Ultrastructural Evidence for Immobilization of an Incompatible Bacterium, Pseudomonas pisi, in Tobacco Leaf Tissue

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ABSTRACT

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Incompatible *(Pseudomonas pisi),* compatible *(P. tabaci),* and saprophytic *(P. fluorescens)* bacteria, 10^9 cells/ml, were injected into tobacco leaf tissue. At 20 minutes, 2, 4, and 6 hours after injection, tissue was examined electronmicroscopically and the extent and manner of bacterial immobilization was determined. After 20 minutes, wall cuticle separated from the wall where incompatible bacteria were in close proximity to the wall surface. This was not noted in control tissue even after 6 hours. After 2, 4, and 6 hours the separated wall cuticle, which was filamentous at 20 minutes, became progressively thicker and more comprehensive by having large numbers of vesicles $\cong$ 25 nm in diameter, dense staining fibrils and membrane fragments integrated into it. During this period this structure appeared to envelope and immobilize some bacteria that had migrated to the plant wall surface. Not all bacteria became ensheathed by the wall cuticle. At 2 and 4 hours after injection, the plasmalemma vesiculated, the wall became swollen, and some elements of the cell wall became more intensely electron-dense. Electronmicrographs suggest migration of some plant cell contents to and through the plant cell wall where they aggregate with remnants of the original wall cuticle to form a bacteria-ensheathing envelope. Some parts of the bacterial cell wall also seem to be incorporated into this structure. After 6 hours, plant cells show typical hypersensitive reaction symptoms. *Pseudomonas fluorescens* induced the separation of cell wall cuticle, which also enveloped bacterial cells, but the ensheathing cuticle remained very fine. Few vesicles were formed and distortion of neither plant nor bacterial cells was detected during the 6-hour course of the experiment. The compatible pathogen, *P. tabaci,* did not induce formation of a bacteria-limiting wall cuticle after 6 hours although host cell cytoplasm was disorganized. Plant cell wall fibrils aggregated loosely around the bacteria, but no vesicles or membrane fragments augmented the fibrillar debris.

In earlier experiments it was noted that the hypersensitive defense reaction (HR) manifested itself in three ways: (i) a cessation of bacterial proliferation within 12 hours (2); (ii) widespread, intense, and rapid cellular disorganization of inoculated tissue (4); and (iii) localization of the incompatible pathogen (8, 9).

It has been suggested (4) that immobilization of bacteria in HR results from tissue desiccation and subsequent cell collapse. Hence generally it is believed that the bacteria are confined to the intercellular space that they occupied at the time cell collapse occurred.

The precise immobilizing mechanism has remained unresolved because electron microscope observations of the HR have been conducted with comparatively low inoculum dosages (10^8 cells/ml), revealing few bacteria (2, 4). A tobacco leaf disk 1 cm in diameter can accommodate in its intercellular space $\sim 4 \times 10^{11}$ bacteria and a 1-cm diameter disk inoculated with 10^8 cells/ml will contain $\sim 10^6$ cells (4). Hence, in our experiments where 10^8 cells/ml or lower concentrations of inoculum were used the vast proportion of intercellular space was devoid of bacteria. This accounts for the low frequency with which bacteria were observed.

To determine more clearly the nature of the bacteria-immobilization reaction, we used an inoculum of 10^9 cells/ml that provided in vivo levels of $\sim 10^7$ cells/leaf disk 1 cm diameter. Tissue infected with the compatible pathogen, *P. tabaci,* reach levels of 10^8 cells/disk (10).

MATERIALS AND METHODS

The bacteria, *Pseudomonas pisi* (a pathogen of pea, *Pisum sativum* L.), *P. fluorescens,* a saprophyte, and *P. tabaci,* a compatible pathogen, were grown on Difco nutrient yeast (0.5%), glucose (1.0%) agar for 16 hours at 28 C. In preparing inocula, bacteria were washed from agar slants with 0.025 M phosphate buffer at pH 6.5, centrifuged, resuspended in fresh buffer, and adjusted photometrically to 10^9 cells/ml.

Nicotiana tabacum L. 'Samsun N-N' plants were grown in vermiculite at 24 C, under a 14-hour day at 16,000 lx, and watered daily with Hoagland's solution. Plants with four-to-six leaves expanded were infiltrated either with buffer or bacterial suspension in buffer at 10^9 cells/ml. Excess water in the leaves generally equilibrated with the surrounding atmospheric moisture within 30 minutes.

Tissue samples (1-mm^2) were cut from bacteria- and buffer-injected leaves after 20 minutes, 2, 4, and 6 hours into a drop of glutaraldehyde (4% glutaraldehyde in 0.066 M sodium cacodylate), fixed at 4 C for 4 hours, and washed 18 hours in sucrose-cacodylate [a mixture of 0.5 M sucrose and 0.2 M sodium cacodylate (1:1, v/v)]. The

tissue was postfixed with 1% OsO_4 in Millonig's buffer (11) at 4 C for 4 hours, dehydrated, and embedded in EPON-812. Ultrathin sections were cut on a Reichert OMU-2 ultramicrotome with a diamond knife and transferred to 38- or 48-μm mesh uncoated copper grids. The sections were stained in 1% uranyl acetate (in 50% ethanol) for 30 minutes at 25 C followed by lead citrate at pH 12 for 15 minutes (12) and examined with a JEOL 100B electron microscope.

RESULTS

The inoculum level of 10^9 cells/ml was sufficient to reveal certain features of the bacterium-plant cell interaction, and not high enough to approximate population levels generally encountered in tissue infected with a compatible pathogen. About half of the intercellular spaces viewed had from one-to-five bacterial cells somewhere along their periphery. Few spaces had more than five bacterial cells.

Ultrastructural features observed 20 minutes after inoculation.—In two experiments, equilibration (loss of water-soaked appearance by the inoculated leaves) occurred in 20 minutes. As the injected water equilibrated with the atmosphere, bacteria migrated to the mesophyll cell surfaces (Fig. 2).

An unusual and heretofore unreported filamentous structure was regularly observed to have separated from the surface of some parenchyma cells bordering intercellular space in close proximity to bacteria (Fig. 2). The structure appears in cross section as a filament, but in reality is probably sheet-like. Since these structures were observed at 20 minutes after bacterial infiltration, but not in control tissues 6 hours after inoculation (Fig. 1), they apparently form rapidly in response to bacteria. They are clearly apparent at 6 hours in *P. fluorescens*-injected tissue (Fig. 12), but are not observed in *P. tabaci*-injected tissue after 6 hours (Fig. 13). This structure consists of fibrils, an amorphous ground substance (probably cutin), and densely-stained particles with an average size of about 25 nm (range, 18-35 nm). It appears to be derived from the comparatively thin outer cuticular layer of those plant cell walls that bordered intercellular space.

Ultrastructural features observed 2 hours after inoculation.—The cuticle seemed to separate from the cell wall and to rupture at those places in close proximity to the bacteria (Fig. 4), and in doing so "extracted" several vesicular dense-staining bodies that are embedded in it (Fig. 3, 4). In addition, some fibrils appeared to be raised at the newly exposed wall surface and others adhered to the separating cuticle (Fig. 4). Lifting off of the cuticle seemed to permit additional dense-staining fibrils and vesicles to collect at the surface and, in addition, deeper layers of the cell wall also stained more intensely (Fig. 3, 9). This phenomenon suggested the possible migration of proteins, phospholipids, or sugar nucleotides into the wall from the cell beneath or perhaps a change in cell wall structure that permitted more intense staining of these osmiophyllic and heavy-metal-adsorbing substances.

Where the wall cuticle was removed, apparently in response to the presence of one or more bacteria, the plasmalemma showed initial signs of vesiculation (Fig. 9). The interface between the cell wall and densely-stained fibrillar region also may reveal membrane-bound vesicles of about the same size as the densely-stained bodies observed on the wall cuticle at 20 minutes (Fig. 3, 9).

That the cuticle actually lifts away and folds back is suggested in Fig. 3. In fact, at the higher magnification (Fig. 3, right inset) two of the small vesicles are on fibrils and appear to have been pulled out of the cell wall.

Occasionally at 2 hours, membrane fragments appeared to be associated with or integrated into the wall cuticle. Some seem to be derived from the membranous blebs that extend from the outer surface of the bacterial cells (Fig. 3, left inset).

Ultrastructural features observed 4 hours after inoculation.—The rather wispy wall cuticle that at 2 hours either just was loosened from the cell wall and extended into intercellular space or partially enveloped some bacterial cells at 4 hours regularly was observed to completely ensheath one-to-several bacterial cells. Furthermore, the sheath-like structure became thicker and more complex when additional amounts of vesicles, fibrils, and membrane fragments were integrated into it (Fig. 5, 6).

The structural integrity of the plant cells was altered by intense vesiculation of the plasmalemma. In addition, rifts in this organelle were evident along its entire length (Insets, Fig. 5, 6). The plant cell wall opposite the ensheathed bacteria had swelled to two or more times normal thickness. The swollen wall appeared loose and net-like in structure through which vesicles and other substances might more easily migrate to the surface (Fig. 5, 6). Some vesicles were observed within the swollen wall (Fig. 10).

Ultrastructural features observed 6 hours after inoculation.—Classical symptoms of HR-associated tissue collapse were generally visible (Fig. 7). The plasmalemma and other normally membrane-bound organelles, e.g. chloroplasts, mitochondria, etc., were severely degenerated and organellar compartmentalization within the cell was barely discernible (Fig. 7). The sheath enclosing the bacteria became thicker with additional vesicles, membrane fragments, and fibrillar material integrated into its structure (Fig. 8). It also is apparent that not all bacteria were immobilized in this fashion. Some seemed simply to be trapped between cells that had collapsed as a consequence of plasmalemma damage and subsequent tissue desiccation (Fig. 7). The number of vesicles appearing at the surface of the plant cell wall increased greatly (Fig. 11).

Sections of tissue that had been inoculated with *P. fluorescens* revealed wall cuticle surrounding bacteria after 6 hours. However, the cuticle remained extremely fine and very few free vesicles were noted (Fig. 12). It was apparent that the interaction between bacteria and plant tissue was extremely mild. Both appeared to be normal at the 6-hour examination.

Six hours after tissue was inoculated with 10^9 cells/ml of a compatible pathogen, *P. tabaci,* there was no evidence of any bacteria-immobilizing wall cuticle. Occasionally wall microfibrils completely surrounded bacteria (Fig. 13); however, no vesicles were observed. The host cell wall appeared to be disintegrating, which freed wall microfibrils, and host-cell cytoplasm was severely degraded.

DISCUSSION

Localization of the incompatible species, *P. pisi,* in tobacco leaf tissue at least in part seemed to be an active process. The localization phenomenon characterized by an external buildup of "granules" between incompatible bacteria and tobacco plant cell walls was first pointed out without specific comment by Goodman (2). In situ agglutination of an avirulent strain of *Erwinia amylovora* in apple xylem vessels has been reported by Huang et al. (7) and a similar in vivo agglutination has been observed in rice inoculated with an avirulent strain of *Xanthomonas oryzae* by Horino (5). Goodman et al. (3) reported the isolation of agglutinating factors from apple and tobacco tissue inoculated with an avirulent strain of *E. amylovora* and *P. pisi,* respectively. The factors isolated from apple and tobacco could agglutinate in vitro only the agglutinin-inducing bacteria. These reports and this paper describe active processes whereby an incompatible pathogen may be immobilized in plant tissue and present morphological evidence for what appears to be new types of plant defense reactions against incompatible bacteria.

In this report, a number of observations are described for the first time. These include detachment of the outer cell wall component, the cuticle, in response to the close proximity of incompatible bacterial cells (Fig. 2-4). Scott (13) has indicated that the leaves of dicotyledenous plants generally exhibit extensive suberization of cells contiguous with intercellular space. Thicknesses of this inner cuticle of as much as 1 μm have been recorded (1).

The process of immobilization of bacteria by means of a rapidly developing sheath is envisaged as follows. First, as the water bearing the inoculum equilibrated with the surrounding atmosphere, the bacteria migrated toward the plant cell wall surfaces. As they approached or perhaps contacted the cell wall, a process was initiated that caused the suberized cuticle to become detached from the wall. If one could visualize the surfaces of an intercellular space at this time, they might appear blistered as in Fig. 14. Then the blisters ruptured, and an area of suberized wall cuticle separated from the wall. In doing so, underlying microfibrils were exposed and some of these adhered to the cuticle as did numerous vesicles that appeared to have been at or near the newly exposed wall surface (Fig. 3, 4). The bacteria were able to move still closer to the wall and perhaps further exchange of metabolites between bacteria and plant cell occurred.

It seems likely that permeability of the plant cell wall was altered by the removal of its suberized coating. The exposed fibrils of the cell wall swelled, permitting them to stain more readily or become impregnated by substances moving out of the plant cell per se and these readily picked up the osmium, uranyl, and lead stains (Fig. 3, 9, 10). Within 2 hours, additional small vesicles were visible that appeared to have migrated from within the protoplast (Fig. 3, 9). The partially detached wall cuticle became more complex as plant and bacterial cell components became integrated into it (Fig. 3, 4). Outward migration of plant cell components intensified at 4 hours and this was made possible by a localized change in character and composition of the plasmalemma and cell wall. The vesiculated plasmalemma appeared to disintegrate and fragments of it were seen moving through the major thickness of the plant cell wall as vesicles (Fig. 5, 6, 10). Fragmentation and eventual disappearance of the plasmalemma in cells undergoing HR was reported by us earlier (2, 4). The bacteria-immobilizing sheath became progressively more complex until it seemed to surround completely some of the bacteria.

Structural changes in the plant cell protoplast, other than vesiculation of the plasmalemma, were not observed until 6 hours after the bacteria were injected into leaf tissue. This observation is in agreement with our previous studies on the appearance of cell organelle distortion during the development of HR (2, 4). That irreparable membrane damage occurred sometime between 20 minutes and 2 hours after inoculation also was determined previously by Huang and Goodman (6) and is substantiated in this report.

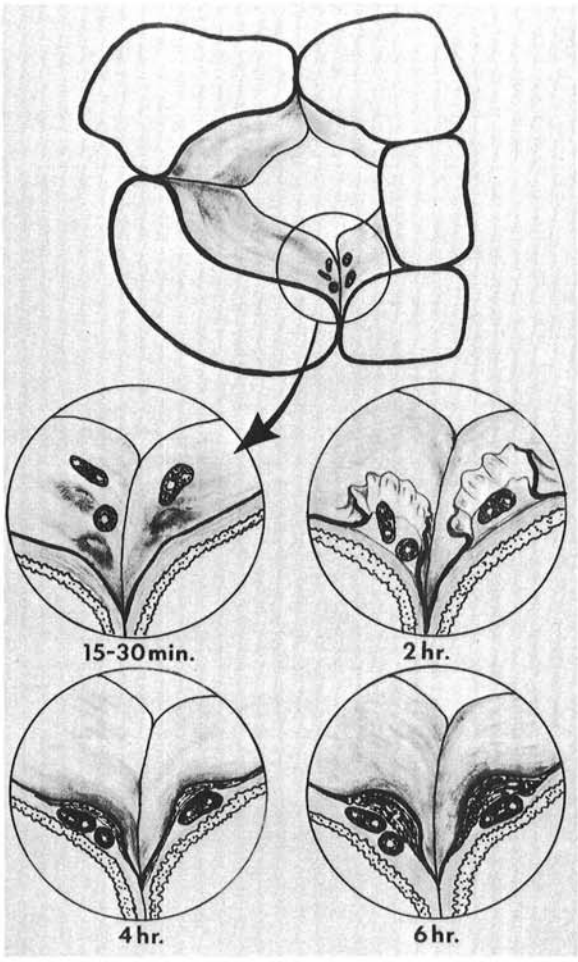

Fig. 14. Idealized representation of the sequence of events that occur in tobacco leaf tissue 0.25-6.0 hours after inoculation with 10^9 cells/ml of *Pseudomonas pisi.* As the buffer-containing inoculum equilibrates, bacteria migrate to plant cell surfaces. During the first 0.5 hours wall cuticle probably swells, separates from the wall where bacteria are in close proximity. Between 0.5 and 2.0 hours afterwards the cuticle breaks and folds back, and from 2-4 hours later materials from tobacco and bacterial cells are integrated into the wall cuticle which completely surrounds some bacteria. The wall cuticle becomes increasingly more complex from 4-6 hours after inoculation.

More difficult to correlate is this early change in plant cell wall and plasmamembrane integrity with the fact that electrolyte leakage in these tissues did not occur until 3-4 hours after inoculation. However, it is possible that the wall and membrane changes noted prior to that time were localized and involved only a comparatively small area of the total plant cell surface (Fig. 14). Furthermore, when an inoculum 5×10^6 or even 10^8 cells/ml is used much less plant cell surface is affected than we observed herein with 10^9 cells/ml. This contention is supported by the fact that intense electrolyte leakage occurs in tobacco tissue inoculated with 10^8 cells/ml after 5-6 hours, whereas 10^9 cells/ml cause leakage after 3-4 hours.

We have shown that inoculum levels of 5×10^6 or greater cause tissue collapse and confluent necrosis (2, 9). Turner and Novacky (14) have presented evidence to suggest that inoculum doses less than 5×10^6 result in the death of one leaf cell per bacterial cell. However, in these instances neither tissue collapse nor confluent necrosis occurred, yet the bacteria were immobilized. It would appear, therefore, that neither tissue collapse nor confluent necrosis is required a priori to localize incompatible bacteria.

Apparently, immobilization of incompatible bacteria may occur in two ways. First, by an active process that actually sequesters bacteria within a complex cuticular sheath. Second, as originally conceived (4) by being trapped between cells when leaf tissues collapse as a consequence of desiccation. We believe that tissue collapse and desiccation are probably of secondary importance in immobilizing incompatible bacteria and occur only when the inoculum level is high enough to cause confluent necrosis. We doubt that this level of inoculum is a common occurrence in nature in nonhost tissues. Hence, we contend that it is under conditions of low inoculum doses, which are more likely to occur in nature, that the active process of bacterial immobilization assumes real importance as a resistance mechanism.

The observations made on tissue injected with *P. fluorescens* are consistent with earlier reports (8, 9, 14) that HR is not induced, but bacteria are nevertheless immobilized. It is known that the saprophyte causes injected leaf tissues to become chlorotic in 4-5 days, but no abnormal effects were noted in 6 hours.

Although the high inoculum dose of *P. tabaci* caused rapid and severe damage to host cell cytoplasm and caused cell wall fibrils to loosen, shred, and partially surround bacterial cells, no confining complex wall cuticle was observed to form. In nature, the compatible pathogen continues to spread as long as conditions for disease development remain favorable.

The basic concepts concerning our observations of the bacteria-immobilizing wall cuticle sheath include: (i) wall cuticle separation appeared to occur rapidly and in response to the physical presence of incompatible bacteria; (ii) though the cuticle became progressively more complex with time, it possessed, after only 20 minutes, some components that seemed to be preformed; (iii) some of these components (e.g. vesicles, deep-staining fibrils, and membrane fragments) increased in quantity with time and became integrated into the wall cuticle, which permitted it to envelop and immobilize bacteria; and (iv) most of the components that formed the complex ensheathing wall cuticle seemed to come from plant cell protoplasts and walls that were deteriorating during the 6-hour course of the experiment.

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Abbreviations for electronmicrographs:
* = area magnified in inset; B = bacteria; BL = blebs; C = chloroplast; CW = cell wall; DB = dense-staining bodies; DSF = dense-staining fibrils; F = fibrils; HRT = hypersensitive reaction tissue; IS = intercellular space; M = mitochondrion; MF = membrane fragments; N = nucleus; Nu = nucleolus; PL = plasmalemma; SW = swollen wall; T = tonoplast; V = vesicle; Va = vacuole; VPL = vesiculated plasmalemma; WC = wall cuticle.

Fig. 1-2. **1)** Control, tobacco leaf tissue 6 hours after infiltration with buffer. All organelles are normal, arrows point to smooth wall surfaces (× 13,500). **2)** Twenty minutes after inoculation with *Pseudomonas pisi* showing wall cuticle containing fibrils, dense-staining bodies (probably same as vesicles), and ground substance (probably cutin). Note fibrillar surface where cuticle has separated from wall (arrows) (× 36,400).

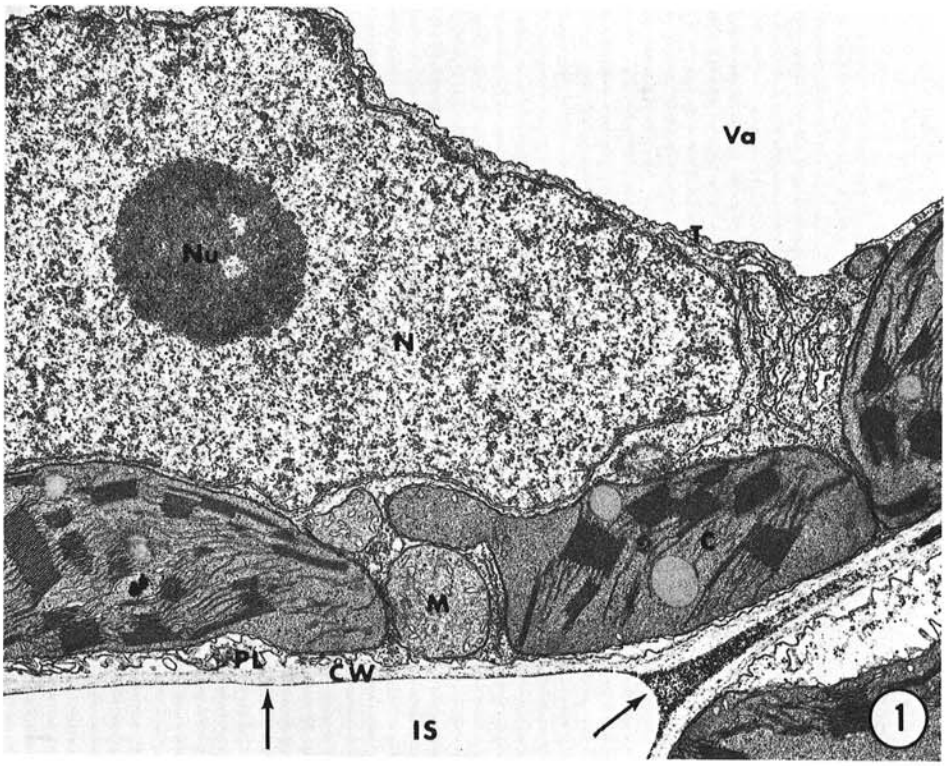
Fig. 3-4. **3)** Wall cuticle separates from two wall surfaces 2 hours after inoculation with *Pseudomonas pisi* to form a continuous sheet (× 16,400). Right inset shows details of cuticle folding back with adhering vesicles and fibrils from wall and underlying dense staining fibrils (× 53,500). Left inset reveals bacterial surface membrane fragments (blebs) being integrated into wall cuticle (× 47,000). **4)** Wall cuticle folds back from surfaces of two neighboring cells 2 hours after inoculation with *P. pisi*. Note close proximity to bacterial cell as well as oblique and cross-section view of wall cuticle revealing both fibrils and vesicles (× 80,300).

Fig. 5-6. **5)** Cells of *Pseudomonas pisi* completely ensheathed by complex wall cuticle along two wall surfaces 4 hours after inoculation (note swollen walls near bacteria) (× 9,000). Inset shows close proximity of bacteria to the tobacco cell surface, net-like swollen tobacco cell wall and vesiculated plasmalemma (× 42,800). **6)** Cells of *P. pisi* completely ensheathed by complex wall cuticle at juncture of two tobacco leaf cells, 4 hours after inoculation (× 19,900). Inset shows vesiculated plasmalemma and swollen wall of the tobacco leaf cell (× 42,800).

Fig. 7-8. **7)** Groups of bacteria trapped between spongy parenchyma cells collapsed as a consequence of HR and other bacteria-ensheathed in complex wall cuticle 6 hours after inoculation with *Pseudomonas pisi* (× 10,900). **8)** High magnification of a portion of Fig. 9 showing vesicles, dense-staining fibrils, and membrane fragments integrated into the complex ensheathing wall cuticle 6 hours after inoculation with *P. pisi* (× 64,000).

Fig. 9-11. A sequence showing the progressive movement of vesicles from inside to outer surface of plant cell wall at 2, 4, and 6 hours after inoculation. **9)** Reveals the presence of vesicles in the wall cuticle or near the interface between the cuticle and the newly exposed cell (× 62,000). **10)** The movement of a vesicle through a swollen tobacco leaf cell wall (× 77,600). **11)** Large numbers of vesicles emerging from the plant cell wall surface (× 93,000).

Fig. 12-13. **12)** A juncture between two tobacco leaf cells wherein *Pseudomonas fluorescens* cells have been localized by a wispy wall cuticle 6 hours after inoculation with 10^9 cells / ml. Tobacco leaf and bacterial cells both seem unaffected (× 14,000). Inset shows the extremely thin nature of the wall cuticle and the comparatively few vesicles and smooth wall surface (× 42,800). **13)** A juncture between two tobacco leaf cells 6 hours after inoculation with 10^9 cells / ml of *P. tabaci*. Cell wall fibrillar composition appears to be shredding and fibrils loosely aggregate around bacteria. Wall cuticle is not comprehensive and vesicles are not in evidence. Arrows denote electron-lucent and fibril-free region around bacterial cells.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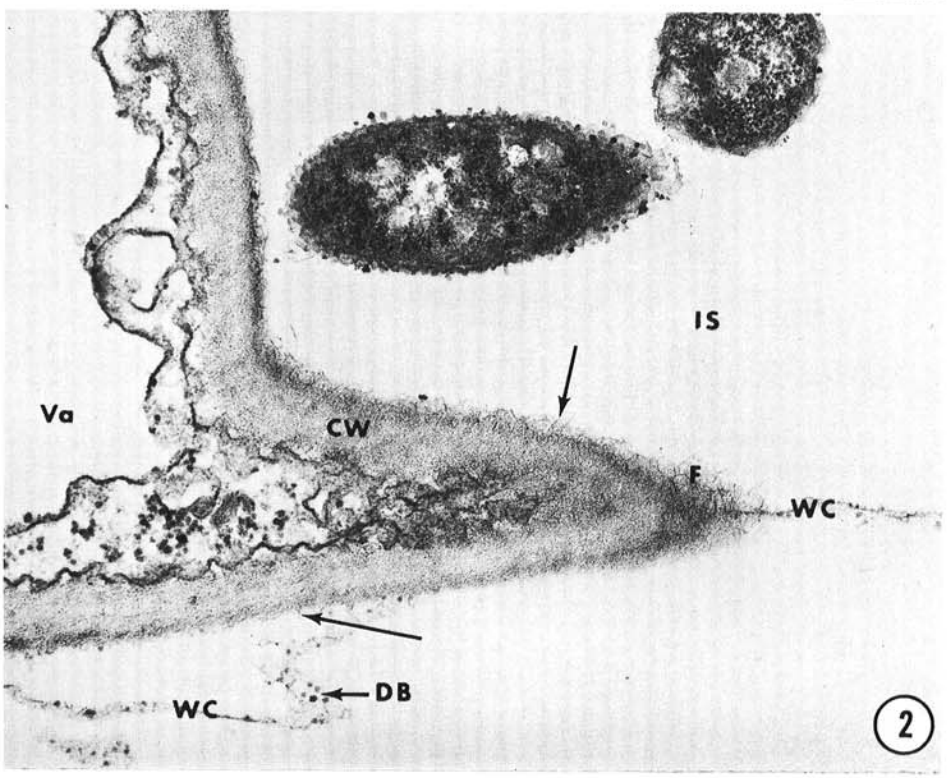
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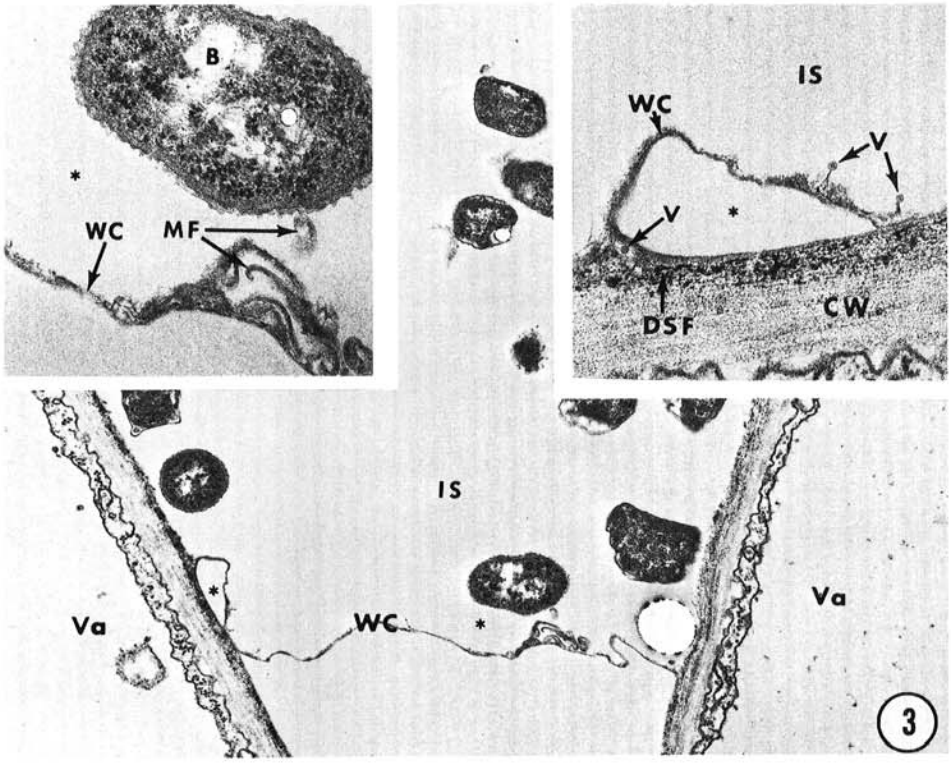
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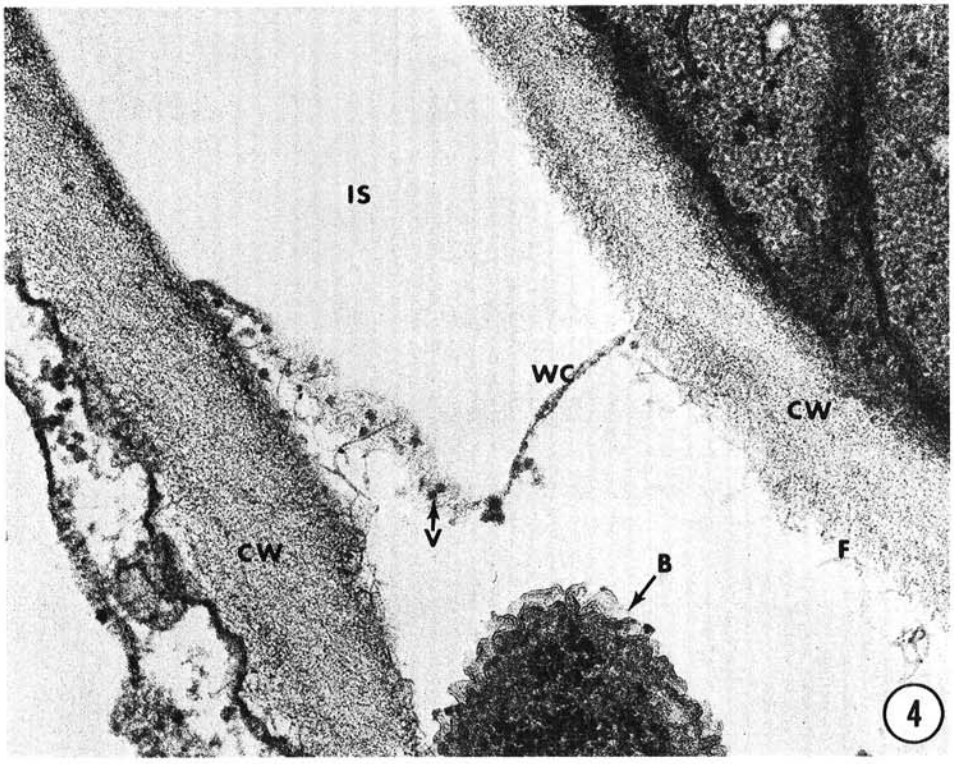
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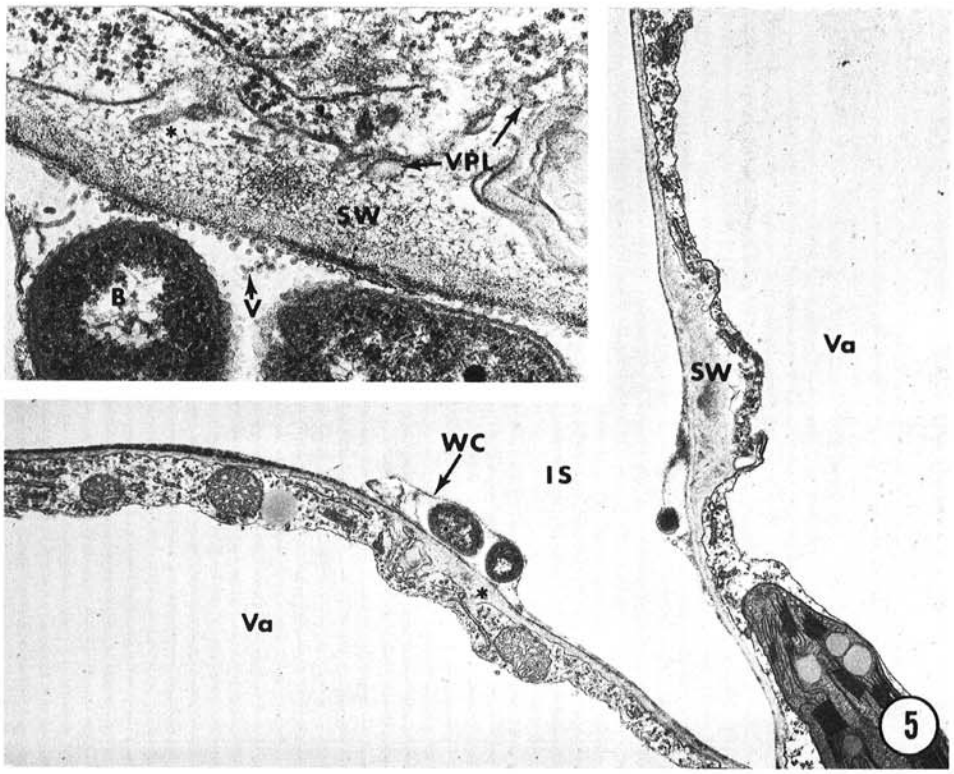
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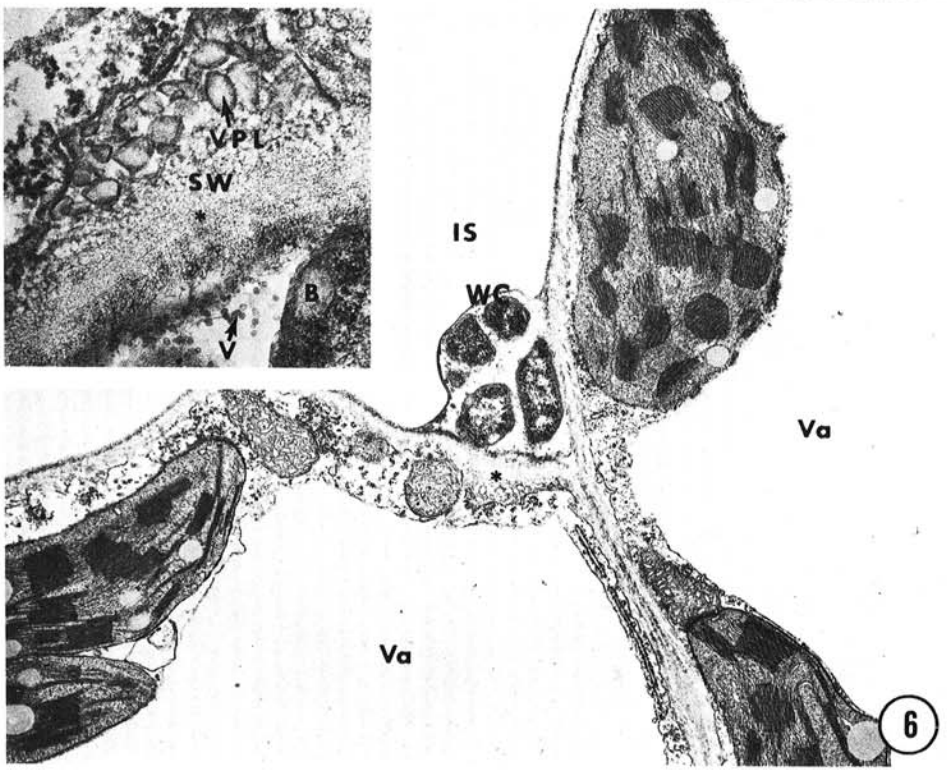
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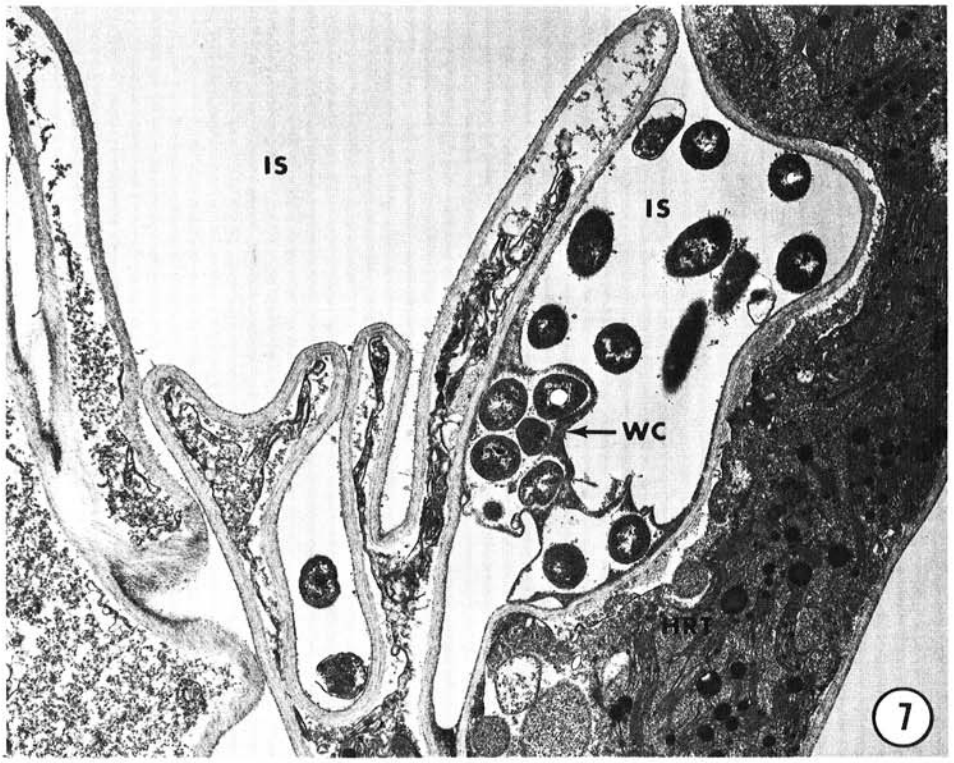
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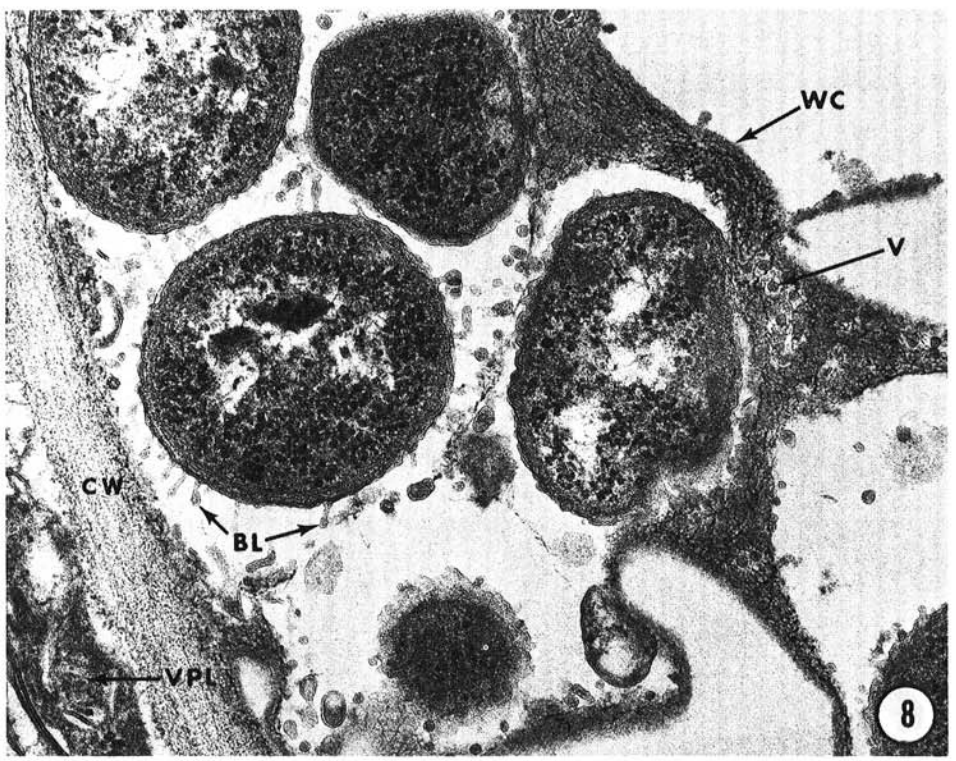
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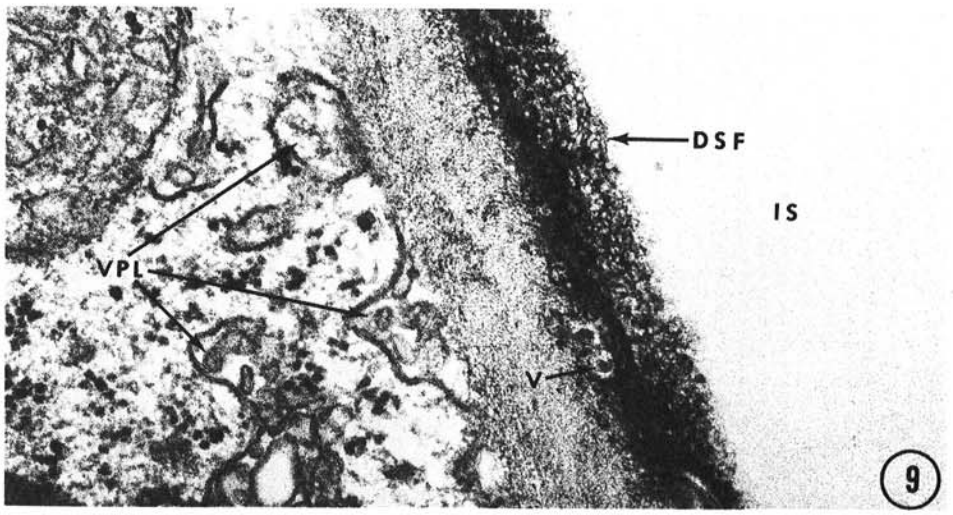
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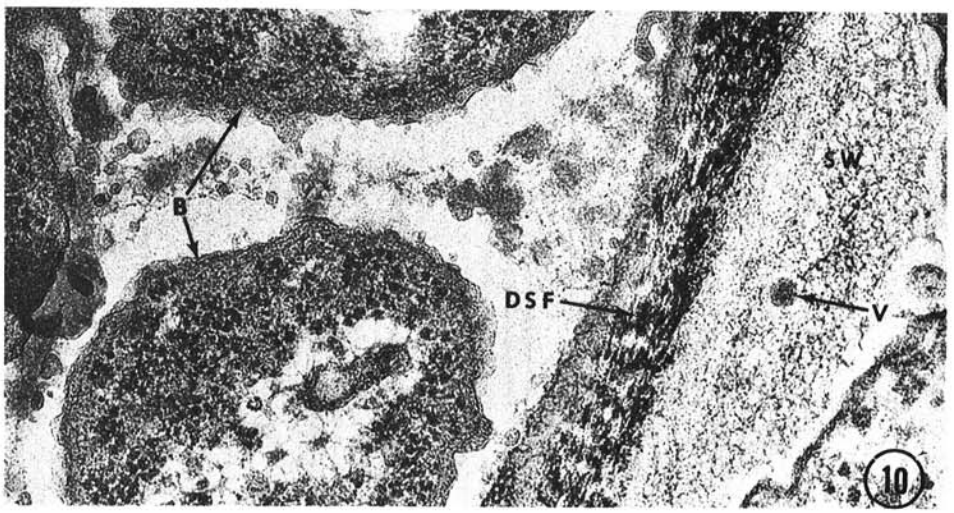
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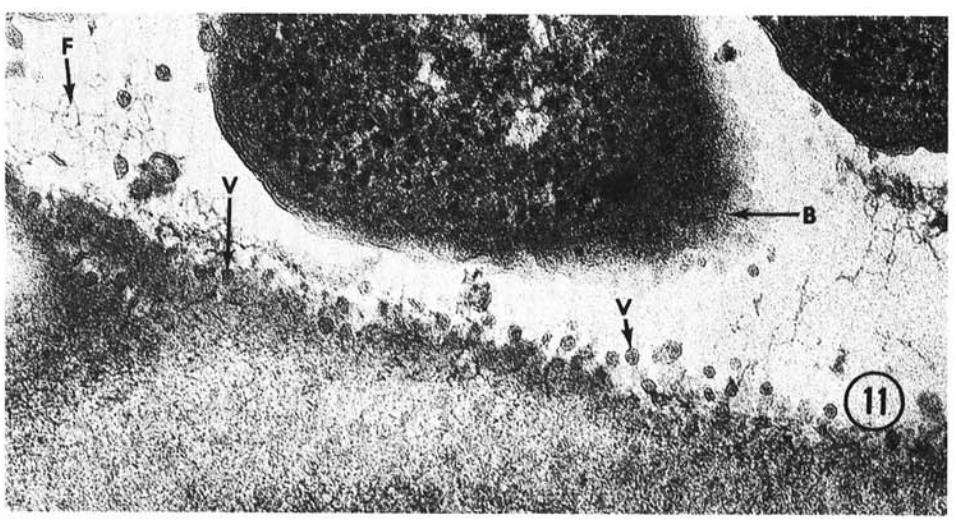
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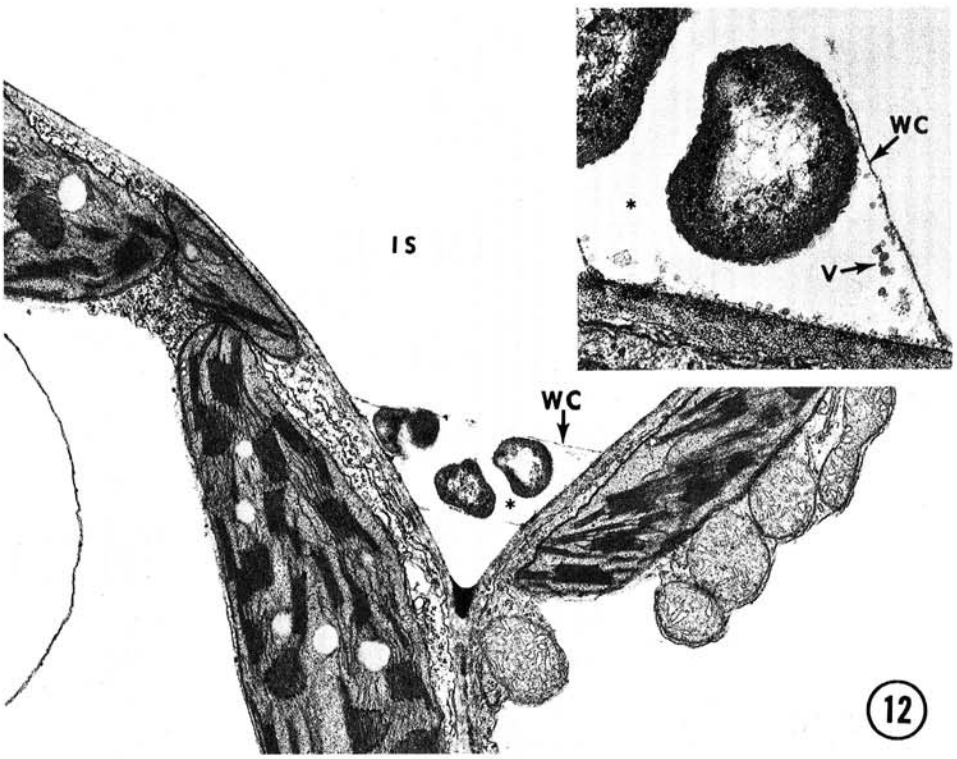
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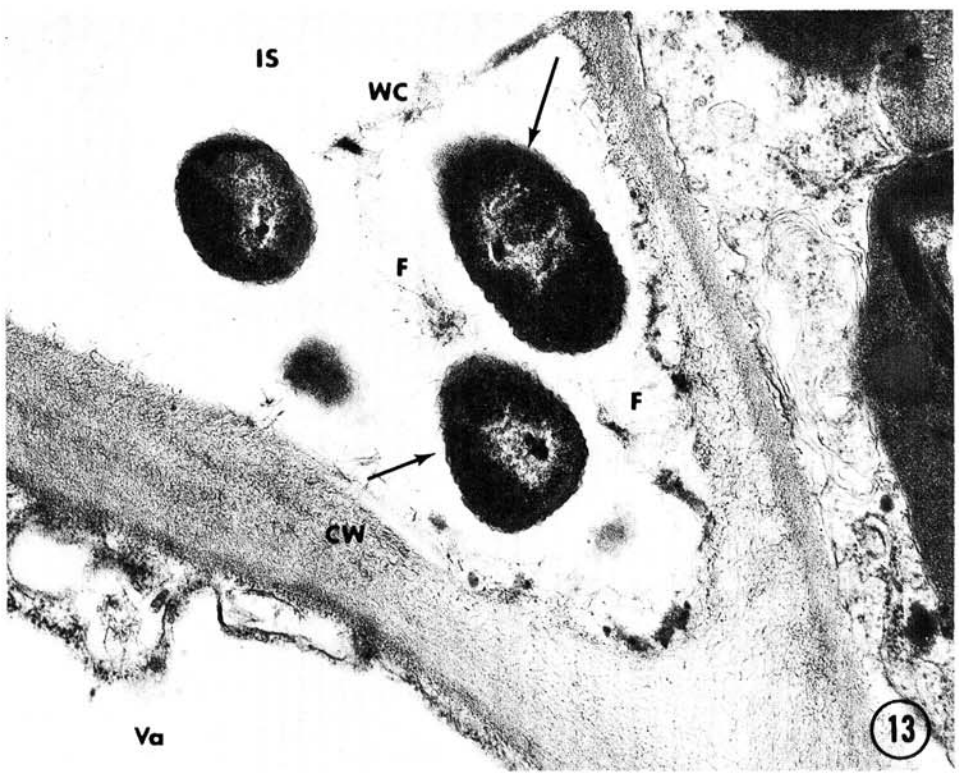
IS
WC
F
F
CW
Va
13