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Ultrastructure of Tapetum Prior to Liberation of Pollen Grains in Trifoliate Orange [*Poncirus trifoliata* (L.) Raf.]

Pear Mohammad, Masaya Shiraishi* and Junko Toda*
Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan

Abstract
The tapetal cells in trifoliate orange were studied prior to liberation of pollen grains under light and transmission electron microscopes. The tapetum was glandular or secretory type and consisted of two-layered flattened cells immediately below the 2-3 layered elongated parietal cells and anther wall. It surrounded the pollen sacs where numerous pollen grains were embedded freely. Many sporopollenin-like granules appeared throughout the tapetal zone releasing from the territory of tapetum and parietal layers. These granules moved through the intercellular spaces and found their ways towards the pollen grains which were finally absorbed by the bacula of grain walls. The multi-layered rough endoplasmic reticulum appeared near these absorption sites. The tapetal cells were devoid of distinct walls or they had only very thin envelopes. The tapetal cytoplasm contained highly dense, irregularly dense and less dense granules within different vesicles.

Introduction
As in other biological forms, so also in the higher plants, special cells are set aside for differentiation into reproductive units, first as spores and subsequently as definitive eggs and sperm (Ledbetter and Porter, 1970). After the development and segregation of sporogenous tissue, the individual spore mother cells begin to take form (Ledbetter and Porter, 1970).

The tapetum is the tissue in the anther in closest contact with the developing pollen grains (Mascarenhas, 1975). These cells apparently serves for the nourishment of the developing pollen mother cells and pollen grains (Fahn, 1982). The products of secretion, evident deep in the interstices between the cells of this tissue, are able to move into the open space around the pollen grains (Ledbetter and Porter, 1970).

In citrus, the tissue lying between the pollen sac and anther epidermis is composed of thin-walled parenchymatous cells. The archesporial cells divide periclinally to produce an outer layer of parietal cells and an inner layer of sporogenous cells (Banerji, 1954). Additional divisions of the parietal cells produce 4 cell layers. The innermost layer form the anther wall. However, these kinds of cytological investigations in citrus have not been followed up during the last few decades. Recently, the ultrastructure of pollen grains of citrus has drawn the attention of many scientists (Kozaki and Hirai, 1981; Ye *et al*., 1981; Martens *et al*., 1989; Mohammad *et al*., 1999). The ultrastructural characteristics of tapetum in relation to parietal cells and their contributing strategies to the development of pollen grains have not been taken into consideration to the best of our knowledge. This study was intended to know the ultrastructural details of tapetum in relation to parietal cells prior to liberation of pollen grains in trifoliate orange.

Materials and Methods
The experiment was conducted in the Citriculture Laboratory, Faculty of Agriculture, Ehime University, Japan during the spring seasons of 1997 and 1998. Anthers of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] was used as the study material. Flowers were collected from a citrus orchard of Yawatahama City, Ehime Prefecture, Japan. Three small branches of each tree were selected every year. The collecting period was immediately prior to the anthesis of flowers. The collected flowers were placed between moist paper, wrapped with a polyethylene sheet and carried to the laboratory in an ice box. After the removal of flower petals, anthers were dissected. To aid fixation, the anther tips were excised. Samples from three plants were used every year.

For light phase microscopy, the anthers were fixed immediately in Karnovsky's solution (Karnovsky, 1965). The fixed samples were dehydrated in a graded ethanol series and infiltrated and embedded in JB-4 resin. Transverse sections of anthers were taken at 3μm thickness with dry glass knives on a Sorval (MT-1) ultramicrotone, stained with toluidine blue O and observed and photographed under a light phase microscope.

For transmission electron microscopy, the samples were pre-fixed in the above mentioned fixative solution, post-fixed in osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin. The appropriate anthers were selected through the observations of semi-thin sections under light phase microscope. The ultra-thin sections of the selected portions were cut and double stained with uranyl acetate and lead citrate. The sections were observed and photographed under a HITACHI H-7100 transmission electron microscope at 100kV. Two-year observations were summarized and presented.

Results
Tapetum in association with pollen sacs and pollen grains: The anther contained four pollen sacs (microsporangia) in four corners and a zone of sterile tissue (ST) as the connective of sacs in the center (Fig. 1 A). The tapetum

was glandular or secretory type and its cells (TC) were flattened (Fig. 1 B). The parietal cells between tapetum and anther wall consisted of 2-3 layers of parenchymatous cells (PC) (Fig. 1 B). However, the parietal layer had only one row of cells in the dehiscence end of pollen sacs. The tapetum contained much cell content but the parietal cells were devoid of cellular details. The parenchymatous cells, especially the cells immediately below the anther wall were slightly elongated inward. The parietal cells were always organized but the tapetal cells degraded in few places even then the tapetal layer surrounded the pollen sacs where the pollen grains (PG) were embedded freely (Fig. 1 B). A large number of less dense droplets were also present with the grains in the pollen sac. The distinct arrangement of parenchymatous (PC) and tapetal cells (TC), and pollen grains (PG) under electron microscope are shown in Fig. 1 C. The parenchymatous and tapetal cells were attached together but the pollen grains of different stages gradually detached from the tapetal cells.

Tapetum with parenchymatous cells and pollen grains under Transmission electron microscope: The tapetum was rich in cell contents where different cell organelles were embedded (Fig. 2 A). The sporopollenin-like granules (arrows) released from the territory of parietal and tapetal layers and extended throughout the tapetal zone (Fig. 2 A). These granules found their ways through the intercellular spaces towards the pollen grains (PG) and finally accumulated on the tapetal cell envelope near the grains. The cell envelopes (CW) were extremely thinner in the tapetum compared to parietal cells (CW) (Figs. 2 B, C). However, although the tapetal cells were devoid of primary cell wall, at least surrounded by membranous envelopes. The membranous envelopes became less dense along the intercellular space. While in few cases, the tapetal cells were completely devoid of cell walls or cellular envelopes. The flow of sporopollenin-like granules towards the grains and the absorption (arrow) of the granules by the bacula were observed (Fig. 2 D). These sporopollenin-like granules also accumulated around the grains. Many multi-layered rough endoplasmic reticulum were found near these absorption sites. The endoplasmic reticulum (ER) was also abundant throughout the tapetal cells and occasionally remained attached with electron dense granules (G) (Fig. 2 E).

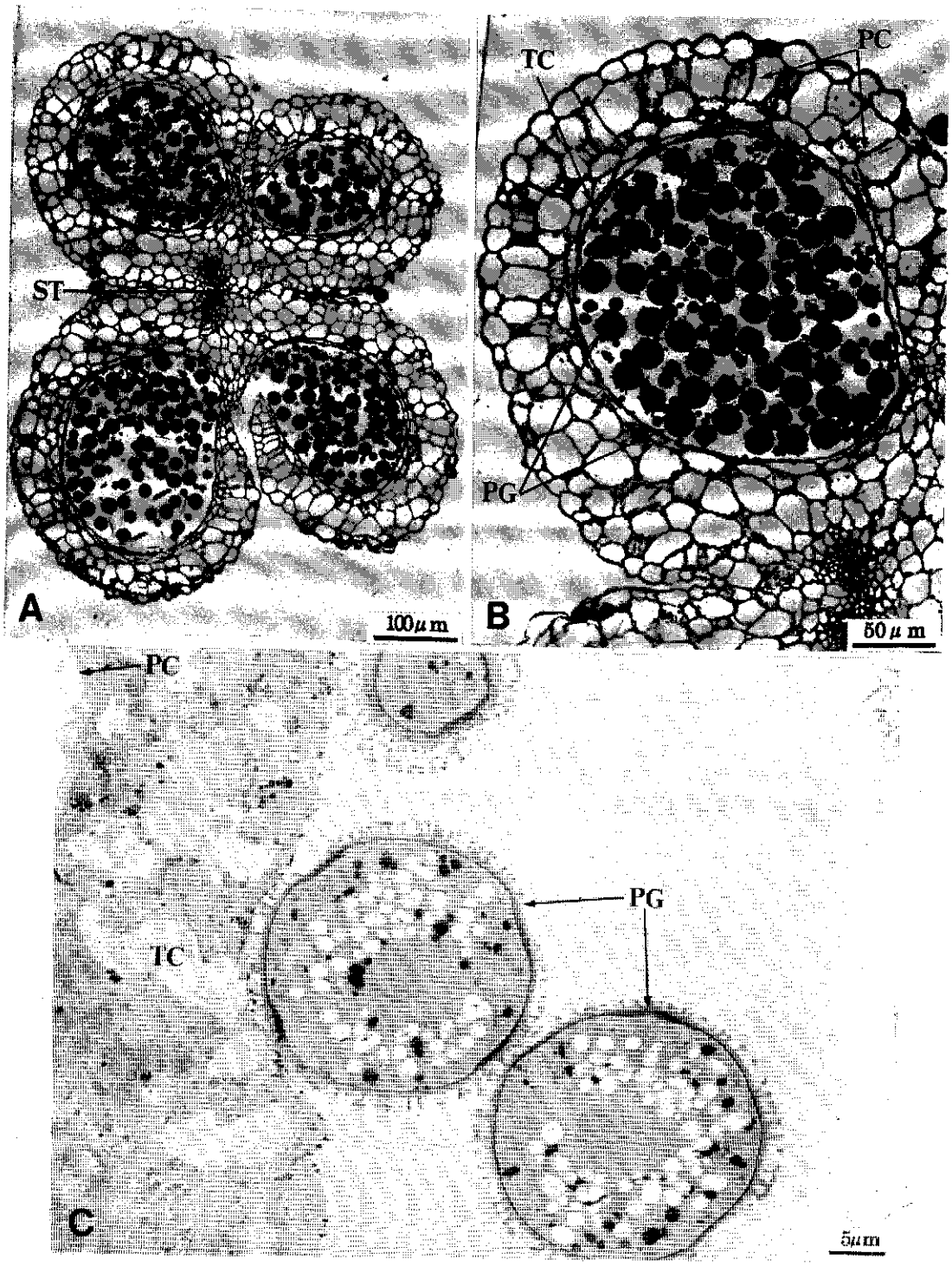
Electron dense granules in the tapetal cells: The tapetal cytoplasm contained electron dense granules of different kinds within vesicles (Fig. 3 A). However, these granules were categorized as highly electron dense, irregularly electron dense and less electron dense. The highly dense granules (HG) were usually in groups in separate vesicle (V) but sometimes they were scattered (Fig. 3 B). The irregularly dense granules (IG) were many but always within the vesicles (V) (Fig. 3 C). The less dense granules (LG) were also within vesicles in group but they were comparatively fewer within each vesicle (V) (Fig. 3 D).

Discussion

The occurrence of 4-chambered anthers in the presen study was consistent with the previously know characteristics of citrus as was reported by Reuther et a (1968). Pollen sacs are usually found in two groups wit two lobes in each group. In trifoliate orange also, the sac were consisted of 4 lobes in 2 groups and the connecti portion located exactly in the center placing the pollen sac in four corners of the anther. The study suggested th tapetal cells in citrus as glandular or secretory type. Th sporadic disorganization of tapetal cells in the prese mature stage of pollen grains probably indicated that th tapetal cells were partially degraded to contribute th development of pollen grains.

The presence of cytoplasm rich tapetal cells and th dominance of cell organelle in the cytoplasm at the matu stage of pollen grains can be considered as striking in th plant species as the tapetum usually disappear in this stag The existence of smaller granules in the intercellular spa was consistent with other plants (Heslop-Harrison, 196 Christensen *et al.*, 1972). However, the present stu revealed that the flow of these granules started from th territory of parietal and tapetal layers extending up to th pollen grains. These granules are responsible agents f producing pollen grain walls for the protection of grains to its germination as has been described by Ledbetter a Porter (1970). In our observation the granules were fou to pass towards the grain wall after release. The granul primarily accumulated near the grains on the tapetal c envelope and finally merged with the grain wall. Th observation established clearly the basis for the assumpti that tapetal cell has unique contribution to the developm of pollen grain walls. It is also interesting in this study th the tapetum contained differently dense granules in cytoplasm. These granules have not yet been reported other plant species.

The tapetal cells in other plants were reported to be th walled which correspondence with the thinner envelopes of tapetum compared to that of parietal cell addition in many places the cell walls were comple absent which probably facilitated the migration of tap cell contents towards the pollen grains. The abundanc rough endoplasmic reticulum in the tapetal cell migh due to the higher physiological activities in these cell enhance the migration of cell contents to the pollen gra However, the observation of multi-layered ro endoplasmic reticulum in association with the elec dense granules stood in support of our opinion about migration of electron dense granules towards the grai In conclusion, the internal structures of tapetum, espec the location of sporopollenin granules release, movement and the observation of differently dense gra structures in the cytoplasm differed considerably in trif



1: Light and electron micrographs showing the pollen sac(s) and the association of tapetum with the parietal cells and pollen grains in trifoliate orange. A: Low magnified view of 4-chambered anther. B: Magnified view of a pollen sac. C: Electron micrograph of tapetum with parenchymatous cells and pollen grains. PC, Parenchymatous cell; PG, Pollen grain; ST, Sterile tissue and TC, Tapetal cell.

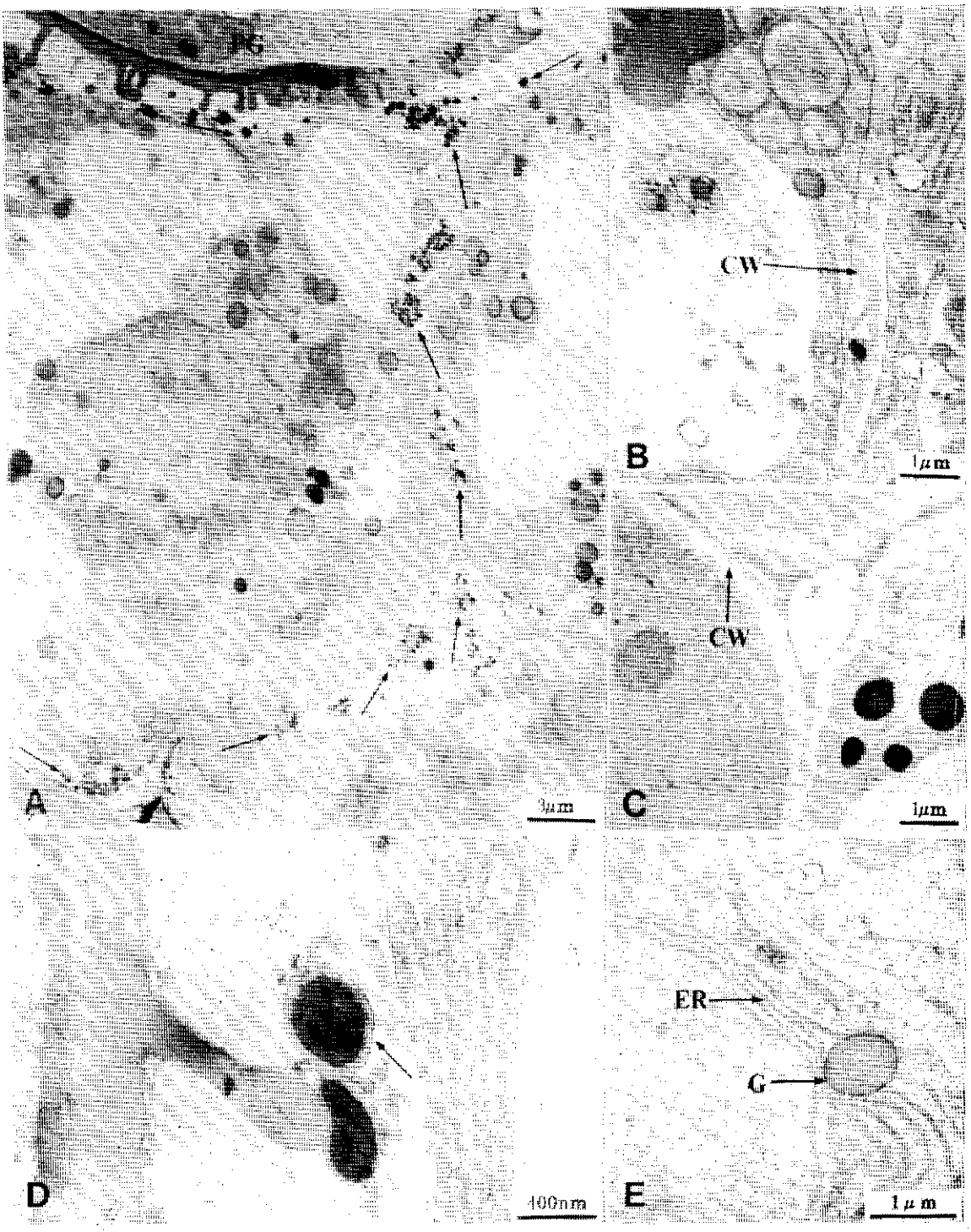
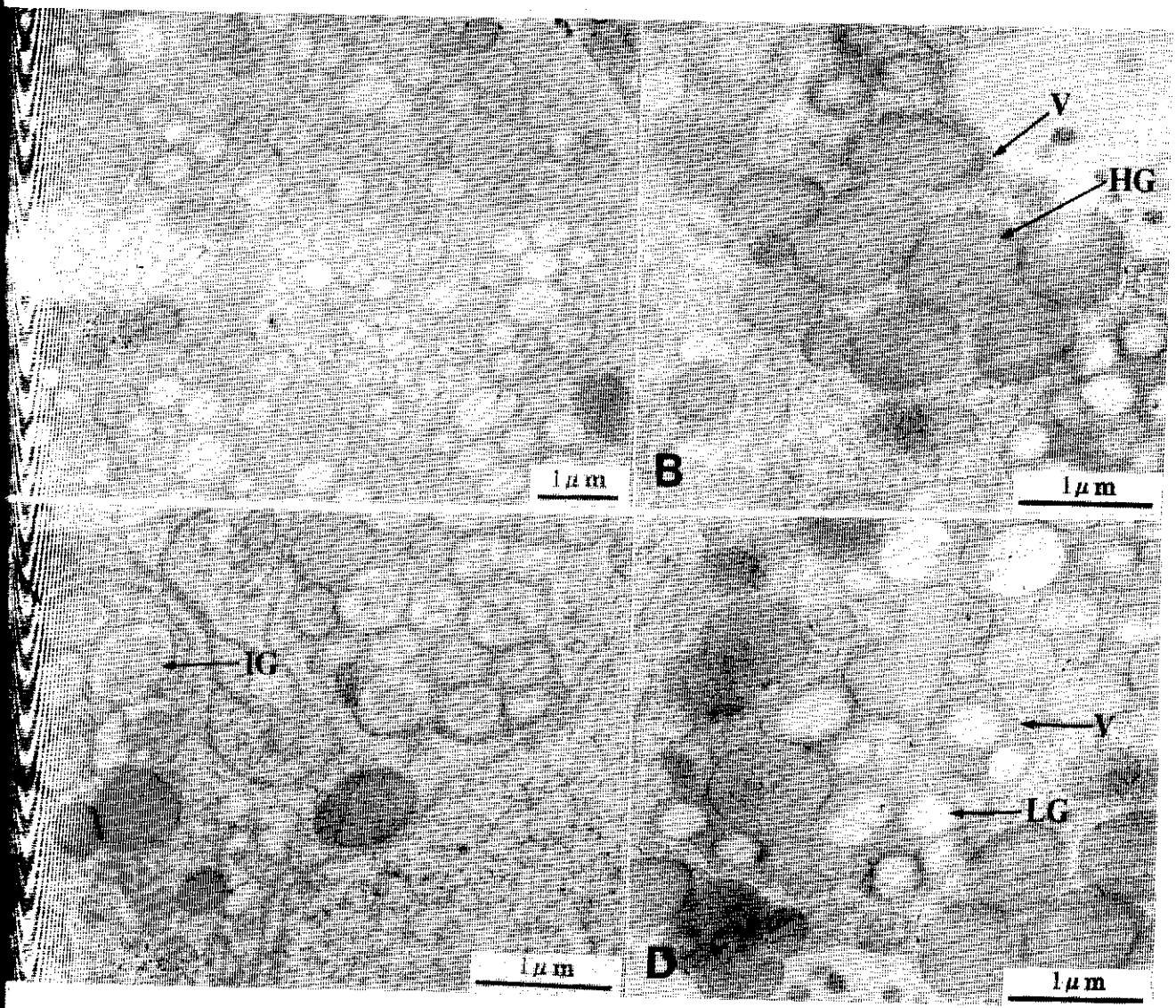


Fig. 2: Transmission electron micrographs of tapetal cells in association with the parenchymatous cells and pollen gr
A: Tapetal cells showing the trend of sporopollenin-like granules (arrows) towards the pollen grains (PG). B:
cell envelope (CW) of tapetum. C: Comparatively thicker cell wall (CW) in the parietal cell. D: Associatio
sporopollenin-like granules with bacula (arrow). E: Association of electron dense granules (G) with endopla
reticulum (ER).



3: Transmission electron micrographs showing differently dense granules in the tapetal cytoplasm. A: Low magnified view of tapetal cells having different granules. B: Highly dense granules (HG) within vesicles (V). C: Irregularly dense granules (IG) within vesicle (V). D: Less dense granules (LG) within vesicle (V).

orange from other plant species. This study thereby warranted the detailed investigations of pollen grain development in relation to tapetal cell in this species for the complete resolution of the ultrastructural details of pollen grain formation in citrus by the expense of tapetal cell contents.

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