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Phloem of Primitive Angiosperms. III. Phloem of Petioles of *Drimys granadensis* (Winteraceae)[1]

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Drimys granadensis (Winteraceae) was chosen for study because of its primitive evolutionary position among angiosperms. Mature sieve elements of the primary phloem of petioles are enucleate and have what we interpret as crystalline P-protein in addition to the more common tubular and fibrillar forms. Companion cells associated with sieve elements retain normal-appearing nuclei and possess bodies of flocculent material within the cytoplasm. The pores connecting mature adjacent sieve elements are of much smaller diameter than those typically encountered in angiosperms. The possession of P-protein and companion cells in *Drimys* argues for the early evolution of these traits in angiosperm phloem.

INDEX DESCRIPTORS: Phloem, sieve-element, P-protein, phloem-protein, *Drimys*.

Drimys granadensis L.f. var. *mexicana* (DC.) A. C. Smith is a species of the Winteraceae, order Magnoliales, of the so-called Ranalian complex. The members of this group possess traits considered to be primitive (Cronquist, 1968), among which is the absence of vessels (Bailey, 1944; see, however, the discussion in Young, 1981). The ancient nature of the Winteraceae has been confirmed recently by the discovery of winteraceous pollen in sediments some 105 million years old from the Lower Cretaceous of Israel (Walker et al., 1983). Therefore, it was felt that *Drimys* would be an excellent genus for our continuing ultrastructural study of the sieve elements of primitive angiosperms (Dute, 1983; Friis and Dute, 1983).

MATERIALS AND METHODS

The material used in this study came from greenhouse-grown seedlings provided by Dr. Richard Pohl of Iowa State University from seeds supplied by Fairchild Botanical Gardens. A voucher specimen has been deposited in the Iowa State Herbarium (Pohl 14,628 ISC). Young petioles near the shoot tips were diced in 0.05 M cacodylate buffer (pH 7.3), then placed immediately into cacodylate-buffered 6% glutaraldehyde for six hours at room temperature. The solution was replaced once with fresh fixative. After thorough rinsing in buffer, samples were postfixed for 4 hours in 2% osmium tetroxide at room temperature, rinsed in water, and stained *en bloc* with a 0.5% solution of uranyl acetate for two hours. Dehydration was in an ethanol-propylene oxide series and embedment was in Spurr's resin (Spurr, 1969). Thin sections were cut on Sorvall MT-1 and LKB Ultrotome III ultramicrotomes, stained with uranyl acetate and lead citrate, and photographed with RCA EMU 3E and Philips 300 electron microscopes. Thick sections (1.5 μm) were cut for light microscopy and stained with toluidine blue 0 prior to viewing.

OBSERVATIONS

In cross-section, the vascular bundles of young petioles are distributed as an arc. Most bundles are collateral, but the smallest possess only phloem (Fig. 1). The most noticeable feature of the primary phloem of all these bundles is the presence of sieve element/companion cell complexes (Fig. 2), a feature shared with the phloem of other angiosperms.

Unlike mature sieve elements (the most common developmental stage found in the young petioles) companion cells retain normal-appearing nuclei (Fig. 3). In addition, and without exception, the companion cell cytoplasm appears to be very active metabolically as evidenced by numerous ribosomes, both free and bound, frequent ER profiles (Fig. 4), and large numbers of mitochondria with dense stromas (Fig. 5).

Mature companion cells possess one or more bodies of flocculent material located in the cytoplasm (Fig. 2, 3, 5). A close examination of the periphery of these structures (Fig. 6) shows them to possess a sharply delimited boundary. For the most part, these bodies lack a surrounding membrane, but occasionally membrane profiles are observed near the surface of a body.

Connections between companion cells and sieve elements are common. Individual callose-lined pores link the sieve-element lumen with a median cavity at the middle lamella. From the cavity two or more plasmodesmata cross the companion cell wall (Fig. 7). The walls of both companion cell and sieve element often are thicker in the region of the connections than elsewhere (Fig. 7).

The mature sieve element has no nucleus, an ofttimes clear lumen, and a relatively thick wall (Fig. 2, 3). On the other hand, young sieve elements have typical cytoplasmic components and a thicker wall (Fig. 2).

Sieve elements are connected to each other by callose-lined pores often filled with dense, fibrillar material (Fig. 8). Closer inspection shows that the plasmalemma also traverses the pore from one sieve element to another (Fig. 9). The callose layer is wider near the mouths of the pores. It is thought (Evert, 1982) that the callose is an artifact of wounding and that these pores are open (lack callose) *in situ*. With this in mind, pore diameter measurements (taken from the center of the pore channel) included the callose lining. This gave an average diameter of 0.22 μm for 32 pores with a range from 0.12 to 0.39 μm. No distinction was made between pores on lateral and end walls.

Figure 10 shows a portion of a nearly mature sieve element about to lose its nucleus. Its cytoplasmic features are typical of sieve elements at or near maturity. Organelles such as mitochondria and plastids are appressed to the plasmalemma. Accumulations of smooth ER are common, mostly associated with the cell periphery, although in Fig. 10 one "stack" is attached to the nuclear surface. These ER accumulations take various forms, such as the lattice-like structure in Figure 11.

Phloem-protein (P-protein) is a typical component of the sieve elements of *Drimys*. In immature elements, P-protein can exist as tubules of 18-19 nm in diameter aggregated into P-protein bodies (Fig. 12, 13, 14). The integrity of these bodies is maintained by attachments between the individual tubules (Fig. 13, arrow).

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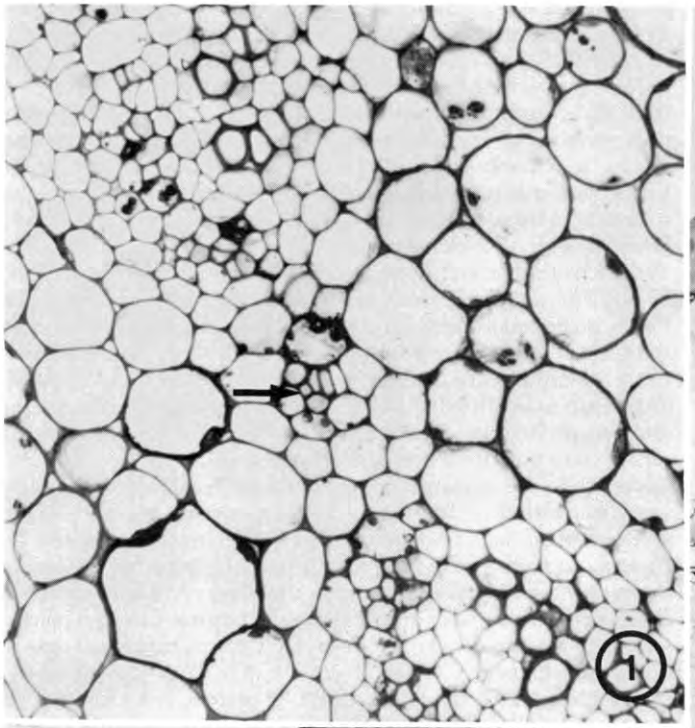
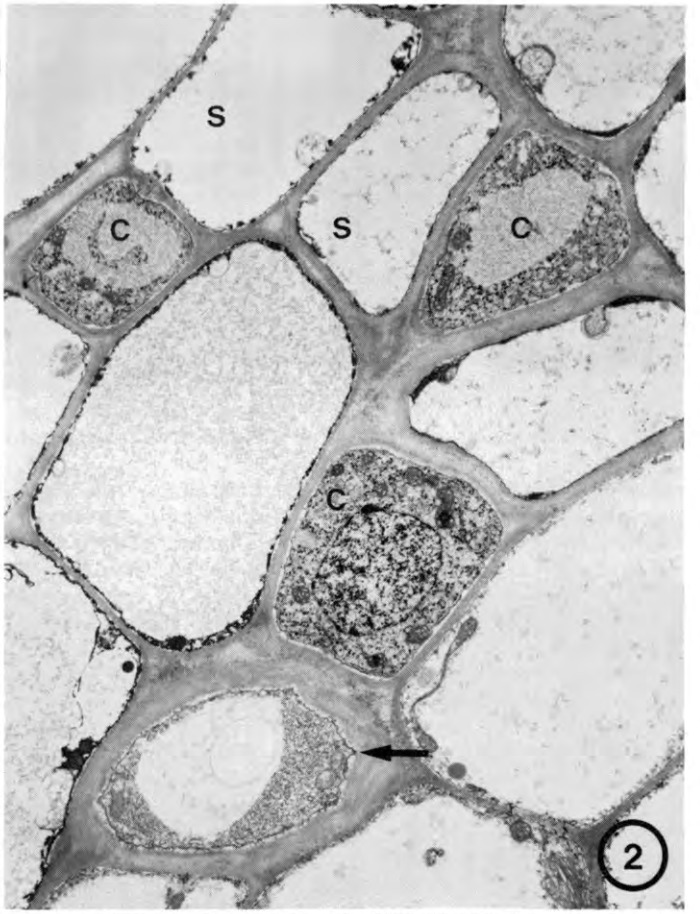
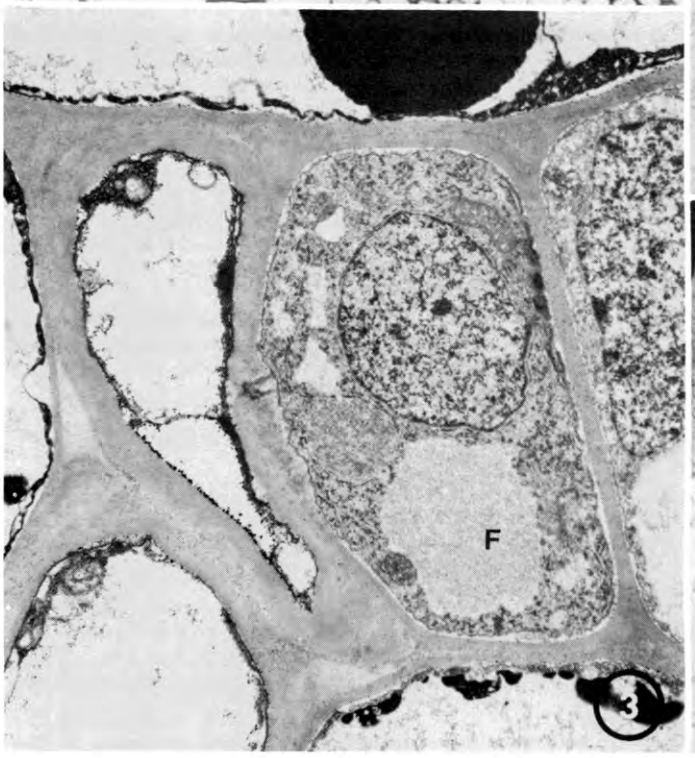






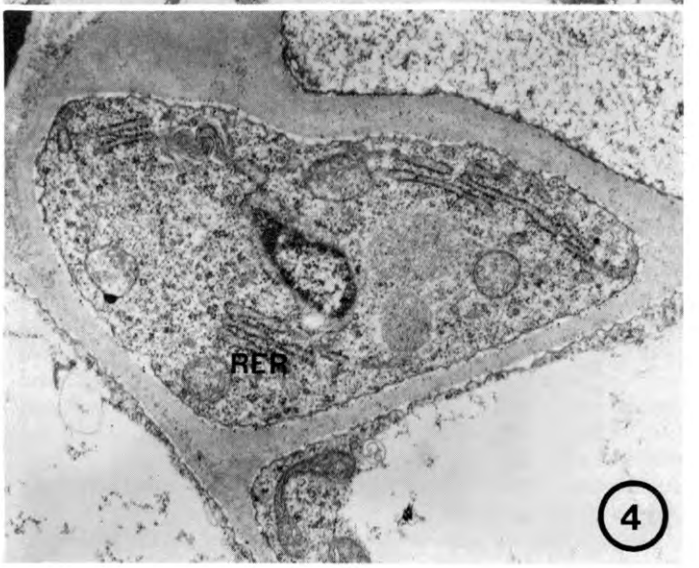


Fig. 1. A light microscopic view of a portion of a petiole in cross-section. Portions of two large bundles flank a small central bundle (arrow) containing only phloem. X 490.

Fig. 2. A portion of the phloem as seen with the electron microscope. The mature sieve elements (S) are enucleate, whereas the associated companion cells (C) possess both nuclei and electron-dense cytoplasm. The young sieve element indicated by an arrow has a very thick wall. X 6290.

Fig. 3. A sieve element/companion cell complex in which the companion cell has both a nucleus and a body of flocculent material (F) in its cytoplasm. X 11,380.

Fig. 4. Another cross section of a companion cell showing conspicuous cisternae of rough endoplasmic reticulum (RER). X 14,960.

In mature sieve elements the P-protein takes the form of fibrils 11-12 nm in diameter. These fibrils possess striations with a repeat of 12 nm (Fig. 15). Masses of fibrillar P-protein are conspicuous especially near the pores of mature sieve elements and constitute the material filling the pores (Fig. 8).

In addition to the fibrillar P-protein, other non-membranous inclusions exist within the lumen of mature elements. Free starch grains, for example, are common (Fig. 15). They originate from plastids that have broken and released their contents (Fig. 16).

One type of plastid inclusion body appears, in cross section, as a cluster of electron-dense particles (Fig. 16, 17). The particles, of 9.5 nm diameter, appear to be closely-packed, although each is separated from its immediate neighbors by a small electron-lucent zone. In longitudinal section, each particle is aligned with many others to form a beaded chain. The entire inclusion body consists of a series of such beaded chains (Fig. 18). Although these particles do not show hollow centers, their structure and arrangement resemble that of phytoferritin observed in the plastids of other species (Hyde, et al., 1963; Robards and Humperson, 1967). These inclusions, like the starch grains and other bodies frequently found within plastids, are released into the sieve element lumen when a plastid bursts.

The largest inclusions found in mature sieve elements are referred to here by the term "stellate inclusion." Under the electron microscope, individual inclusions vary greatly in electron density. In the less dense examples, a substructure is quite apparent, consisting of many 15 nm diameter tubules that appear to radiate spirally from the inclusion's center (Fig. 19). Transverse sections of the tubules show them attached to one another to form interlocking rings encircling empty areas (Fig. 20). Longitudinal sections of such tubules occasionally show closely spaced striations. Stellate inclusions have been observed solitary in the lumen, as well as associated with plastid material near the pores (Fig. 21) as the result of surging.

DISCUSSION

Esau (1965) defines companion cells as specialized parenchyma cells associated with the sieve tubes of angiosperms. The two cell types are closely related in that they arise from the same meristematic precursor but are easily distinguished because the companion cell remains nucleate at maturity. With the advent of electron microscopy, the mature companion cell was characterized as having a dense cytoplasm containing numerous mitochondria, rough ER, ribosomes, and plastids (Evert, 1977). Also characteristic are the connections between cells, consisting of pores in the sieve-tube member wall and branched plasmodesmata within the companion cell wall (Behnke, 1975; Evert, 1977). This structural intimacy between the two cell types implies a physiological relationship involved with nutrient transport, a relationship suggested by various physiological and histochemical studies (Geiger et al., 1973; Gilder and Cronshaw, 1973). Cells similar to companion cells, called albuminous cells, are associated with the sieve elements of gymnosperms. However, sieve elements and albuminous cells generally show no ontogenetic relationship (Behnke, 1975).

Cytologically, the cells we have designated in *Drimys* petioles as companion cells fit the definition very well. Although we observed no meristematic precursors in the act of division, some examples were seen of mature sieve element/companion cell pairs that in cross-section appeared to have developed from a common precursor (Fig. 2). This observation agrees with those of other authors (e.g. Esau and Cheadle, 1984) who have noted companion cells in the secondary phloem of *Drimys* stems.

We have referred to the conducting elements in the petiolar phloem of *Drimys* throughout this paper as sieve elements rather than sieve-tube members or sieve cells because we made no distinction between pores on end and lateral walls. The degree of specialization of sieve areas plus the vertical stacking of sieve elements (both necessary for the definition of sieve-tube members) are better observed with light microscopy. Nevertheless, the pore size is of interest. A diameter of 0.22 μm is very small compared to the 1.99 μm average for the sieve plate pores in the secondary phloem of stems of 148 dicotyledonous species. It is somewhat smaller than the 0.48 μm average for the lateral sieve-area pores in stems of 128 species (both averages calculated from Table 5, Esau and Cheadle, 1959). Rather, it compares favorably with sieve-element pores in lower vascular plants (Evert, 1976; Kruatrachue and Evert, 1978; Warmbrodt and Evert, 1978, 1979). The small pore diameter observed in our study is not due merely to the petiolar location of the sieve elements investigated. Esau and Cheadle (1984), using light microscopy, found that the pores in the secondary phloem of *Drimys winteri* stems were also very small (from 1μm to less than 0.5 μm in diameter). As we have noted in the observations, the pore width increases toward the lumens, and our values, taken from the center of the pore channel, represent minimum values. Thus, the measurements of Esau and Cheadle are in reasonable agreement with ours. It is interesting to note that, despite the small pore size, Esau and Cheadle consider both *Drimys winteri* and *D. lanceolata* to possess sieve-tube members in the secondary phloem of the stem. According to these authors, the larger pores are clustered in large, well-defined sieve areas. The walls bearing these specialized sieve areas are considered to be sieve plates, a structure found only in sieve-tube members.

According to Evert (1977, 1982), P-protein is a characteristic component of dicotyledonous sieve-tube members, but is lacking in all lower vascular plants and gymnosperms, and many, but not all monocots. Spanner and Moattari (1978) stated that P-protein of normal appearance existed in *Drimys winteri* although they provided no photos. We have confirmed their observations in this paper. As in other species of the Ranalian complex so far investigated in this laboratory (Friis and Dute, 1983), both the tubular and fribrillar forms of P-protein are present. However, this observation does call into question the recent claim by Behnke and Kiritsis (1983) that the tubular form of P-protein is not found in *Drimys* or in other primitive

Fig. 5-10.

Fig. 5. A longitudinal view of a companion cell/sieve element pair. The companion cell is typified by a large number of mitochondria (M) with dense stromas as well as by the aforementioned flocculent material. X 8,810.

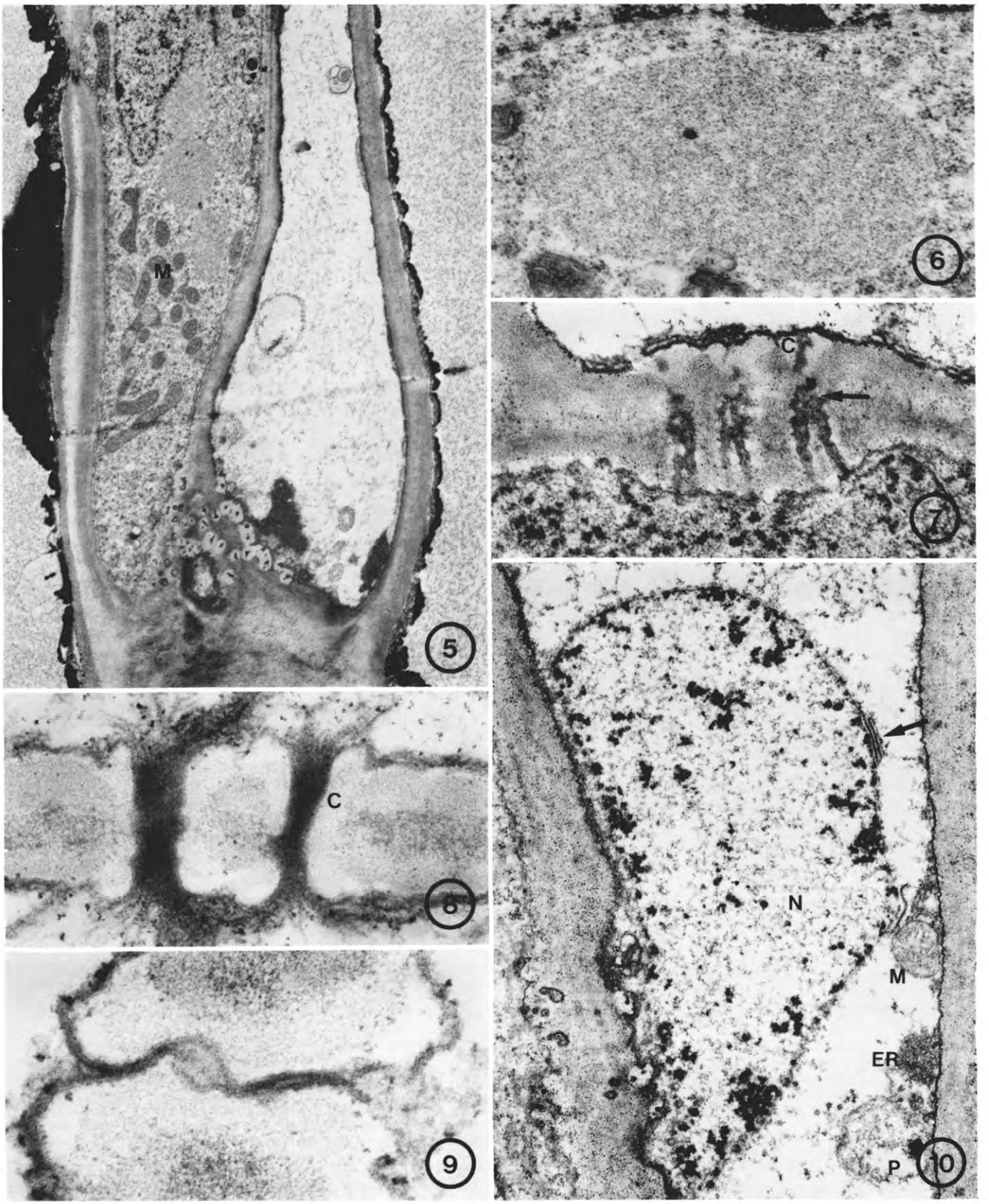
Fig. 6. A more detailed view of a body of flocculent material in a companion cell. X 30,680.

Fig. 7. The common wall of a companion cell/sieve element pair. The wall is thickened where traversed by cytoplasmic connections. The channels from both cells converge upon a median cavity (arrow). Callose (C) is associated with the channel on the sieve-element side. X 31,100.

Fig. 8. Pores, lined with callose (C), which connect mature sieve elements. In this instance, the pores are tightly-packed with a fibrillar material (P-protein) which can be seen to extend into the cell lumens. X 87,400.

Fig. 9. A high magnification of a portion of a pore. Membrane profiles, probably a continuation of the plasmalemma traverse the channel. Note how the callose cylinder (hence the actual width of the pore) increases in diameter toward the lumen of the cell. X 137,350.

Fig. 10. A portion of a sieve element with a degenerating nucleus (N). Compare the distribution of chromatin in the nucleus of the sieve element with that in the nuclei of companion cells in Figures 3 and 5. The remaining organelles and membranes of the sieve element show the peripheral distribution typical of such cells at or near maturity. ER, endoplasmic reticulum; P, plastid; M, mitochondrion; arrow, stack of ER on nuclear envelope. X 15,750.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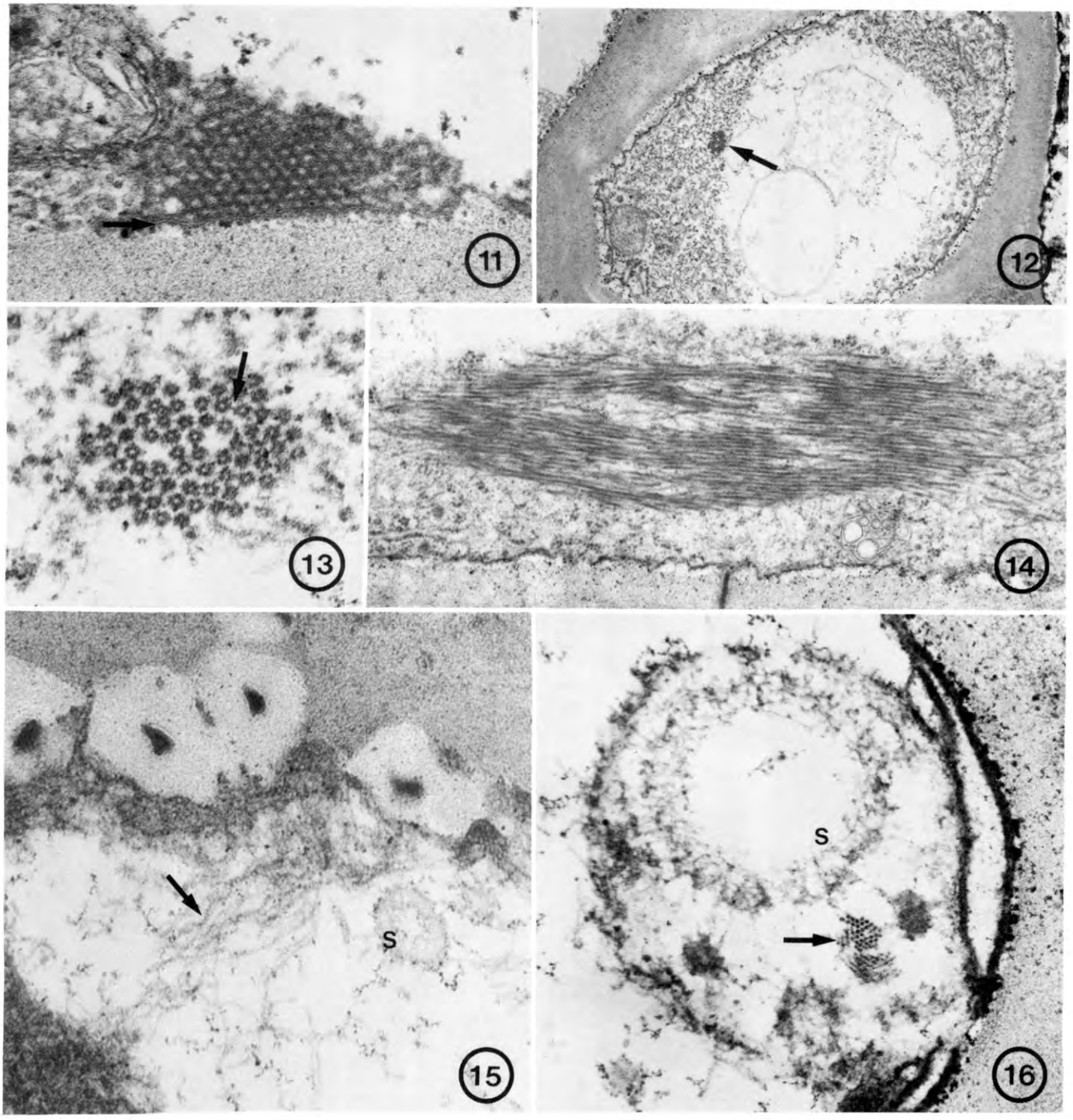

Fig. 11. A detailed view of a lattice-like accumulation of ER adjacent to the plasmalemma (arrow). The light areas represent the intracisternal regions of the ER. X 87,640.

Fig. 12. A cross-section of an immature thick-walled sieve element. The arrow indicates a P-protein body. X 15,150.

Fig. 13. A detailed view of the P-protein body in Figure 12. The tubular nature of the subunits is obvious. A spokelike structure (arrow) connecting the tubules is barely visible. X 132,800.

Fig. 14. Longitudinal view of a P-protein body. X 25,690.

Fig. 15. High magnification of a portion of Figure 5. Fibrillar P-protein (arrow) is located adjacent to sieve-element pores. A starch grain (S) is located nearby. X 66,350.

Fig. 16. A plastid in a mature sieve element. Both a starch grain (S) and clusters of electron-dense particles (arrow) are present. The plastid membrane is ruptured in a couple of places. X 53,700.

angiosperms.

The stellate inclusions represent the most striking objects observed in this study. Structures of similar morphology, formerly called extruded nucleoli, have been noted in the sieve elements of a number of basically woody dicot families of the dicotyledons (Deshpande and Evert, 1970; Esau, 1978; Behnke, 1981). Studies have shown that these inclusions are not released from the nucleus (Deshpande and Evert, 1970; Nehls et al., 1978; Esau, 1978), but rather develop in the cytoplasm. Cytochemical studies indicate these structures to be proteinaceous with no trace of nucleic acids (Ilker and Currier, 1975; Nehls et al. 1978). These protein inclusions possess properties similar to P-protein in the same sieve elements and are referred to by others as crystalline P-protein bodies (Behnke, 1981). Such bodies already have been observed in the Winteraceae (Behnke, 1981; Behnke and Kiritsis, 1983; Esau and Cheadle, 1984), but until now a detailed view of the substructure of crystalline P-protein in this family had not been provided.

The sieve elements of the primary phloem of *Drimys granadensis* petioles possess P-protein of various forms and are associated with companion cells. These are features they share with other dicotyledonous angiosperms (Evert, 1977). With the possible exception of pore size (complexity of sieve areas was not investigated), the ultrastructure of *Drimys* sieve elements does not appear to be primitive or gymnosperm-like. This conclusion agrees with earlier work concerning *Liriodendron* and *Magnolia* (Dute, 1983; Friis and Dute, 1983). If these taxa are assumed to be primitive, then certain features typical of angiosperm phloem must have evolved very early, earlier even than vessel members.

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Fig. 17-21.

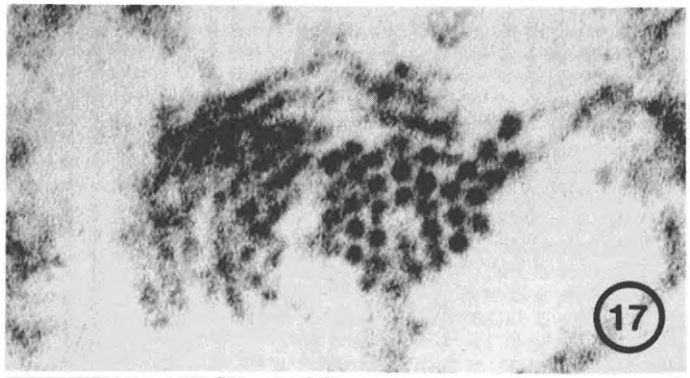
Fig. 17. A closer view of the particle clusters in Figure 16. X 232,850.

Fig. 18. A longitudinal view of a particle cluster showing the beaded-chain effect. X 220,100.

Fig. 19. A stellate inclusion. X 63,100.

Fig. 20. A detailed view of another stellate inclusion. The tubular nature of the subunits is evident (arrow). The tubules join one another to encircle areas devoid of material. X 209,000.

Fig. 21. Portions of two mature sieve elements in cross-section. The lumen of one cell is filled (at this level) with plastid material, fibrillar P-protein, and portions of a stellate inclusion, perhaps due to surging of the contents. The arrow indicates that the stellate inclusion is in contact with P-protein. The lumen of the other sieve element is clear. X 20,120.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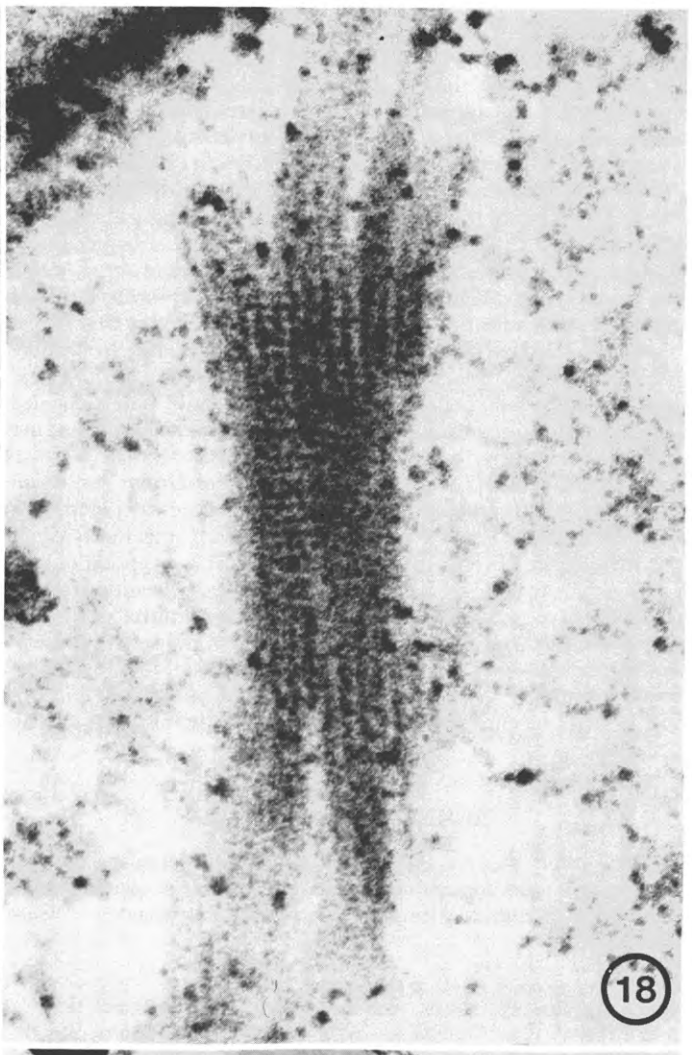
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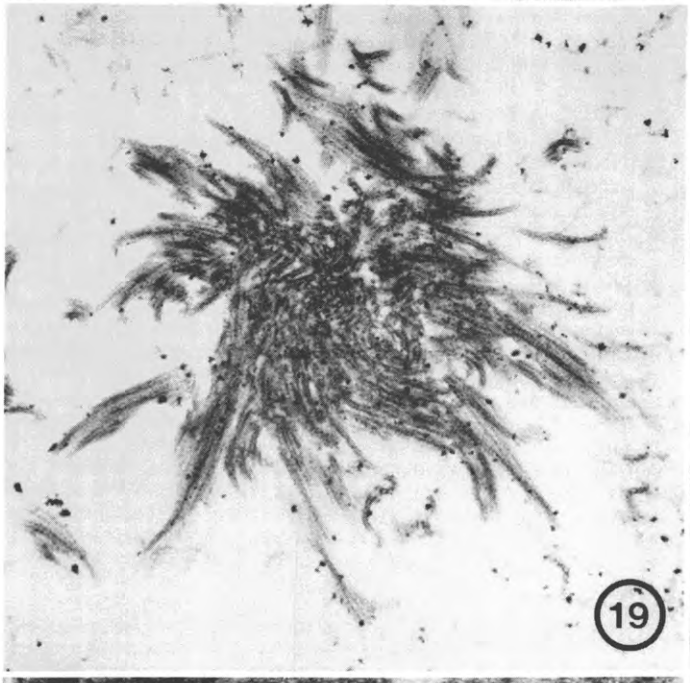
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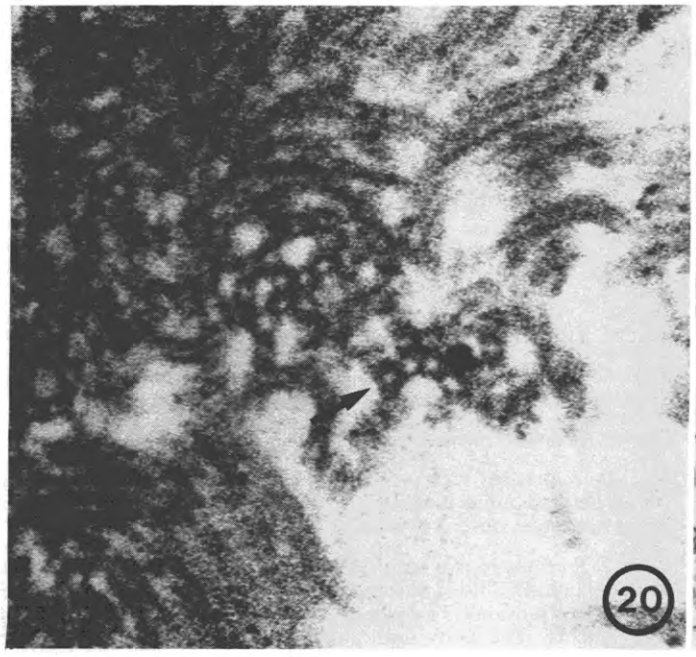
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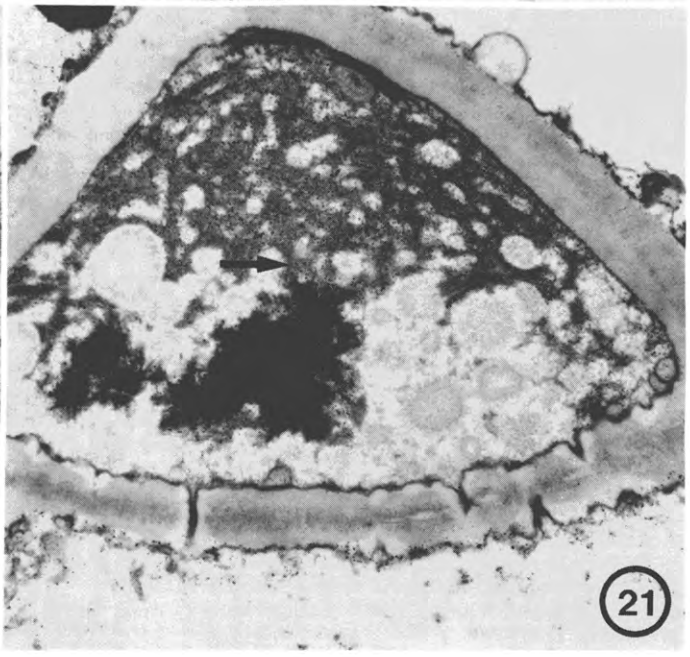
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