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MICROSPOROGENESIS IN HORDEUM COMPRESSUM:
MICROSPORE AND TAPETUM FROM
THE TETRAD STAGE TO POLLEN MITOSIS

presented by

Joanne Holland Whallon

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Botany & Plant Pathology

Major professor

Date Nov. 6, 1979

O-7639

MICROSPOROGENESIS IN *HORDEUM COMPRESSUM*: MICROSPORE AND TAPETUM FROM THE TETRAD STAGE TO POLLEN MITOSIS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1979

ABSTRACT

MICROSPOROGENESIS IN HORDEUM COMPRESSUM:
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Microsporogenesis from the tetrad stage until the time of the first pollen mitosis was investigated in Hordeum compressum by light microscopy as well as by transmission and scanning electron microscopy. H. compressum is the first barley species, and the first wild grass species, to be so investigated. It was found that both microspore and tapetum bear a strong general resemblance to the microspore and tapetum of other grasses for which comparable information is available. The development of the microspore wall, however, appears to differ in several details from that reported for other grasses, and may represent a different ontogenetic pathway. In the tapetum, differences in the timing of senescence compared to the cultivated species was apparent. A previously unreported type of cytoplasmic structure was observed in some tapetal cells. The ultrastructure of the developing tapetal orbicular wall supports the hypothesis that the wall is an evolutionary specialization rather than either a vestigial capacity of a tissue that was once sporogenous, or a random, nonspecific configuration of excess sporopollenin.

ACKNOWLEDGMENTS

The successful completion of this dissertation has been made possible by many people, and to all of them I extend my sincere thanks. I am particularly grateful to the members of my committee: to Dr. William Tai, my major professor, for introducing me to the study of ultrastructure, for permitting me then to wander far afield from his major interests, and for his constant encouragement; to Dr. Gary Hooper for patient instruction and enlightening discussions; and to Drs. Clifford Pollard and William Hooker for long years of interest and support.

Mrs. June Mack provided me with much valuable advice on the fine points of transmission electron microscopy.

Plates 47-51 were taken by Royce Hazard, who also instructed me in the use of the scanning electron microscope.

The pollen fertility assessment was done by Dr. Rye-Ho Huang.

Part of this work was supported by a grant from the Michigan State University Electron Optics Committee, and the work was made much easier by the purchase of equipment for my use by the Department of Botany and Plant Pathology.

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Introduction

From the early 1860's until the early 1960's, the study of pollen was largely the province of taxonomists. As a result of their efforts, descriptions of the pollen morphology of thousands of species entered the literature. Even with the relatively limited resolution and depth of field of the light microscope, it became apparent that the pollen wall is a highly complex structure. Cytologists augmented the information supplied by the taxonomists, adding some details on the developmental processes.

In recent years pollen studies have taken a new direction. Not only are we able to see the sculpturing of the exine much more clearly, but we are also able to observe the ultrastructural details of its development, to examine the living cell hidden inside, and to follow developments in associated anther tissues, particularly the tapetum. In addition, it has become possible to begin a correlation of biochemical studies with ultrastructural details.

At least some stage of microsporogenesis has by now been studied at the ultrastructural level in several species representing several different families. A partial listing would include Beta vulgaris (Hoefert, 1969), Capsicum annuum (Horner and Rogers, 1974), Citrus limon (Horner and Lersten, 1971), Cosmos bipinnatus (Dickinson, 1976), Helleborus foetidus (Echlin and Godwin, 1969), Ipomoea purpurea (Godwin, Echlin and Chapman, 1967), Lilium longiflorum

(Heslop-Harrison, 1968b; Dickinson, 1970, 1976), Parkinsonia aculeata (Larson and Lewis, 1962), Pinus banksiana (Dickinson, 1971), Silene pendula (Heslop-Harrison, 1963), Sorghum bicolor (Christensen, Horner and Lersten, 1972), Tradescantia bracteata (Mepham and Lane, 1970), and Zea mays (Skvarla and Larson, 1966). Somewhat less attention has been devoted to the tapetum, but nevertheless Antirrhinum maius (Lombardo and Carraro, 1976), Avena sativa (Steer, 1977a,b), Beta vulgaris (Hoefert, 1971), Helleborus foetidus (Echlin and Godwin, 1968), Paeonia tenuifolia (Marquardt, Barth and von Rahden, 1968), Pinus banksiana (Dickinson and Bell, 1972, 1976), Scilla non-scripta (Rodriguez-Garcia, 1978) and Tradescantia bracteata (Mepham and Lane, 1969) are among those in which this tissue has been investigated.

Subsequent to these ultrastructural studies has come renewed interest in the adaptive significance of the large numbers of variations encountered, and a concomitant interest in dysfunctions that might result in a reduced production of viable pollen. As a result of these investigations we now have, for example, evidence that the pollen wall acts as a repository for a number of proteins, some derived from the gametophyte and others from the sporophyte, which ensure the success or failure of the pollen-stigma interaction (Heslop-Harrison, Heslop-Harrison, Knox and Howlett, 1973; Dickinson and Lewis, 1973; Heslop-Harrison, Knox and Heslop-Harrison, 1974; Heslop-Harrison, 1976) and we also know that the causes of sterility

are many and varied, from improper development of the intine (DeVries and Ie, 1970) to failure of tapetal mitochondria (Warmke and Lee, 1977; Lee, Gracen and Earle, 1979).

Barley (genus Hordeum), although it is an economic crop of considerable significance, has up to this time not been the subject of such investigations. The present work is an ultrastructural study of part of the sequence of events of microsporogenesis in H. compressum, a species that has not undergone heavy selection pressure for cultivation.

Materials and Methods

Eleven cloned plants of Hordeum compressum were maintained in field and greenhouse over a three-year period. Spikes which had not yet begun to emerge from the boot were cut, stripped of enclosing boot tissues and plunged intact into fixative. Occasionally anthers were dissected out before fixation, but as this did not noticeably improve fixation and did greatly complicate the process of following the developmental sequence, it was not done as a general rule.

Because satisfactory fixation of grass anthers is difficult to achieve, a variety of different fixatives and buffers was tried. The most successful results were obtained with a solution of 5% glutaraldehyde either in 0.1M phosphate buffer at pH 7.4 with 6-10% sucrose, or in 0.01M phosphate buffer at pH 7.4 with 6 drops of 0.1M $CaCl_2$ per 40 ml. of buffer. Fixation was carried out in the cold for 10-18 hours. Following two 30 minute rinses with buffer, spikes were postfixed in 1 or 2% OsO_4 for 4-12 hours, rinsed in water and dehydrated either in a graded acetone series (30 minute steps) or, for scanning electron microscopy (SEM), with three five-minute rinses of acidified dimethoxypropane (Johnson, Hooper, Holdaway and Rasmussen, 1976).

Anthers prepared for transmission electron microscopy (TEM) were embedded in Epon, sectioned with a diamond knife on a Reichert ultramicrotome, double-stained with uranyl acetate and lead citrate

and viewed in either a Philips 300 or a Philips 201 electron microscope operating at 60 KV. Monitor sections were stained with toluidine blue and photographed with a Zeiss Photomicroscope II using phase contrast optics. Anthers prepared for SEM were critical-point dried in a Sorvall critical-point drying apparatus, mounted on a stub using Scotch brand double sticky tape, coated with gold alloy in a sputter coater and viewed in an I. S. I. Super II. The anthers were mechanically broken open or cut with a razor blade after mounting on the stub but before gold coating.

What appeared to be developmental abnormalities within the anthers appeared fairly frequently, compounding the problems resulting from troublesome fixation. These imperfect anthers upon study yielded many valuable insights that are indirectly a part of this dissertation, but their presence made determination of the events of normal microsporogenesis difficult. The final decision as to whether a given observation should or should not be included as normal was thus influenced by the quality of fixation, by how well the observation in question fitted into a continuum with earlier and later stages whose identification was more secure, and by the availability of duplicate observations, but these criteria could not be applied equally in all instances.

Results

Pollen Fertility

A total of 2,402 grains was collected, stained and counted. Of these, 1,988 or 78.6%, gave a positive response to I_2-KI.

Light Microscope Observations of the Gametophyte

Tetrad Stage

Sections through anthers which had completed meiosis (Figures 1, 2) revealed locules almost completely filled with tetrads in a variety of non-spherical shapes surrounded by a considerable amount of callose. The size of the tetrads relative to the size of the locule ensured that each tetrad was contiguous with the tapetum. Furthermore, the tetrads displayed some polarity of orientation, so that many of the individual microspores were also adjacent to the tapetum. This was particularly evident in cross-section, where the common wall between two adjacent cells frequently coincided with the locule radius. In longitudinal section, one division plane was often more or less parallel to the long axis of the anther, with the second one perpendicular to it.

In each cell of the tetrad, a centrally located nucleus, with one or two nucleoli, and some small vacuoles could be seen. No cytoplasmic connections were visible between any of these haploid cells.

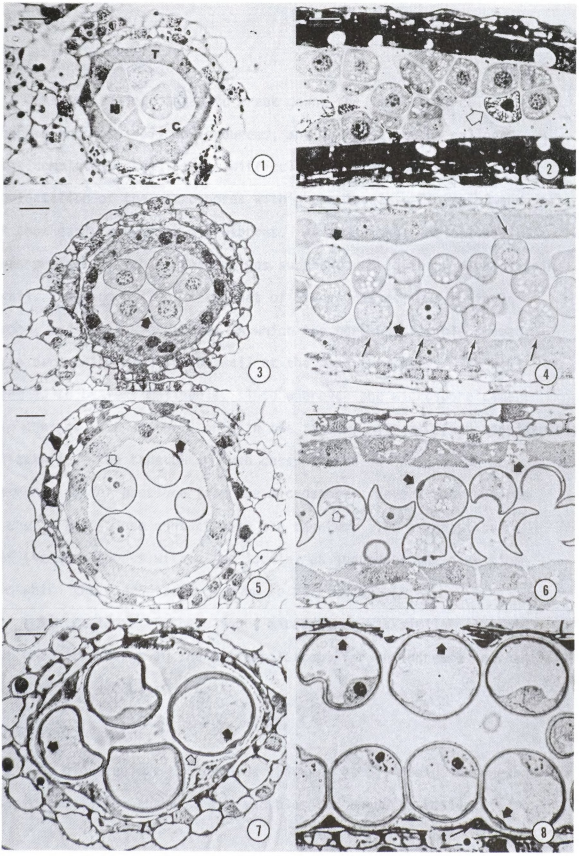
Figures 1-8. Stages of microspore development, phase contrast microscopy. C, callose, M, microspore, T, tapetum; solid arrow indicates pore. Bar = 10 μm.

Figures 1 and 2. Tetrad stage. Microspores still enclosed in callose wall. Open arrow in Figure 2 indicates a tetrad with one degenerating microspore.

Figures 3 and 4. Early vacuolate microspore stage. Callose has disappeared, microspores have rounded, up wall is evident. Note close association of microspores with tapetum (Figure 4 thin arrows).

Figures 5 and 6. Midvacuolate microspore stage. Microspore in Figure 5 shows nuclear displacement (open arrow). Orbicules are visible on inner tapetal face. Two layers of microspore wall are visible in Figure 6 (open arrow). Tapetal cells in Figure 6 are pulling apart.

Figure 7 and 8. Late vacuolate stage/pollen mitosis. Large vacuole occupies central area of each microspore, nucleus is located opposite pore region, tapetum is degenerate; 'tapetal membrane' lines locule (open arrow).


T
C
1
2
3
4
5
6
7
8

Early Vacuolate Stage

As the callose began to break down, the individual cells became progressively more rounded, and by the time the callose was completely gone, it was difficult to discern any special orientation of the microspores within the locule. Figures 3 and 4 show early vacuolate microspores. At this stage, no callose was present. Each microspore was surrounded by a thin, dark-staining wall. Hordeum pollen, like that of other grasses, has a single pore, and those microspores in which the pore was visible showed a dark thickening of the wall at the pore periphery. The wall seemed to be very malleable, since wherever one microspore came in contact with another, or with the tapetum, it was often considerably flattened. The tapetum in such cases showed no indentation or other signs of pressure from the microspore. Several microspores were in very close contact with the tapetum. The orientation of the microspores within the locule, as judged by the direction in which the pores faced, seemed to be random.

The contents of these young microspores was similar to that of the microspores in the tetrads, except for an increase in the size of the vacuoles.

Midvacuolate Stage

In midvacuolate microspores (Figures 5, 6), found in somewhat older and larger anthers, the vacuoles had begun to coalesce,

and some nuclei showed signs of lateral displacement. The direction of nuclear displacement seemed random, with respect both to the cell itself and to the anther as a whole. The orientation of the pores at this stage, however, was much less random than previously; more often than not they faced outward rather than inward. The wall was much thicker and more intensely stained, and the pores appeared correspondingly more developed. The microspores seldom made contact with each other or with the tapetum, presumably owing to locule enlargement, and when they did they were much less deformed than in the early vacuolate stage.

Development in the latter part of this stage (Figure 6) was marked by increasing vacuolization, more nuclear displacement and additional thickening of the microspore wall. Two layers of the wall, with an intervening space, could be resolved. A common sight at this and later stages was the almost total collapse of some of the microspores, presumably a fixation artifact.

Late Vacuolate Stage - Pollen Mitosis

By the time of pollen mitosis (Figures 7, 8), a single large vacuole filled the center of each microspore, leaving only a thin layer of cytoplasm adjacent to the plasma membrane. Most of the nuclei were in late prophase or prometaphase, some were in interphase, and a few had completed mitosis. Each nucleus had migrated to a position approximately opposite the pollen pore. The pores themselves, as well as a considerable amount of adjacent wall

surface, were in close contact with the inner lining of the locule. The pressure of the still-enlarging microspores had pushed aside the remnants of the tapetal cells, and frequently impinged on the middle layer, or, more rarely, on the endothecium.

The layers of the pollen grain wall were much thicker than earlier, and, in contrast to the previous stages, stained dissimilarly, with the inner layer being much darker than the outer one. The dark inner layer passed uninterrupted beneath the pore, and in the pore region, was greatly thickened and protruded into the pore proper, where the operculum appeared to be resting directly on it.

The maximum number of microspores seen in locule cross sections was six. Where the microsporangia became narrower, the number decreased to four, and eventually, at the ends of the anthers, to one. Although the locule did not appear crowded, packing was fairly close, such that in all stages the individual microspores wre probably held in position by the presence of adjacent cells.

From the time of tetrad formation until pollen mitosis, both microsporangia and microspores enlarged considerably. The anthers increased in both length and width by at least 100%, while the diameters of the microspores increased 150 - 200%. From the latter figures it can be calculated that the volume of the individual microspores increased 20 to 25 times. These values are only approximate, since the measurements were made on thick sections

of Epon-embedded tissue. The fixation and embedding procedures cause shrinkage of the anthers, and it is not known what factors influence the shrinkage, nor which tissues are the most susceptible to shrinkage.

In the tetrad stage, it was not uncommon to see tetrads in which one or more of the member cells showed clear signs of degeneration (Figure 2). After the callose had disappeared, however, all the microspores of a given locule usually displayed the same characteristics -- that is, either all appeared to be in good condition, or all did not, and whatever abnormality was displayed by one was displayed by all.

Light Microscope Observations of the Tapetum

From the time the microspores were in the tetrad stage, through the midvacuolate stage, the tapetal cells formed a very prominent layer owing to the fact that, unlike all the other cells of the anther wall, they were not vacuolated. There was no evidence of tapetal dimorphism - all the cells were approximately the same size and shape, and all were binucleate.

The tapetum of Hordeum compressum is of the parietal or secretory rather than the plasmodial or invasive type: that is, at no time does it separate from the anther wall and become interspersed with the microspores in the locule. Like all parietal tapeta, it breaks down at some point after meiosis has been completed.

In the light microscope this breakdown first became evident prior to pollen mitosis, during the latter part of the midvacuolate stage (Figure 6), and was evidenced by a somewhat disorganized appearance of the cells, by their separation from each other, and by indentations caused by the enlarging microspores. By the time of pollen mitosis (Figures 7, 8), very little could be seen of the tapetal cells except patches of dark-staining and apparently degenerate cytoplasm.

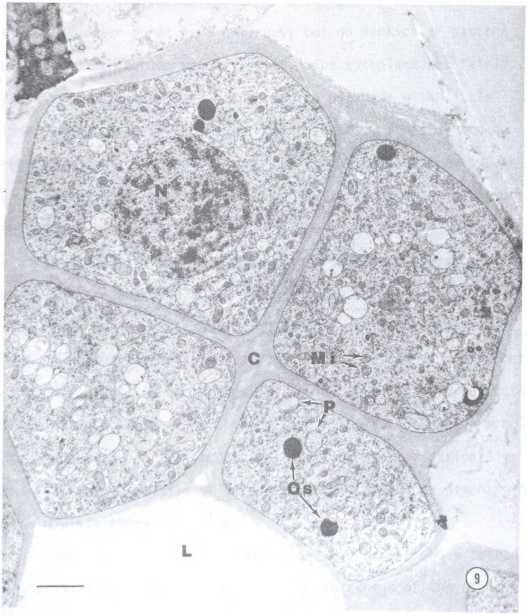
A unique feature of the tapetum in this and some other plants is the development on the locule face of structures called orbicules or Ubisch bodies (Ubisch 1927, Kosmath 1927). The first detectable sign of these in the light microscope was the appearance, at the beginning of the midvacuolate stage (Figure 5), of small granules lining the entire locule. These increased in size as the microspores matured. By the time of pollen mitosis (Figures 7, 8), hollow centers could be seen in some of them. At that time also, they appeared to be attached to a thin, dark layer which in some places seemed to rest on, and in other places had pulled away from, the remnants of the tapetal cells. This layer, which has been given the name 'tapetal membrane' (Banerjee, 1967), with its attached orbicules lined the entire inner wall of the locule.

Electron Microscope Observations of the Gametophyte

Tetrad Stage

The most prominent feature of the young tetrad cells was

Figure 9. Tetrad of microspores encased in callose. C, callose; L, locule; Mi, mitochondria; Os, osmiophilic bodies; P, plastids. Bar = 2 µm.

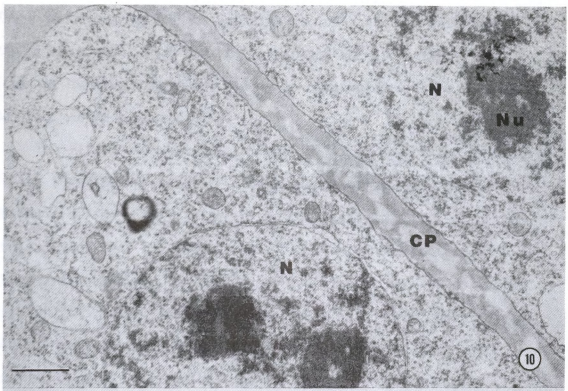

N
C
Mi
P
Os
L
9

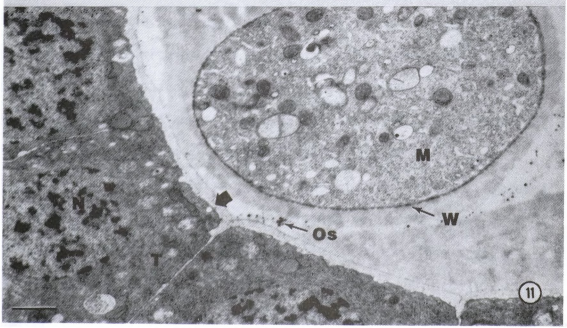
the centrally located nucleus (Figures 9, 10) with two nucleoli (Figure 10). The chromatin appeared to be in a partially dispersed state. Nuclear pores were numerous, but no particular pattern of their distribution was discerned. The cytoplasm was fairly dense and contained small mitochondria, plastids with little internal membrane development, free ribosomes, numerous small vesicles, some rough endoplasmic reticulum (ER) and some smooth ER. Dictyosomes were infrequent and quite small, consisting of only three or four plates about one μm in diameter. There were also in each cell several osmiophilic droplets or bodies around which a membrane could not be distinguished. These ranged in size up to one μm in diameter, and did not seem to occupy any particular location within the cell. Also present were a number of small organelles whose identity could not be established with certainty, but which were thought to be promitochondria or proplastids. The plasma membrane was smooth for the most part; occasionally indentations suggestive of vacuoles either forming or discharging were visible there.

Each individual tetrad member, as well as the whole tetrad, was totally enveloped by callose. The thickness of the callose sheath between any two adjacent tetrad members was about one μm. No cytoplasmic connections of any kind were seen traversing the callose walls between these haploid cells. Outside the callose sheath were more osmiophilic bodies, smaller in size (ca. 500

Figure 10. Two microspores of a tetrad. CP, remnants of cell plate; N, nucleus; Nu, nucleolus. Bar = 1 μm.

Figure 11. Microspore with new wall developing under special callose wall. M, microspore; N, nucleus of tapetal cell (nucleus of microspore is out of plane of section); Os, osmiophilicbodies; T, tapetum; W, developing microspore wall; arrow indicates probable pro-orbicule lying just under tapetal plasma membrane (cf. text p. 51 and Figures 31, 40). Bar = 1 μm.


N
Nu
CP
N
10


M
W
Os
N
T
11

nm) but otherwise similar to those within the microspore cytoplasm. These were usually seen between the callose and the remnants of the primary wall which had once separated the sporogenous cells from the tapetum, and occasionally in other places, such as between adjacent tetrads (Figure 11).

The tetrads which were examined fell into one of two groups. In the first and presumably younger group a close examination of the area immediately exterior to the plasma membrane (Figure 10) revealed that the callose sheath was in direct contact with the plasma membrane. Some sections contained membranes or what appeared to be small membrane-bound vesicles or both, just outside the plasma membrane. Around the tetrads in the second group however (Figure 11), the callose was not in direct contact with the plasma membrane, but was separated from it by a narrow electron-translucent space within which a variety of structures could be seen. Some cells had collections of membrane-bound vesicles in this area; others had what looked like thin fibrils extending from the plasma membrane toward the callose; still others had both vesicles and collections of fibrils (Figures 12-15). Paramural bodies (i.e. multivesicular bodies adjacent to the wall) were also seen (Figure 13). In most instances, however, this space between the plasma membrane and the callose wall was largely filled except for some lateral discontinuities by a layer of amorphous, osmiophilic material (Figures 16-18). The source of this material

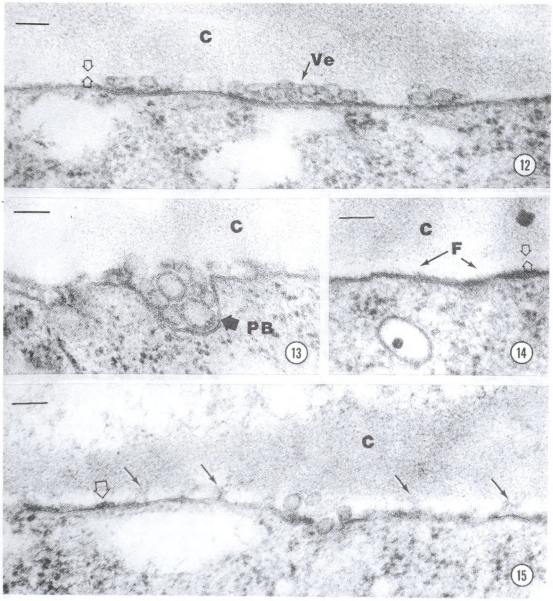
Figures 12-15. Early wall development in tetrad stage. C, callose; F, fibrils; PB, paramural body; Ve, vesicles. 0.2 Bar = 0.2 μm.

Figure 12. Vesicles lying outside plasma membrane. Note thin horizontal line between open arrows (see discussion), here and in Figure 14.

Figure 13. Microspore with paramural body.

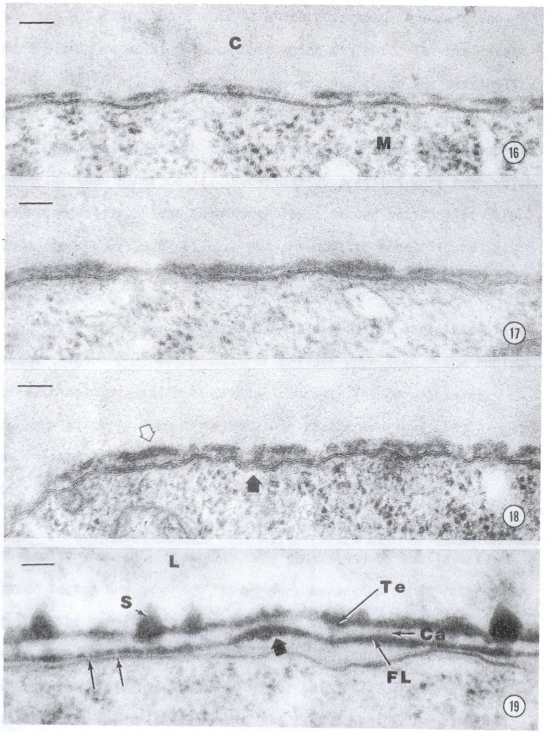
Figure 14. Fibrils extend from area of plasma membrane toward callose.

Figure 15. Microspore with vesicles, fibrillar deposits (thin arrows) and some amorphous material (open arrow).


C
Ve
12
C
PB
13
C
F
14
C
15

Figures 16-18. More advanced wall development in tetrad stage. C, callose; M, microspore; possible development of two (open arrow) and three (closed arrow) layers. Bar = 0.2 μm.

Figure 19. Exine of early vacuolate microspore. Ca, cavus; FL, foot-layer; L, locule; S, spinule; Te, tectum; thin arrows indicate channels in foot-layer, heavy arrow indicates lamella. Bar = 0.2 μm.


C
M
16
17
18
L
S
Te
Ca
FL
19

was not apparent, but the cells which possessed it were often more highly vacuolated than those which did not. The stellate configuration of the vacuoles, however, indicated the possibility of a fixation artifact (Matile, 1975).

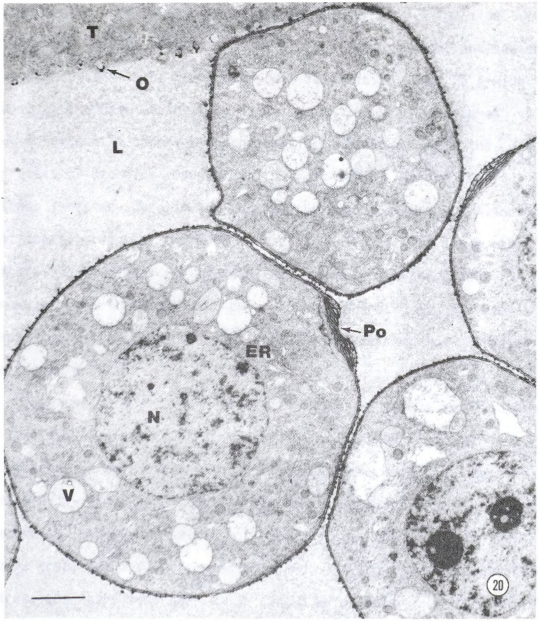
Some cells had much thicker accumulations of this osmiophilic material than other cells. Occasionally profiles were seen which were suggestive of the development of a second osmiophilic layer separated from the first by a non-osmiophilic area (Figure 18).

Early Vacuolate Stage

By the time the microspores had reached the early vacuolate stage (Figure 20), no remnants of callose were seen. The nucleus of each cell was still centrally located. The chromatin was perhaps slightly more dispersed than at the previous stage. ER profiles were somewhat more frequent, and sometimes appeared in pairs. There were many more vacuoles of all sizes.

Surrounding each microspore at this stage was a very definite rudimentary exine (Figures 19, 20) consisting of two layers, an outer tectum and an inner foot-layer, separated by an intervening space, the cavus. Spinules protruded above the tectum, and columellae were seen to continue inward from some of the spinules and to end in the foot-layer. The width of this wall, from the base of the foot-layer to the top of the tectum, but not including the spinules, measured between 80 and 100 nm; the spinules protruded above the tectum an additional 80 nm. Both tectum and foot-layer,

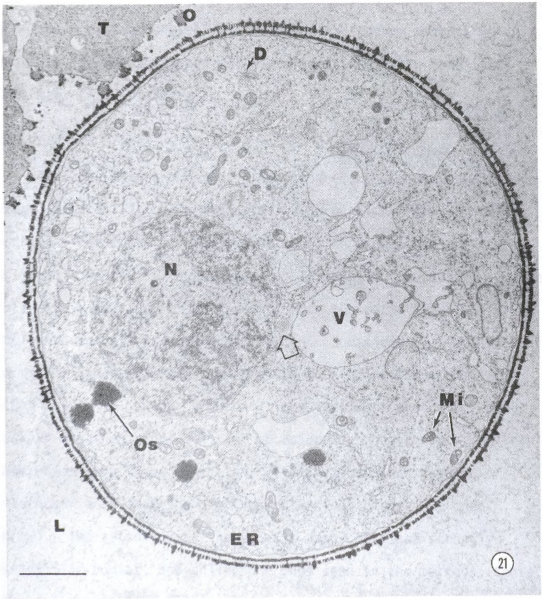
Figure 20. Early vacuolate microspores. ER, (rough) endoplasmic reticulum; L, locule; O, orbicule; Po, pore; T, tapetum; V, vacuole. Bar = 2 μm.


T
O
L
Po
ER
N
V
20

but particularly the former, had an irregular and discontinuous appearance. The inner edge of the foot-layer (i.e., the side toward the plasma membrane) was very smooth and regular, and seemed in some places to be demarcated by a faint layer about 25 Å wide. Also at the base of the foot-layer smooth lamellar structures were frequently observed which sometimes seemed to be in the process of becoming embedded in the foot-layer. The width of these lamellae approximated that of the plasma membrane. Although it constituted the innermost part of the wall at this stage, the foot-layer was only rarely in direct contact with the plasma membrane; for the most part the two were separated by an uneven space of moderate electron density. The plasma membrane presented a smooth profile.

At the pore area, the foot-layer branched into a multi-layered structure (Figures 20, 27). Each layer consisted of a lamella thickened on both sides with an electron-dense material; the more externally positioned lamellae had greater amounts of this encrusting material. The lamellae did not extend across the center of the pore area, but met and curved downward, leaving a clear, undecorated area, the 'pore membrane' about two μm in diameter. In the center of the 'pore membrane' a small decoration, the incipient operculum, could be seen. The lamellae were located in, or probably caused, a raised ring or annulus around the area of the 'pore membrane'. Beneath the 'pore membrane' and the annulus the plasma membrane

Figure 21. Early midvacuolate microspore. D, dictyosome; ER, (rough) endoplasmic reticulum; Mi, mitochondria; N, nucleus; O, orbicule; T, tapetum; V, vacuole; evagination of nuclear membrane at open arrow. Bar = 2 μm.


T
O
D
N
V
Mi
Os
L
ER
21

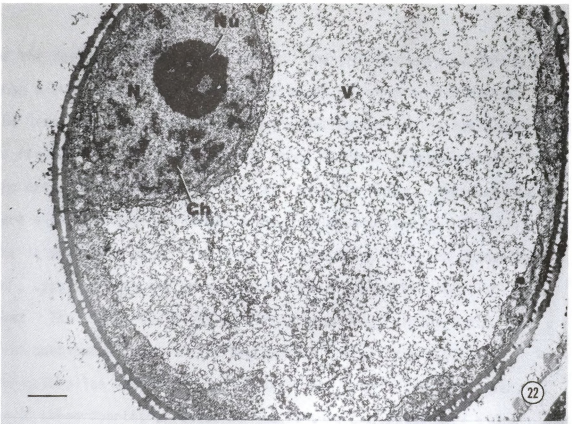
was considerably withdrawn, and the space thus created was filled with material continuous with and apparently identical to the previously mentioned material of moderate electron density which underlay the non-apertured wall, and additionally in some cases with many small vesicles.

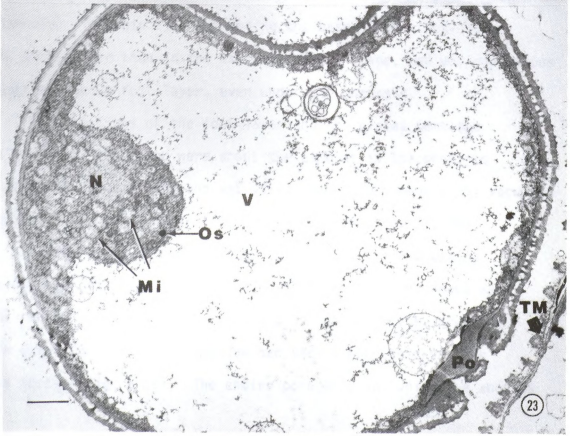
Midvacuolate Stage

In those microspores which had progressed to the midvacuolate stage (Figure 21), there were still a large number of very small vacuoles, but there were also some much larger vacuoles, and some of these appeared to be autophagic. The nuclei, frequently somewhat laterally displaced, contained very fine chromatin fibrils. Nuclear evaginations were quite common. Sometimes it was difficult to discern whether these evaginations made connections with any other organelle or not; at other times they were extensive enough to have attached ribosomes, and occasionally they contained within themselves structures consistent with the idea that budding of mitochondria or plastids might be occurring (Figure 21, open arrow). The cytoplasm of these cells also contained strongly osmiophilic bodies, but unlike the ones seen in the tetrads, these were not smooth in outline.

At this stage (Figure 24) the thickness of the wall was almost triple what it had been in the early vacuolate stage (Figure 19), an increase which was observed in foot-layer, cavus and tectum, but not in spinules. From the base of the foot-layer to the top

Figures 22 and 23. Microspores at time of pollen mitosis. Ch, chromatin; Mi, mitochondria; N, nucleus; Nu, nucleolus; Os, osmiophilic body; Po, pore; TM, tapetal membrane; V, vacuole. Bar = 2 μm.


Nu
N
V
Ch
22


N
V
Os
Mi
TM
Po
23

of the tectum measured about 250 nm. The spinules were no larger than previously and again projected about 80 nm above the tectum. The tectum no longer seemed to be discontinuous, but it was interrupted at frequent intervals by channels about 50 nm in diameter connecting the external environment of the cell with the cavus. These channels were almost always unbranched and occupied about one-third of the interspinule area. The spinules themselves were not channelled, nor, usually, were the areas of the tectum immediately beneath them. The cavus itself was electron-translucent except for a thin continuous layer in its approximate center, and except for the columellae connecting tectum and foot-layer. The foot-layer, about three fourths as thick as the tectum, contained only a few channels. The thin line subtending the base of the foot-layer was much darker than in the early vacuolate stage, and was continuous under the whole foot-layer, even under the channels.

The positions of the foot-layer and the plasma membrane relative to each other were about the same as in the previous stage. The plasma membrane was still smooth, but many small vacuoles could be seen lying immediately beneath it (Figures 21, 28).

In the early vacuolate stage (Figure 27) the encrusting deposits on the pore lamellae were about the same thickness as the tectum and foot-layer. By the midvacuolate stage, however, the outer three or four lamellae had become much thicker than the tectum (Figure 28). The entire pore area including the annulus

had increased in width and depth, but the number of individual lamellae remained the same. Although the tectum of the annulus bore the same pattern of channels as did the rest of the tectum, the encrusted lamellae differed from the foot-layer in that they contained no channels at all.

As the midvacuolate stage progressed, the exine nearly doubled in width, to about 400 nm (Figure 25). Again, this increase reflected changes in size of tectum, cavus and foot-layer, but not spinules. The channels in the tectum and foot-layer had not narrowed. The dark line at the base of the foot-layer was still apparent, even under the channels. Both the inner side of the tectum and the outer side of the foot-layer were much more irregular than previously. The cavus seemed to contain some fibrillar material.

Late Vacuolate Stage - Pollen Mitosis

The very large, centrally located vacuole characteristic of this stage pressed all the other cell organelles against the pollen grain wall (Figures 22, 23). The cytoplasm thus compressed was heavily populated with organelles, including large numbers of dictyosomes, and some osmiophilic deposits. The nucleus, surrounded by a thin layer of cytoplasm, protruded into the vacuole. No unusual chromosomal features were apparent.

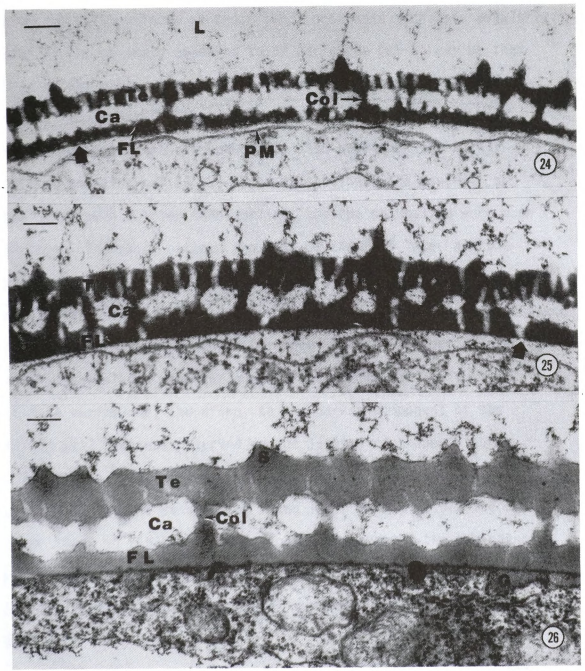
The exine at this stage measured about 700 nm in thickness (Figure 26). Except for a thin layer lining the cavus, the channels

Figures 24-26. Stages of exine development. Ca, cavus; Col, columella; FL, foot-layer; G, granular material between plasma membrane and foot-layer; L, locule; PM, plasma membrane; S, spinule; Te, tectum. Bar = 0.2 μm. Note thin dark line subtending foot-layer in Figures 24 and 25 (arrows).

Figure 24. Early midvacuolate stage.

Figure 25. Late midvacuolate stage.

Figure 26. Pollen mitosis.


L
Ca
FL
Col
PM
24
Ca
25
Te
Ca
Col
FL
26

and the outer side of the tectum, it was noticeably less osmiophilic than in earlier stages. All the surfaces were smoother; the spinules, although still pointed at their tips, protruded much less angularly. Some of the channels appeared to be occluded, but it may be that they merely passed out of the plane of the section. Although many of the channels seemed to be narrower than those of the earlier stages, that appearance may also have been the result of their passing out of the plane of section. The contents of the cavus were coarser than earlier, and the columellae were much thicker. The space between the base of the foot-layer and the plasma membrane had narrowed, and was filled with a granular substance of moderate electron density. This same granular substance continued into the area under the pore, filling the entire area between the plasma membrane and the 'pore membrane' (Figure 29). It must correspond, therefore, to the darker component of the pollen wall that was observed in the light microscope (Figures 7,8). (Thus, the inner of the two layers in Figures 7 and 8 is apparently not the same as the inner layer in Figure 6. The dark inner layer in Figures 7 and 8 probably represents the beginning of the endexine, which by the intensity of its stain is obscuring the foot-layer, i.e., the inner of the two layers visible in Figure 6.)

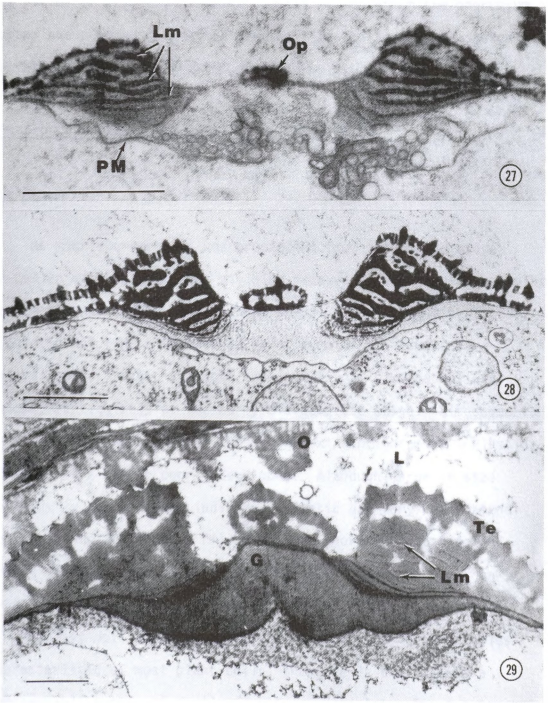
In the pore area (Figure 29) the lamellae were so thickened as to have almost obliterated the spaces between them, although

Figures 27-29. Stages in pore development. G, granular material; L, locule; Lm, lamellae; O, orbicule; Op, operculum; PM, plasma membrane; Te, tectum. Bar = 1 μm.

Figure 27. Early vacuolate microspore.

Figure 28. Midvacuolate microspore.

Figure 29. Microspore at pollen mitosis.


Lm
Op
PM
27
28
O
L
Te
G
Lm
29

The space between the outermost lamella and the tectum remained. The pillow of granular material (endexine?) on which the operculum rested had protruded into the pore so that the top of the operculum was almost level with the top of the annulus.

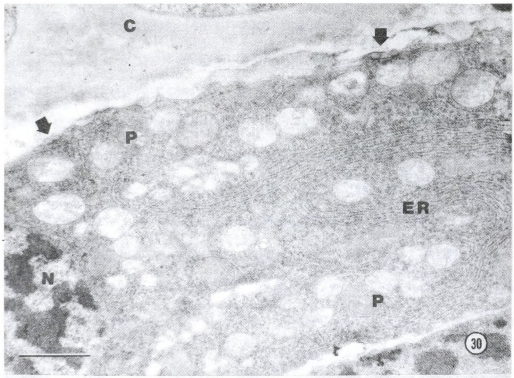
Electron Microscope Observations of the Tapetum

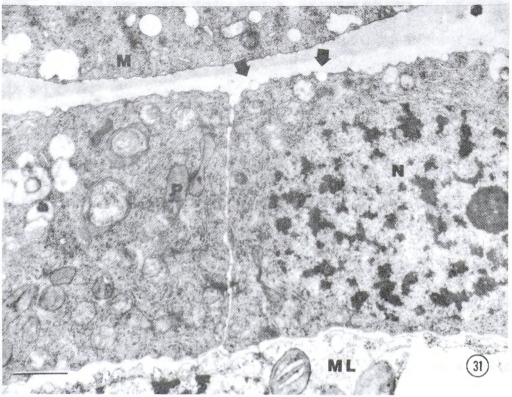
Cytoplasm

As microsporogenesis was proceeding from the tetrad stage to pollen mitosis, the most striking feature of the tapetal cytoplasm was the abundant rough ER (Figures 30-35). There was a very heavy population of free ribosomes as well, some of which were in a helical rather than a polysome configuration. Dictyosomes were common, particularly at the early midvacuolate stage. Mitochondria were abundant and always in the 'orthodox' configuration. Plastids, however, were rather poorly developed. Although large in size and many in number, each individual plastid had very few internal membranes and was largely filled with an amorphous substance of medium electron density. Occasional inclusions, some electron-dense and some electron-transparent, were contained within some of the plastids. Although there were none of the large vacuoles characteristic of most plant cells, these cells did possess a considerable number of small vacuoles or vesicles. The two tapetal nuclei were usually more or less centrally located on the cell's

Figures 30-38. Tapetal maturation.

Figures 30 and 31. Tapetum at microspore tetrad stage. C, callose; ER, (rough) endoplasmic reticulum; M, microspore; ML, cell of middle layer; N, nucleus; P, plastid; incipient orbicules at heavy arrows. Bar = 1 μm.


C
P
ER
N
P
30


M
P
N
ML
31

long axis. The proportion of euchromatin to heterochromatin (i.e., dispersed vs. condensed) in these nuclei was about the same as that of the other cells in the anther wall, and much lower than that in the microspores.

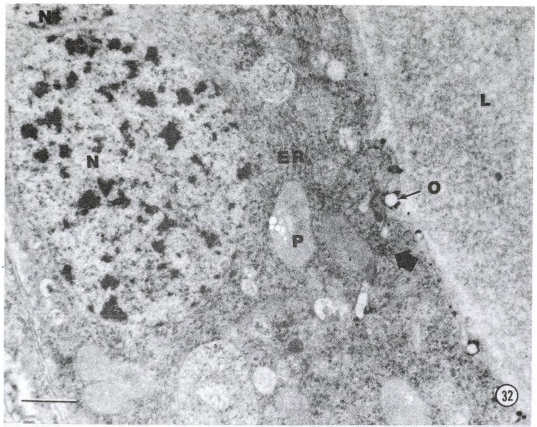
Although none of the various types of organelles was confined exclusively to one area of the cell, their distribution was not totally random. In the early tetrad stage (Figure 30), the stacks of rough ER tended to enclose pockets of cytoplasm containing mitochondria and plastids. By the early vacuolate stage (Figure 32) this arrangement was no longer so apparent, and by the midvacuolate stage (Figures 33, 34) nearly every plastid seemed to be closely associated with, and sometimes almost surrounded by, an ER cisterna. These cisternae were often smooth, but sometimes rough and occasionally bifacial.

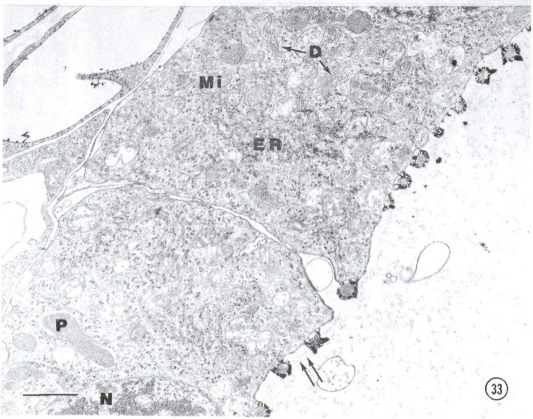
Some of the features seen in the early midvacuolate stage are illustrated in more detail in Figures 34-36. The smooth cisternae ensheathing the plastids, and cytoplasmic connections between cells, are prominent. Unusual subcellular structures, which appeared to be a combination of cytoplasmic plates and flattened vesicles, were observed in several tapetal cells of this anther. These structures have tentatively been named 'cytoplasmic plate complexes.' They consisted of flat or curved cytoplasmic plates surrounded by a flattened sac whose boundaries paralleled those of the cytoplasmic plates (Figures 34-36). At their ends

Figures 32 and 33. Tapetum at early vacuolate and early midvacuolate microspore stages. D, dictyosome; ER, (rough) endoplasmic reticulum; L, locule; Mi, mitochondrion; N, nucleus; O, orbicule; P, plastid. Bar = 1 μm.

Figure 32. Early vacuolate microspore stage. Cytoplasm of adjacent cells confluent at heavy arrow.

Figure 33. Early midvacuolate microspore stage. Remnants of primary wall at double arrows.


N
N
ER
L
O
P
32

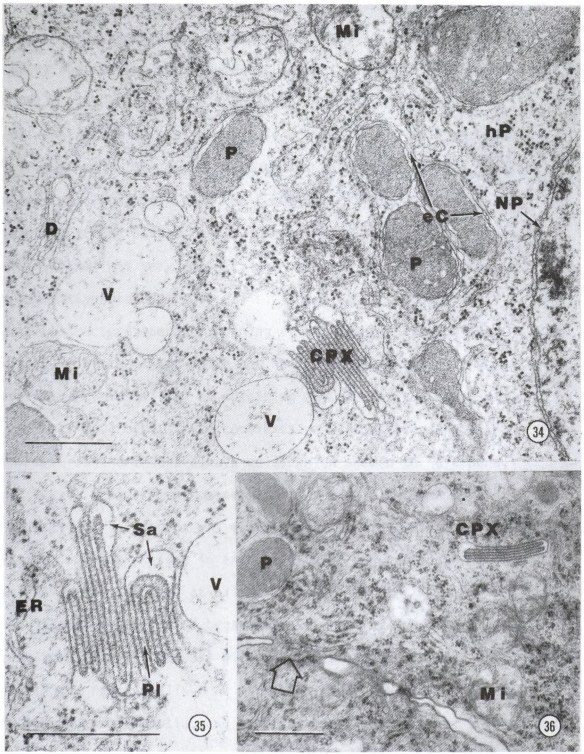

D
Mi
ER
P
N
33

Figures 34-36. Detail of tapetal cytoplasm at early midvacuolate stage. CPX, cytoplasmic plate complex; D, dictyosome; eC, ensheathing cisternae; ER, (rough) endoplasmic reticulum; hP, helical polysome; Mi, mitochondrion; NP, nuclear pore; P, plastid; Pl, plate area of cytoplasmic plate complex; Sa, sac or cisternal area of cytoplasmic plate complex; V, small vacuole. Bar = 0.5 μm.

Figure 34. Overview of cytoplasm.

Figure 35. Cytoplasmic plate complex enlarged from Figure 34.

Figure 36. Adjacent cells connected by cytoplasmic band (arrow). Cytoplasmic plate complex in upper cell.


Mi
hP
P
eC
NP
D
P
V
CPX
Mi
V
34
Sa
V
ER
PI
35
CPX
P
Mi
36

the sacs were dilated and frequently continuous with larger vesicles which usually appeared to contain some fibrillar material. Occasionally some of the sacs seemed to connect with the ER (Figures 34, 35). The membranes of both the plates and the surrounding sacs were trilaminar, as were those of the larger vesicles; the non-cytoplasmic face of these membranes (and of the plasma membrane) stained more intensely than the cytoplasmic face did (Figure 35). The width of the plates (about 30 nm) and of the cisternal areas (about 15 nm) was very regular, much more so than that of the ER and dictyosomes in these cells. There was a suggestion of some substructure, perhaps running at right angles to the long axis, in both the cytoplasmic and non-cytoplasmic areas. There were no ribosomes within or adjoining any part of the complexes.

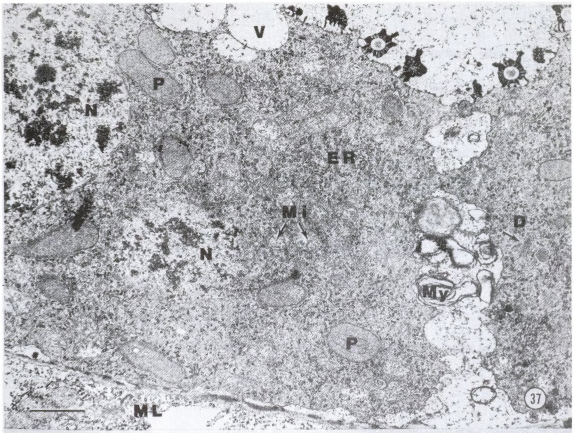
The frequency of these complexes was low, since in any one thin section there were always cells which seemed to have none, and two was the maximum number seen in a single section of a single cell. No attempt was made to determine by serial sections the total number present in an entire cell, or whether there were any cells that had none at all.

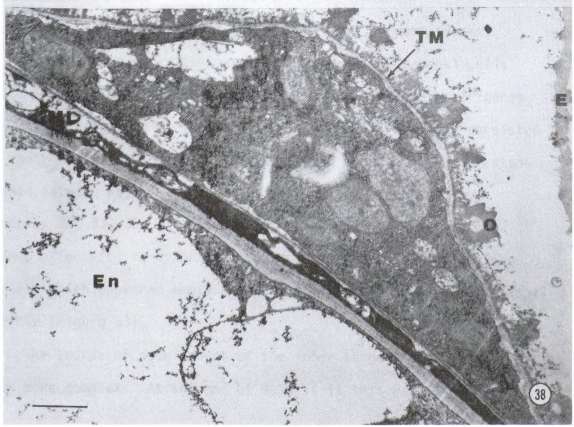
Tapetal degeneration began to be evident in the late mid-vacuolate stage (Figure 37) with the appearance of 'myelin' figures between the radial walls. At that time there was a great increase in the number of plastids and some decrease in the number of mitochondria. By the time of pollen mitosis (Figure 38) the tapetal

Figures 37 and 38. Stages in tapetal degeneration. D, dictyosome; E, microspore exine; En, endothecial cell; ER, (rough) endoplasmic reticulum; Mi, mitochondria; My, myelin figure; N, nucleus; O, orbicule; P, plastid; TM, tapetal membrane; V, vacuole. Bar = 1 μm.

Figure 37. Late midvacuolate stage.

Figure 38. Advanced degeneration at time of pollen mitosis.


V
P
N
ER
MI
D
N
MY
P
ML
37


TM
E
D
O
En
38

cytoplasm had been reduced to about one-tenth of its former amount. The plasma membrane did not rupture, and some organelles within the cells were still identifiable.

Premature degeneration and other abnormalities were not as common in the tapetum as in the microspores. No examples were seen of degeneration which was only premature: all prematurely degenerating tapetal cells displayed a type of degeneration different than that which normally began in the late midvacuolate stage. Abnormal tapetal degeneration was not always accompanied by abnormal microsporogenesis; sometimes the developing microspores appeared normal (Figure 9).

Cell Walls

At the end of meiosis II, thin primary walls were present at the radial and outer tangential surfaces of the tapetal cells (Figure 31). The radial walls subsequently disintegrated (Figures 32, 33) although remnants of the middle lamella frequently persisted at their distal ends, and broad cytoplasmic connections were established between adjacent cells, making the tapetum a syncytium (Figures 32, 33, 36, 37). The outer tangential plasma membrane showed a tendency to withdraw at intervals from its wall, leaving pockets which appeared empty except for the presence of an occasional vesicle (Figure 33).

The course of development of the inner tangetial wall was much more complex. At the end of meiosis II this wall appeared

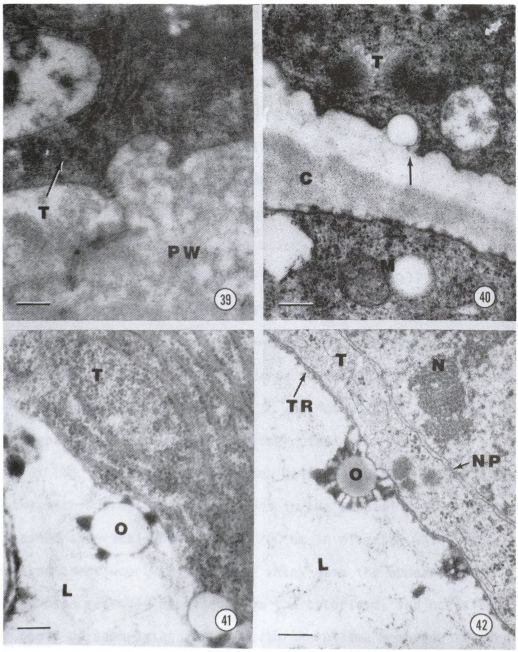
Figures 39-42. Orbicule development. C, callose; L, locule; M, microspore; N, nucleus; NP, nuclear pore; O, orbicules; PW, remnant of primary wall; T, tapetum; TR, tapetal reticulum. Bar = 0.2 μm.

Figure 39. Late tetrad stage.

Figure 40. Late tetrad stage. Note electron-dense material at orbicule surface (arrow). Microspore has some wall development.

Figure 41. Early vacuolate microspore stage. Orbicule has prominent spinules.

Figure 42. Early midvacuolate microspore stage. Orbicule with spinules and channels. Incipient tapetal reticulum on locular face of tapetal plasma membrane.


T
PW
39
T
C
M
40
T
O
L
41
T
N
TR
NP
O
L
42

much thicker than any of the other tapetal walls, but appeared to be undergoing a process of dissolution and was fibrillar and granular in appearance (Figures 39, 40). The middle lamella remained intact, and as mentioned previously, osmiophilic bodies were trapped between it and the callose of the microspores. The middle lamella subsequently disappeared, but the fibrillar remnants of the rest of the wall were still apparent even at the time of pollen mitosis (Figures 33, 37, 38).

Shortly after the tapetal wall began disintegrating, the tapetal plasma membrane acquired a very active configuration, with a large number of pockets or depressions. Small homogeneous droplets or spherical bodies of a medium electron density about 150 nm in diameter were present in many of these depressions (Figure 39). These droplets appeared to have a boundary of some sort, but not a trilaminar one. It was not possible to identify any comparable structures within the cytoplasm.

At the late tetrad stage, when wall development was apparent in the microspores, the depressions in the tapetal plasma membrane contained spherical bodies about 200 nm in diameter which were electron-translucent (Figure 40). Many gave the appearance of having been recently expelled from the cytoplasm, and occasionally one could be seen lying just beneath the plasma membrane (Figure 11). Small patches of a moderately electron-dense material were frequently seen close to the surface of these droplets (Figure

40).

By the early vacuolate stage, the spherical bodies measured about 300 nm, and were still electron-translucent (Figure 41). Accretions of electron-dense material, presumably sporopollenin or protosporopollenin, decorated the periphery of all the droplets identifying them with certainty as developing orbicules. In size and shape these accretions resembled the spinules on the developing exine in the same anthers. The subsequent development of these orbicules closely paralleled that of the tectum of the developing microspore exine. In the early midvacuolate stage, the orbicular covering, 60 to 100 nm thick, contained both channels and spinule projections (Figure 42); the contents of the orbicule were no longer electron-translucent. The channels, which had a diameter of about 45 nm, and thus were a little narrower than those in the tectum, appeared to be open to the exterior but not to the interior. As the midvacuolate period progressed the covering thickened (Figure 43). The orbicular contents lost their uniformity and became more electron-translucent at their peripheries. By the time of pollen mitosis (Figure 44) the orbicular covering was indistinguishable from the pollen tectum in width (about 300 nm), size of channels (25-45 nm) and spinule-type projections. The center of the orbicules at that stage contained fibrillar material comparable to that in the locule itself, and the channels appeared patent. Although there was frequent direct contact

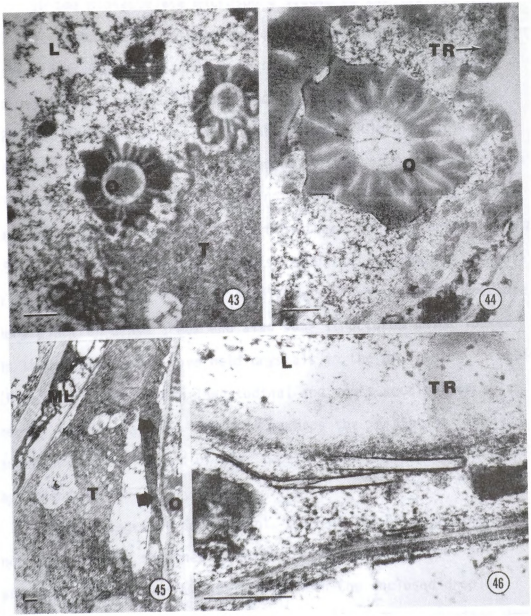
Figures 43-46. 'Tapetal membrane' development. L, locule; ML, cell of middle layer; O, orbicule; T, tapetum; TR, tapetal reticulum. Bar = 0.2 μm.

Figure 43. Late midvacuolate microspore stage.

Figure 44. Orbicule at time of pollen mitosis. Tapetal reticulum well developed.

Figure 45. Tapetal cell at time of pollen mitosis. Note layer between reticulum and plasma membrane containing crystal-like structures (arrows).

Figure 46. Detail of crystal-like structure.


L
T
43
TR
O
44
T
O
45
L
TR
46

between the orbicules and the pollen exine at this time, there were never any signs of actual merging of the two.

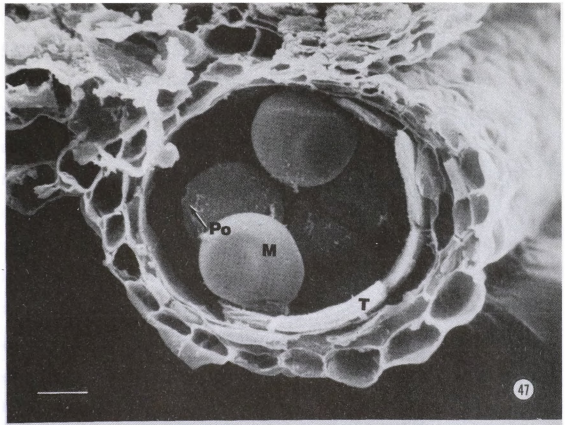
In the midvacuolate microspore stage, on the locular face of the tapetal cells between the orbicules, an incipient electron-dense reticulum was visible (Figure 42). This layer continued to develop and eventually became quite conspicuous (Figures 38, 52). At the time of pollen mitosis (Figure 38) this reticulum with the attached orbicules seemed to form an integral unit which lined the entire locule. It did not rest directly on the tapetal cell remnants, but was separated from them by a region approximately 150 nm in width (Figure 38). This region was not empty; it contained a material of light electron density within which there were some very electron-dense areas and also some structures which looked as though they might have held crystals in the process of formation (Figures 45, 46). Both longitudinal and cross sections of this region looked the same. This is perhaps the initiation of the outer of the two layers of the 'tapetal membrane' as described by Banerjee (1967).

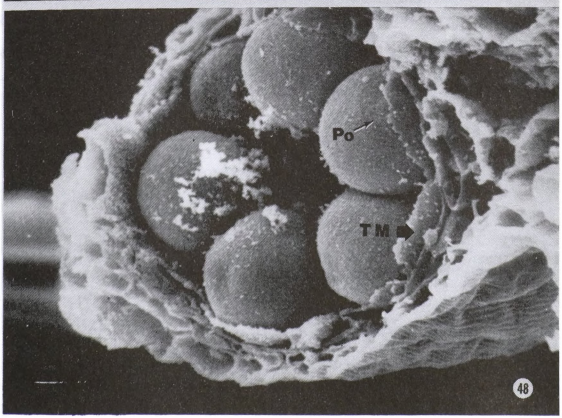
At all stages of development, many of the orbicules were not quite wholly sheathed by sporopollenin. In the area not sheathed, contact was maintained between the enclosed droplet and the tapetal plasma membrane (Figure 42). These openings were still present to some extent at pollen mitosis.

Figures 47 and 48. Cross-sections of individual locules. M, microspore; Po, pore; T, tapetum; TM, tapetal membrane. Bar = 1 μm.

Figure 47. Tapetum not yet degenerate.

Figure 48. Tapetum degenerate. 'Tapetal membrane' adhering to surface of pollen grain.


Po
M
T
47

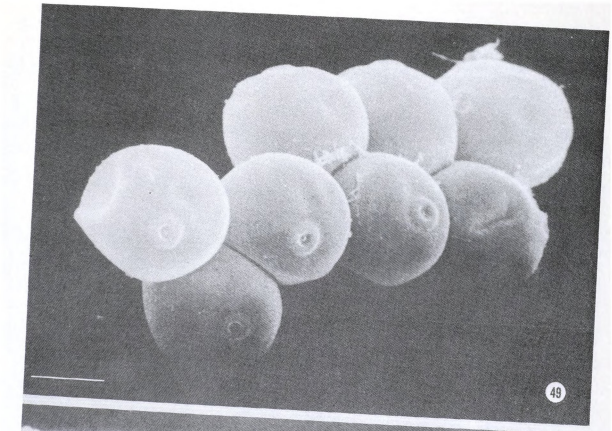

Po
TM
48

Scanning Electron Microscope Observations of Whole Anthers

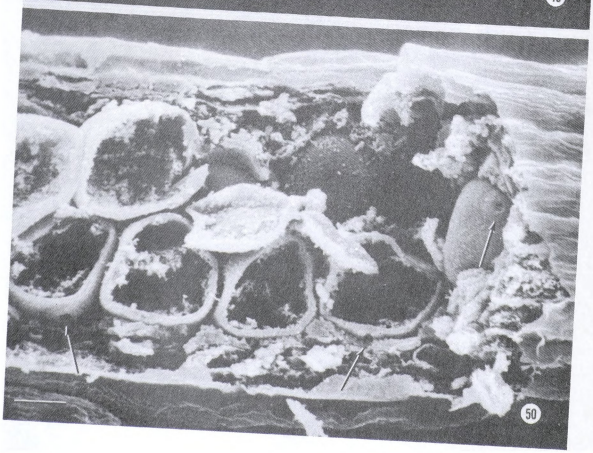
Several of the observations made in the light and transmission electron microscopes were corroborated with the SEM. The close packing of the microspores within the anther was much more striking than in comparable TEM sections (Figures 47-50). Orientation polarity was clearly evident (Figures 49, 50); microspores which did not have their pores in contact with the tapetum were uncommon. The closeness of the association between the microspores and the 'tapetal membrane' was especially apparent. Where microspores had been dislodged, indentations left in the tapetum by the annuli were clearly visible (Figure 53), and in some places patches of the reticulum could be seen clinging to the microspores (Figure 48). The orbicules varied in size, some being about twice as large as others; their distribution appeared to be random (Figure 51).

Figure 49. Microspores dislodged from an anther. Note orientation of pores. Bar = 1 µm.

Figure 50. Longitudinal section of anther. Note orientation of pores (arrows). Bar = 1 µm.



49

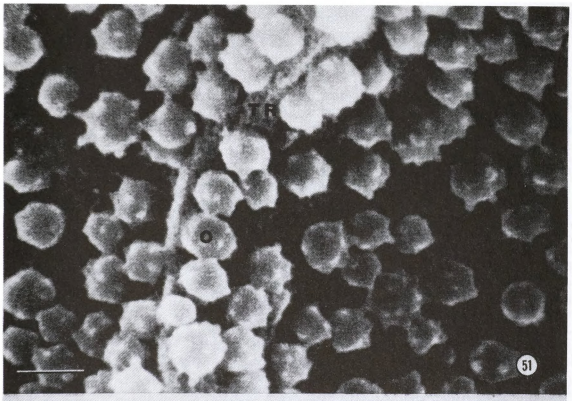


50

Figures 51 and 52. Orbicules. O, orbicule; T, tapetum; TR, tapetal reticulum. Bar = 1 μm.

Figure 51. Orbicules on the locular face of the tapetum.

Figure 52. Tangential section of the locular face of a tapetal cell.


TR
51

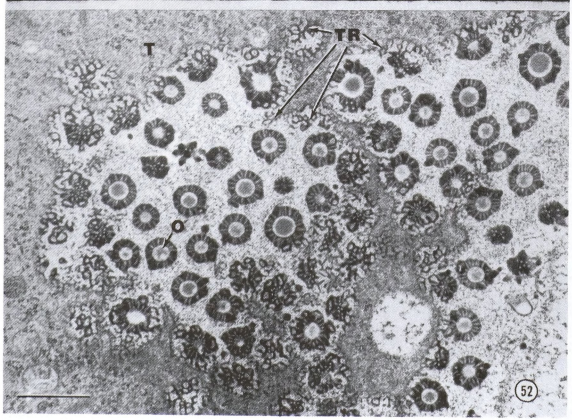
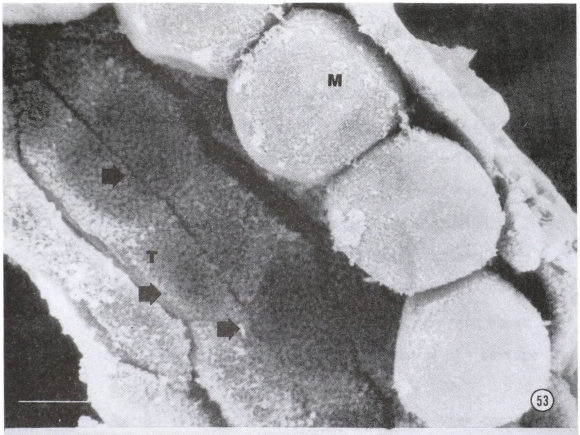
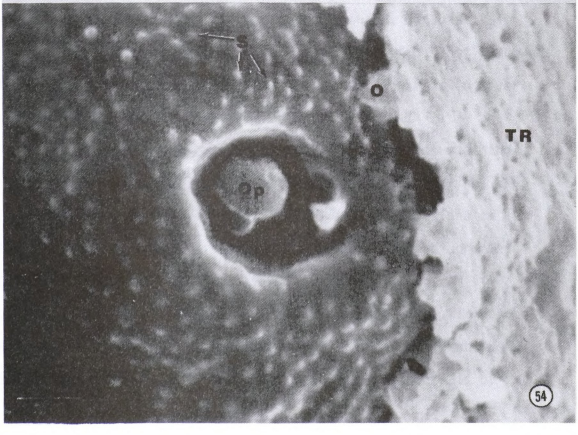

T
TR
52

Figure 53. Open anther. Note close packing of microspores, and indentations in tapetum where microspores have been dislodged (arrows). M, microspore; T, tapetum. Bar = 1 µm.

Figure 54. Single microspore with attached reticulum. O, orbicule; Op, operculum; S, spinules; TR, tapetal reticulum. Bar = 1 µm.


M
T
53


O
TR
Op
54

Discussion

When viewed from the outside, the mature pollen grain of H. compressum is one of the least elaborate in the plant kingdom. It is spherical, or nearly so, and its most conspicuous feature is its single pore. The pore is surrounded by a raised rim, or annulus, and has a small spherical decoration, the operculum, at its center. The surface of the grain, including the annulus and the operculum, is smooth except for a dense population of small spinules, more or less evenly distributed, which rise about 50 nm above the surface and occupy about one-fourth of the surface area.

At maturity the pollen wall has an inner and an outer component, the intine and the exine. It is the exine which, because it is composed mainly of the extraordinarily resistant family of compounds collectively called sporopollenin, is capable of persisting over extremely long periods of time with little change.

A cross-section of the gametophyte of H. compressum at the first mitosis shows the wall to be composed solely of exine, the intine having not yet developed. The several subdivisions of the exine can be distinguished at this stage and are diagrammed in Figure 55. The outermost layer is the tectum and from it extend the spinules that are apparent in surface view. Immediately beneath the tectum is a space, the cavus, and beneath that the foot-layer. The cavus is traversed at irregular intervals by

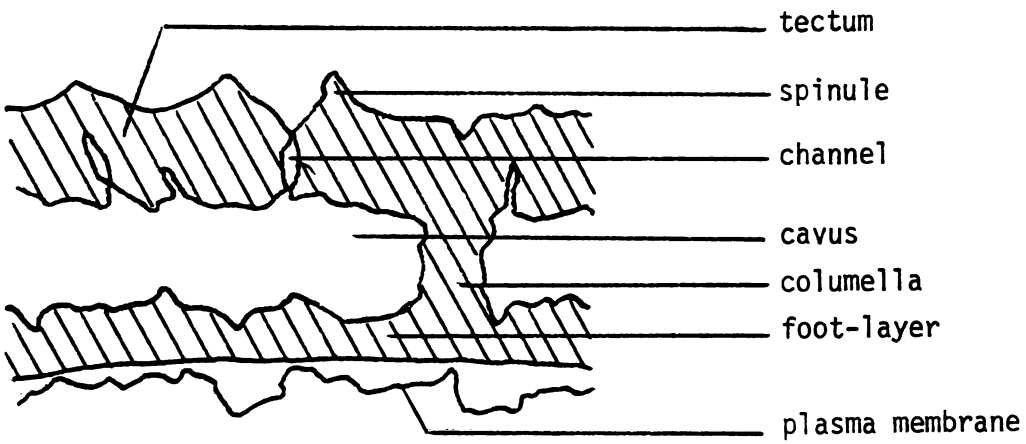


Figure 55. Diagram of the microspore wall at the time of pollen mitosis.

rather thick columns, called columellae. Other authors (Skvarla and Larson, 1966) have concluded that originally the spinules are the tips of the columellae, and that during the expansion which the exine undergoes as the microspore enlarges, the spinules and columellae sometimes become separated. The entire unit, columella plus spinule, is a baculum. All the aforementioned areas of the exine, with the exception of the cavus, exhibit the same staining reaction, and together they comprise the ektexine. A number of narrow channels traverse the tectum, connecting the cavus with the external environment. The foot-layer is also channelled, but much less extensively so. Beneath the foot-layer, occupying most of the space between it and the plasma membrane,

is a moderately osmiophilic granular material.

The pollen walls of other species have the same basic subdivisions, although the final product may be more or less elaborate than that of H. compressum. The development of this highly complex wall has been traced in several species, and in all cases has been found to begin while the microspores are still joined in a tetrad formation. For the purposes of discussion, the developmental process can be divided into three stages: (1) the early development of the layers lying parallel to the plasma membrane, (2) the development of the radial components, and (3) the growth of the various components.

The initial sign of wall development, a layer laid down outside the tetrad microspore plasma membrane underneath the special callose wall, is called the primexine; the word was coined by Heslop-Harrison (1963) to indicate the importance of this material in the development of the mature exine. The term 'primexine' is somewhat ambiguous, since in most cases it does not refer to a structure with clearly defined characteristics, but to a structure or structures whose characteristics vary considerably from species to species. It is usually finely fibrillar in nature, although in some species with certain fixatives, especially $KMnO_4$, it may appear translucent (Heslop-Harrison, 1968a,b). In some species it is of uniform consistency and distribution (Christensen, Horner and Lersten, 1972) and in others not (Horner and Pearson, 1978).

It has been shown in some species to be cellulosic in nature (Heslop-Harrison, 1968a,b), although variation in staining reactions from species to species suggests the possibility of other compositions. Its upper and lower surfaces are said to act as a template for the formation of the tectum and foot-layer of the mature exine (Heslop-Harrison, 1968a), while the bacula (or columellae) arise as radially oriented, dense, rod-like structures within it (Heslop-Harrison, 1968a; Christensen, Horner and Lersten, 1972; Horner and Pearson, 1978).

A surprising variety of configurations was observed in the initial stages of wall development in H. compressum. Some of these are particularly interesting because of their similarities to wall development in other types of cells. For example, membrane-bound vesicles lying outside the plasma membrane can be found in both normally growing tissue, such as Avena coleoptiles (Heyn, 1971, 1972), and experimental tissue, such as callus. In Andrographis paniculata callus (Bowes and Butcher, 1967) vesicles are embedded in the walls. In carrot callus (Halperin and Jensen, 1967) one of the first signs of wall breakdown is the de novo appearance of small membrane-bound vesicles in the region of the middle lamella. Furthermore, around both the dividing and non-dividing cells of proembryos derived from carrot callus, the cell wall is separated from the plasma membrane by an electron-translucent space, as was the case for some of the H. compressum microspores.

Within this space, in addition to vesicles, a thin, faint, horizontal dense layer is visible, similar to the one sometimes seen around the microspores (Figures 12, 14). This may be the structure which Skvarla and Larson (1966) labelled the tectum template in Zea mays microspores. Its presence in carrot callus, whose cells do not develop a tectum, would presumably nullify that interpretation.

The microspores showing early wall development were also the ones in which paramural bodies were seen. Paramural bodies have been found in Z. mays tetrads (Skvarla and Larson, 1966) at about the same stage of development, and indeed are a common feature in many types of plant cells (Roland, 1973). Although it is far from certain, the paucity of dictyosomes in H. compressum microspore tetrads would tend to indicate that these particular paramural bodies were plasmalemmasomes (derived from the plasma membrane) rather than lomasomes (derived from cytoplasmic membranes). According to Marchant and Robards (1968) plasmalemmasomes are more likely than lomasomes to be involved in enzymic processes, and may function in secondary modifications of the cell wall. On the other hand, whether these vesicles were moving out of the cell, or perhaps into it as has been suggested by Thomson (1967), and what their relationship is to the vesicles lying outside the plasma membrane, is really a matter of conjecture.

Some of the H. compressum microspores exhibited a type or stage of early wall development that seems to correspond to the

primexine described in Silene pendula and some other species (Heslop-Harrison, 1968a), where a thin electron-translucent area lying parallel to the surface of the plasma membrane is interrupted at intervals by radially oriented deposits of a fibrillar nature (Figure 15). But in H. compressum wall development in apparently more advanced microspores still encased in callose consisted of a discontinuous layer of amorphous osmiophilic material (Figures 16-18). This material was not on the surfaces of the electron-translucent areas, but within them, and radially directed rods, rather than being prominent, were not visible. It would seem, therefore, that in H. compressum the electron-translucent areas may represent sites where a substance secreted by the microspore has caused a localized degradation of callose, and that within these electron-translucent areas chemical reactions occurring between the breakdown products and materials secreted by the cell result in the deposition of the amorphous osmiophilic substance. Also, materials from the locule may diffuse through the callose and enter into this reaction; there are conflicting reports about the impermeability of callose (Mepham, 1970; Southworth, 1971). The fibrillar areas in Figure 15 could be residual areas of callose.

Alternatively, preformed osmiophilic material may be discharged from vesicles into the translucent areas. Arguing against this possibility is the fact that the profile of the plasma membrane was not as convoluted as would be expected in a cell actively

engaged in such secretion.

The dense osmiophilic material varied in width from cell to cell, but even when it attained its maximum width, lateral discontinuities persisted in it. This is contrary to the situation reported for Sorghum bicolor (Christensen, Horner and Lersten, 1972) and some other species where the primexine becomes a uniform layer. If this osmiophilic layer in H. compressum is a primexine, as seems likely, then we can assume that sporopollenin will be laid down on its upper and lower surfaces, that bacula will develop, most probably in the discontinuities, and that the primexine will ultimately disappear leaving the profile of the developing exine seen in Figure 19.

There is the possibility of another ontogenetic pathway. The osmiophilic layer in Figure 18 is separated from the plasma membrane by a less darkly-staining region, and in at least one area there seem to be two osmiophilic layers separated by a spacer layer. These configurations could be interpreted to mean that the initial layer of dense material is the rudimentary tectum, and that following the secretion of its components two other layers are secreted: a 'spacer' layer that initially occupies the region that will become the cavus, followed by another osmiophilic layer which will become the foot-layer. Should this interpretation be correct, it would raise the possibility that H. compressum is totally lacking a primexine, and would tend to corroborate

the observations of Rowley and Dahl (1977) who found no primexine in Artemisia vulgaris. In that event, the role that has been postulated for the primexine, namely that it is a necessary intermediate and probably a template in the development of the mature exine(Heslop-Harrison, 1968 a,b), would have to be reassessed.

In various plants the eccentric location of certain organelles, usually but not always ER, has been associated with the laying down of primexine and with the imprinting upon it of a template which will control the pattern of the mature exine (Heslop-Harrison, 1963; Skvarla and Larson, 1966; Vazart, 1970). No such organelle association was seen in this study. The possibility of microtubule involvement has not been ruled out, however, since fixation was carried out in the cold, which would have caused the depolymerization of any microtubules that might have been present.

Radially oriented structures usually begin to appear while the microspores are still encased in the special callose wall, although not necessarily at the same time as that the concentric components of the primexine appear. In many species, including Z. mays (Skvarla and Larson, 1966) and S. bicolor (Christensen, Horner and Lersten, 1972), bacula, or columellae as the case may be, evidently arise as thickenings within the primexine. In species that possess spines (e.g. Helianthus annuus) unambiguous spine (but not spinule) templates may be present as outward extensions of the primexine (Horner and Pearson, 1978). The ontogeny of

the bacula in H. compressum is unresolved. As mentioned earlier, it is generally held that the bacula are merely columellae whose tips or spinules project above the tectum. Yet the fact is that the profiles of the spear-shaped spinules as they appeared in the early vacuolate microspore stage of H. compressum often gave the impression that they were separate entities which had come to rest in the discontinuities of the tectum: their density was slightly different than that of the tectum and foot-layer, they had a smooth outline which sometimes did not connect to the tectum, and, unlike the tectum and foot-layer, they displayed an osmiophlic boundary layer. Although they frequently did seem to extend to the foot-layer, or to rest atop columellae, such was not always the case. Sometimes a spinule was displaced slightly to one side of a columella; at other times no columella was visible beneath a spinule. The very strong resemblance of the spinules in size, shape and staining characteristics to similar structures on the developing tapetal orbicules is a curious fact, and one that makes a primexine origin doubtful. A more likely explanation would seem to be that both the rudimentary exine and the surface of the orbicules have specific areas that for one reason or another act as nucleating centers for the crystallization of precursors that are present in the locular fluid. This would be consistent with the points mentioned, and also with the facts that (1) the spear-like structures appeared comparably well developed on all

parts of the exine as well as on the tapetum, and (2) no spear-like structures were seen in the exine of those tetrads still encased in callose.

Following the dissolution of the special callose wall, all components of the ektexine underwent considerable growth, and the tectum and foot-layer became channelled. Both phenomena, but particularly the latter, are poorly understood. It has been shown that sporopollenin precursors are formed in the tapetum of at least some plants (Brooks and Shaw, 1978; Rodriguez-Garcia, 1978), and it is presumed that they are secreted by the tapetum into the locule after which they polymerize on any suitable surface. Nothing observed in H. compressum could be considered inconsistent with this mode of action. Initially in this species only the rudimentary exine and tapetal orbicules present suitable surfaces for sporopollenin polymerization. At a later stage the plasma membranes on the inner tangential face of the tapetal cells also become covered with sporopollenin.

There is evidence that microspores themselves are also capable of secreting sporopollenin precursors and probably contribute at least part of the exine (Echlin, 1971; Gabara, 1974). This is especially likely to be true in plants where the exine is highly developed before the microspores are released from the callose (e.g. Helianthus annuus (Horner and Pearson, 1978), Tradescantia bracteata (Mepham and Lane, 1970)). It seems likely that

in H. compressum the microspore contributes to the sporopollenin of the foot-layer at least, since it appears to be built up from the inside, and to the endexine when it ultimately develops.

Much attention has been focused on the structure and function of the 'tapes' or 'lamellae' which have been observed frequently in the developing foot-layer, particularly in the pore area, and also, but much less frequently, in the tectum and orbicules. Some investigators claim that sporopollenin is deposited only on lamellae (Heslop-Harrison, 1975). Lamellae were clearly visible in H. compressum, especially during the early vacuolate period, in the foot layer of the non-apertured wall and of the pore, and as reported for other species, they seemed to become embedded and subsequently obscured in the foot-layer of the non-apertured wall, but remained visible in the pore area. It is not clear whether the dark line subtending the foot-layer in the midvacuolate stage is such a lamella or is a separate phenomenon. It is thought that the lamellae are produced by the plasma membrane and travel from the plasma membrane to the foot-layer (Dickinson, 1976). Whether this is also true in H. compressum has not been determined but it may be true since some lamellae were seen very close to the plasma membrane.

The evidence from a great number of investigators suggests that for sporopollenin polymerization to occur one of two things must be present, either a special surface or pre-existing sporopol-

lenin. Sporopollenin is not deposited on every available surface. It is frequently not deposited on the tapetal cells themselves, and it is never deposited on the microspore plasma membrane. On the other hand, once it has been deposited in the tectum or on the tapetal orbicules, it usually continues to thicken without the obvious intervention of a new polymerizing surface. (For an exception see Echlin and Godwin, 1968). These facts have caused Mepham (1970) to advance the theory that the polymerization process may require two enzymes, an initiator and a chain elongator. Apparently the tapetal orbicules, at least some primexines and the lamellae, contain an initiator or an initiating site. It remains enigmatic that the foot-layer should require successive layers of lamellae, and it would seem that in those species where repeated layers of lamellae are produced some other purpose must also be served by them. It is worth noting that the production of layers of lamellae is not unique to pollen grain walls: a similar configuration has been observed during the development of new walls around tomato protoplasts (Willison and Cocking, 1972).

The debate in the literature at present centers on whether lamellae are essential for polymerization of sporopollenin. The lamellae have been reported often enough that the reality of their existence is no longer questioned; it is assumed that they are of unknown composition. The possibility that the lamellae themselves could be the fixed ultrastructural representation of sporopollenin

precursors in the process of polymerization does not seem to have been given much consideration. In view of the difficulty of locating a source for the lamellae, particularly for those which occur in the orbicules, it would seem reasonable to consider such a possibility.

The channels that penetrated the tectum and the foot-layer were on the whole fairly straight (although a few were branched), non-tapering, and of similar size. Many of them persisted as the tectum and foot-layer thickened. (In the late midvacuolate and late vacuolate stages some appeared to be occluded, but may have simply passed out of the plane of the section). It is not known how such channels arise. Rowley (1976) has reviewed three processes which may result in tectal perforation, which presumably could be applied to the foot-layer as well. The first of these is incomplete coalescence of the tectum as it spreads over the primexine. Perforations thus formed can be distinguished by their size variations as well as by their shapes: funnel-form in longitudinal section and angular in cross-section. The H. compressum channels do not fit this description. A second possibility is that a once complete tectal sheet may become reticulate by stretching, as has been reported for Sparganium (Banerjee, Rowley and Allessio, 1965). Stretching cannot be invoked in the case of H. compressum since it would not explain the channels, identical to those of the exine, that were found on the tapetal orbicules, which did

not enlarge. Nor did the channels continue to increase in size or number as would be expected from continuing microspore enlargement.

The third mechanism, and the one strongly favored by Rowley, is the exclusion of sporopollenin from certain areas by the presence of rod-shaped entities which he considers to be part of a plasma membrane glycocalyx. Work done by Roland and Vian (1971) using periodic acid Schiff-thiocarbohydrazide-silver protein (PATSP) and phosphotungstic acid (PTA) at low pH indicates the presence of a coat on the plasma membrane of plant cells similar to that described in animal cells (Rambourg, 1971; Winzler, 1970), as well as the presence of strands connecting the plasma membrane to the cell wall. Rowley and coworkers (Rowley, 1973a, 1976; Rowley and Dahl, 1977; Rowley and Skvarla, 1975), applying similar techniques to microspores of several species, have found, among other things, that microchannels in Epilobium and Artemisia stain with lanthanum nitrate and other cations, indicating the presence of acid polysaccharides. It is not clear whether this hypothesis can be applied to grasses. The idea that a rodshaped element may be responsible for sporopollenin exclusion is appealing because it would explain why the channels persist rather than become occluded as one might expect if they were awash in sporopollenin precursors. In H. compressum, very narrow, somewhat opaque channels were seen in the early vacuolate stage (Figure 19). Whether these expanded to become the channels visible in later stages is very

uncertain.

The function of the channels in grasses is unknown. Studies done on a variety of species have shown that molecules with molecular weights as high as 500,000 can cross both aperturate and non-aperturate walls (Rowley, 1973b, 1975). Thus there is at present no reason to assume that the channels in H. compressum are not capable of transporting materials. Since all areas of the cell inside the wall are metabolically active, it would seem advantageous for all areas to have easy access to metabolites. On the other hand, it would be premature to dismiss the possibility that the channels primarily serve some other function, or that they might be in whole or in part artefactual. Inducible channels have been reported in the nexine of Epilobium angustifolium (Rowley, 1973a, 1976).

If we assume that the channels are real structures engaged in transport, then, in H. compressum at least, more transport apparently occurs between the locule and the cavus than between the cavus and the microspore. Among the things we might reasonably expect to find transported into the cavus are sporopollenin precursors, because the columellae increase considerably in both length and width. If the columellae do not grow by accretion of new material, then they must do so as a result of material flow from the tectum or the foot layer or both. No attention has been given to either the growth of the columellae or the increase in width of the cavus. Does the cavus enlarge because the columellae grow, or

is the situation the reverse? Is it possible, for example, that the cavus contains a hygroscopic carbohydrate which swells as a consequence of absorbing water from the locule, thus forcing the tectum and foot-layer farther and farther apart? Although the cavus of acetolyzed pollen is empty (hence its name), and although at the time of pollen mitosis the contents of the cavus of H. compressum appeared similar to the contents of the locule, in earlier stages its characteristics were different from those of the locule and we have to assume that the contents of the two areas were not the same and were not freely interchangeable. We need not expect to find the contents of the cavus to be the same for all species, identical in each and every species, since it apparently serves a function - storage of material derived from the tapetum - in some species (Heslop-Harrison, Heslop-Harrison, Knox and Howlett, 1973; Howlett, Knox and Heslop-Harrison, 1973) which it does not serve in grasses, where its function is presently unknown.

Although exine development has been investigated at the TEM level in a number of species, fairly complete information is available for only two grass species, Sorghum bicolor (Christensen, Horner and Lersten, 1972; Christensen and Horner, 1974) and Zea mays (Skvarla and Larson, 1966). Some information on later stages is available for Poa annua (Rowley, 1962, 1964) and Triticum aestivum (DeVries and Ie, 1970), and there are a few isolated pictures

available of Avena sativa (Gunning and Steer, 1975; Steer, 1977), Agropyron intermedians (Young et al., 1979), and Phleum pratense (Rowley and Skvarla, 1974). Since Sorghum, Zea and Hordeum are strikingly similar although not identical at the time of pollen mitosis - there are some subtle differences in channelling and surface texturing (Andersen and Bertelsen, 1972; Grant, 1972; Rajendra et al., 1978) - it is disconcerting to find that some stages of early exine development in H. compressum resemble those of S. bicolor, while others are more similar to those of Z. mays, especially so since the latter was fixed in $KMnO_4$. It is highly likely, however, that identical preparative techniques would go a long way toward bringing the three grasses into closer agreement while making it possible to determine exactly what the differences are. The problem of interpretation, not only for the grasses but also for all the species that have been studied, is compounded by an almost total lack of cytochemical data. What seems to be needed is a series of extensive cytochemical studies such as those done recently by Rowley and Dahl (1977) on Artemisia. Such studies which, in addition to locating sporopollenin, make possible the identification of proteins, lipids and various classes of carbohydrates, ought to resolve many of the ambiguities and contradictions that presently exist.

The ultrastructure of the microspore cytoplasm and nucleus was unremarkable and, as far as can be determined from the scant

literature available, similar to that of other grasses. The usual organelles were present, with the exception of dictyosomes, which were scarce until the time of pollen mitosis. The lack of dictyosomes is surprising in a cell whose major activity seems to be the making of a complex wall, particularly if a cellulosic primexine is in fact being produced. In Artemisia (Rowley and Dahl, 1977) the initial exine structures contain large amounts of protein, a fact that might be consistent with a dearth of dictyosomes. It may be that dictyosome appearance at the time of pollen mitosis is a preparation for the secretion of the intine, which in all species is reported to be cellulosic and which in H. compressum is laid down after the first pollen mitosis. The nature of the osmiophilic bodies seen in several microspores and the occasional appearance of pairs of parallel ER cisternae are without explanation at present. Growth in cell size was concomitant with increasing vacuolization and was accompanied by the migration of the nucleus to the side of the cell away from the germinal pore.

It was abundantly clear that at the time of pollen mitosis the majority of the microspores were so oriented that the pore was in contact with the tapetum; most of the microspore tetrads also seemed to have one surface near the tapetum. Although Banerjee and Barghoorn (1971) have advanced the view that the microspores become oriented after their release from the callose, it seems reasonable to suppose that the pore ordinarily develops adjacent

to the tapetum and remains there throughout the period of pollen development. This has been proposed by Christensen and Horner (1974) as one of several polar events occurring within the developing microspore. The random orientation of the microspores in the early and midvacuolate stages could easily have resulted from disturbances during fixation. But whether or not the position of the pore adjacent to the tapetum has any functional significance is a moot point. The factor determining pore location has been looked for in several species, and in some grasses has been reported to be, or to be related to, the orientation of the meiotic spindle (Rajendra et al., 1978). Thus the pore-tapetum relationship could be the consequence of a polarization several generations of cells earlier and have no particular significance to the plant at this stage.

The tapetum itself is one of the most puzzling of all plant tissues. Its various unusual physical features combined with its location make clear that it plays a highly specialized and significant role which must somehow be concerned with the development of the microspore, although very little progress has been made toward determining what that role is. The tapetum is the site of synthesis of sporopollenin precursors in some species (Brooks and Shaw, 1978; Rodriguez-Garcia, 1978) and in some species is the site of synthesis of recognition proteins which are later transferred to the pollen grain and function in pollen-stigma

interactions (Heslop-Harrison, Heslop-Harrison, Knox and Howlett, 1973; Dickinson and Lewis, 1973; Heslop-Harrison, Knox and Heslop-Harrison, 1974). Maheshwari (1950) suggested that the tapetum is important in the nutrition of the developing microspore, and it is frequently labelled a "nurse" tissue; that designation seems inappropriate since the tapetum has neither chloroplasts nor starch reserves. The tapeta of different species follow a sequence of events which is in a general sense fairly predictable, but correlation of these events with the development of the microspore is complicated by variation in their timing from species to species.

Among the uncommon features which characterize the tapetum of H. compressum and many other species are: (1) a binucleate condition; (2) the lack of an extensive vacuole system; (3) a high proportion of ER, predominantly rough; (4) dissolution of the primary walls followed by a period when the inner tangential and radial sides of the cells have no walls whatsoever; (5) subsequent development on the inner tangential face of a highly unusual wall; and (6) broad cytoplasmic connections between many of the tapetal cells.

The first three of these six, although noteworthy, are not extraordinary. The binucleate or multinucleate condition is a type of polyploidy found in many different kinds of plant and animal cells, and there are no satisfactory explanations to account for it. In addition, some of the individual nuclei in the tapetum

have more than two nucleoli, and during the karyokinesis which results in the binucleate condition, the chromosomes appear unusually large (Whallon, unpublished). Both observations suggest that each individual nucleus may also be polyploid.

The absence of a large vacuole may be correlated with the lack of wall structure and with the proximity of the locular fluid. Since the vacuole and the wall together function in osmoregulation, any plant cell without a wall and with a large vacuole could be expected to burst. Thus, the absence of a vacuolar system may be necessary if the cell is to survive without a wall. A large vacuole also usually serves as a repository of waste products. At or shortly after the time that a vacuolar system might ordinarily be developing, the plasma membrane comes into direct contact with the locular fluid as a result of wall dissolution. Presumably such waste products as the cell needs to get rid of can then be excreted directly into the locule. Once the tapetal reticulum has formed, contact between the plasma membrane and the locule must be severely restricted, and it is possible that the consequent build-up of poisons contributes to the cell's death. It is not likely to be the primary cause of death since all tapetal cells, even those in species where a reticulum does not develop, senesce prematurely.

Many of the speculations about tapetal function center around the large amount of ER, particularly rough ER, that develops in

these cells. It is generally accepted that the tapetum is a secretory tissue, and although sporopollenin is suspected of being the primary product, definitive proof of this is lacking. Unusually close association between ER and plastids, such as that shown by the ensheathing cisternae in Figure 34, has been reported in A. sativa (Steer, 1977b), where the ER also ensheaths the mitochondria. In Lycopersicon peruvianum the association is even more intimate (Pacini and Cresti, 1976).

It is possible that the 'cytoplasmic plate complexes' represent a localized area of modified ER. Previous published studies have not shown any similar structures, with the possible exception of the 'zytoplasmavesikel' described by Marquardt et al. (1968). The complexes bear some resemblance to dictyosomes, but their association with the larger vesicles, and their enclosed cytoplasmic plates, indicate that they are something other than specialized dictyosomes. Morphologically they are somewhat similar to the so-called 'dense compartments' found in megakaryocytes of stressed rats (Bentfeld-Barker and Bainton, 1977); the function of dense compartments is also unknown.

The tapetum of H. compressum bears a strong overall resemblance to those of A. sativa and T. aestivum. But apparently in A. sativa, and perhaps in T. aestivum, the tapetum reaches its fullest development after the pollen wall has completed most of its growth (Steer, 1977b; DeVries and Ie, 1970). This is not true in H. compressum,

nor in many other species (Echlin, 1971). Intine development also precedes tapetal degeneration in T. aestivum, but follows it in H. compressum.

One of the most unusual features of the maturing anther is the layer of orbicules which completely covers the inner tangential wall of the tapetum. These structures, whose function remains an enigma, are present in many, but not all, species. The ontogeny of the orbicules is approximately the same in all species, although there is some dispute about whether the droplets or cores (pro-orbicules) upon which the sporopollenin aggregates are performed in the tapetum (Echlin and Godwin, 1968; Steer, 1977a). In H. compressum, the first-formed droplets could not be seen within the tapetal cytoplasm (Figure 39), although the later-formed, electron-translucent droplets could (Figures 40, 11); it is not possible to conclude that the earlier droplets were in fact pro-orbicules. There have been attempts to dismiss the orbicules as an accidental product of the tapetal cell's ability to secrete sporopollenin precursors from all its surfaces in combination with the peculiar environment presented by the locule (Dickinson and Bell, 1972). However, it is apparent in grasses, more so than in some other species, that the patterning of the orbicule is identical to that of the tectum on the developing pollen grains. Thus, there is clearly something special about the orbicule as a polymerizing surface, since sporopollenin which accumulates

on other tapetal surfaces either has a different pattern (e.g., the tapetal reticulum in H. compressum) or lacks a pattern altogether (e.g. that produced at the outer tangetial surface in A. sativa). An examination of Figure 51 shows that the orbicules vary in size, some being at least twice as large as others.

The 'tapetal membrane' which developed beneath the orbicules as the tapetal cells senesced consisted, at the time of pollen mitosis, of two layers, an inner (i.e., toward the locule), fenestrated reticulum, and an outer, non-fenestrated, backing layer. The 'membrane' seems to serve the function of enclosing the pollen grains in a sac, for reasons unknown. A similar structure, called the 'peritapetal membrane', develops in non-grass species at the outer tangential side of the tapetum (Heslop-Harrison, 1969). In most species remnants of the tapetal cells are ultimately deposited on the pollen grains as either 'pollenkitt' - a sticky homogenous substance composed largely of carotenes, characteristic of entomophilous species, - or as tryphine - a nonhomogenous substance composed of remnants of cytoplasmic elements. The presence of the 'tapetal membrane' on the inner side of the tapetum would seem to prevent any tapetal remnants from reaching the microspores unless the backing layer becomes fenestrated at some later time.

In grasses, there is a very close association between the orbicules and the reticulum of the tapetal membrane, but this

does not necessarily imply a functional relationship. Some species (e.g. Pinus banksiana, Beta vulgaris) with a peritapetal membrane also have orbicules on the locular face of the tapetum.

In the plasmodial or invasive type of tapetum, the cytoplasm of adjacent cells becomes confluent as a result of breakdown of the plasma membranes (Mepham and Lane, 1969). Such an occurrence has not usually been mentioned as characteristic of parietal tapeta, although it has been reported to occur in Pinus banksiana by Dickinson and Bell (1972), and has been mentioned in passing by Young et al. (1979) with regard to Agropyron intermedians. Broad cytoplasmic bands connecting adjacent cells were clearly present in H. compressum from the early vacuolate microspore stage onwards.

Good progress has been made toward an understanding of events in the microspore, and there is every reason to believe that progress will continue to be good. An approach that I believe might be fruitful, especially with regard to the developing wall, would be to look for similarities between the microspore and other cells of the plant. But no concepts of microsporogenesis can be satisfactory until we also understand the tapetum, and at present we know very little about the tapetum. Considerable difficulty arises because there are some very great differences among the tapeta of those species which have been studied to date, and also because the tapeta of some species contain highly anomalous structures,

such as the nucleolar tubules of Beta vulgaris (Hoefert, 1971). Ultrastructural studies need to be done on many more species. A technique for isolating tapetal cells so that biochemical assays on a time course could be performed, especially if combined with ultrastructural studies, ought to prove extremely valuable.

Summary

From combined light and electron microscopic observations on developing anthers of H. compressum, it appears to be true that:

1. The microscope resembles that of other grasses in number and kind of organelles, pattern of vacuolization, migration of the nucleus, and orientation relative to the tapetum.
2. In the development of the microspore, a primexine may be lacking; bacula probably arise after callose has dissolved from precursors present in the locule. Lamellae are present in both the apertured and non-apertured parts of the wall during the period of sporopollenin deposition. The exine channels are not the result of incomplete tectal and foot-layer spreading, nor of stretching consequent to an increase in size of the microspore.
3. In the tapetal cells, which are parietal (secretory) and binucleate, extensive ER develops, and there is no large vacuolar system. Broad cytoplasmic bands connect the tapetal cells to one another. A cytoplasmic structure found in some tapetal cells during the midvacuolate stage may represent a previously undescribed organelle, or an undescribed configuration of a known organelle.
4. Tapetal degeneration occurs earlier than in A. sativa or T. aestivum. The development of the 'tapetal membrane' prior to rupturing of the tapetal plasma membrane may prevent any remnants of the tapetal cells from reaching the pollen as tryphine.

5. Tapetal pro-orbicules are preformed in the tapetal cytoplasm; the subsequent development of the orbicules is practically identical to that of the microspore tectum. This fact combined with the pattern of sporopollenin in the tapetal reticulum, supports the hypothesis that the orbicules represent an evolutionary specialization rather than a vestigial capacity of a tissue that was once sporogenous, or a random, non-specific configuration of excess sporopollenin.

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