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Histochemical Studies on the Female Gametophyte and Embryo of *Agathis robusta* (C Moore) F M Bailey

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Various metabolites, at different stages of female gametophyte and embryo development in *Agathis*, were localized histochemically. The contents of megaspore mother cell are denser in the chalazal half than in the micropylar half. PAS-positive granules evenly distributed in the functional megaspore are absent during the free nuclear stage of the gametophyte. The free nuclear and the young cellular gametophytes show low amount of total proteins and RNA until the embryo starts differentiation. The mature egg nucleus stains intensely for proteins, but it is Feulgen-and pyronin-negative except for nucleoli. The cytoplasmic inclusions in the egg are rich in total proteins and RNA. Starch grains abundantly present in neck cells are absent in the jacket cells. The thick inner wall of the jacket with simple pits stains intensely for polysaccharides. During embryogenesis both embryo and endosperm (female gametophyte) store proteins, starch and lipids.

Key Words : *Agathis robusta*, **Archegonia, Embryo, Female gametophyte, Megaspore mother cell, Histochemistry**

Introduction

The large and multicellular female gametophyte of gymnosperms bears the gametes and nourishes the developing embryo. An interesting aspect of the study of the female gametophyte is to establish (*i*) the stage at which the reserve materials are deposited, (*ii*) which part has maximum concentration of food, (*iii*) what is the nature of the stored material, and (*iv*) how the embryo obtains its nutrition.

Histochemical studies on gymnosperms have been made by various workers and some of the important reports are by de Sloover (1961, 1964) on megasporogenesis and female gametophyte of *Encephalartos poggei*, Shimamura (1935) on the egg and free nuclear proembryo of *Cycas revoluta*, and Tâkao (1959, 1960) on archegonium and embryogenesis of *Pinus thunbergii*. Deposition of proteins, starch and fats in the endosperm has been observed in *Picea abies*, *Pinus sylvestris* (Hâkansson 1956), *Ginkgo biloba* (Favre-Duchartre 1956, 1958, Dexheimer

1973) and *Ephedra* sp. (Moussel 1974). Konar & Moitra (1980) have recently reviewed the histochemical and ultrastructural work on the female gametophyte of gymnosperms.

The paper reports some significant results of histochemical studies during progressive stages of development of the female gametophyte and embryo of *Agathis robusta*.

Material and Methods

Female cones at various stages of development were collected from the Arboretum of the Forest Research Institute, Dehra Dun and fixed in Farmer's fluid or FAA. After dehydration, infiltration and embedding, sections were cut at 10μ on an AO Spencer Rotary Microtome and stained as follows: Periodic acid-Schiff's reagent for insoluble polysaccharides (McMannus 1948), Mercuric bromophenol blue for total proteins (Mazia et al. 1953), Feulgen staining for DNA (Gomori 1952), Pyronin Y for RNA (Tepper & Gifford 1962), IKI for starch (Johansen 1940), and Sudan III for lipids (Gomori 1952).

Observations

Megasporangium

The cells of the megasporophyll which give rise to the ovule take up uniformly faint stain for total proteins and RNA. The parenchymatous cells of the cone axis contain proteins and starch grains; the latter being absent in the megasporophyll. Soon after the emergence of cones from the covering scales, a few hypodermal cells become differentiated in the megasporophyll and these cells show higher content of total proteins and RNA. At this stage protein and starch grains are not visible in the cells of the central cone axis.

The megaspore mother cell shows PAS-positive granules in the chalazal region, and its large nucleus is rich in proteins (figure 1 A) and RNA. Presence of starch grains in the chalazal region of the megaspore mother cell was also reported in *Encephalartos poggei* (de Sloover 1961), *Pinus sylvestris* (Willemse 1968) and *Ginkgo biloba* (Stewart & Gifford 1967). It is apparent from these studies that starch accumulates much in advance at a site where the functional megaspore is to be formed.

The functional megaspore shows a few randomly-distributed PAS-positive granules (figure 1 B), which are absent in the free nuclear gametophyte. The latter and the young cellular gametophyte have low level of total proteins and RNA, while in the female gametophyte their contents continue to remain low till embryo differentiation.

At the time of pollen tube entry the cells of the nucellar tip, which are full of starch grains (figure 1 C), stain very faintly for total proteins and RNA, but nuclei stain brightly for these substances. Deep in the nucellar tissue, only a few cells surrounding the pollen tube show the presence of PAS-positive granules.

Archegonium

At the foam-stage of the archegonium, the nucleus of the central cell possesses one or two prominent nucleoli which are brightly stained for total proteins (figure 1 D) and RNA. The chromatin is diffused throughout the nucleus and stains weakly with Feulgen as compared to gametophytic cells (figure 1 F). The cytoplasm is moderately-rich in proteins (figure 1D).

After the central cell has reached its full size, vacuoles begin to disappear and, simultaneously, small spherical bodies start appearing. As their number

increases, some larger protein-rich bodies, (figure 1 E) and granular inclusions, rich in RNA, become visible (figure 1 G). In the mature archegonium RNA and total protein content of the cytoplasm is very high.

In the newly formed egg nucleus the chromatin threads and the nucleolus are intensely stained for total proteins (figure 1 E). However, staining with Feulgen and pyronin is extremely low. As the egg nucleus matures, the chromatin contracts, and is eventually no more visible. The mature egg nucleus stains intensely for total proteins. It is Feulgen- and pyronin-negative except for the nucleoli which stain faintly.

During later stages of archegonium development, the neck cells accumulate large amount of PAS-positive granules (starch) (figure 1H) which are absent in the jacket cells. In *Cunninghamia lanceolata*, Singh et al. (1976) have also reported the presence of starch in the neck cells.

Initially, in the jacket cells the level of total proteins and RNA is low, but it is higher during later stages and numerous small rounded bodies, rich in proteins, appear in these cells. In the nuclei the chromatin takes intense Feulgen stain. The inner walls of the jacket stain brightly with PAS than the other walls and show simple pits (figure 1 H, I) which facilitate the translocation of materials from the jacket to the egg.

Embryo Development

The cytoplasm around the free nuclei of the proembryo stains homogenously for proteins (figure 2 A) and RNA and their intensity is higher than that in the egg cytoplasm. It stains faintly with PAS, indicating low amount of polysaccharides (figure 2 B). The nuclei show low level of proteins and RNA in comparison to the surrounding cytoplasm. The nucleoli, on the other hand, are brightly stained (figure 2 A), but take very faint stain with Feulgen as compared to the nuclei of the surrounding gametophytic cells. The chromosomes, however, take intense stain with Feulgen during division.

In the cellular proembryo, the cap cells show little amount of polysaccharides (figure 2 C), but are rich in total proteins (figure 2 D) and RNA. The nuclei of the embryonal cells have considerably more RNA and total proteins than the cytoplasm (figure 2 D) and small PAS-positive granules are also observed in these cells. The suspensor cells are vacuolate and show low amount of total proteins as compared with the cap and the embryonal cells. At this stage the nuclei of the proembryo are brightly stained for Feulgen and resemble those of the gametophytic nuclei in intensity.

During early stages of embryo development (when embryonal mass is 6 to 8-layered), a few embryonal cells at the distal end accumulate proteins (figure 2 F). All the cells contain starch but its accumulation shows a progressive increase from the distal end to the proximal end (figure 2 E). With further development, these proteinaceous granules start disappearing gradually from the distal cells. The nuclei and the cytoplasm show moderate but uniform staining for RNA and total proteins in contrast to intense staining of the nucleoli (figure 2 F, G).

In the young embryo (when two regions can be recognized viz., a distal region having irregularly arranged cells with dense cytoplasm, and a proximal region which is composed of flat, rectangular, vacuolate cells that are arranged in regular rows), the cells of the distal region show complete absence of starch (figure 2H). These are, however, richer in total

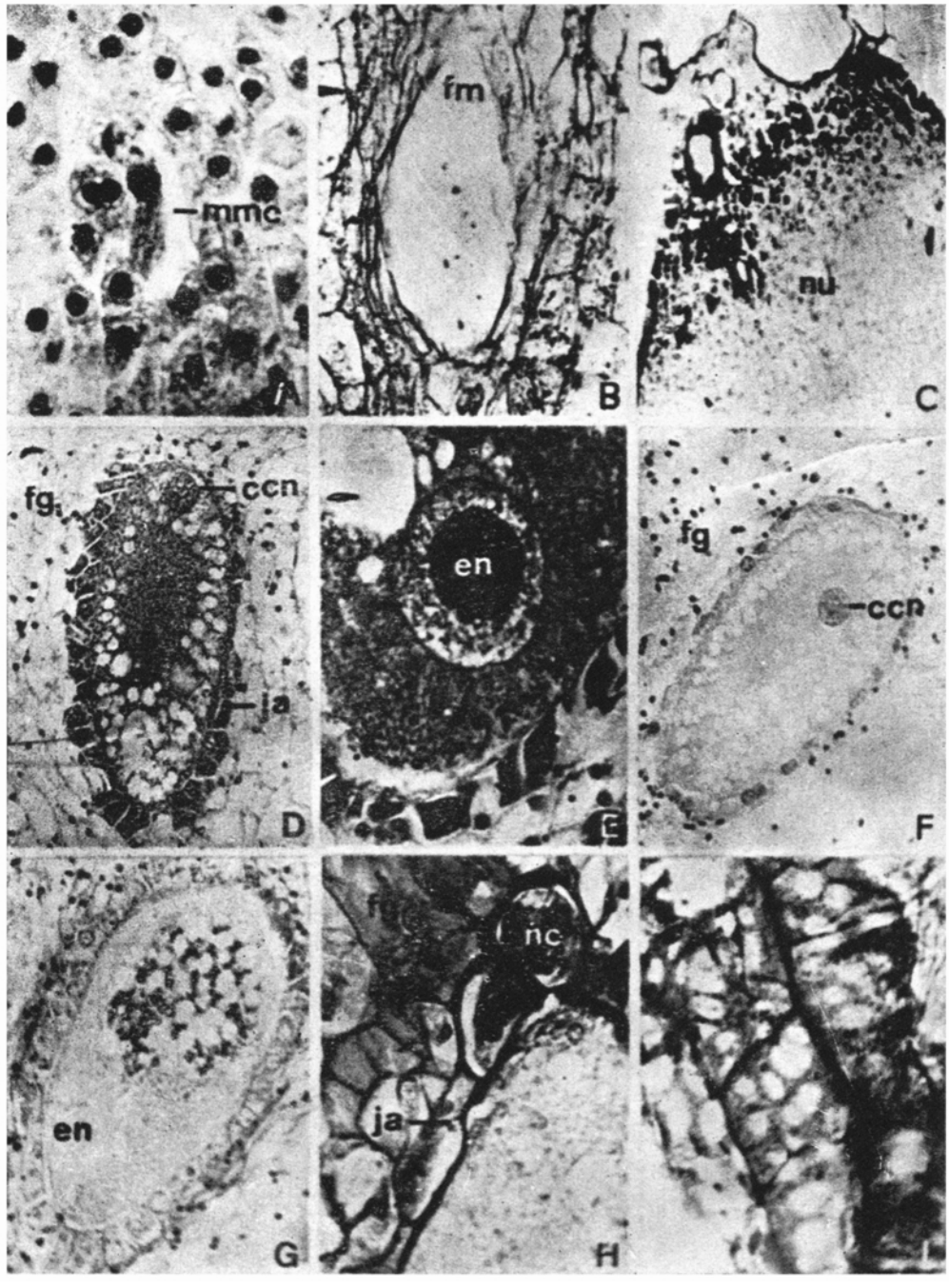

Figure 1 A—I, (**A**) Megaspore mother cell stained for total proteins (× 470); (**B**) Functional megaspore stained for insoluble polysaccharides (× 425); (**C**), Micropylar portion of the nucellus stained for insoluble polysaccharides (× 65); (**D**), Archegonium at the foam stage stained for total proteins (x 144); (**E**), Mature archegonium stained for total proteins showing intensely stained chromatin in the egg nucleus and protein bodies in the cytoplasm (×150); (**F**), Archegonium at the foam stage stained with feulgen (× 120); (**G**), Archegonia stained for RNA. Note Pyronin-negative egg nucleus and RNA rich inclusions in the cytoplasm (× 162); (**H**), L.s. neck of the archegonium showing PAS-positive granules in the neck cells (× 520); (**I**) Paradermal section of archegonial jacket showing simple pits (PAS staining) (× 530)

ccn, central cell nucleus; **en,** egg nucleus; **fg,** female gametophyte; **fm,** functional megaspore; **ja,** jacket; **mmc,** megaspore mother cell; **nc,** neck cell; **nu,** nucellus

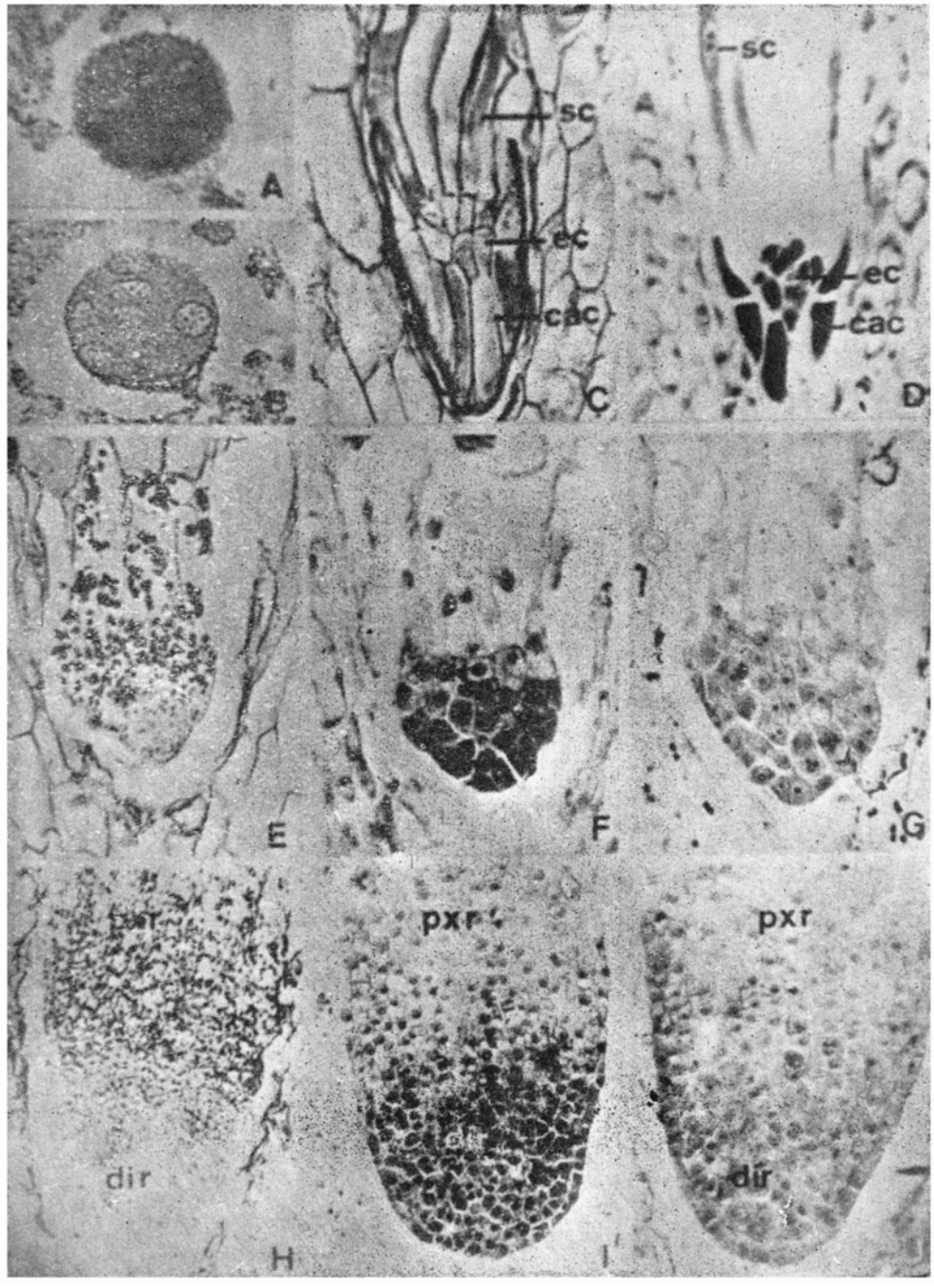

Figure 2 A–J, (**A**), Free nuclear proembryo stained for total proteins. Note brightly stained nucleoli (× 336); (**B**), Same, stained for insoluble polysaccharides. Only cytoplasm is faintly stained (× 365); (**C**), Proembryo showing cap, embryonal and suspensor cells stained for insoluble polysaccharides (× 216); (**D**), Same, stained for total proteins. Note intense staining reaction in the cap cells (× 122); (**E**), Young embryo stained for insoluble polysaccharides. Note PAS-positive granules (starch) in all embryonal cells (× 168); (**F**) Same, stained for total proteins (× 172); (**G**), Same, stained for RNA (× 166); (**H**), Embryo showing differentiation into proximal and distal regions, stained for insoluble polysaccharides. Note the absence of PAS-positive granules in the distal region (× 120); (**I**) Same, stained for total proteins showing higher protein content in the distal region (× 138); (**J**) Same, stained for RNA (× 168)

cac, cap cell; **dir**, distal region; **ec**, embryonal cell; **pxr**, proximal region; **sc**, suspensor cell

proteins than those of the proximal region (figure 2 I). The amount of protein and RNA decreases from the embryonal apex to the suspensor. The nucleoli are, however, rich in RNA (figure 2 J).

In a young dicotyledonous embryo, the cytoplasm and the nuclei stain with bromophenol-blue. The nuclei of the provascular strands are stained more intensely than the other nuclei (figure 3 B). The cells of the root cap region towards suspensor are lightly stained for proteins (figure 3 A). Protein content of the embryo increases and reaches its maximum in the mature embryo. Minute PAS-positive grains are distributed in all the cells of the embryo (figure 3 C) except in procambial strands, shoot and root apices. Their concentration is greater in the cells of the root cap region (figure 3 D). As the embryo matures, slight increase in the quantity of starch concentration is observed and in the mature embryo the cells are filled with starch, protein and lipid. RNA is generally localized in nuclei and is very sparse in the cytoplasm. The nuclei of the root and shoot apices are less pyroninophilic (figure 3 E, F) and also stain lightly with Feulgen.

Female Gametophyte during Post-fertilization Stages

Concomitant with the development of the embryo, significant histochemical changes occur in the female gametophyte. Prior to embryo formation the gametophyte is made up of large, thin-walled, vacuolate cells with a very low content of cytoplasmic proteins and RNA.

At the free nuclear proembryo stage small PAS positive granules start accumulating in the cells of the central part of the female gametophyte (which later forms the corrosion cavity). These cells stain intensely for proteins compared to other gametophytic cells. As the suspensor cells elongate and the embryo is gradually pushed down, the cells of the gametophyte break down in the corrosion region. When the embryo comes to lie in the central part of the gametophyte, a few layers of cells surrounding the embryo show small PAS-positive granules although 1 or 2 layers of cells lying in direct contact with the embryo are devoid of them. Later these cells also break down and, till embryo differentiation, cells of the female gametophyte show complete absence of insoluble polysaccharides and proteins in the cytoplasm.

By the time the cotyledons are differetiated in the embryo, small PAS-positive granules appear in the gametophytic cells, first in a few layers of cells adjacent to the embryo and then gradually in most of the other cells. A few layers of cells at the basal and the peripheral regions of the gametophyte either do not contain these granules or only a few may be present. The proteins appear first in the peripheral region of the female gametophyte (figure 3G), and gradually in the central region of the gametophyte. The protein content of the female gametophyte increases with the maturity of embryo and reaches its maximum in the mature seed.

When the embryo is fully differentiated the gametophytic cells are full of protein granules (figure 3H), small starch grains (figure 3I) and lipid bodies.

Nucellar Tissue during Post-fertilization Stages

At the cellular gametophytic stage the nucellus is 8-10 cells thick except at the micropylar region where it is considerably thicker. Three or four layers of cells, immediately around the gametophyte, contain very little starch. Protein content continues to be very low throughout the

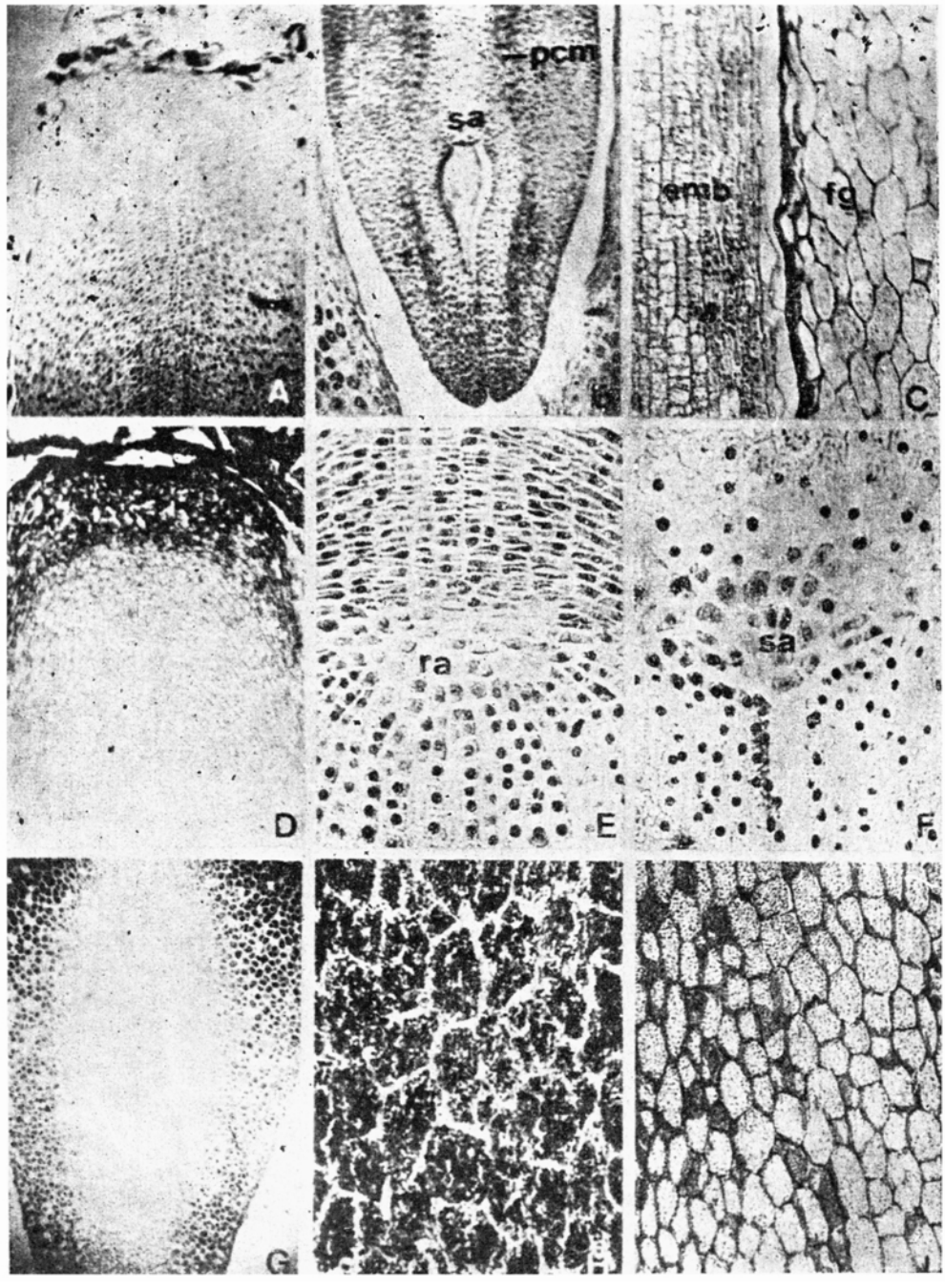

Figure 3 A–I, **(A)**, L.s. of mature embryo stained for total proteins (root cap region). The terminal cells of root cap show lesser proteins (× 78); **(B)**, Same, showing shoot apex and cotyledons (× 74); **(C)**, Part of embryo and female gametophyte stained for insoluble polysaccharides (× 168); **(D)**, L.s. part of mature embryo stained for insoluble polysaccharides (root cap region). Note higher concentration of PAS-positive granules (starch) in the terminal cells of root cap (× 68); **(E)**, Portion of mature embryo stained for RNA (root apex region) (× 354); **(F)**, Same (shoot apex region) (× 368); **(G)**, Lower portion of female gametophyte stained for total proteins. The peripheral cells of the gametophyte show higher content of proteins (× 24); **(H)**, Portion of female gametophyte at mature embryo stage stained for total proteins (× 213); (I), Same, stained for insoluble polysaccharides (× 140) **emb**, embryo; **fg**, female gametophyte; **pcm**, procambium; **ra**, root apex; **sa**, shoot apex

development of the nucellus. The cells of the nucellar tip packed with starch grains (at the time of pollentu been try) show very few grains at later stages (developing embryo stage), and finally disappear.

Discussion

Protein content which is low in the young archegonium increases and reaches its maximum at maturity. The cytoplasmic inclusions of the archegonium, rich in total proteins and RNA, are comparable to the small inclusions present in the members of the family Pinaceae. Proteid vacuoles rich in proteins have been reported in *Cycas rumphii* (de Silva & Tambiah 1952), *Ginkgo biloba* (Favre-Duchartre 1956), *Pinus cembra*, *P. montana*, *P. sylvestris* (Stopes & Fuji 1906), *P. thunbergii* (Tâkao 1959) and *Cephalotaxus drupacea* (Favre-Duchartre 1957, Singh 1961). According to Tâkao (1959) the proteid vacuoles probably provide nutrition to the developing embryo which is in agreement with our findings.

The Feulgen-negative egg nucleus has also been observed in *Ginkgo biloba* (Shimamura 1935) and *Pinus thunbergii* (Shimamura 1935, Tâkao 1959). In *Ginkgo biloba* (Lee 1955), *Cycas revoluta* (Shimamura 1935), *Zamia umbrosa* (Bryan & Evans 1956) and *Cephalotaxus drupacea* (Singh 1961) the large egg nucleus shows a small globule of Feulgen-positive chromatin. It is perhaps the large volume of the nucleus that is responsible for this negative reactivity as in some angiosperms where the presence of DNA was demonstrated by fluorescence microscopy and ultra-violet microspectrophotometry (Georgieva 1966, Makhanets 1966, Woodcock & Bell 1968, Bannikova 1971).

The stainability at the free nuclear embryo stage indicates that the cytoplasm surrounding the free nuclei contains mainly proteins and RNA with some amount of amorphous polysaccharides. Probably these substances are utilized for the embryo formation. In *P. thunbergii* Shimamura (1956) confirmed the presence of RNA in the cytoplasm surrounding the free nuclei, and Tâkao (1960) observed that protein stainability of this cytoplasmic area reaches its maximum at 4-nucleate stage and disappears at the 8-celled stage.

The present study indicates that the cells of the female gametophyte, which are about to lyse to form the corrosion cavity during the elongation of suspensor, contain more proteins than the rest of the gametophytic cells and this region also shows small PAS-positive granules. With the breakdown of cells in the corrosion region, the metabolites are converted into simpler substances which are then utilized by the developing embryo. Tâkao (1960) has made similar observations in *Pinus thunbergii*.

The female gametophyte as well as embryo store reserve food which is utilized at the time of germination. Metabolites start accumulating in the gametophyte only after the cotyledons are initiated in the embryo. Konar (1958) and Tâkao (1960) observed the deposition of proteins in the gametophyte after the cotyledons are initiated. According to Konar (1958) in *P. roxburghii*, alcohol-soluble nitrogenous substances in the embryo and gametophyte increase and are converted into proteins after differentiation of the cotyledon initials. In *Picea abies* and *Pinus sylvestris*, Hâkansson (1956) reported that as the suspensor begins to elongate, the starch grains start appearing in the cells around the apex of the pro embryo and then in the other cells of the gametophyte. In all these cases, accumulation of metabolites starts during the post-fertilization stages, whereas in *Ginkgo biloba* (Favre-Duchartre

1956, 1958, Dexheimer 1973) rseserve foods are accumulated before fertilization. In *Ephedra* (Moussel 1974) starch accumulates in the micropylar region of the female gametophyte during pre-fertilization stages and, during embryo development, the micropylar part of the gametophyte is more or less devoid of storage products which accumulate in the lower half of the gametophyte.

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