Ultrastructural Modifications in Apple Stems Induced by Erwinia amylovora and the Fire Blight Toxin

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ABSTRACT

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Ultrastructural changes in apple stem tissues induced by *Erwinia amylovora* and its toxin were investigated by electron microscopy. In bacteria-inoculated apple stem tissues, there was no significant ultrastructural change in host cells 24 hours after inoculation even though bacteria were found in xylem vessels and in the intercellular spaces between collenchyma and parenchyma cells. The earliest ultrastructural modification was plasmolysis of xylem parenchyma cells. This took place about 48 hours after inoculation and was followed by aggregation of cytoplasm and disorganization of subcellular organelles. Cytoplasm became a dark amorphous coagulum and lysigenous cavities developed in xylem by the third day after inoculation. In toxin-treated stem tissues, plasmolysis of xylem parenchyma cells took place 2 hours after treatment with 200 μg/ml of toxin and was the first sign of ultrastructural change. Four to five hours after the toxin treatment, membrane systems of subcellular organelles as well as the plasmalemma and tonoplasts were intensely deranged. These observations suggest that the ultrastructural changes induced by the fire blight toxin are much the same as those induced by the pathogen.

Fire blight of pear and apple was the first plant disease shown to be caused by a bacterium. The disease has been studied extensively since the discovery of the bacterium as a plant pathogen by Burrill in 1881 (1). Several aspects of the disease, however, have remained either uninvestigated or unclear. One of these is the damage caused by the bacterium to host cells at the subcellular level.

Goodman and Burkowicz (3) have studied electron-microscopically the ultrastructural changes of the interveinal area of apple leaf tissue infiltrated with the fire blight bacterium, *Erwinia amylovora*. They reported that membranes of subcellular organelles of the leaf tissues disintegrated rapidly in response to the inoculation by either a virulent or an avirulent strain of the pathogen. They concluded that the response of the leaf tissue to both strains was a hypersensitive type reaction rather than normal disease development. An earlier study of Plurad et al. (9) also showed that inoculation of *E. amylovora* into the interveinal apple leaf tissue does not result in systemic infection and growth of the bacteria is restricted. On the other hand, inoculation of the bacterium into the broken xylem vessels always results in blight development (2). It is therefore believed that successful infection of *E. amylovora* occurs initially in vascular tissue of leaf veinlets, petioles, or stem.

The pathogenesis of the disease has not been fully understood. Goodman et al. (4), however, have recently reported the isolation of a toxin from the fire blighted apple slices. The toxin, amylovorin, caused a wilting effect on many hosts but not on nonhost plants and is believed to be responsible for the development of symptoms associated with the fire blight disease.

The objectives of this study were to compare the ultrastructural effects of a virulent isolate of *E. amylovora* and amylovorin on the susceptible apple tissues (petioles and stems) and to elucidate the possible disease process engendered by the bacterium.

MATERIALS AND METHODS

The youngest fully unrolled leaf laminae of vigorously growing Jonathan apple shoots (7-9 cm in length with four to five expanded leaves) were excised at the leaf base and the petiole tips thereby exposed were inoculated with 0.01 ml of a bacterial suspension or with sterile distilled water. The bacterial suspension, 10^8 cells/ml of a virulent isolate of *Erwinia amylovora* [isolate E9, (7)] was prepared from 24-hour cultures grown on Difco nutrient agar fortified with 0.5% yeast extract and 1% glucose. The bacteria-inoculated shoots were collected at 6-hour intervals for 3 days and 2-mm sections of the stem just below the inoculated petioles were fixed in 4% glutaraldehyde. To prepare toxin-treated stem tissues, the bases of apple shoots of 5-7 cm in length were placed in fire blight toxin solution at concentration of 200 μg/ml. The toxin was prepared according to the procedures described previously (4). The toxin-treated apple shoots were collected hourly for 6 hours and a 2-mm section of the stem immediately below the petiole of the youngest fully unrolled leaf was fixed. All fixed stem sections were embedded in Epon according to the procedures described previously (6). Thick sections of 1 μm of the embedded plant tissue were stained with 0.1% toluidine blue on a

slide warmer for 30 seconds for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an RCA-3G or JEOL 100B electron microscope.

RESULTS

Ultrastructure of normal apple stem.—Cross sections of the control apple stem revealed epidermal, cortical and vascular tissue. Epidermal cells, covered by a cuticular layer, have well developed endoplasmic reticulum (ER) and other subcellular organelles normally found in living cells (Fig. 1). Cortex consists of collenchyma (Fig. 2) and parenchyma cells (Fig. 3). The former have thickened corners of walls and are found under the epidermis, whereas the latter have thin walls and are arranged loosely with large intercellular spaces beneath the collenchyma cells. In the vascular tissue, there are sieve tubes, phloem parenchyma, vascular cambium, vessel and xylem parenchyma (Fig. 4, and 5). All cells in control tissue, with the exception of differentiating and mature sieve and vessel elements, posses typical unit plasma membranes which are appressed to the cell wall. Tonoplast, chloroplast and mitochondrial membranes are intact (Fig. 6) as well as nuclear and microbody membranes (Fig. 2).

Ultrastructure of apple stem inoculated with Erwinia amylovora.—Within 24 hours after inoculation, bacterial cells were found predominantly in vessels (Fig. 7) but also in the intercellular spaces between collenchyma and parenchyma cells in cortical tissues (Fig. 8) of the stem. At this stage, in xylem parenchyma and vessel no ultrastructural changes were noted. However, the cytoplasm in some cortical cells appeared slightly aggregated (Fig. 8). The cells possessed normal nuclei, mitochondria, chloroplasts, rough ER, Golgi apparatus and microbodies. The plasma membranes were in close contact with the cell walls and intact tonoplasts bordered the vacuoles (Fig. 7, 8).

Ultrastructural modifications became apparent 48 hours after inoculation mainly in vascular tissues and pith although epidermal and cortical tissues were also affected. These included first and foremost plasmolysis and intense aggregation of cytoplasm. Plasmodesmata seemed to have been pulled out of cell walls (Fig. 9), the mitochondrial matrix was decreased (Fig. 10), and chloroplasts and nuclei were disorganized (Fig. 11, 12). Occasionally bacteria were found inside the phloem parenchyma cells. These bacteria were surrounded by an electron lucent area, probably a polysaccharidal capsule (Fig. 13).

The first visible symptoms, wilting and browning of the inoculated petioles, were observed 54-60 hours after inoculation. However, the stem tissue sampled for electron microscopy appeared to the unaided eye to be symptomless at this time. At the subcellular level, organelles in some cells of this apparently normal stem tissue were degraded to such extent that their fine structures could no longer be discerned. In addition, the cytoplasm had become a dark amorphous coagulum. Lysigenous cavities were found in xylem (Fig. 14) and some intercellular spaces were filled with bacteria.

Ultrastructure of Erwinia amylovora cells in inoculated apple stem.—Cells of *E. amylovora* always possessed distinct cell walls and in many instances, three electron dense layers were apparent (Fig. 15). Virulent bacteria also formed a "protective" layer consisting of an electronlucent zone and fine filaments which projected from bacterial cell surface (Fig. 15). These observations suggest that most virulent cells do not degenerate even at 72 hours after inoculation and are surrounded by capsular material.

Ultrastructure of apple stem treated with fire blight toxin.—At the electron microscopic level, plasmolysis of xylem parenchyma cells was the first sign of ultrastructural change. This took place 1-2 hours after apple shoot bases were placed in 200 μg/ml toxin solution. The subcellular organelles of the cells, however, remained normal (Fig. 16). Four to five hours later, the cytoplasm was plasmolyzed so severely that plasmodesmata seemed to have been pulled out of their "sockets" in the cell walls. Membrane systems of other organelles as well as the plasmalemma and tonoplasts were severely deranged (Fig. 17).

DISCUSSION

Our results have shown the presence of bacterial cells in the xylem vessels and the intercellular spaces between cortical collenchyma and parenchyma tissues of the stem within 1 day after inoculation. At this time the walls of the xylem vessels were not altered structurally indicating that the bacteria in xylem vessels could not move out into the surrounding tissues. This suggests that *E. amylovora* translocated from the incision of the inoculated petioles to stem through both xylem vessels and the intercellular spaces of the cortex. These two pathways, however, are not the only means by which bacteria can translocate in the infected plants. Lewis and Goodman (8) have placed *E. amylovora* on the healthy upper leaf surface of apple and found that movement of the bacteria from the inoculated leaves into stems was stopped by girdling. This suggests that the bacteria may travel in the sieve tubes of the phloem tissues. Hence, it would seem that the pathway of translocation taken by the bacteria depends on the inoculation method employed.

Our earlier study (7) showed that avirulent bacteria of *E. amylovora* were also found in xylem vessels and in the intercellular spaces of cortical tissue of inoculated Jonathan apple shoots when the inoculation technique described herein was employed. These avirulent bacteria, however, were localized in the petiole and did not translocate more than 5 mm from the point of inoculation. The failure of the avirulent bacteria to translocate from petiole to stem was due to a hypersensitive reaction of host cells characterized by rapid degeneration of subcellular organelles in parenchyma cells and an agglutination reaction in xylem vessel characterized by the formation of bacterial aggregates. Neither of these reactions was observed in the research we report here with a virulent bacterium.

Seemüller et al. (10) have reported that pectinases and other cell wall degrading enzymes are not involved in the pathogenesis of fire blight disease. In this study we did not observe any wall degradation in vascular tissues similar to that in veinlets of cabbage leaves induced by *Xanthomonas campestris* (11) nor the separation of middle lamellae as noted in potato petioles caused by

Corynebacterium sepedonicum (5). *Erwinia amylovora,* however, did form lysigenous cavities in xylem tissue. We have not determined the exact mechanism involved in the cavity formation. However, it is possible that loss of turgor and collapse of xylem parenchyma may cause twisting and rupture of the walls of xylem vessels.

The first sign of ultrastructural changes in parenchyma cell of bacteria-inoculated tissues was plasmolysis. This was followed by aggregation of cytoplasm and disruption of organelle membranes. Similar changes were also observed in toxin-treated stem tissues suggesting that the ultrastructural modifications in both instances are the same. It is apparent that the fire blight toxin is responsible for the ultrastructural changes found in the tissue inoculated with *E. amylovora* and the effect of the toxin is probably on the plasma membrane and other organellar membranes of the cell.

That toxigenicity of *E. amylovora* is the basis of pathogenesis in host tissue is further supported by the fact that avirulent strains are nontoxigenic (4). However, the basis for the toxin-induced plasmolysis is as yet unknown.

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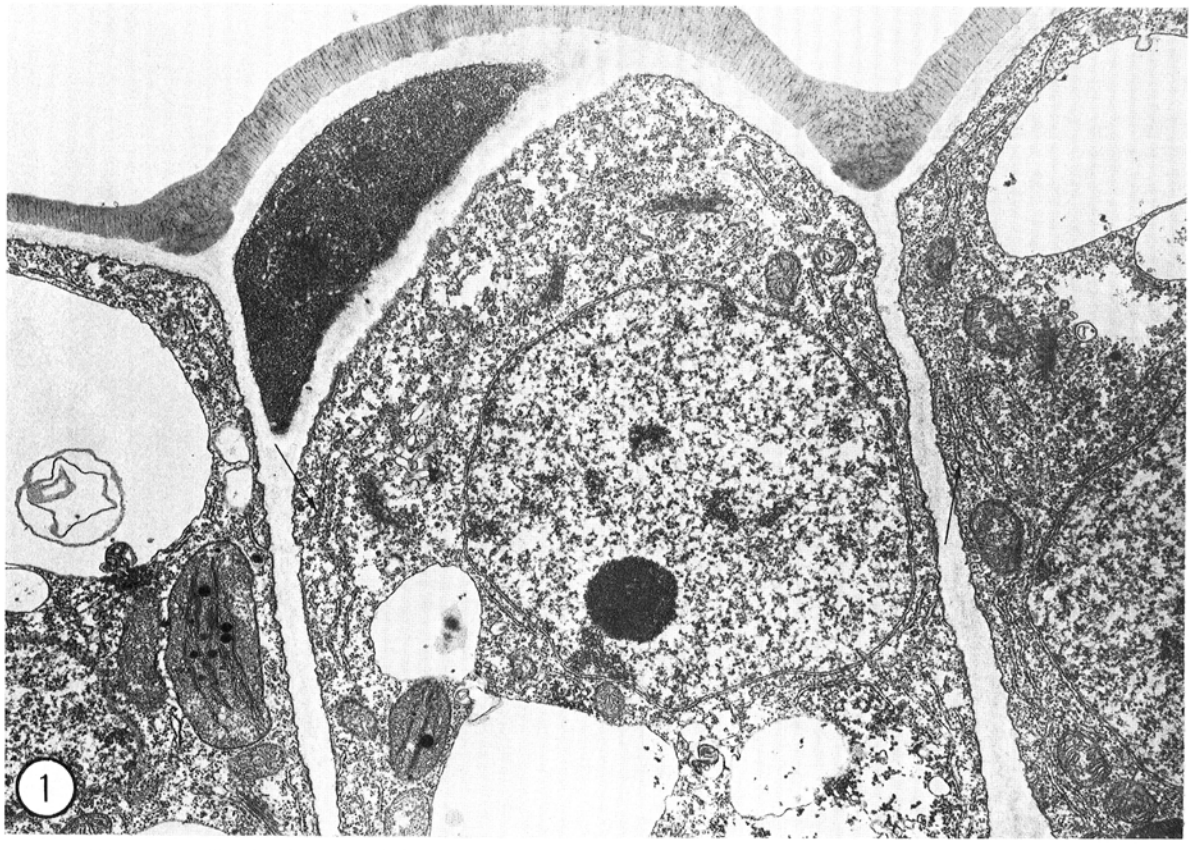
Fig. 1-3. Epidermis and cortex of control apple stem for comparison with similar tissues inoculated with *Erwinia amylovora* or treated with the fire blight toxin. **1)** Epidermal cells are covered by a cuticular layer and have various subcellular organelles with unusually well-developed endoplasmic reticulum (arrows) (× 10,800). **2)** Collenchyma cells are characterized by thickened corners of the cell wall (× 7,200). **3)** Parenchyma cells have a thin cell wall and relatively large intercellular spaces (× 7,200).

Fig. 4-6. Vascular tissues and subcellular organelles in the parenchyma cell of control apple stem for comparison with similar *Erwinia amylovora*-infected or the fire blight toxin-affected tissues and organelles. **4)** Sieve tubes of phloem tissue have thickened walls. Immature sieve tube member (IM) has cytoplast. Subcellular organelles disintegrate in intermediate stage (IN) and disappear after maturation (MA) with exception of endoplasmic reticulum (arrows) (× 7,200). **5)** Vessel members of xylem tissue are characterized by the presence of secondary wall (arrow) in spiral thickenings. They are devoid of cellular content. Parenchyma cells which contain various organelles are also present (× 1,800). **6)** Parenchyma cell possesses plasma membranes which are appressed to the cell wall. Tonoplast and membrane systems of chloroplasts and mitochondria are intact (× 48,600).

Fig. 7-9. Cross section of apple stem inoculated with *Erwinia amylovora.* **7)** Bacterial cells were found predominantly in xylem vessels 18 hours after inoculation. No ultrastructural changes were noted in xylem or xylem parenchyma cells (× 11,400). **8)** Bacterial cells were also found in the intercellular space of cortical tissue. Both bacteria and plant cells were normal in appearance with the exception of slight cytoplasmic aggregation (arrow) in some plant cells. The tissue was prepared 24 hours after inoculation (× 7,200). **9)** Plasmolysis is apparent in xylem parenchyma cells A and B; however, aggregation of cytoplasm is intense in cell B. Note that the plasmodesmata have been pulled out of the cell wall (arrow). The tissue was prepared 48 hours after inoculation (× 10,000).

Fig. 10-13. Ultrastructural changes in apple stem tissue 48 hours after inoculation with *Erwinia amylovora.* **10)** The mitochondrial matrix was decreased and cytoplasm was aggregated in an epidermal cell (× 23,500). **11)** The chloroplast in a parenchyma cell had lost its bounding membrane and granal arrangement. Vestigial cytoplasm was vaguely apparent (arrows) (× 11,400). **12)** The nucleus in a parenchyma cell had lost its envelope and the nuclear area was filled with filaments of nucleic acid (arrow) (× 23,500). **13)** Bacterium (B) might be found in a phloem parenchyma cell. The bacterium was usually surrounded by an electron lucent area (× 32,400).

Fig. 14-17. *Erwinia amylovora*-inoculated and fire blight toxin-affected tissues. **14)** Light micrograph of a lysigenous cavity in xylem tissue. The tissue was prepared 60 hours after inoculation with *E. amylovora* (× 750). **15)** Presence of fine filaments (probably polysaccharide) and electron lucent zone on the surface of bacteria in the intercellular space or cortical tissue. The tissue was prepared 48 hours after inoculation with *E. amylovora* (× 32,400). **16)** Xylem parenchyma cells were plasmolyzed 2 hours after the stem was treated with the fire blight toxin at concentration of 200 μg/ml (× 8,400). **17)** Xylem parenchyma cells were plasmolyzed severely and the subcellular organelles were disorganized 4 hours after the stem was treated with the fire blight toxin at concentration of 200 μg/ml. Plasmodesmata had been pulled out of the cell wall (arrow). Note similarity between Fig. 9 and 17 (× 9,600).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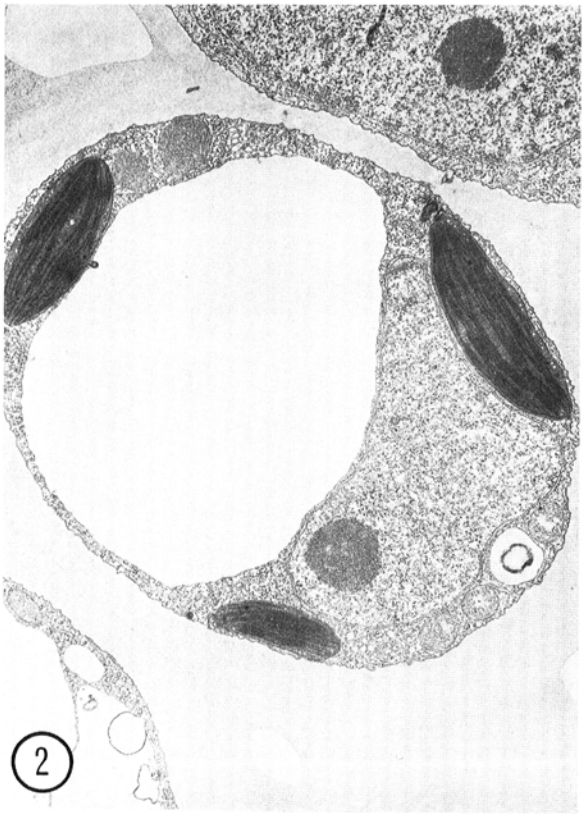
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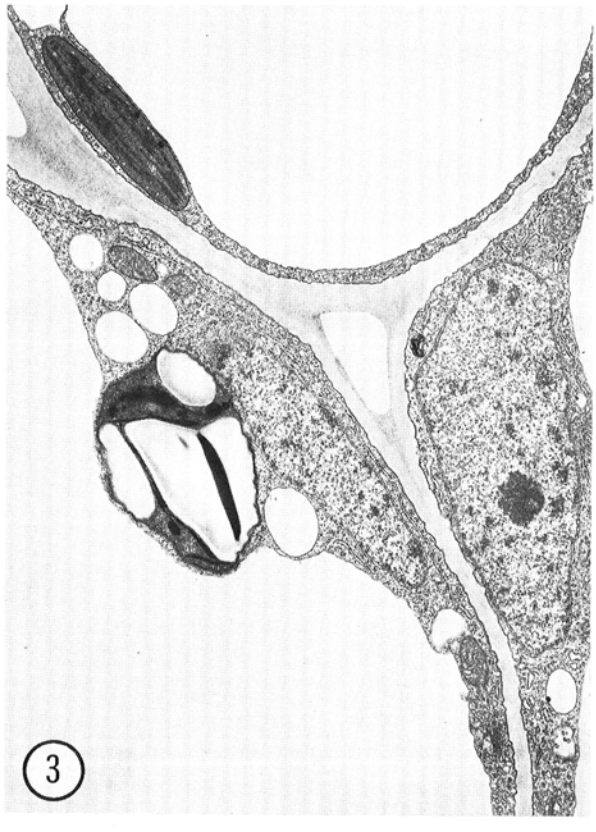
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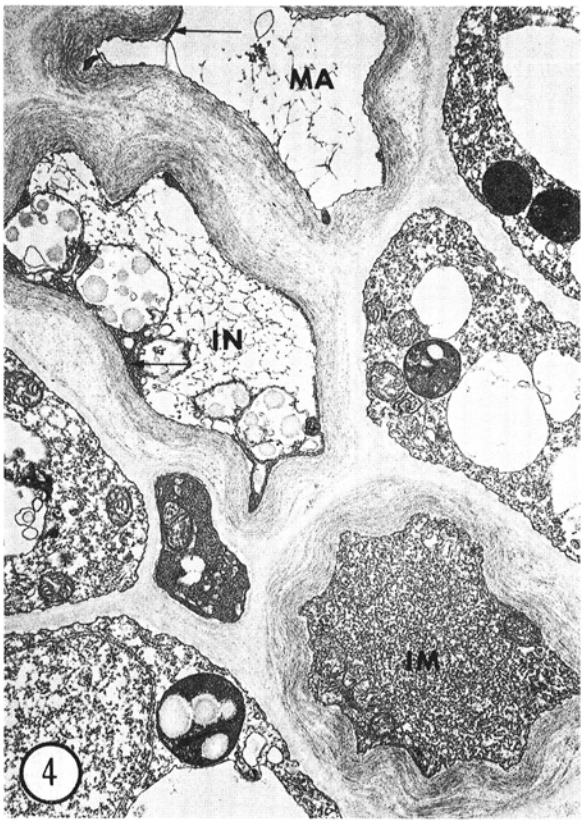
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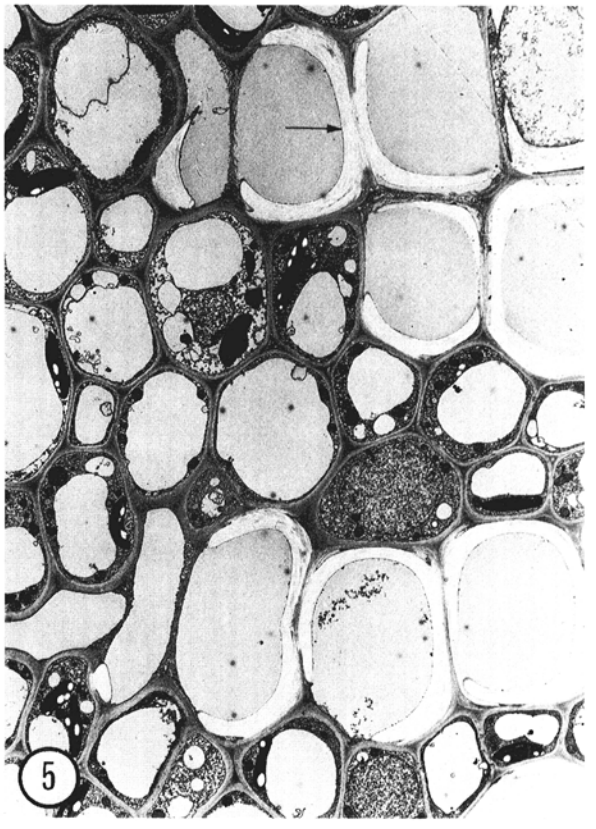
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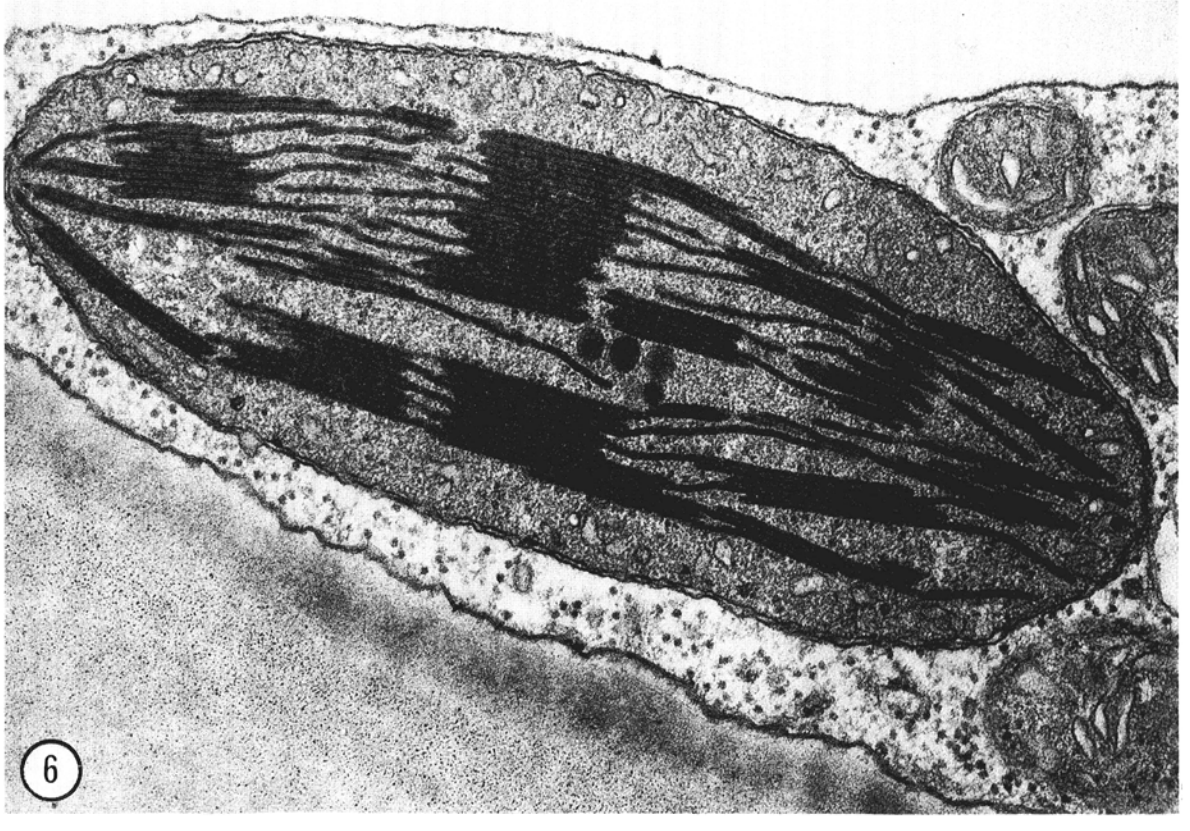
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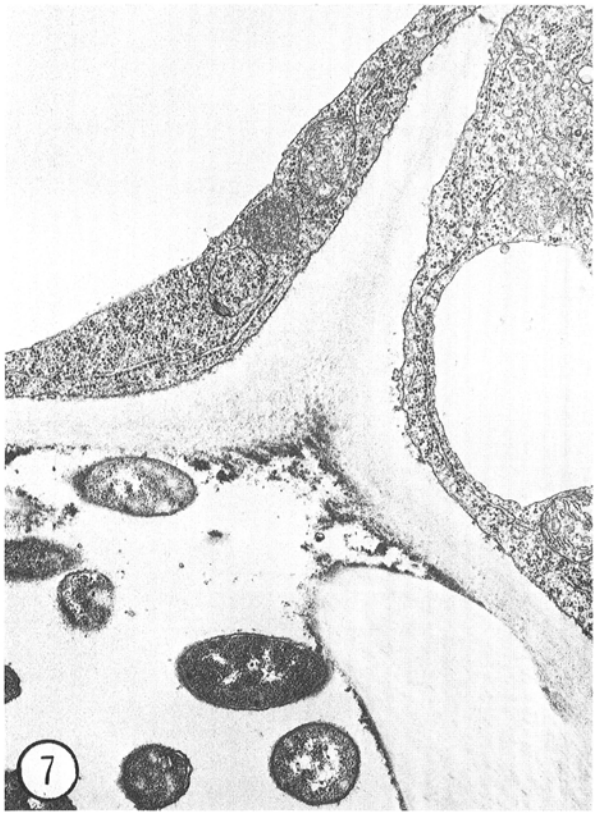

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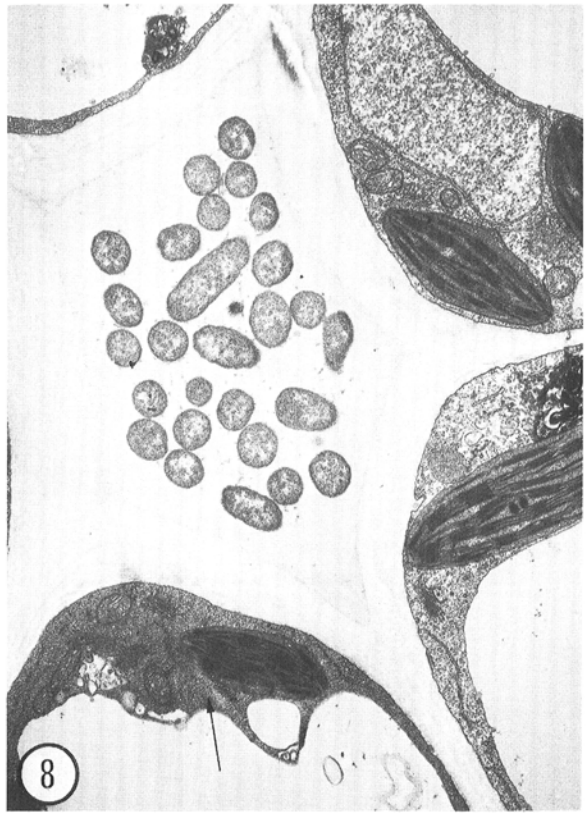

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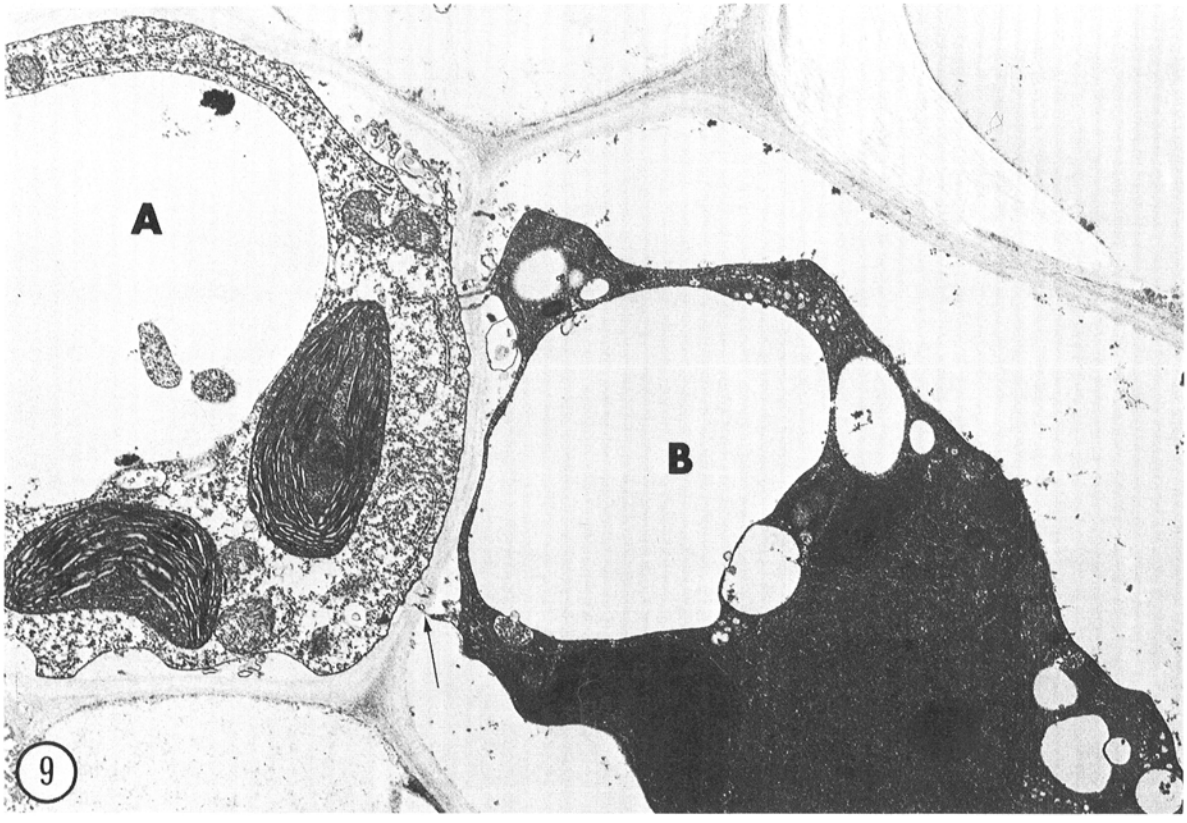

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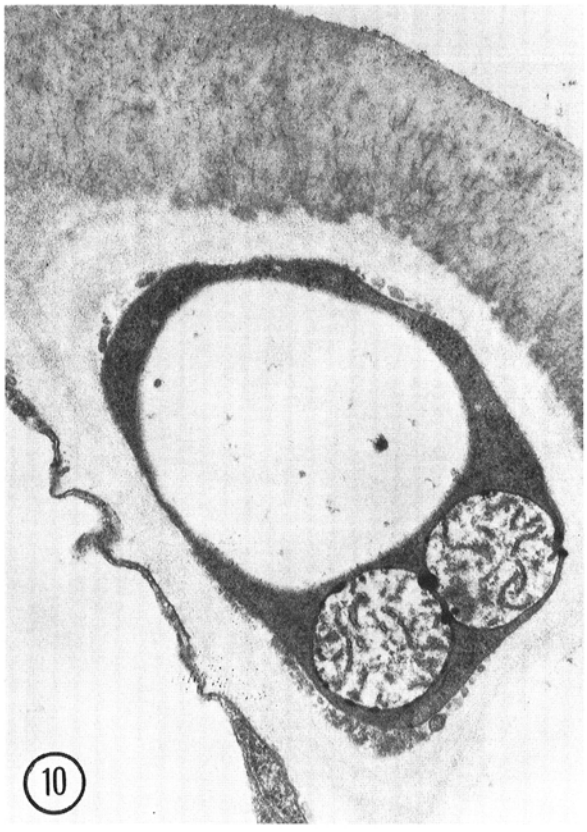
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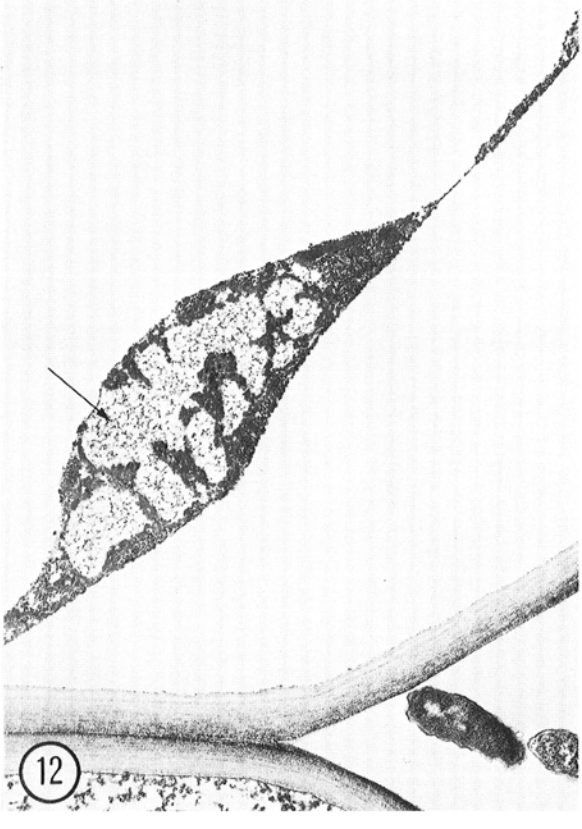
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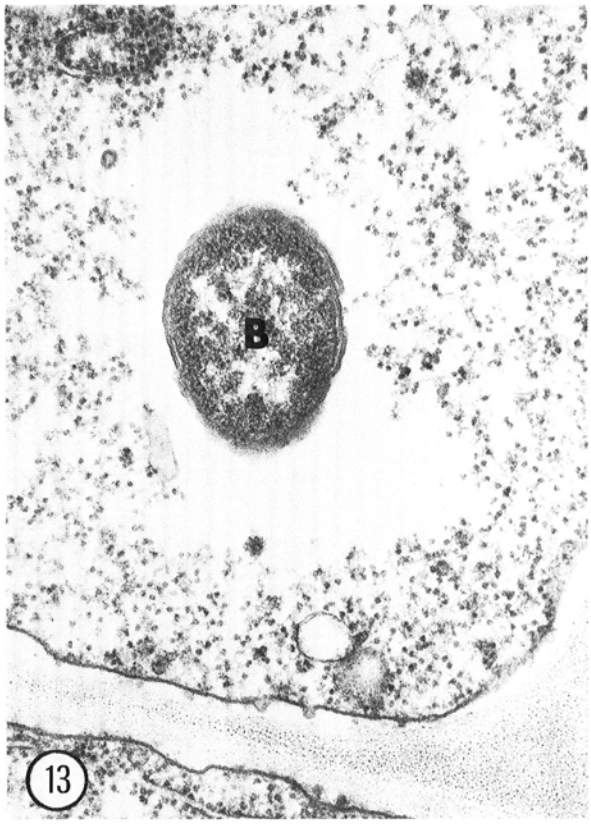
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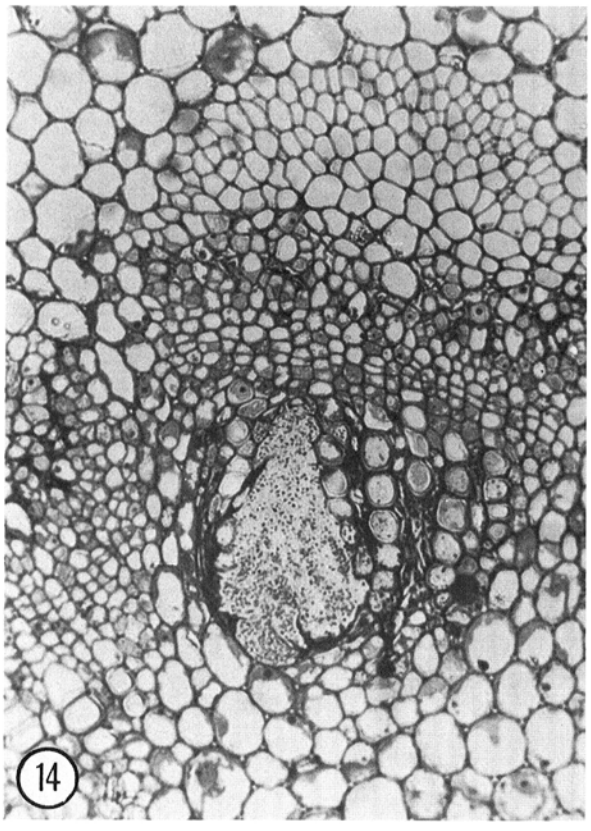
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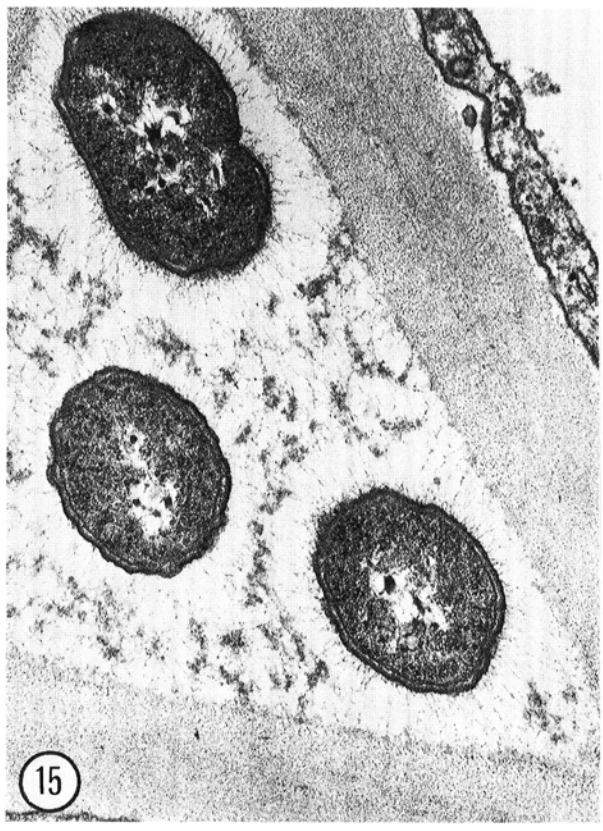
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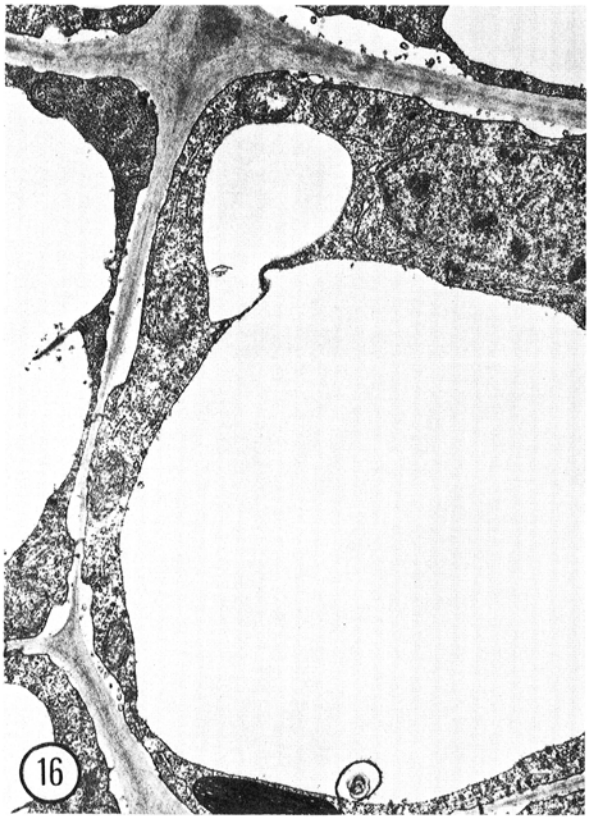
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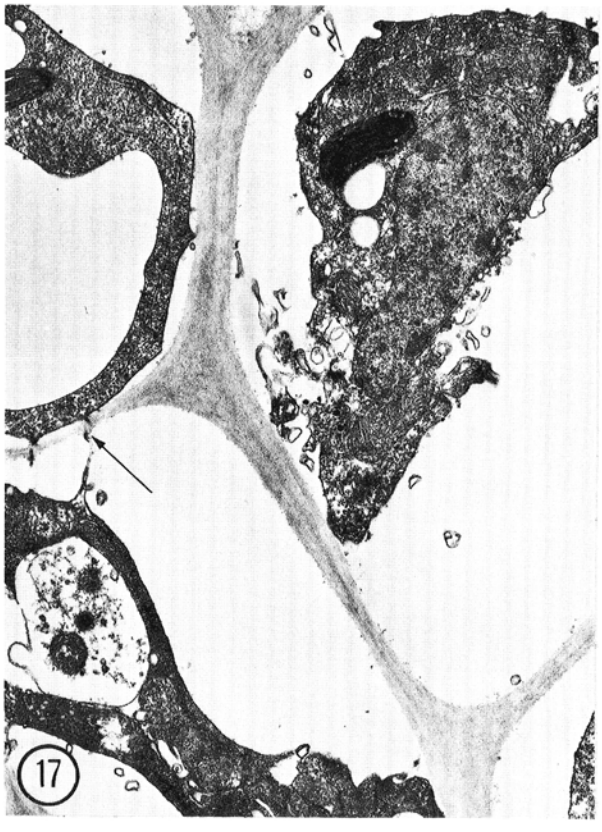
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