

Xylem Parenchyma Plasmolysis and Vessel Wall Disorientation Caused by *Erwinia amylovora*

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ABSTRACT

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Ultrastructural changes induced in Jonathan apple leaf petioles by inoculation with 5×10^4 cells of *Erwinia amylovora* were monitored after 24 and 48 hr. Although no external symptoms were visible within 48 hr, ultrastructural alterations were detected after 24 hr. These included xylem parenchyma plasmolysis, deposition of a vessel-occluding ground substance, and the appearance of brushlike fibrils on the inner surfaces and spiral secondary thickenings of the xylem cell walls. After 48 hr, the vessel-occluding substance appeared in more vessels and became more dense and xylem parenchyma protoplast material aggregated and shrank still further. Then the brushlike fibrils appeared to become more granular and seemed to aggregate with bacterial extracellular polysaccharide, possibly accounting

for the denser deposits of vessel-occluding material. The electron micrographs suggest that the often-reported lysigenous cavities develop due to the collapse of plasmolyzed xylem parenchyma cells. Release of bacteria into this newly formed intercellular space followed the rupture of xylem vessels that had lost the physical support formerly provided by the xylem parenchyma cells. The xylem vessels also showed what seemed to be wall dissolution. These ultrastructural observations revealed xylem parenchyma plasmolysis to be an early symptom of pathogenesis and substantiated previous reports that xylem vessels are sites for early proliferation of *E. amylovora*.

The wilting and tissue necrosis caused in rosaceous plant species by *E. amylovora* (Burrill) Winslow et al, 1920, is indicative of cellular destruction. However, there is no consensus on either the precise nature and site of early pathogenesis or how *E. amylovora* elicits those effects. Although as early as 1926 Pitman and Creuss (13) seemed to dispel the notion that tissue degradation was a reflection of pathogen-produced wall-macerating enzymes, the concept was kept plausible by the detailed cytological studies of Rosen (14,15), which suggested the activity of wall-destroying enzymes. However, the recent study by Seemüller and Beer (16) appears to have ruled out, with a degree of finality, the concept of wall maceration by pathogen-elaborated enzymes.

How then do we explain the several vivid descriptions, using light microscopy, of xylem wall rupture, middle lamella and wall dissolution (2,12,15), and the development of lysigenous cavities (2,4)? The latter are locales of collapsed parenchymatous tissue resulting in a cavity filled with bacterial cells. Huang and Goodman (6) suggested that the earliest stage of ultrastructurally detectable disease development by *E. amylovora* is accompanied by plasmolysis of xylem parenchyma, followed by the development of lysigenous cavities (12) in the affected areas (2). It was also observed that the polysaccharide produced by the pathogen, when absorbed by apple stem tissue, caused development of symptoms much like those caused by the pathogen (6). However, Sjulín and Beer (18) were unable to discern "directly detrimental" effects by the polysaccharide that could explain the early stages of pathogenesis. The current study and others (18,19) were conducted to detect more precisely the earliest ultrastructural changes that develop following inoculation of apple stem (8) and petiole (6) tissue with a virulent strain of *E. amylovora*. The objective of this report was to delineate ultrastructurally the necrosis feature of the fireblight syndrome. Research from our laboratory (4,6,19) demonstrated that xylem parenchyma plasmolysis, lysigenous cavity formation (precursors of necrosis) and xylem vessel

occlusion, each a facet of the fireblight syndrome, could be induced in Jonathan stem and petiole tissue by either the virulent bacteria per se or their extracellular polysaccharide (EPS). In this report the designations EPS and amylovin, the polysaccharide produced by *E. amylovora* in green apple fruit tissue (5), are used interchangeably. Recent findings (1) suggest that their molecular sizes and antigenic determinants are similar.

MATERIALS AND METHODS

Culture of the pathogen. The inoculum used was the highly virulent E9 isolate of *E. amylovora*. Lyophilized cultures were suspended in pH 7.0 phosphate-buffered saline (PBS) and after 30 min were transferred to a medium consisting of nutrient agar (1.5%), yeast extract (0.5%), and glucose (1.0%) in petri dishes (NYGA) that were incubated at 27 C for 48 hr. A typical colony was resuspended in PBS and immediately streaked on NYGA slants. The bacterial concentration of the inoculum, 10^8 cells per milliliter, obtained from slants was established in a Beckman spectrophotometer at $A_{500nm} = 0.10$. The E9 strain makes copious quantities of EPS, which was identified as a primary virulence character (1,3).

Host tissue. Our previous studies generally involved stem tissue (8) needle-inoculated with E9 midway between the apex and the first node. However, this procedure precluded precise administration of the inoculum. Further, stem tissue presented too large a mass of cells in which to monitor the development of pathogenesis. Hence, we inoculated the petiole of the youngest fully unrolled leaf (6) of a vigorous shoot of a greenhouse-grown 1-yr-old whip of the cultivar Jonathan. The leaf lamina was removed with a razor blade and a 0.5- μ l droplet containing 5×10^4 *E. amylovora* cells was placed on the surface with a Gilson Pipetman (Rainin Instrument Co. Inc., Mack Rd., Woburn, MA 01801). Controls were inoculated with a 0.5- μ l droplet of PBS. When the inoculation was performed at 0900-1000 hours in the greenhouse on a sunlit day, the droplet of inoculum is absorbed in 2-4 min.

Electron microscopy. Petioles were collected at 6, 12, 24, and 48 hr after inoculation and the second and third 2-mm segments from the cut surface were fixed in 4% glutaraldehyde and embedded in Spurr's medium as previously reported (6,19). Ultrathin sections

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were cut with a diamond knife on a Reichert OM-U2 (Wein, Austria; Hacker Instrument Co. Inc., Box 646, West Caldwell, NJ 07007) ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL-100B electron microscope (Jeol U.S.A. Inc., 11 Dearborn Road, Peabody, MA 01960).

RESULTS

Our observations were made exclusively on the second 2-mm petiole segment from the bacteria-inoculated petiole apex. These revealed no structural changes at 6 and 12 hr after inoculation. After 24 hr, however, several ultrastructural changes were detected in xylem and xylem parenchyma cells that were not apparent in control petiole tissue (Figs. 1-6). Although no externally visible symptoms of infection were apparent on the petiole 24 hr after inoculation, the ultrastructural modifications detected included intense plasmolysis of xylem parenchyma cells. Plasmolysis was most frequently noted in those parenchyma cells that bordered xylem vessels with lumens containing bacteria (Figs. 3-5). In addition, at 24 or 48 hr the xylem vessels containing bacteria almost always contained some granular ground substance (Figs. 3-5, 7,8). Occasionally this vessel-occluding substance could be seen in xylem vessels of inoculated petioles even though no bacteria were visible (Fig. 6). Here too, neighboring xylem parenchyma cells exhibited plasmolysis. Neither xylem parenchyma plasmolysis nor vessel-occluding ground substance was ever detected in control (water-inoculated) petiole tissue (Figs. 1 and 2). A third ultrastructural manifestation of infection was the appearance of a brushlike array of fibrils extending from the surface of the secondary wall and the helical secondary wall thickenings (Figs. 3-6). A high magnification of this structure, which, in the opinion of the authors, has never been reported before, is shown in Figs. 6 and 12.

At 48 hr after inoculation, further ultrastructural changes were observed. However, visible signs of infection on the petiole were confined to a pinching of the petiole apex; this appears more as an indication of desiccation of the apical 1 mm of cells of the inoculated petiole. Both control and bacteria-inoculated tissue revealed this symptom, yet neither revealed external symptoms of discoloration or necrosis at this time. The electron microscope revealed intensified cellular disruption; the plasmolyzed protoplasts of xylem parenchyma cells were even further aggregated and distorted (Figs. 9 and 10). In some instances the protoplasts were barely discernible, having shrunk to a thin band along the parenchyma cell wall (Fig. 10). The intensified plasmolysis and protoplast aggregation greatly diminished the turgor in several files of parenchyma cells, which allowed the adjacent xylem vessels to twist and rupture (Fig. 10). Rupture of the xylem vessels released bacteria into what had been intercellular space and the additional space that developed from the collapse of xylem parenchyma. This appears to be the manner in which the lysigenous cavities developed. It is into these cavities that bacteria were released (Fig. 10) and rapid growth occurred, and from which subsequent widespread transintercellular space movement of bacteria ensued some 48 hr or more after inoculation. At 48 hr we also observed bacteria being discharged from ruptured xylem vessels (Fig. 11). Some bacteria appeared to have gained entry into xylem parenchyma cells and to be growing there (Fig. 11). It is conjectured that their initial entry into these parenchyma cells occurred through cell wall lesions that developed much as they did in xylem vessels.

Finally, 48 hr after inoculation, the brushlike fibrillar structure on the wall surfaces of xylem vessels became granular in nature. Part of it appeared to become distributed through and contribute to the increased density of the ground substance in the vessel lumen in which the bacteria were embedded (Figs. 7 and 8). This has suggested that the increased density of the ground substance may reflect the agglutination of bacterial EPS and xylem wall fibrillar material.

DISCUSSION

Several features concerning pathogenesis by *E. amylovora* in apple tissue have remained anomalies. For example, how do lysigenous cavities develop if the pathogen does not degrade plant

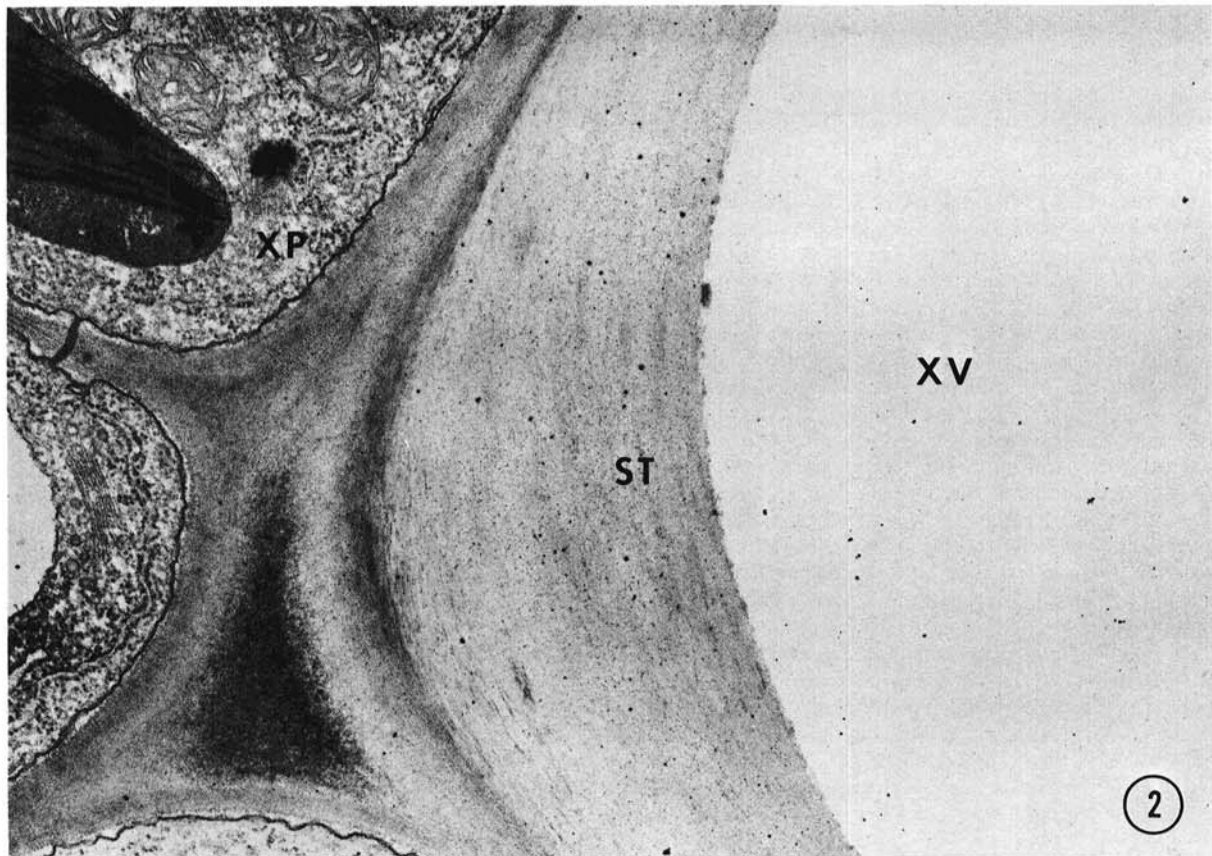
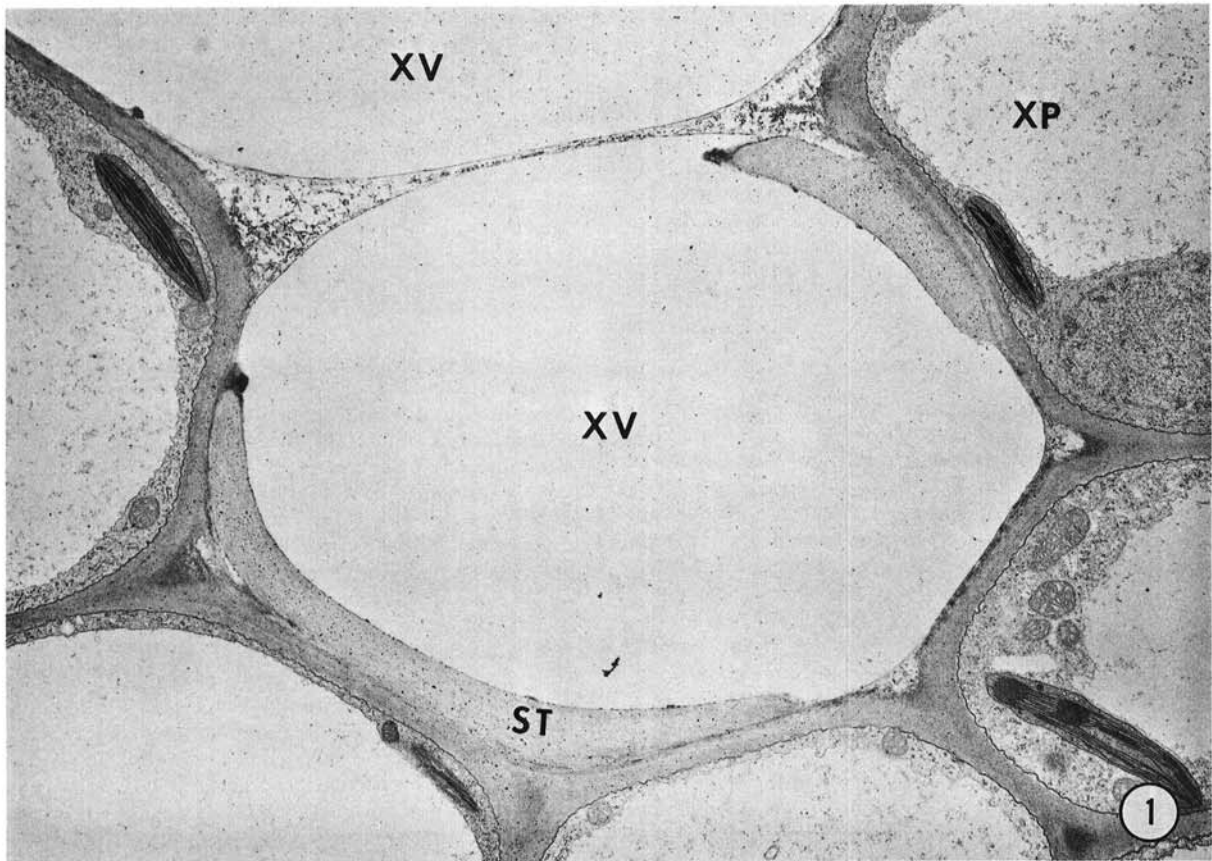
cell walls? Does bacterial EPS have a direct toxigenic effect on host cells? Is there a causal relationship between the observed pathogen-induced plasmolysis and EPS? As indicated previously, we use the terms amylovorin and EPS interchangeably (1,20). We believe that this report and others (1,7,18,20) now provide sufficient information to answer, at least in part, these questions.

Our previous reports that early systemic transport and growth of *E. amylovora* may occur in xylem vessels have been confirmed (18). In fact, the lumen of the xylem vessel may be the preferred and usual site of early pathogenesis. How these xylem-locked bacteria emerge into intercellular space and how this space gives rise to the oft-observed lysigenous cavities is made clear in Figs. 9 and 10. Intercellular space was enlarged by the collapse and shrinkage of xylem parenchyma cells. Nelson and Dickey (11) reported that the exact manner by which bacteria move from vessel elements into the surrounding tissues had not been determined. We suggest that the parenchyma cells lose their turgor due to damage from EPS-induced plasmolysis. This contention is supported by the study of Suhyada and Goodman (19) and verifies a previous report by Huang and Goodman (6). Plasmolysis of xylem parenchyma was also reported by Bachmann (2). Release of bacteria from the xylem vessels appears to be caused by a twisting and subsequent rupture of the vessels. This occurs following the collapse of the vessel-supporting xylem parenchyma cells. Once in the enlarged intercellular space, and nourished with substrate from collapsed xylem parenchyma and ruptured vessels, the bacteria grow rapidly. Visual symptoms of petiole reddening and necrosis usually appear 72 hr after inoculation.

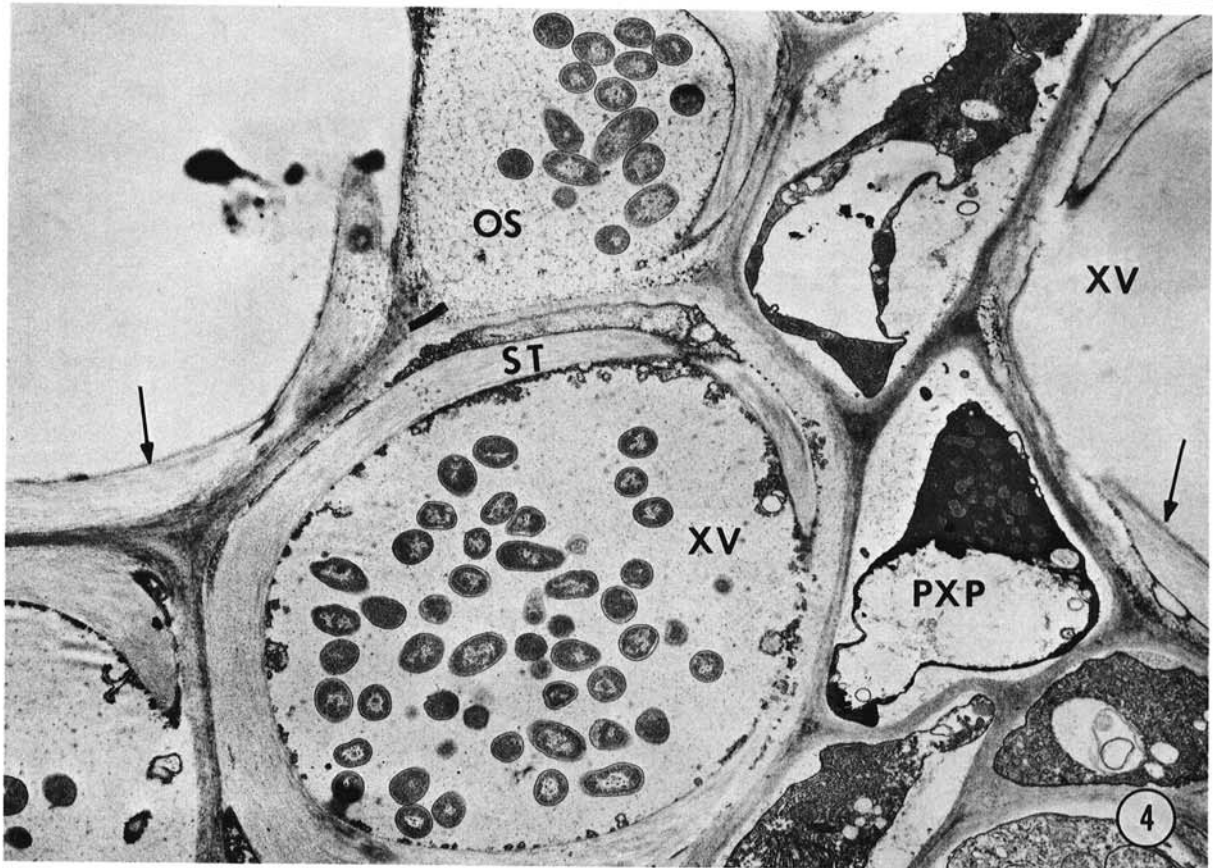
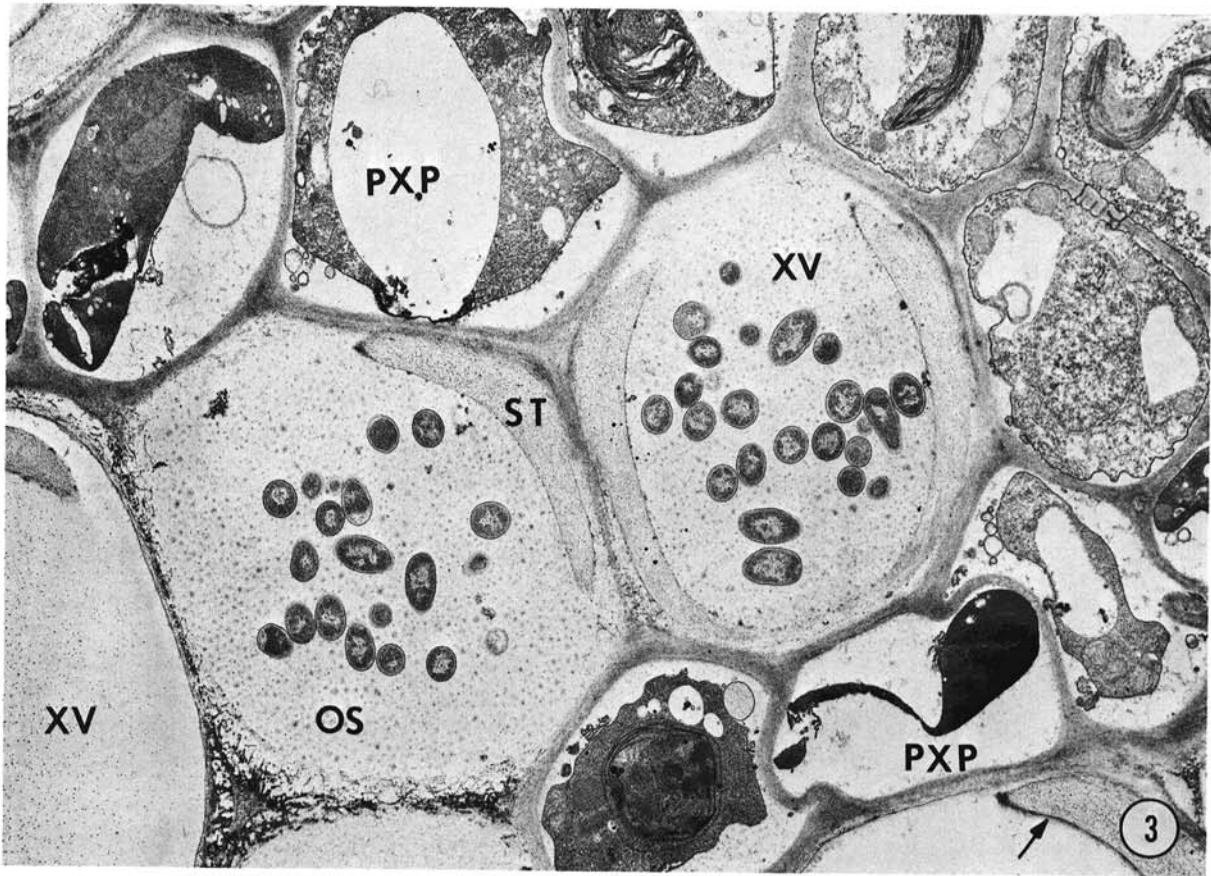
It seems apparent that xylem parenchyma plasmolysis and plasmalemma vesiculation in these cells (6) occurs in direct response to bacterial EPS (20). Although Sjulín and Beer (17) surmised that amylovorin (EPS) did not cause plasmalemma dysfunction, our observations suggest otherwise. Their contention is based on failure to detect electrolyte leakage. However, this study demonstrated that not all xylem vessels or xylem parenchyma cells are uniformly affected as was also observed by Bachmann (2). Further, xylem parenchyma cells comprise an estimated 10% of either petiole or stem cylinder volume. Hence electrolyte leakage from comparatively few parenchyma cells might be difficult to detect. Finally, their measurements of electrolyte leakage in leaf and stem tissue (of *Cotoneaster pannosa*) were made considerable distances from where we presume the effect of the amylovorin they applied was exerted.

Our studies (6,8,18,19) emphasized that the effect of either the bacteria or their extracellular polysaccharide must be localized in those tissues where high concentrations of the bacteria and/or amylovorin are present. We noted that EPS taken up by cut shoot bases (6,19) must exert its effect in the basal 1 cm of the shoot because excision of that part of the stem tissue permits the shoots to regain turgor on reimmersion in water. This occurs providing EPS-induced wilt has not ensued more than 4 hr. The actual damage by either *E. amylovora* or EPS to these shoots; ie, xylem vessel plasmolysis and xylem occlusion (6,19) occur almost exclusively where the bacteria or EPS are located. Figure 5 shows xylem parenchyma plasmolysis in cells bordering vessels whose lumens contained no bacteria but revealed both ground substance and brushlike fibrils. It is our opinion that these symptoms reflect the presence of bacteria either above or below the thin section under observation as bacteria were seen in subsequent serial sections. Attention is called however, to the fact that vessel occlusion and plasmalemma vesiculation can be induced by EPS *per se* (19).

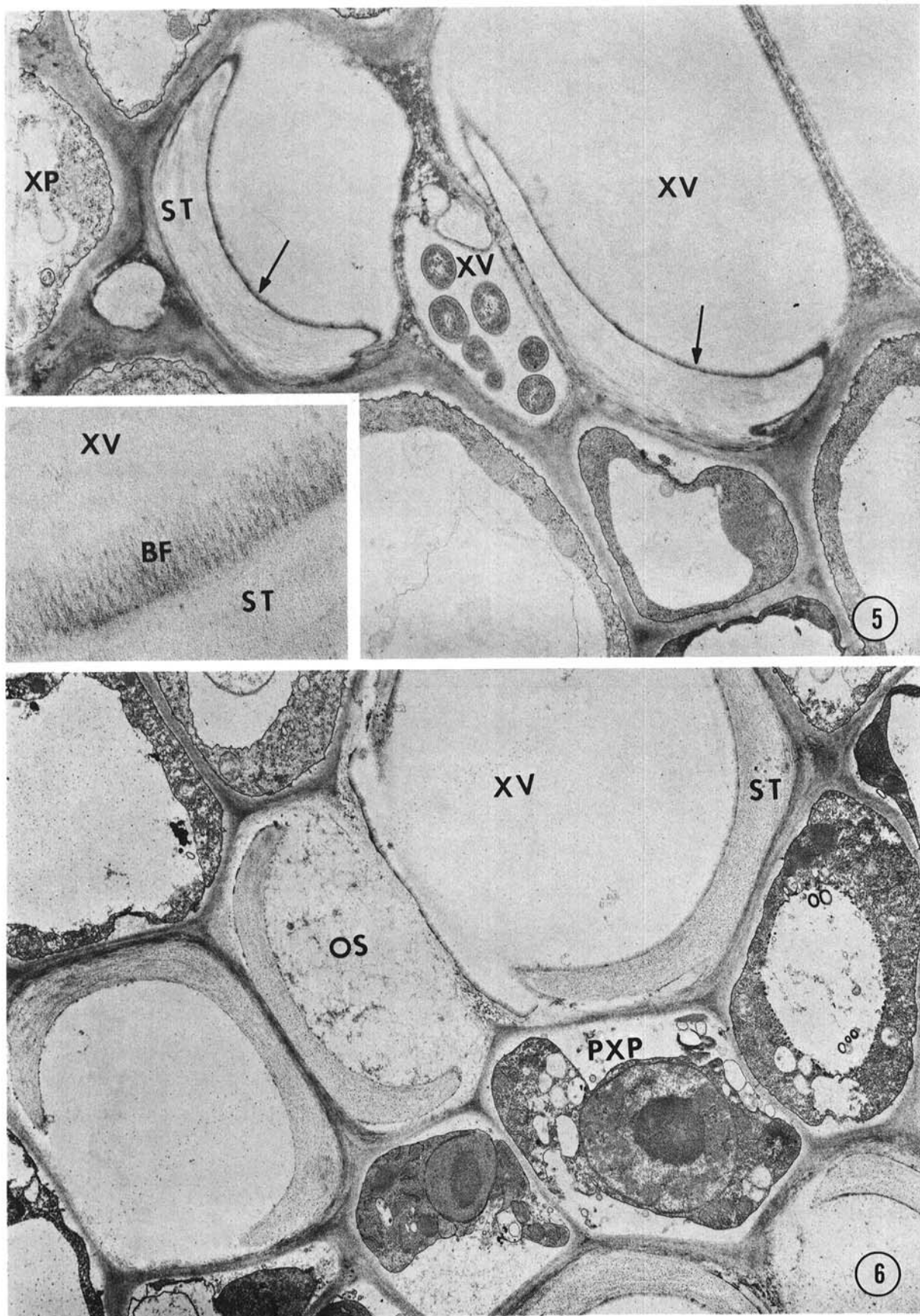
The observation of a fibrillar brushlike structure (Fig. 12) that became prominent 24 hr after inoculation is, in our opinion, being reported for the first time. This structure appears to be of host cell origin and is seen most clearly on vessel walls and their thickenings before these vessels have significant numbers of bacteria or levels of ground substance in them. These fibrils were never observed in control petiole tissue (Figs. 1 and 2) and seem to be an early response to the presence of bacteria or bacterial metabolites in neighboring vessels, Figs. 5 and 12. Their brushlike quality is most pronounced in vessels 24 hr after inoculation. However, after 48 hr



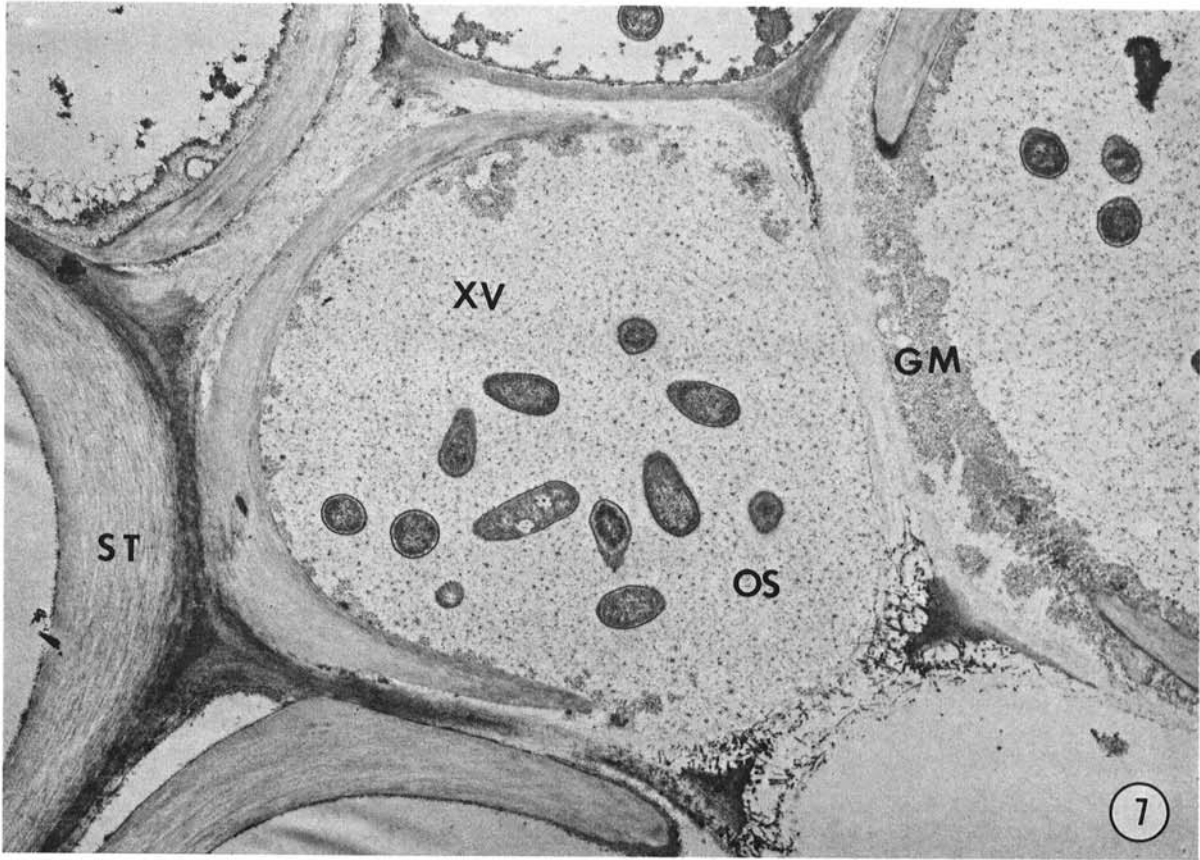
Figs. 1-2. 1, Control apple leaf petiole tissue 24 hr after inoculation with phosphate-buffered saline. Xylem vessel (XV) lumens are entirely clear, the surface of the secondary thickening (ST) of the XV is smooth, and five surrounding healthy xylem parenchyma (XP) cells show no signs of plasmolysis ($\times 2,600$). 2, Higher magnification of similar control tissue ($\times 42,000$).



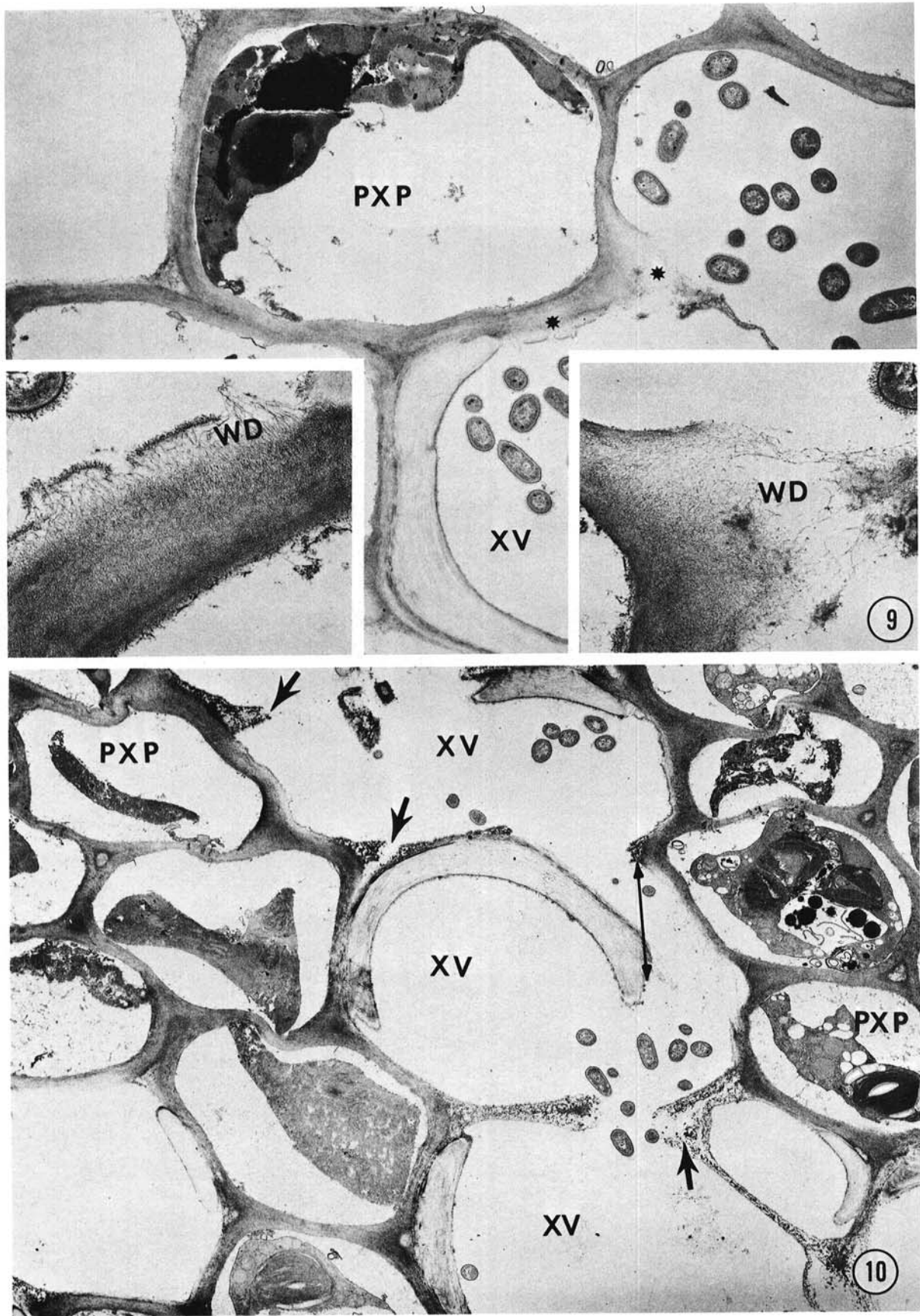
Figs. 3-4. 3, Infected apple leaf petiole tissue taken after 24 hr, 2 mm from the site of inoculation with 5×10^4 *Erwinia amylovora* cells. Six of eight xylem parenchyma (XP) cells surrounding three xylem vessels (XV) which are free of bacteria are also free of occluding substance (OS). The bacteria-free XV do show brushlike fibrillar arrays of the interface of the vessel wall and lumen (arrow) ($\times 2,600$). 4, Section of tissue taken under the same conditions as Fig. 3 revealing almost identical results from another experiment and a completely separate embedment. However, the brushlike fibrils (arrows) are more prominent ($\times 2,600$).



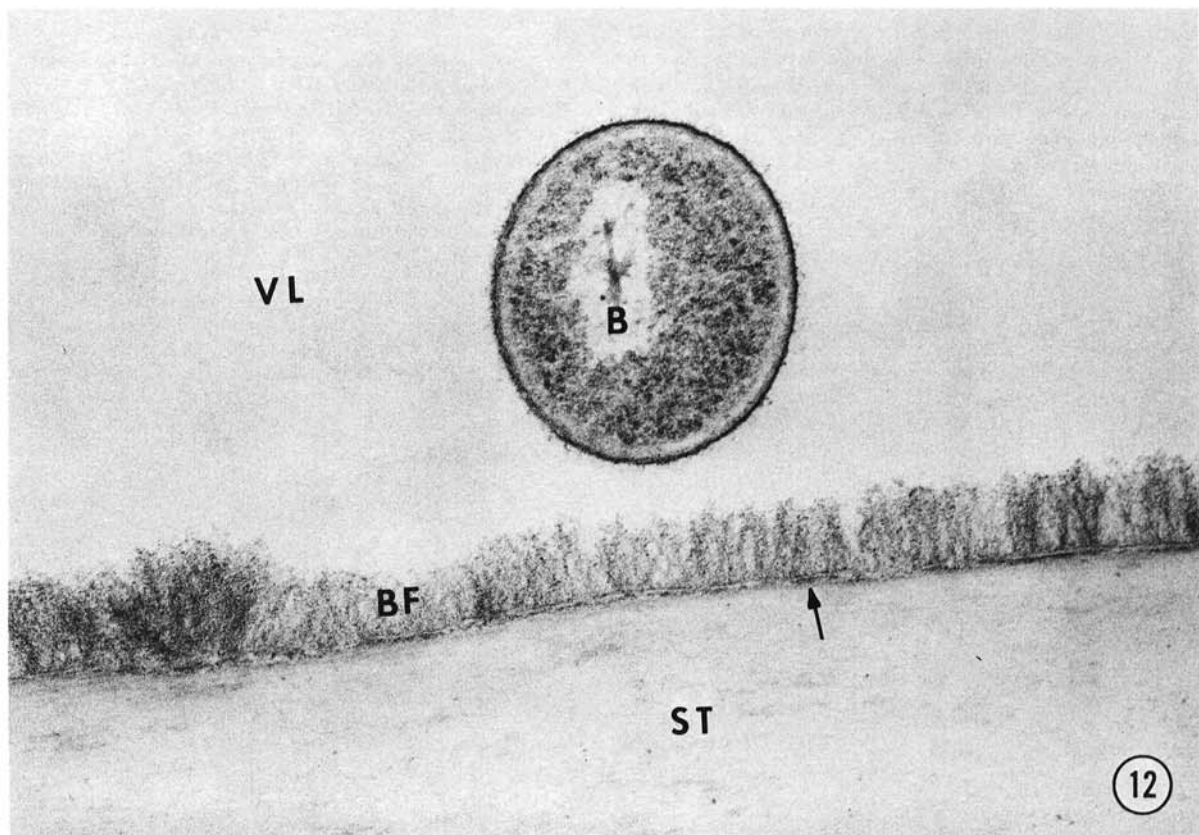
Figs. 5-6. 5, Apple leaf petiole tissue after 24 hr, 2 mm from the site of inoculation with 5×10^4 cells of *Erwinia amylovora*. The xylem vessel (XV) in the center contains several bacterial cells and a slight amount of ocluding substance. However, the two bordering XV are free of both ocluding substance (OS) and bacterial, yet the brushlike fibrillar array (BF) is clear in these vessels (arrows) ($\times 9,000$). A high magnification of BF is noted in the inset ($\times 50,000$). 6, A section of tissue neighboring that shown in Fig. 5. However, of the three XV shown only the center one has OS but no bacteria are present. The XV to the right reveals BF whereas the one to its left does not. The XV to the right reveals BF whereas the one to its left does not. All of the xylem parenchyma (XP) show varying degrees of plasmolysis. Subsequent serial sections revealed bacteria in the center XV ($\times 9,000$).



Figs. 7-8. 7, Apple leaf petiole tissue after 48 hr, 2 mm from the site of inoculation with 5×10^4 cells of *Erwinia amylovora*. Two xylem vessels showing heavier deposits of occluding substance (OS) and that the brushlike fibrils (BF) have become more granular (GM) ($\times 9,000$). 8, A higher magnification of the GM which appears to concentrate in the vicinity of vessel wall secondary thickening (ST) ($\times 21,600$).



Figs. 9-10. 9, Apple leaf petiole tissue after 48 hr, 2 mm from the site of inoculation with 5×10^4 cells of *Erwinia amylovora*. The two xylem vessels contain bacteria and the neighboring PXP is intensely plasmolyzed ($\times 7,200$). Two areas of the secondary wall of these cells (asterisks) have been magnified to show what appears to be wall dissolution (WD) ($\times 21,600$). 10, Apple leaf petiole tissue after 48 hr, 2 mm from the site of inoculation with 5×10^4 cells of *E. amylovora*. A file of XV is bordered by files of intensely plasmolyzed xylem parenchyma (PXP). Both middle lamellar, secondary wall and secondary wall thickenings have ruptured permitting bacteria to spill into what will become enlarged intercellular space. The rupture of XV clearly occurs as a consequence of the collapse of XP. This may be accentuated by wall dissolution which also is apparent in middle lamellar (arrows) material ($\times 4,500$).



Figs. 11–12. 11, Apple leaf petiole tissue taken after 48 hr, 2 mm from the site of inoculation with 5×10^4 *Erwinia amylovora* cells. A xylem vessel discharging bacteria into intercellular space (IS) created by the collapse of both ray parenchyma cells (RP) and a large area of xylem parenchyma (XP) opposite the bacteria (not shown) ($\times 9,000$). 12, A single bacterial cell in xylem vessel lumen (VL) near the surface of secondary wall thickenings (ST) that shows the nature of the brushlike fibrils (BF). Note also what appears to be a double membrane at the ST-BF interface (arrow) which may be wall cuticle ($\times 52,000$).

many more vessel lumens show vestigial fibrillar material at their peripheries (Figs. 7 and 8) and in addition contain vessel-occluding ground substances. The vestigial fibrillar material at this time appears more granular and seems to contribute to the density of the ground substance.

Our interpretation of the ontogeny of the lumen-occluding substance and the sequence of its development is as follows: 18–24 hr after inoculation, the bacteria begin to multiply in a limited number of vessels, which, as a consequence, also showed early stages of lumen occlusion. Neighboring vessels may be free of both bacteria and ground substance; however, the walls and surfaces of their spiral secondary thickening are covered by fibrils that have a brushlike orientation. In one instance (Fig. 5) we saw comparatively few bacteria in a xylem vessel lumen and only a minimal amount of occluding ground substance. In this vessel (see arrows) the brushlike fibrils also were apparent. This is probably an early stage of occluding ground substance formation. Note the fibrillar array in the two neighboring vessels.

We believe that these fibrils develop in xylem vessels in response to the presence of bacteria in the lumen of neighboring vessel elements. They may reflect the effect of activated macerating enzymes in the xylem wall. Morre (9) described a sequence of events for cell wall dissolution and enzyme secretion during leaf abscission that may be applicable. It is possible that the wall dissolution seen in Figs. 9 and 10 is also a consequence of a stimulated endogenous wall macerating enzyme system. As the number of bacteria in vessels increase and the numbers of vessels containing bacteria increase, the fibrillar orientation changes to a granular configuration (Figs. 7 and 8). This metamorphosis occurs only in vessels where bacteria are present or are nearby. We believe that the increased density of the occluding substance and the transformation of the fibrils to a granular form may reflect the aggregation of these two components into a gellike structure. A similar phenomenon was described by Morris et al (10) for *Xanthomonas campestris* EPS and cabbage cell wall polysaccharide. In fact, these authors suggest that the conformation of the fibrils plays a role in recognition of host by the pathogen. Van der Molen et al (21) described gel-filled vessels in banana infected by *Fusarium oxysporum* f. sp. *lycopersici*, which seemed identical in appearance with gels described by Suhayda and Goodman (19) that were induced either by amylovorin or *E. amylovora* per se. Van der Molen et al (21) were unsure whether the gel reflected “de novo synthesis as well as swelling of preformed wall structures.”

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