodermal zone assumes intense meristematic activity, and groups of brachy-sclereids differentiate in the mesocarp. Druses of calcium oxalate are commonly seen in the outer part of the ovary wall (Fig. 16 D).

As the pericarp matures, the inner subdermal cells become sclerosed, acquire prismatic crystals and constitute the stony endocarp (Fig. 16C). Cells of the middle zone enlarge considerably and several trichosclereids are also differentiated. The exocarp is smooth and tanniniferous. Each of the three compartments of the endocarp contains two seeds and has a vertical line of dispersal (Fig. 17 A, B).

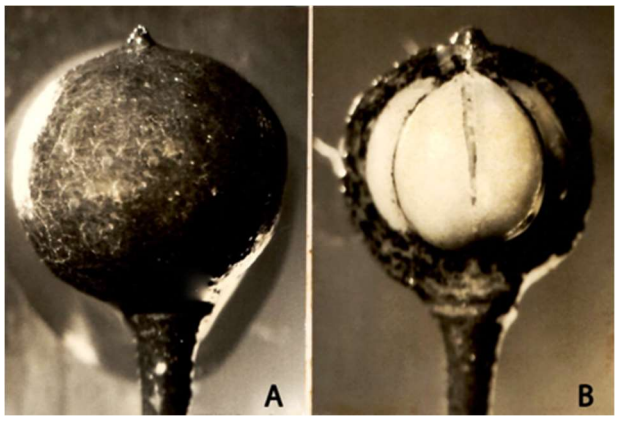

Fig. 17. *Bischofia javanica.* Fruit and seed. **A**. A fruit still green and fleshy (x10). **B**. Fruit with exocarp and mesocarp partially removed to reveal the stony endocarp with opening of a locule along the line of dispersal (x10).

DISCUSSION

The ovules in *Bischofia javanica* are anatropous, bitegmic and crassinucellate. However, we report for the first time that both the integuments are initiated by divisions in the dermal layer. In unitegmic ovules the integument develops from the dermal cells but in bitegmic ovules the inner integument is usually dermal in origin and the outer is sub-dermal. Bor and Bouman (1974) and Bor and Kapil (1975) have, however, recorded the unique occurrence of subdermal initiation of inner as well as outer integument in *Euphorbia milii, E. geniculata*, *Codiaeum variegatum* and *Chrozophora* sp.. Close resemblance in the ontogeny of the outer and inner integuments in *B. javanica* ovule gives the impression that these are homologous appendages. The suppression of the outer integument towards the funiculus, which has been considered as indicative of lack of such a homology in the Euphorbiaceae appears to be a derived feature in the family. The placental obturator, so characteristic a feature of the Euphorbiaceae *sensu lato* (Singh, 1972), is also well-developed in *Bischofia*.

In *B. javanica*, the activity of chalazal and micropylar axial meristems and periclinal divisions in hypodermal nucellar cells result in building up a massive and symmetrical nucellus. Such nucelli recall the pattern described in *Pelargonium hortorum* (Tsai *et al*., 1973) and *Gossypium hirsutum* (Lintilhac and Jensen, 1974). In *Daphniphyllum himalayense* the densely cytoplasmic hypodermal layers of nucellus persist in the mature seed in the form of food-laden perisperm (Bhatnagar and Kapil, 1983). It is noteworthy that the meristematic cells of the nucellus subsequently become modified into hypostase and persist to play an active role in the nutrition of the embryo sac. Cells of the micropylar axial meristem have starch grains that are utilized for early development of the proembryo. In *B. javanica* the archesporial cells in the ovular primordia sometimes fail to divide, but a massive nucellus is nevertheless formed in the ovules.

The formation of megaspore tetrads and the variation and patterns observed during the process are important to establish taxonomic relationships. In the present study on *B. javanica* variation in the number of archesporial cells was observed and linear, T-shaped or inverted T-shaped tetrads were seen.

The development and structure of the embryo sac in *B. javanica* simulate the course of ontogeny recorded in the majority of the Euphorbiaceae. The synergids and antipodals are ephemeral.

The precise mode of endosperm development in *B. japonica* involves initial free-nuclear divisions, wall formation at the periphery when 100 or more nuclei are formed, and centripetal growth resulting in cellularization of the entire endosperm. Such a pattern is common in Euphorbiaceae sensu lato/ Phyllanthaceae (Kapil and Bhatnagar, 1972).

The Onagrad type of embryogeny observed in *Bischofia* has also been reported in the Euphorbiaceae (Kapil and Bhatnagar, 1994). The absence of caruncle in *Bischofia* supports its inclusion in the family Phyllanthaceae.

It is noteworthy that the divisions in zygote and subsequently in the basal cell and its derivatives are always unequal. After each division, the daughter cell formed in proximity of the embryo sac wall is larger and more vacuolated. This process can be correlated with the functional aspects of differentiation of various parts of the embryo. The suspensor cells in *B. javanica* are large, vacuolated, and occasionally coenocytic with multinucleolated nuclei, indicating their absorptive and secretory nature. It is also significant that in two- celled proembryo the cell *cb* divides earlier than *ca.* In *Bischofia,* second division precedes in *ci,* as if the stimulus for mitosis moves from the micropylar to the chalazal part of the proembryo.

In *B. javanica* embryos with unequal cotyledons, folded cotyledons and lobed cotyledons have been observed. Tricotylous embryos were also spotted but their frequency was around three per cent among embryos dissected out from randomly selected seeds. Such embryos have three equal-sized cotyledons or one of them is larger than the other two, which are equal-sized and curved.

The reasons of pleiocotyly have not been deciphered. It is debatable whether the phenomenon is genetically determined or is a response to environmental factors. In the present study, no significant differences were noticed among embryos excised from seeds obtained from trees with trifoliate leaves, growing wild or in cultivation at Dehradun (subtropical climate), and from those with pentafoliate leaves growing in the J.C. Bose Indian Botanical Garden, Howrah (tropical climate). Therefore, in *B. javanica* this character seems to be a genetically stable and relatively free from the effect of climatic factors.

Although references to pleiocotylous embryos in literature are less frequent, most of the reports are incidental. Consequently, very few anatomical descriptions are given. Generally, the number of hypocotyledonary bundles increase proportionally in such embryos. For example, in *Trianthema triquetra* (Bhambie and Nigam, 1976) there are six vascular bundles below the cotyledonary node of normal seedlings, but nine in tricotylous ones. Diarchy is completely or partially replaced by triarchy in *Honkenya peploides* (Pyykkö, 1974), but in *Azadirachta indica* (Kumar, 1976) the root vasculature in dicot and tricot seedlings is similar, i.e., diarch. Such facts indicate that the increase in number of cotyledons is not merely due to splitting of the existing cotyledons or their primordia but has wider evolutionary implications.

In *B. javanica* the dicotylous embryos have four procambial strands (four median cotyledonary and two intercotyledonary) in the hypocotyl region, but the tricotylous embryos have five bundles (three of median cotyledonary and two intercotyledonary). This contrasts with the observations made by Verdus (1976) who described eight hypocotyledonary bundles in normal seedlings of *B. javanica.* He envisaged the existence of a pseudocycle in vascular structure of the seedling in the Euphorbiaceae. According to him, the four hypocotyledonary bundles (Phyllanthus type) have given rise to an eight-bundle state in *Bischofia;* later this number decreases to six (hexafasciculary Euphorbia type) and finally to four (tetrafasciculary Euphorbia type). This pseudocyclic condition of vascular structure is correlated with a similar pseudocycle in evolution of the size of Phyllanthaceae and it continues until the giant cotyledons of *Jatropha curcas*, and then by a decrease (overevolved phase; *Euphorbia)*, returning to the ancestral small type.

Pleiocotyly occurs commonly in association with nine (or eight) vascular bundles. It seems more likely that tetrafascicular condition with large cotyledons observed in *Bischofia* has given rise to at least two distinct lines: (i) tetrafascicular state with small cotyledons in Phyllanthoideae, and (ii) the hexafascicular condition in *Euphorbia* which further led to tetrafascicular condition within this genus and in the rest of the Euphorbioideae. *Bischofia,* therefore, does not seem to constitute a turning point during a pseudocyclic evolution in the Euphorbiaceae as believed by Verdus (1976).

B. javanica is unique in Phyllanthaceae in possessing compound leaves that bear marsupiform acaridomatia that functionally host mites (Rozario, 1995; Wurdack, 2004).

Phyllanthaceae is believed to be consistent with sub-family Phyllanthoideae of Euphorbiaceae *sensu lato* (Malpighiales) excluding Putranjiviaceae (Kathriarachchi *et al.,* 2005). The affinities of *Bischofia javanica* with Euphorbiaceae have generally been accepted on the basis of embryological evidence. In Euphorbiaceae, tricarpellary, syncarpous ovary consisting of three locules, each enclosing one or two ovules, is present (Webster, 1994; Radcliffe-Smith, 2001). In *B. javanica*, two ovules are present. Young ovular primordium, irrespective of its place of initiation from the placenta, becomes distinguishable into three zones – dermatogen, one or two layered sub-dermatogen and a central core or corpus. Developing ovules curve either because of more frequent anticlinal divisions at the abaxial side or due to unilateral cell enlargement on one side. Involvement of both processes causing the anatropy of the ovule has also been observed in *Daphniphyllum himalay*ense (Bhatnagar and Kapil, 1983).

The fruit of *B. javanica* has been variously described as a berry (Gilman *et al.,* 2018) or a drupe (Hayden, 2020). Such descriptions seem to be based on external examination of material. The removal of mesocarp clearly reveals the 3-lobed, hard endocarp with median vertical line of dispersal in each lobe, indicating loculicidal dispersal mechanism. According to Wurdack *et al.* (2005) and Gagliardi *et al.* (2014) in Euphorbiaceae (sensu stricto), Phyllanthaceae and Picrodendraceae, despite certain other differences, fruit type is a schizocarp in the investigated species. Although in the present study, real time observations on seed dispersal could not be carried out, based on morphological details, it can be surmised that the fruit in *B. javanica* is a 3-loculed, loculicidal capsule or schizocarp.

In all the euphorbiaceous plants investigated to date the cells of the outer epidermis of inner integument elongate radially, and their walls become thickened and pitted. The typical exotegmic palisade layer is a most characteristic feature of the Euphorbiaceae (Gagliardi *et al.*, 2012). Corner (1976), in his classic work on Seeds of Dicotyledons, however, has pointed out that the exotegmal cells develop into a palisade of radially elongated prismatic cells, whereas in the Phyllanthoideae these modify into tangentially or radially elongated woody fibres. However, these fibres are produced by formation of spirals of ribbon-like thickenings, whereas palisade cells are formed by the deposition of secondary thickenings over the primary wall. The differences in the type of exotegmen, therefore, seem to be related to the condition of cells in different regions of the seeds. It would hardly be justifiable to split existing families or to separate taxa such as *Bischofia* on the grounds of differences in exotegmic structure.

The spiral thickenings in exotegmen in the broad central part of the seed seems to be an unique evolutionary adaptation. It allows flexibility and shape alteration for compact arrangement of six trigonal seeds inside a capsule with hard endocarp. As the dry fruits fall to the ground and moisture becomes available, these spiral thickenings may expand and help in splitting of the capsule compartments along the lines of dispersal in the hard endocarp.

Profuse flowering, pollination not dependent on biotic factors, high rate of fruit/seed set, effective dispersal mechanism (present work), long seed viability in soil bank, low seed predation, high seedling growth in shade or light (Yamashita *et al.*, 2003) are some of the reproductive features that can aid invasive potential of *B. javanica*. It would be interesting to investigate if any of the many secondary metabolites in the seed also impart allelopathic properties to the species.

ACKNOWLEDGMENTS

We are thankful to the Forest Research Institute, Dehra Dun and Botanical Survey of India, Kolkata for their help in collection of material from their gardens.

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