# Localized Cell Wall Appositions: Incompatibility Response of Tobacco Leaf Cells to Pseudomonas pisi

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ABSTRACT

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Two hr after tobacco leaf tissue was inoculated with *Pseudomonas pisi* (nonpathogenic to tobacco), the bacteria were localized in close proximity to plant cell walls. The plasmalemma in cells opposite the bacteria is convoluted and separates from the inner wall layers. Three to 4 hr later, loose microfibrillar material accumulated in the area between the plasmalemma and the inner wall layers directly opposite the localized bacteria. This accumulation was called a cell wall apposition. Six hr later the cell wall appositions were well organized massive structures that invaginated the plasmalemma. They appeared to be striated, an effect which reflected variations in packing density of the component microfibrils; membrane-bound vesicles frequently were found embedded within the layers of the electron-dense microfibrils. The vesicles originated from the convoluted plasmalemma opposite the attached bacteria and appeared to carry and deposit the microfibrillar materials that form the cell wall apposition. During the first 6 hr the general membrane damage that usually is associated with the hypersensitive reaction in cells was not noticeable. These observations suggested that pronounced cell wall appositions consist of newly synthesized cell wall material that is formed as a part of a complex series of reactions that lead to tobacco leaf cell death caused by the incompatible bacteria.

When tobacco leaves are inoculated with *Pseudomonas pisi* (a species which is not pathogenic on that plant) at $1 \times 10^7$ or $1 \times 10^8$ cells/ml, a series of reactions occur that lead to confluent tissue necrosis characteristic of a hypersensitive reaction (HR). Goodman and Plurad (3) reported that as early as 7 hr after inoculation the membrane system of the cellular organelles as well as the plasmalemma and the tonoplast have been extensively damaged. They attributed tissue collapse of the inoculated leaves to an extensive damage of the cell membrane systems. Goodman et al. (2) reported that 20 min after inoculation, cells of *P. pisi* are found in the proximity of cell walls, and at these points the mesophyll cell cuticle detaches from the underlying wall structure. Two to 4 hr later the bacteria are localized by the cuticle either at junctions between mesophyll cells or randomly along their exposed surfaces. The plasmalemma directly opposite these localized bacteria is highly vesiculated. Four to 6 hr after inoculation the wall cuticle became more complex and electron-dense and ensheathed the wall-attached bacteria. When the compatible pathogen *P. tabaci* was inoculated under similar conditions, only loose microfibrillar material was found around the bacteria (2). Goodman et al. (4) reported that when the concentration of *P. pisi* was reduced from $1 \times 10^9$ cells/ml to $1 \times 10^7$ cells/ml, an elliptically-shaped microfibrillar structure was found directly opposite the localized bacteria 6 hr after inoculation. This structure, which we have termed wall apposition, was formed only between the plasmalemma and the inner wall layers of the mesophyll walls. Furthermore, it was observed only where bacteria were attached or were in close proximity to the cell walls. Sequeira et al. (10) recently reported that avirulent and incompatible isolates of *P. solanacearum* attached themselves to and are localized on the surfaces of the walls of tobacco leaf cells. Their electron micrographs generally confirm our observations.

In this study we have monitored the formation of the cell wall apposition over a 6 hr time period in greater detail than reported previously (4, 8).

## MATERIALS AND METHODS

Tobacco plants, *Nicotiana tabaccum* L. cv "Samsun NN" were grown in controlled-environment chambers at 24 C under 14 hr day length as described previously (2). Fully-expanded leaves of 7-wk-old plants were inoculated with suspensions of *P. pisi*, a pathogen of pea, at a concentration of $1 \times 10^7$ cells/ml, according to the procedure of Klement et al. (5). Leaves infiltrated with sterile distilled water served as controls. The plants were returned to the growth chamber and leaf samples 1 $mm^2$ were excised 2, 3, 4, and 6 hr after inoculation, for electron microscopic examination. The tissue was fixed in 3.0% glutaraldehyde and 1.0% osmium tetroxide in 0.2 M phosphate buffer pH 7.3. Samples were left for 3 hr in the glutaraldehyde at 4 C and for 3 hr in the $OsO_4$ solutions at room temperature. The samples were dehydrated in a graded series of ethanol and embedded in Spurr's low viscosity resin (11). Thin sections were cut with a Reichert

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OMU-2 ultramicrotome and mounted on uncoated copper grids. The sections were stained with uranyl acetate (aqueous solution, 0.5%) for 45 min, then with Reynold's lead citrate (9) for 7 min, and examined with a JEOL 100B electron microscope.

## RESULTS

**Ultrastructural changes 2 and 3 hr after Inoculation.**—*Pseudomonas pisi* cells were found in the intercellular spaces of tobacco mesophyll parenchyma either in close proximity to the walls (Fig. 1) or at cell junctions embedded in a microfibrillar material (Fig. 2). In some instances the bacteria were immobilized by an electron-dense filament (Fig. 3, 4) believed to be of cuticular origin (2). The most prominent features observed in the first 2-3 hr after inoculation were the separation of the plasmalemma from the cell wall's inner surface and its extensive vesiculation in areas directly opposite the bacteria (Figs. 1-4). In the electron-lucent space between the plasmalemma and the inner wall layers loose aggregates of microfibrils mixed with electron-dense membrane-bound vesicles were observed (Fig. 4). This is a highly localized response of the mesophyll cells to the presence of bacteria and has been observed only directly opposite the bacteria. We have termed this fibrillar aggregation as "wall apposition".

**Ultrastructural changes 4 hr after inoculation.**—At this time the *P. pisi* cells were frequently embedded in a mixture of microfibrillar and amorphous material (Fig. 5, 6). The wall appositions were well formed and numerous vesicles were associated with these structures (Fig. 5, inset). A gradation in electron density of the wall apposition was regularly observed (Fig. 6) as the microfibrillar layers close to the mesophyll wall were more densely packed than those closer to the plasmalemma of the mesophyll cells.

**Ultrastructural changes 6 hr after inoculation.**—The wall appositions became highly organized dome-shaped structures, with definite striated patterns (Fig. 7-9). As previously indicated they were found without exception between the invaginated plasmalemma and the inner cell wall layers directly opposite the localized bacteria. Large numbers of membrane-bound vesicles were associated with both the plasmalemma and the wall appositions. Many of these vesicles were embedded within the wall apposition (Fig. 9, and inset) and others contained microfibrils similar to those found in wall appositions (arrow). Numerous microfibrils also were found loosely arranged around the bacteria in the intercellular spaces (Fig. 8, and inset). They appeared to have originated from the upper wall layers of the mesophyll cells. It was clear that cytoplasmic and organellar disruption at 6 hr (Fig. 9) was not nearly as extensive when plants were inoculated with $10^7$ cells/ml of *P. pisi* as with $10^9$ cells/ml (2).

## DISCUSSION

A common ultrastructural response of plant tissues infected by pathogens (fungi, nematodes, or viruses) is the swelling of the host walls in close proximity to the point of interaction. In addition to the swelling response, various modifications of the host walls also have been observed, such as formation of papillae at the site of penetration of host walls by fungi or cell-wall alternations in tissues infected by nematodes or viruses (12).

The morphological similarities of the wall appositions formed by tobacco cells in the presence of *P. pisi* and the papillae formed as a response to the direct penetration of host cells by fungal pathogens are striking. Politis (7) reported that papillae were formed by the epidermal cells of oat leaves in response to appressoria of the fungus, *Colletotrichum graminicola*. The dome-shaped, electron-dense papillae were formed prior to or during penetration of host walls. Aist and Israel (1) observed that 85% of the cysts of *Olpidium brassicae* induced papilla formation in root hair cells of cabbage in what appeared to be an induced-resistance reaction. The efficacy of papillae and our wall appositions as invasion-inhibiting structures has yet to be demonstrated; both appear to be pathogen-induced.

Although the formation of papillae frequently has been associated with the penetration of host walls by fungal pathogens, little is known about their chemical composition. In some instances, histochemical tests have indicated the presence of the polysaccharide, callose (12).

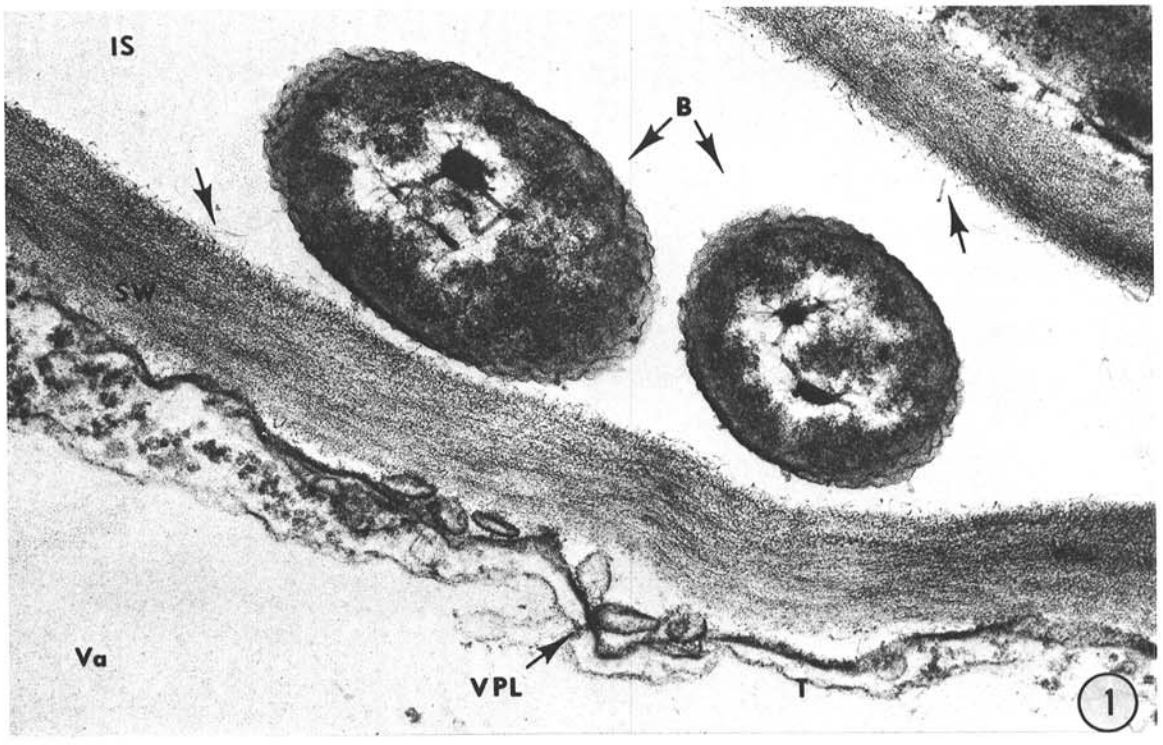
We have detected similar ultrastructural wall alternations of host cells in response to bacteria. The fact that wall apposition formation begins as early as 3 hr after inoculation (Fig. 4) suggests that this phenomenon is a direct response of the plant cell to the attached bacteria.

The lower inoculum concentration used in this study, $10^7$ cells/ml vs. $10^9$ cells/ml used previously (2) permitted us to view some of the early details of wall apposition development. At the lower inoculum level the drastic ultrastructural modifications generally accompanying HR were delayed, which permitted us to observe some synthetic processes of the cell in greater detail. The wall appositions observed by Goodman et al. (2) were not nearly so well defined as those presented herein. These workers used $10^9$ cells/ml, which we believe caused the HR to develop more rapidly, thus curtailing the extent to which wall appositions were formed. Our observations of dilated rough ER (Fig. 4, 6), vesicles, and large numbers of polyribosomes associated with regions of wall apposition, are suggestive of intensified synthetic processes.

Willison and Cocking (13) observed microfibrils, consisting primarily of cellulose at the outer surface of the plasmalemma of freshly isolated tobacco protoplasts. Earlier work with tomato fruit protoplasts cited by Willison and Cocking (13), indicated that microfibrils

Abbreviations used in electron micrographs: B = bacteria, BL = blebs, C = chloroplast, CL = cuticular layer, CW = cell wall, ER = endoplasmic reticulum, G = Golgi bodies, IS = intercellular space, M = mitochondrion, MF = microfibrils, PL = plasmalemma, R = ribosomes, SW = swollen wall, T = tonoplast, V = vesicle, Va = vacuole, VPL = vesiculated plasmalemma, WA = wall apposition.

Figure 1, 2 hr after inoculation; Fig. 2-4, 3 hr after inoculation; Fig. 5-6, 4 hr after inoculation; Fig. 7-9, 6 hr after inoculation.



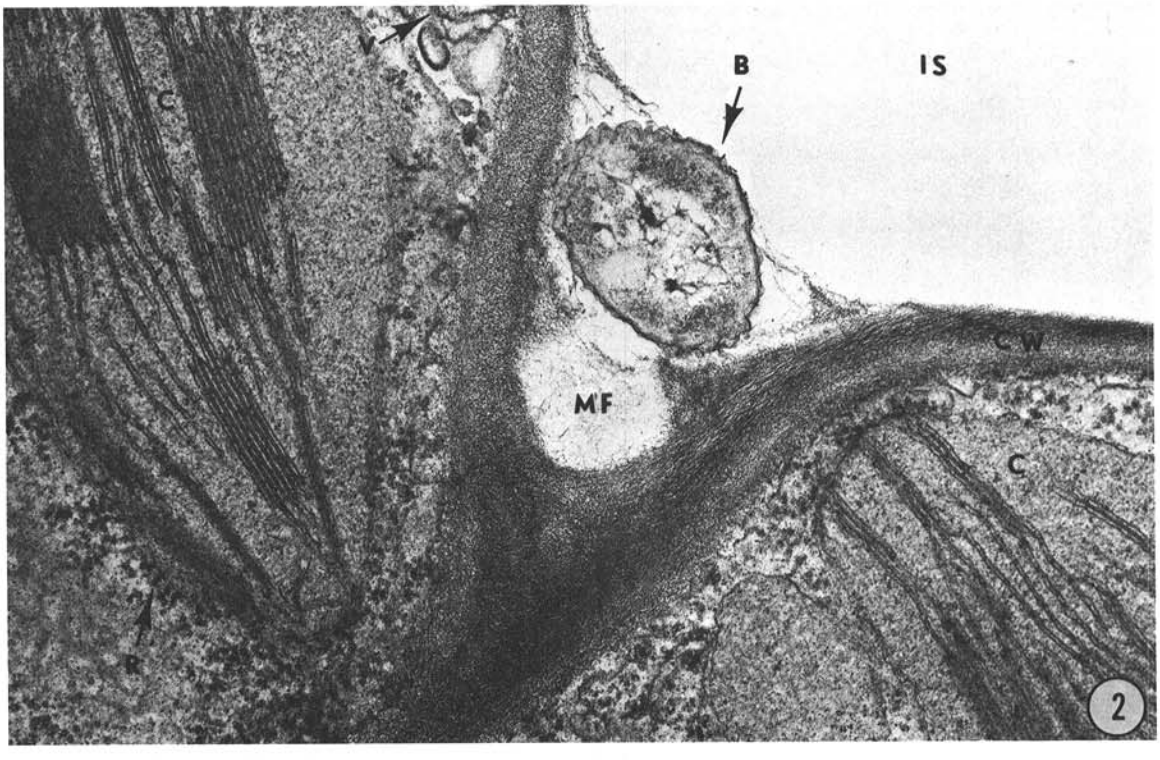
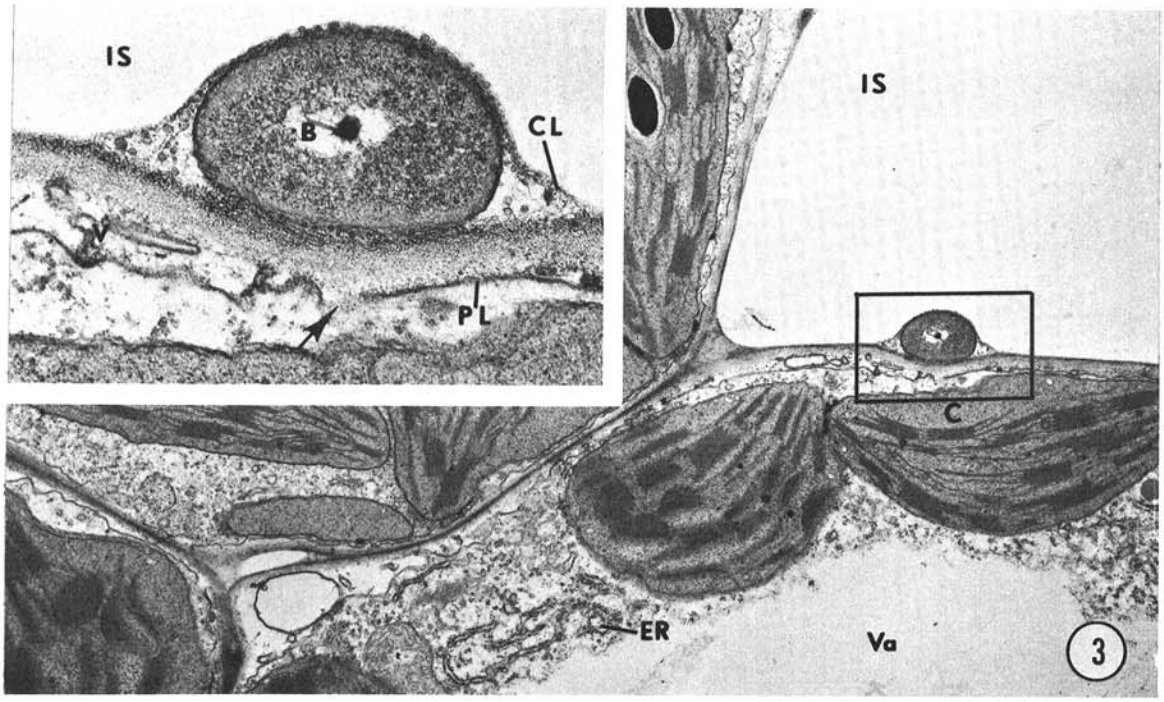




**Fig. 1-2.** 1) Cells of *Pseudomonas pisi* in intercellular space of tobacco leaf cells, 2 hr after inoculation. The walls appear swollen with numerous electron-dense microfibrils (arrows). Note convoluted plasmalemma (×45,000). **2)** A *P. pisi* cell 3 hr after inoculation embedded within electron-dense microfibrils in a cell junction. Electron-dense membrane-bound vesicles (arrows) (×54,000).



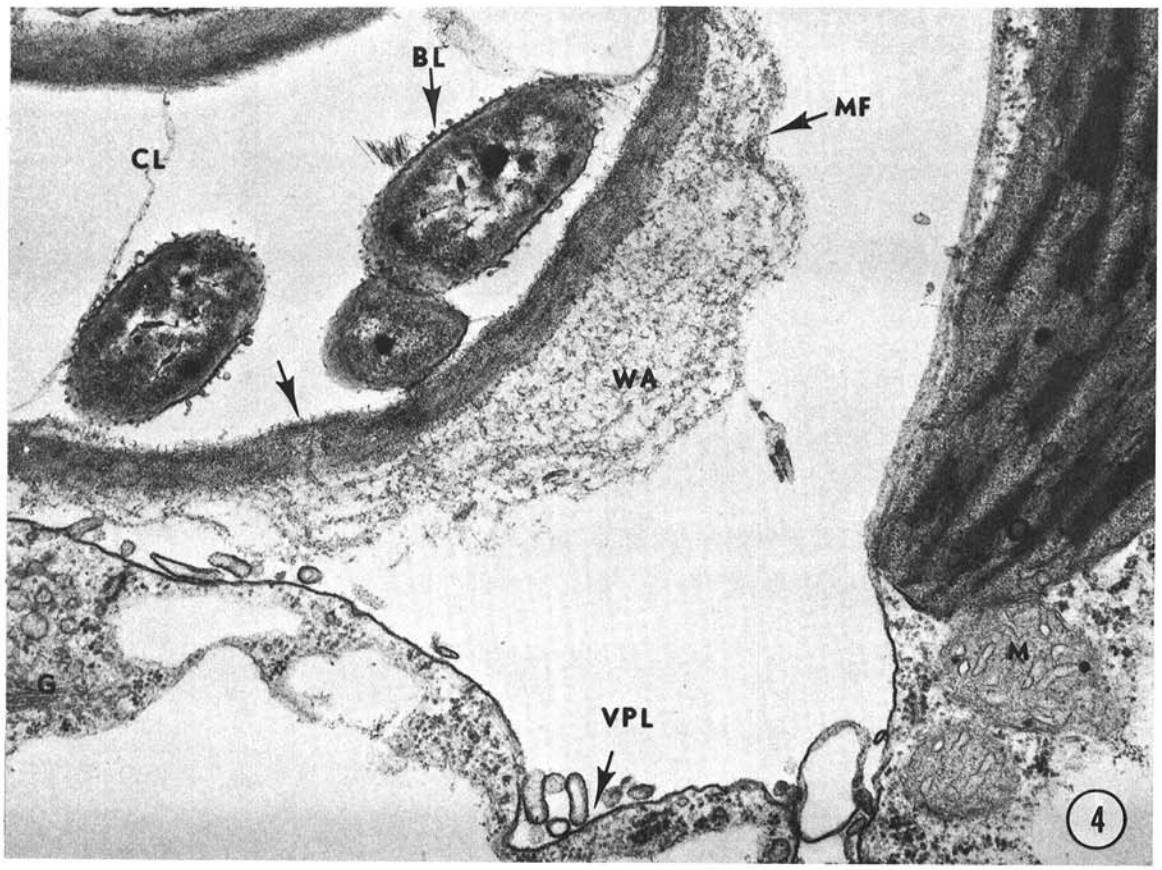




**Fig. 3-4.** 3) A localized bacterium of *Pseudomonas pisi* 3 hr after inoculation in intercellular space of tobacco leaf (×11,400). Note the increased electron-density of the upper wall layers and the accumulation of small vesicles at the bacterium-plant wall interface. 4) The highly convoluted plasmalemma has separated from the inner wall layers of mesophyll cells. Loose microfibrillar material has accumulated opposite the localized bacteria which is the early stage of wall apposition (×36,600).

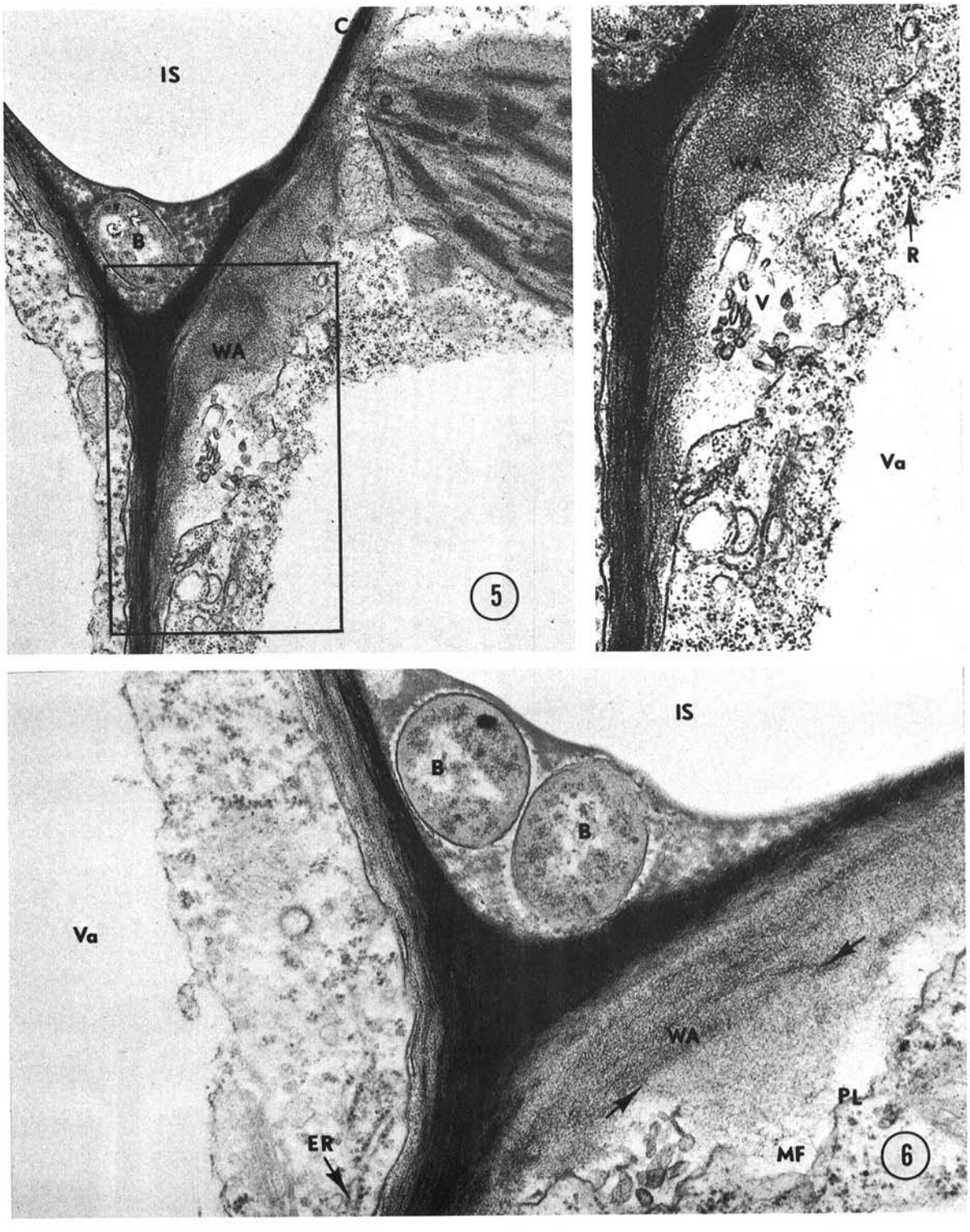
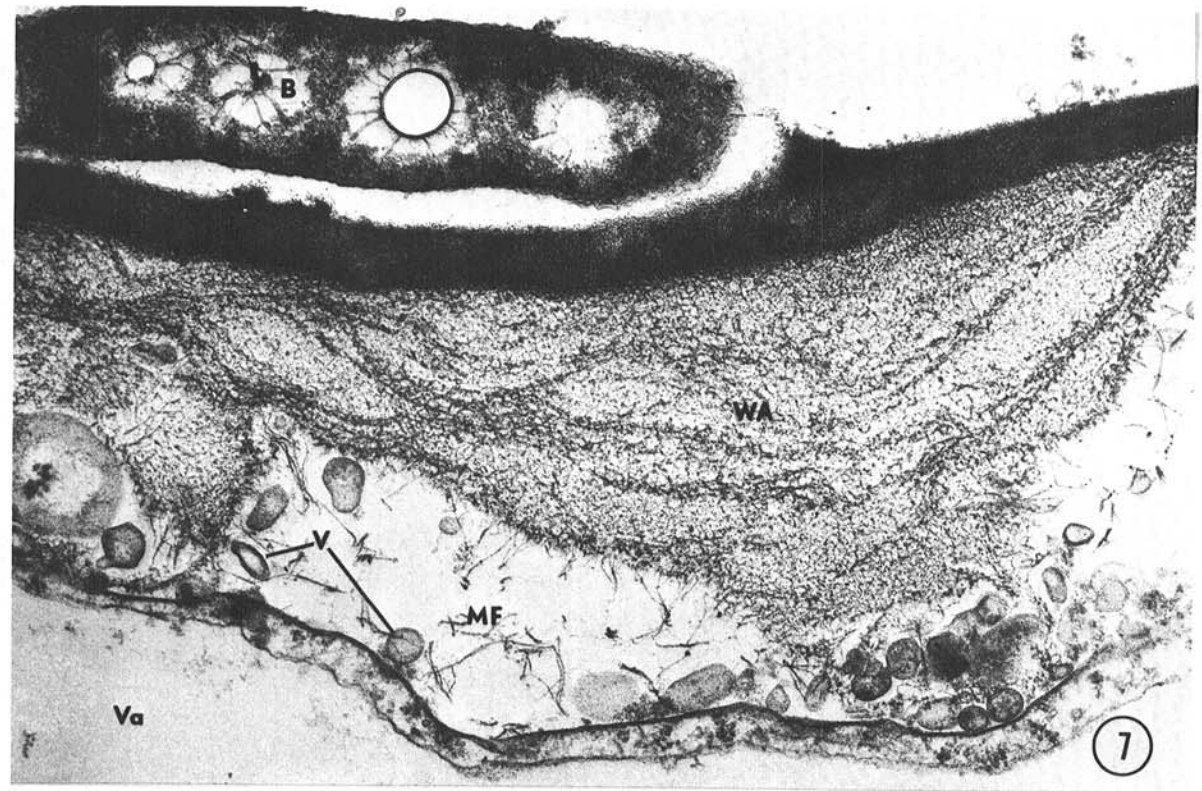



**Fig. 5-6.** Bacterial cells of *Pseudomonas pisi* embedded in a mixture of amorphous and microfibrillar material at a cell junction. By 4 hr after inoculation wall appositions are well formed structures associated with numerous vesicles (inset). **5)** [(×17,000), inset (×22,500)]. **6)** (×45,000).



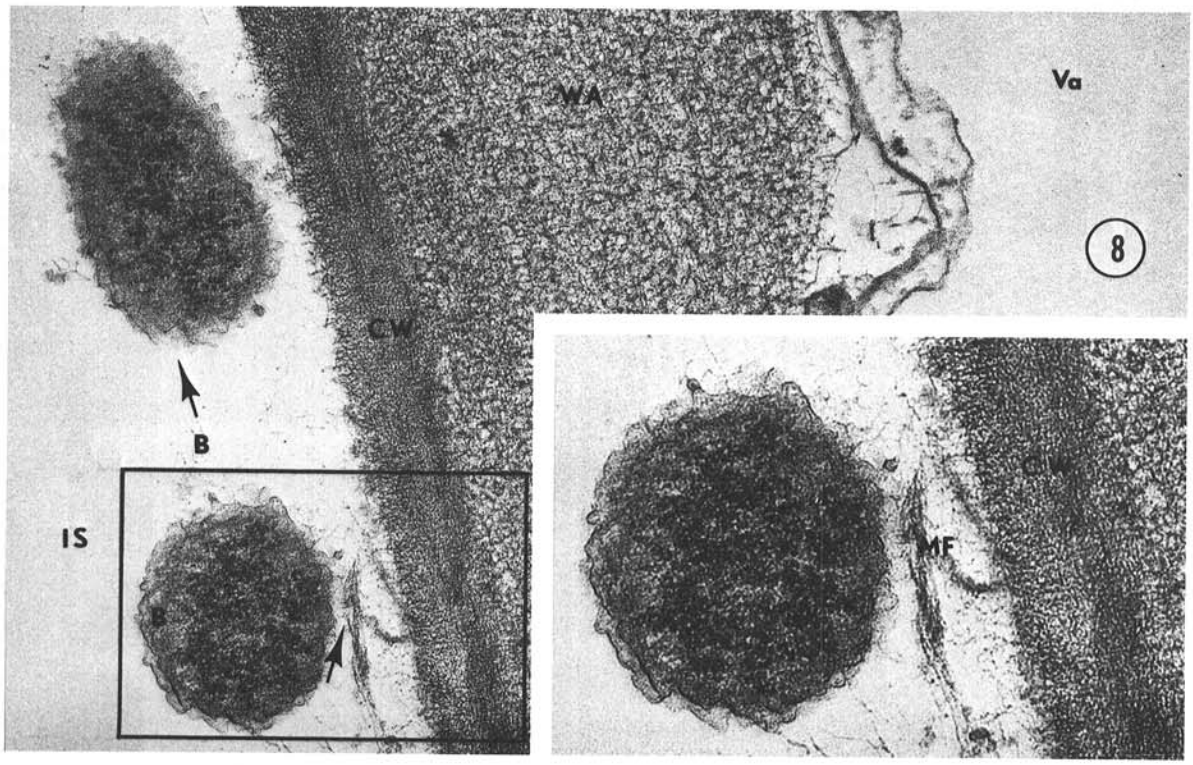




**Fig. 7-8.** 7) Well developed wall apposition beneath a localized bacterium of *Pseudomonas pisi* 6 hr after inoculation. Note the symmetrical formation of this structure. Numerous microfibrils intermingle with membrane-bound vesicles (×36,000). 8) *Pseudomonas pisi* cells in the intercellular space of tobacco leaf cells. Massive wall apposition, (×52,500). Numerous microfibrils and wall cuticle form a physical contact between the bacteria and plant cell wall (arrows), (inset, ×101,500).

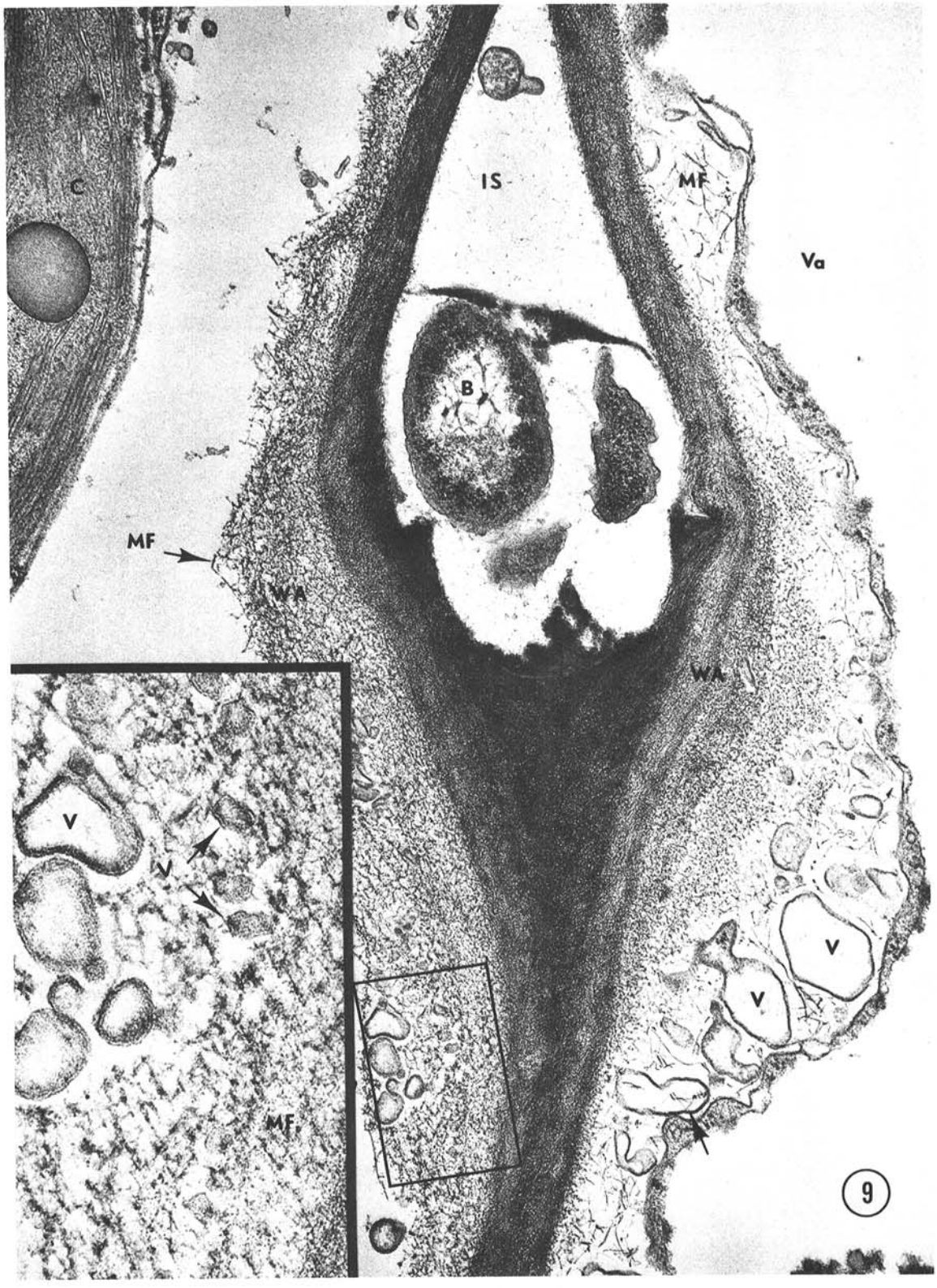



**Fig. 9.** Expression of HR symptoms 6 hr after inoculation. The walls of neighboring cells are collapsing. In a cell junction only portions of localized bacterial cells are shown. The protoplast of the cell on the left has pulled away from the inner wall layers while wall appositions are well developed on both cells. Some membrane-bound vesicles are embedded in the wall apposition (inset) (×115,000).

appear as early as 3 hr after protoplast isolation. We also detected wall apposition in tobacco mesophyll cells 3 hr after inoculation with bacteria.

The membrane-bound vesicles (Fig. 9) arising from the vesiculated plasmalemma of the mesophyll cells contain microfibrils that appear identical to those deposited in the successive layers of microfibrils in the wall apposition. It is likely that the majority of these microfibrils are synthesized at the plasmalemma-mesophyll cell wall interface and are then transported and deposited exocytotically along the inner wall layers. Collapsed vesicles were observed embedded within the region of apposition. Additional data (Politis and Goodman, *unpublished*) suggest that the Golgi dictyosomes and the highly dilated ER produce numerous vesicles that migrate towards the plasmalemma. It is possible, therefore, that these vesicles also may carry microfibrils, polymer precursor, or enzymes (6) each contributing to the formation of wall apposition.

As early as 2 hr after inoculation (Fig. 1, arrows) and up to 6 hr later, microfibrils accumulated in the intercellular spaces in the vicinity of bacteria (Figs. 2, 8, and inset). Goodman et al. (2) suggested that in the presence of bacteria the outer presumed cuticular layer of the mesophyll cell wall detaches and exposes the underlying fibrillar layers (Fig. 4, arrows). Hence, at least some of the loose microfibrils observed in Fig. 4 may be released at the time the cuticle separates from the wall.

That the mesophyll cell walls are relatively intact 6 hr after inoculation is an indication that the microfibrillar components of the wall apposition may have been synthesized during the development of HR, rather than the result of enzymatically altered or swollen cell walls. We contend that the wall appositions are part of a complex series of defense reactions which culminate in the physical localization of the incompatible bacterium and the death of the adjacent plant cell. Still unresolved is the chemical nature of the wall apposition. Histochemical work in progress may shed additional light on this complex host cell wall: bacterial interaction.

## LITERATURE CITED

1. AIST, J. R., and H. W. ISRAEL. 1977. Timing and significance of papilla formation during host penetration by Olpidium brassicae. Phytopathology 67:187-194.
2. GOODMAN, R. N., P.-Y. HUANG, and J. A. WHITE. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, Pseudomonas pisi, in tobacco leaf tissue. Phytopathology 66:754-764.
3. GOODMAN, R. N., and S. B. PLURAD. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1:11-15.
4. GOODMAN, R. N., D. J. POLITIS, and J. A. WHITE. 1977. Ultrastructural evidence of an "active" immobilization process of incompatible bacteria in tobacco leaf tissue. Pages 423-438 *in* Solheim and Raa, eds. Cell wall biochemistry related to specificity in host-plant pathogen interactions. Columbia University Press, New York. 487 p.
5. KLEMENT, R. G., L. FARKAS, and L. LOVREKOVICH. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.
6. MATILE, P. 1975. The lytic compartment of plant cells. Springer-Verlag. 183 p.
7. POLITIS, D. J. 1976. Ultrastructure of penetration by Colletotrichum graminicola of highly resistant oat leaves. Physiol. Plant Pathol. 8:117-122.
8. POLITIS, D. J., and R. N. GOODMAN. 1977. Localized responses of tobacco mesophyll cells, induced by Pseudomonas pisi, during the hypersensitive reaction. Proc. Am. Phytopathol. Soc. 4:(Abstr.) (In press).
9. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212.
10. SEQUEIRA, L., G. GAARD and G. A. DE ZOETEN. 1977. Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiol. Plant Pathol. 10:43-50.
11. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:32-43.
12. WHEELER, H. 1975. Plant pathogenesis. Springer-Verlag. 106 p.
13. WILLISON, J. H. M., and E. C. COCKING. 1975. Microfibril synthesis on the surfaces of isolated tobacco mesophyll protoplasts, a freeze-etch study. Protoplasma 84:147-159.