Early Proliferation and Migration and Subsequent Xylem Occlusion by *Erwinia amylovora* and the Fate of its Extracellular Polysaccharide (EPS) in Apple Shoots

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ABSTRACT

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Growth in intercellular space of a virulent isolate of *Erwinia amylovora* injected into shoot tissue of the susceptible apple cultivar Jonathan is limited to the wound site during the initial 48 hr after inoculation. During this period, systemic proliferation and migration of the pathogen occurs detectably only in mature annular and helical vessels. Occlusion of the vessels is caused by a dense fibrillar material associated with the presence of bacteria and is believed to be largely bacterial extracellular polysaccharide (EPS). Absorption of EPS by cut bases of apple shoots results in vessel occlusion similar to that seen in tissue infected by bacteria. Our electron micrographs indicate that the site of early bacterial proliferation and systemic migration is in mature xylem vessels rather than in the cortex and pith as reported in the earlier literature.

Additional key words: vascular transport, extracellular polysaccharide.

The wilting of young shoots followed by reddish-brown discoloration and necrosis of tissue are symptoms characteristic of infection by *Erwinia amylovora* in susceptible apple cultivars. Studies on the physiology of the disease caused by this vascular pathogen implicate a high-molecular-weight extracellular polysaccharide in the production of the wilting symptom (1,6,7,11,21,22,28).

High-molecular-weight polysaccharides produced by a large number of pathogenic bacteria and fungi are known to interfere with water transport in the vascular tissue of host plants (4,5,10,14,19,24,26–29). The molecular weights of these polysaccharides determine the specific plant tissues that will be affected. Hodgson et al (10) found that macromolecules of molecular weight <52,000 accumulated in the leaflets of treated tomato cuttings and caused leaf wilt, whereas those with molecular weights >52,000 remained in the main stem and resulted in shoot wilt. Van Alfen and Allard-Turner (25) demonstrated that alfalfa petiole and stem vascular tissues had different sensitivities to the disruption of water flow by dextran. Water conduction through petiole-stem junctions and leaf veinlets of alfalfa was stopped by 8 pmoles and 0.4 picomoles, respectively, of dextran with a molecular weight of 2×10^6 daltons; whereas in stems, water flow although reduced, was not shut off by 150 pmoles of dextran.

The production of extracellular polysaccharides (EPS) by plant pathogenic bacteria in host plants has been correlated with virulence in *Pseudomonas solanacearum* (4,8,14,16,28,29), *Xanthomonas campestris* (23,24), and *E. amylovora* (1,3,28). A high-molecular-weight polysaccharide (EPS) obtained from culture filtrates of *E. amylovora* and purified by gel chromatography had a molecular weight of approximately 40×10^6 daltons (1). By using a modified line rocket assay Ayers et al (1) established antigentic identity between the EPS from culture filtrates and the polysaccharide isolated from ooze on infected apple fruits. When used in the shoot wilt bioassay (1) EPS induced wilting in shoots of susceptible apple cultivars similar to that

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caused by the polysaccharide obtained from infected fruit and at identical concentrations and time intervals. Transmission electron microscope (TEM) studies of apple shoots infected by *E. amylovora* and of apple shoots treated with polysaccharide isolated from ooze suggest that virulent cells rapidly enter the xylem vessels and produce an occluding polysaccharide in the vessels that is, at least in part, responsible for the observed wilting (11).

The objectives of our study were threefold: first, to determine the tissue in which bacterial proliferation occurred initially; second, to relate this proliferation to polysaccharide production; and third, to attempt to determine whether there was a link between polysaccharide production by the bacteria and vascular occlusion and wilt.

MATERIALS AND METHODS

Lyophilized cultures of *E. amylovora* were resuspended in nutrient yeast glucose broth (NYGB) (22) and streaked on nutrient yeast glucose agar (NYGA) plates containing 50 mg of triphenyl tetrazolium chloride (TTC) per liter to obtain typical single-colony isolates (16). Virulent E9 colonies on TTC agar are large and mucoid with pink centers and wide white margins. In contrast, avirulent E8 colonies are small and nonmucoid with dark red centers rimmed by narrow white margins (16). Following incubation at 27 C for 48 hr, colonies typical for a given strain were selected, streaked on NYGA slants, stored at 4 C and used within a 2-wk period. When required, a slant was incubated at 27 C for 24 hr and a bacterial suspension was prepared by washing the slant with 5 ml of 0.001 M phosphate buffer, pH 7.0. Bacterial concentrations were adjusted on a Beckman spectrophotometer to an absorbance of 0.10 at 500 nm, which corresponded to 10^8 cells per milliliter.

Young Jonathan apple shoots were inoculated 5 cm below the apex in a puncture wound created by a 0.41-mm-diameter (27-gauge) hypodermic needle. Five microliters of 0.001 M phosphate buffer solution, pH 7.0, containing 5×10^5 cells of either the E9 or E8 strain was placed in the wound. Control shoots were inoculated only with buffer. The inoculated trees were kept in the greenhouse and the shoots were harvested 24 and 48 hr after inoculation. Jonathan shoots 5 cm in length also were treated for 4 hr with polysaccharide by placing their freshly cut bases into 100 μg/ml EPS isolated from the E9 strain of *E. amylovora* (1). Control shoots were placed in a 0.001 M phosphate buffer solution, pH 7.0. During the exposure to EPS the shoots were maintained in a growth chamber at 25 C with 70% relative humidity and a light intensity of 9.8×10^3 watts per centimeter (22).

Infected, EPS-treated, and control shoots were sectioned into 5-mm pieces. The inoculation point was at the center of the first 5-mm segment; additional sections were collected from 3 cm above to 3 cm below the inoculation point. The segments were immediately placed in vials containing 1 ml of a 1% osmium solution in 0.067 M Sorenson's phosphate buffer solution, pH 7.0, and held at 4 C for 72 hr. The specimens were washed twice for 0.5 hr with 3 ml of distilled water and then dehydrated in a graded series of ethanol-water: 20, 40, 60, 80, 95, and 100%, each for 0.5 hr. The ethanol was replaced with a graded amyl acetate-ethanol series 30, 50, 70, 90, 100, and 100%, for 0.5 hr. The samples were critical-point dried in a Parr pressure bomb.

The samples were sectioned in to 1-mm pieces, mounted on 15 × 5-mm stainless steel stubs (Structure Probe Inc.) and coated on a sputter coater (Polaron Instruments Inc., Doylestown, PA 18901) with either gold or a platinum-palladium mixture, prior to being viewed on a JOEL JSM-35 Scanning Electron Microscope at 15 or 25 kV.

Thin sections of 5-cm Jonathan shoots treated with EPS were also observed by transmission electron microscopy (TEM). After polysaccharide treatment, the shoots were cut into three 2-mm sections beginning at the shoot base and placed in a 4% glutaraldehyde fixative. Following fixation, the second and third segments were prepared by the procedure previously described (12). Ultrathin sections were viewed on a JOEL 100B transmission electron microscope at 100 kV.

RESULTS

Vessel ontogeny and function. During primary growth the young apple shoot has a large central pith area composed of storage parenchyma cells (Fig. 1). The external cell layer is the epidermis from which trichomes protrude. At higher magnification the individual components of the vascular bundle are distinct (Fig. 2). Along the inner surface of the bundle in the region abutting the pith are mature annular and helical xylem vessels that together compose the protoxylem. The major function of these vessels is to transport water during primary growth. Immature and mature scalariform, reticulate, and pitted vessels, which compose the metaxylem, are exterior to the protoxylem. The majority of these vessels are immature during primary growth and function in water conduction after secondary growth is initiated. The xylem components originate and differentiate from the cells of the vascular cambium. Outside the cambium are the phloem cells that function in the transport of nutrients. A group of thick-walled sclerenchyma cells compose the bundle cap. These provide structural support for the young shoot.

Mature helical xylem vessels (Fig. 3) are without protoplasts and have secondary thickenings inside their primary wall. Mature scalariform and reticulate vessels (Fig. 4) are also without protoplasts. The secondary thickenings of their walls differ from those of annular and helical vessels in that distinct slitlike or oval pits are clearly visible in the thickened cell wall. Immature vessels in the metaxylem may contain either intact or degenerating protoplasts. At full maturity these vessels will be devoid of protoplasts and protoplast fragments.

Location of the bacteria at 24 hr. Twenty-four hours after inoculaton, virulent bacteria were detected up to 5 mm from the inoculation point in mature annular and helical xylem vessels that were the predominant elements of the xylem invaded by the bacteria (Figs. 5–8). They were rarely observed in scalariform or reticulate vessels or other components of the metaxylem. Some helical vessels were totally occluded by bacteria (Figs. 5 and 6), whereas others had only a few bacteria scattered in the vessel lumen (Figs. 7 and 8). Bacteria in xylem vessels were frequently associated with a fine weblike, fibrous material (Figs. 6 and 11). This fibrous material was also found in vessels of infected shoots in the absence of bacteria but never in control tissue. Whether the fibrous material is totally of bacterial origin or in part elaborated by the plant is not known. Vessels in an advanced state of infection were totally occluded by dense fibrous material in which bacteria were clearly distinguishable (Figs. 9 and 10). Colonization of tissues other than the xylem vessels was not observed further than 1–2 mm from the inoculation point.

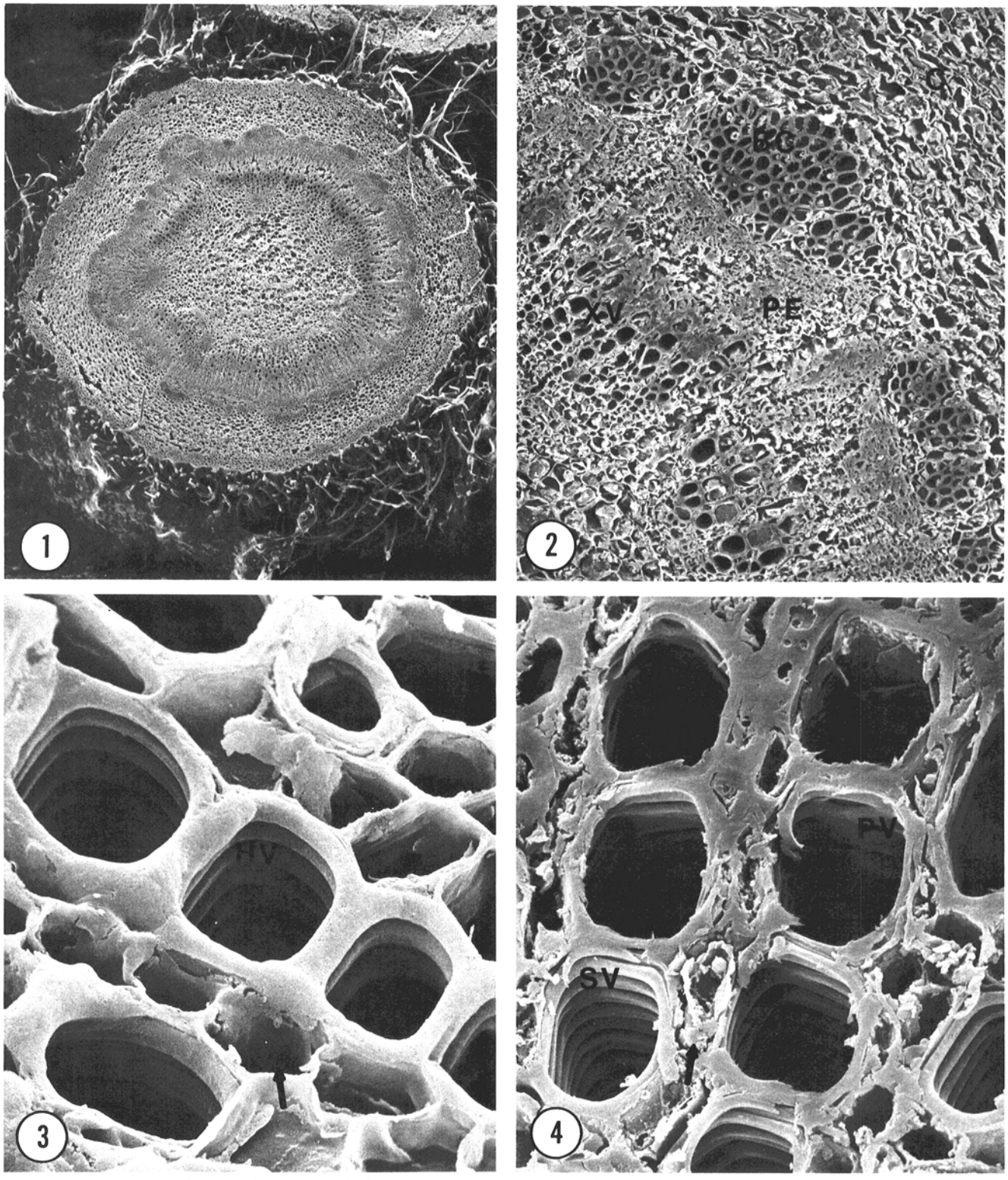
Location of the bacteria at 48 hr. Forty-eight hours after inoculation the number of mature helical xylem vessels occluded by bacteria embedded in dense fibrous material (Figs. 12–15) had increased. The fibrous material had a density ranging from loose and weblike to a densely fibrous matrix. Bacteria also were embedded in a dense solid material (Figs. 16–19) that resembled material in aerial strands exuded from the surface of shoots from infected apple and pear trees (15). This solid matrix with embedded bacteria totally occluded the lumen of many vessel elements. The increase in the number of bacteria observed in xylem vessels at 48 hr coincided with the observed increase in densely fibrous material in the vessels. At this time bacteria were still not observed outside the xylem vessels.

Wound-site bacterial growth. Young shoots infected with *E. amylovora* exude a sticky matrix (often referred to as ooze) from wounds and natural openings such as lenticels (Figs. 20–23). This material can be observed in wounds as early as 12–24 hr after inoculation, but is most evident 36–48 hr after infection and is a reflection of bacterial proliferation. As seen in the scanning electron microscope (SEM) at low magnification, this matrix appears to be amorphous (Figs. 20 and 21), but at higher magnifications, masses of bacteria can be seen embedded in a fibrillar structure (Figs. 22 and 23).

The bacteria proliferate rapidly at the wound site and are exuded to the surface of the shoot from the wound. The fibrillar material

observed is similar to that found in occluded vessels (Figs. 9–15). A similar egress of bacteria in a fibrillar matrix has been observed from stomates of peach leaves infected with *Xanthomonas pruni* (17). Colonization of intercellular space by bacteria in the cortex or pith was restricted to 1–2 mm from the point of inoculation even at 48 hr after inoculation.

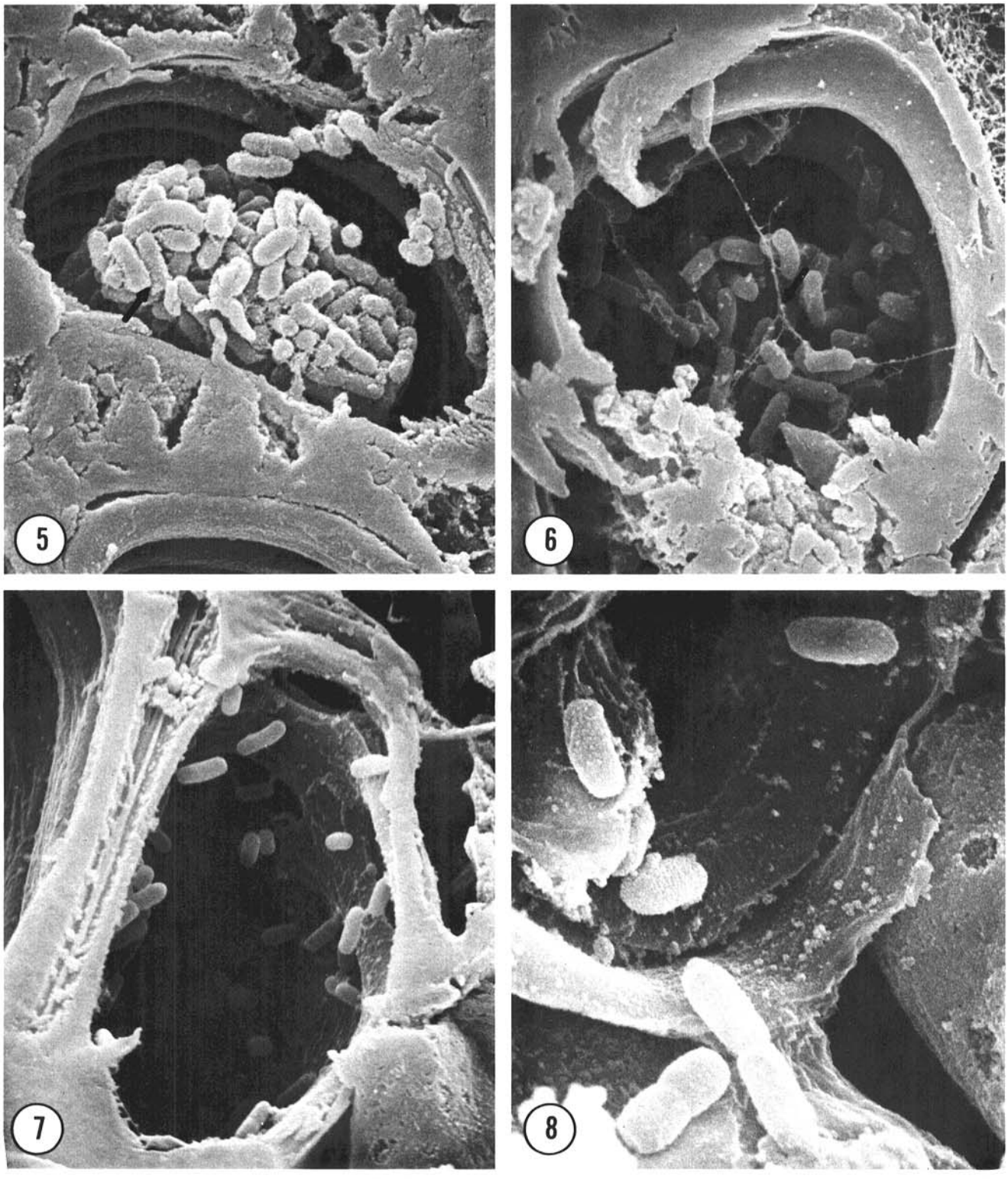
EPS-deficient bacteria. Shoots inoculated with the EPS-deficient avirulent E8 strain were examined by SEM at 24 and 48 hr after inoculation and only a few bacteria were found in either xylem vessels or at the wound site. The bacteria that were observed were



Figs. 1–4. Vascular anatomy of healthy Jonathan apple tissue. **1,** Young Jonathan shoot tissue, excised 5 cm below the apex, showing a central pith area bordered by vascular bundles. The vascular system is surrounded by the cortex, which is bordered by a thin layer of epidermal cells (×32). **2,** Individual vascular bundles with bundle caps (BC). Mature and developing xylem vessels (XV) are adjacent to the pith. The vessels develop from the vascular cambium, which divides the bundle in half. Along the exterior of the cambium are the phloem elements (PE). Darkly colored cells located between the phloem and the cortex (C) are sclerenchyma cells that form the bundle cap (BC) (×265). **3,** A file of mature helical vessels (HV) lined on either side by xylem parenchyma cells (arrow). Mature helical vessels function in water transport (×2,381). **4,** A group of mature scalariform (SV) and pitted (PV) vessels adjacent to xylem parenchyma (arrow). Large pits in the wall are visible in the secondary thickenings (×1,455).

located directly adjacent to the wound. Occlusion of vessels by either bacteria or fibrous material was not detected. Apparently the E8 cells were unable to proliferate and colonize the tissue. The few cells observed were localized at the wound site (13).

EPS-induced vessel occlusion. Shoots treated with EPS solutions were also examined by SEM (Figs. 24–27), which revealed that mature water-conducting vessels in these shoots were more uniformly occluded by dense fibrous material than were vessels in bacteria-inoculated stem tissue. This fibrous material closely resembled the occluding material found in vessels initially infected with *E. amylovora* (Fig. 9, 10, 14, and 15). The EPS had in many instances shrunk away from the wall of the vessel (probably a dehydration artifact); however, it retained the imprint of the secondary thickenings of the vessel on its surface (Figs. 2, 4, and 25). It is presumed that the vessels showing blockage are the ones most heavily involved in water transport.

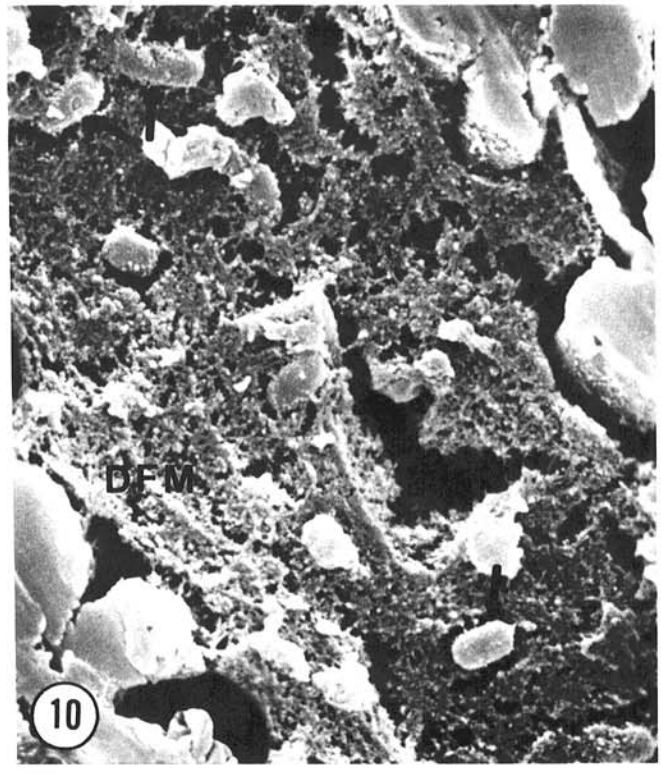


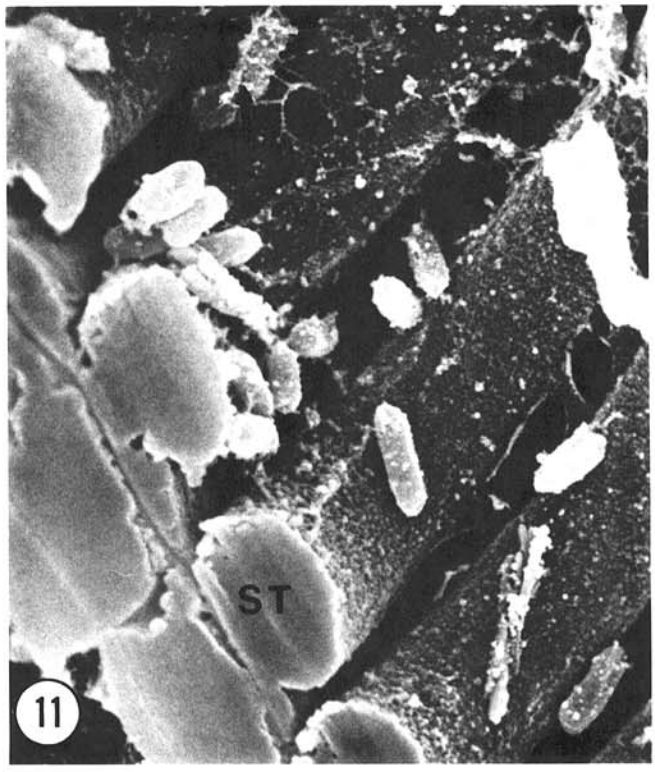
Figs. 5–8. Appearance of bacteria in xylem vessels 24 hr after inoculation with the virulent E9 isolate of *Erwinia amylovora*. **5,** A mass of bacterial cells (arrow) occluding a helical xylem vessel located 5–10 mm from the inoculation point (×7,130). **6,** Bacterial cells occluding a helical xylem vessel within 5 mm of the inoculation point. Fine fibrous strands (arrow) can be seen around the bacteria (×8,740). **7,** Bacterial cells in a mature pitted vessel within 5 mm of the inoculation point (×7,130). **8,** Enlargement of bacterial cells in a mature pitted vessel within 5 mm of the inoculation point (×5,870).

Transmission electron micrographs of EPS-treated shoot tissue confirm the SEM results and are shown in Figs. 28 and 29. Mature helical vessels observed only 4 hr after the uptake of EPS solutions are occluded by material that has a fine fibrillar or loose gellike character. Ray parenchyma cells adjacent to the blocked vessels were plasmolyzed. Vesiculation of the plasmalemma is also evident at the paired pits in the wall between the parenchyma cells and the vessels.

DISCUSSION

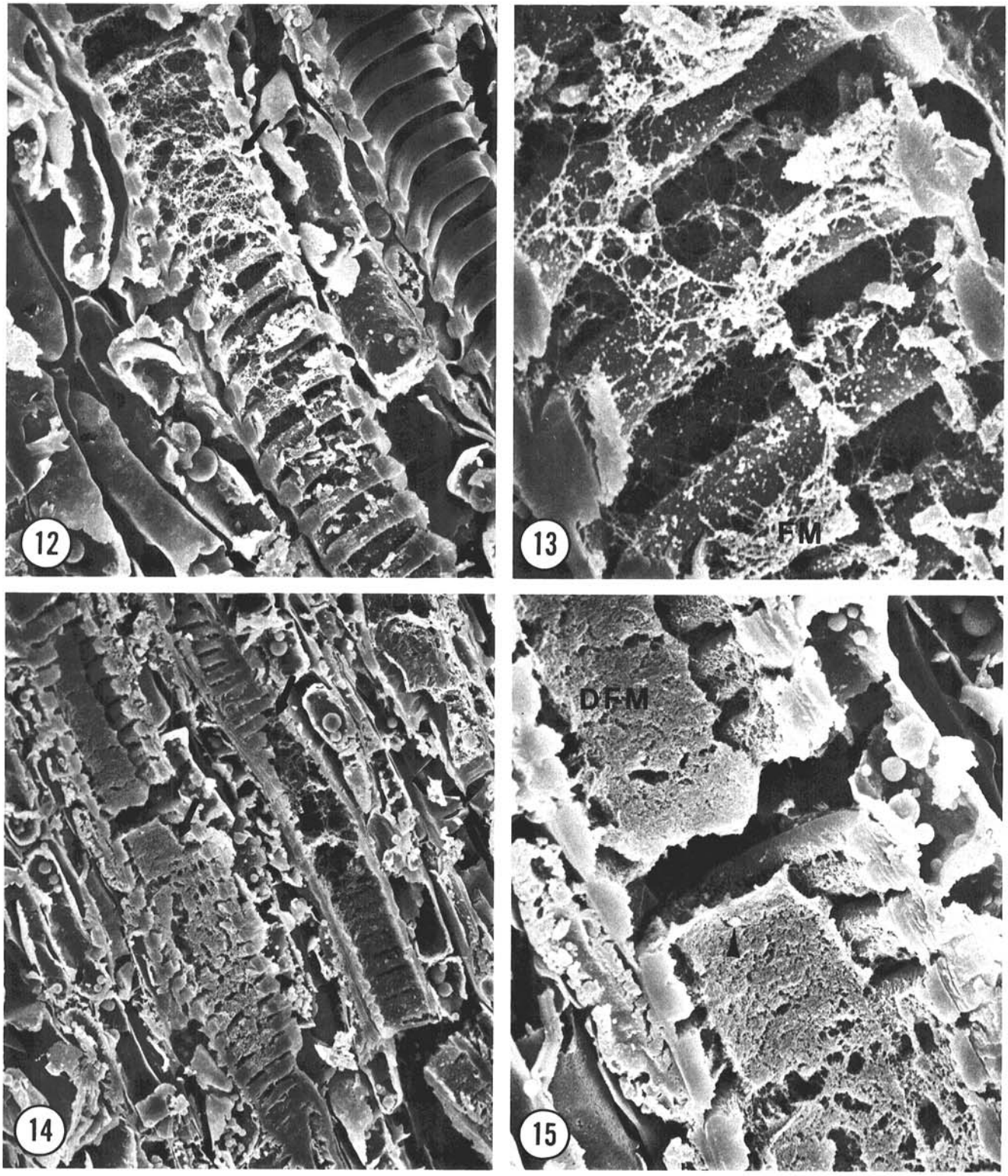
We have interpreted our electron micrographs as suggesting that virulent *E. amylovora* cells colonize young Jonathan shoot tissue





Figs. 9–11. Apple shoot xylem vessels in an advanced state of infection 24 hr after inoculation with the virulent E9 isolate of *Erwinia amylovora*. **9,** Three adjacent helical vessels (HV) within 5 mm of the inoculation point containing bacteria. The vessel in the lower left corner is totally occluded with a dense fibrous material (DFM) in which bacteria are embedded. The other two vessels contain bacteria but are without the fibrillar material. Some fine fibrous material is associated with the bacteria in these two vessels (×2,645). **10,** Higher magnification showing bacteria (arrows) embedded in a dense fibrous material (DFM) (×8,740). **11,** Higher magnification of bacteria in a helical vessel (HV) laying on secondary thickenings (ST). Fine fibrous material (FM) associated with the bacteria appears to originate from the bacterial cells (arrow) (×9,445).

randomly and intensely at the wound site. However, at 1–2 mm from that site, detectable proliferation and movement occurs in mature xylem vessels up to 5 mm from the wound site within 24 hr after inoculation. Bacteria neither colonized nor migrated through immature scalariform, reticulate, or pitted vessels, which contained both protoplasts and end walls. Inoculation of the puncture wound in the shoot presented equal opportunity for infection to occur in intercellular space as well as xylem vessels. Yet exclusive of the wound region the bacteria selectively colonized and migrated through the xylem elements rather than intercellular space of either pith or cortical tissue. No zoogleal mass movement was evident. Our results support the earlier studies of Huang and Goodman (11) who reported that *E. amylovora* cells rapidly invade xylem following inoculation into apple petiole tissue. The data presented

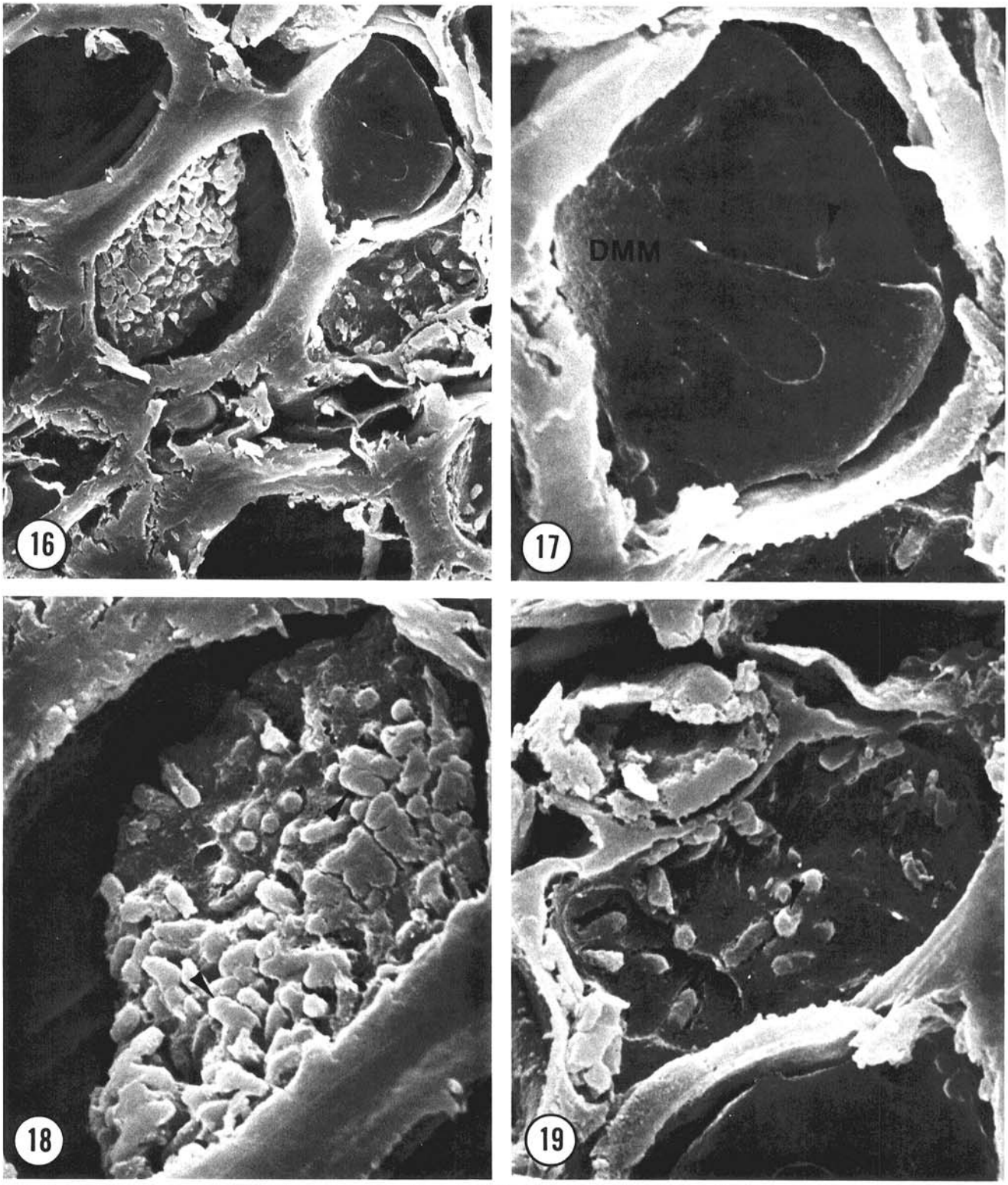


Figs. 12–15. Infected xylem vessels 48 hr after inoculation with the virulent E9 isolate of *Erwinia amylovora*. **12,** Bacteria and fine fibrillar material (arrow) observed in a helical vessel 5–10 mm from the inoculation point (×1,322). **13,** Bacteria (arrow) and the associated fine fibrillar material (FM) (×5,290). **14,** Two mature helical vessels (arrows) 5–10 mm from the inoculation point. The upper vessel contains bacteria and loose fibrillar material; the lower vessel is occluded by dense fibrous material (×776). **15,** Enlargement of the occluding, dense, fibrillar material (DFM). A single bacterial cell is seen embedded (arrow head) in this material (×2,645).

here support the contention that the initial site of systemic migration and proliferation in apple shoot tissue is in mature xylem vessels in contrast to earlier reports that bacteria first colonize and migrate through the intercellular space of the cortex and pith (2,20). Our findings support the recent work of Hockenhull (9) who used a leaf inoculation technique coupled with immunofluorescence microscopy to demonstrate that the early site of tissue colonization by bacteria in hawthorn was the vascular system rather than intercellular space.

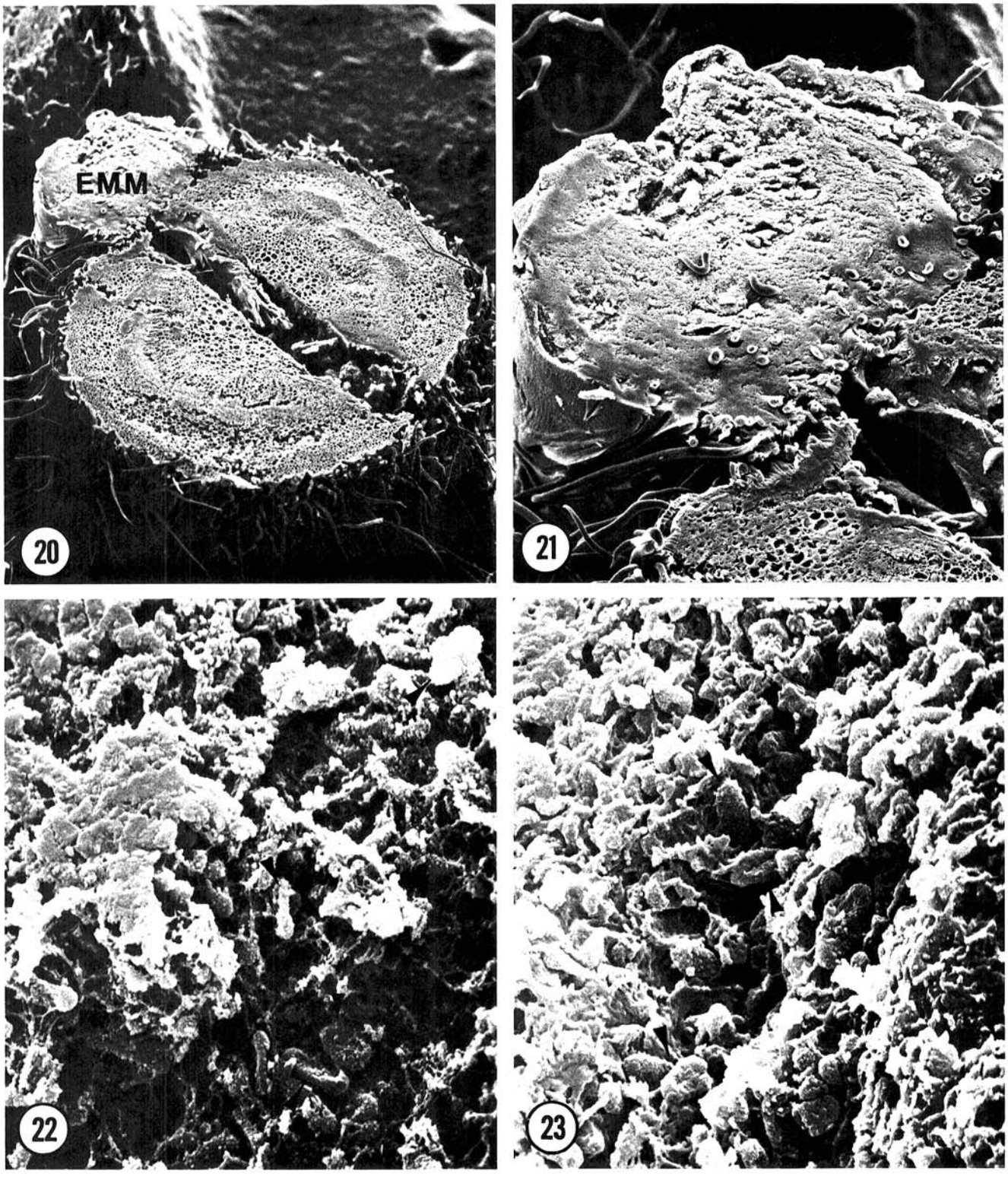
Occlusion of the vascular elements by bacteria was more pronounced 48 hr after infection and coincided with the clearly distinguishable symptoms of wilt observed in inoculated shoots. Despite the rapid colonization of the wound site by bacteria, as evidenced by the formation of ooze, bacteria 2 mm from the inoculation point were, nevertheless, restricted to the xylem vessels.

There is positive correlation between virulence in *E. amylovora*



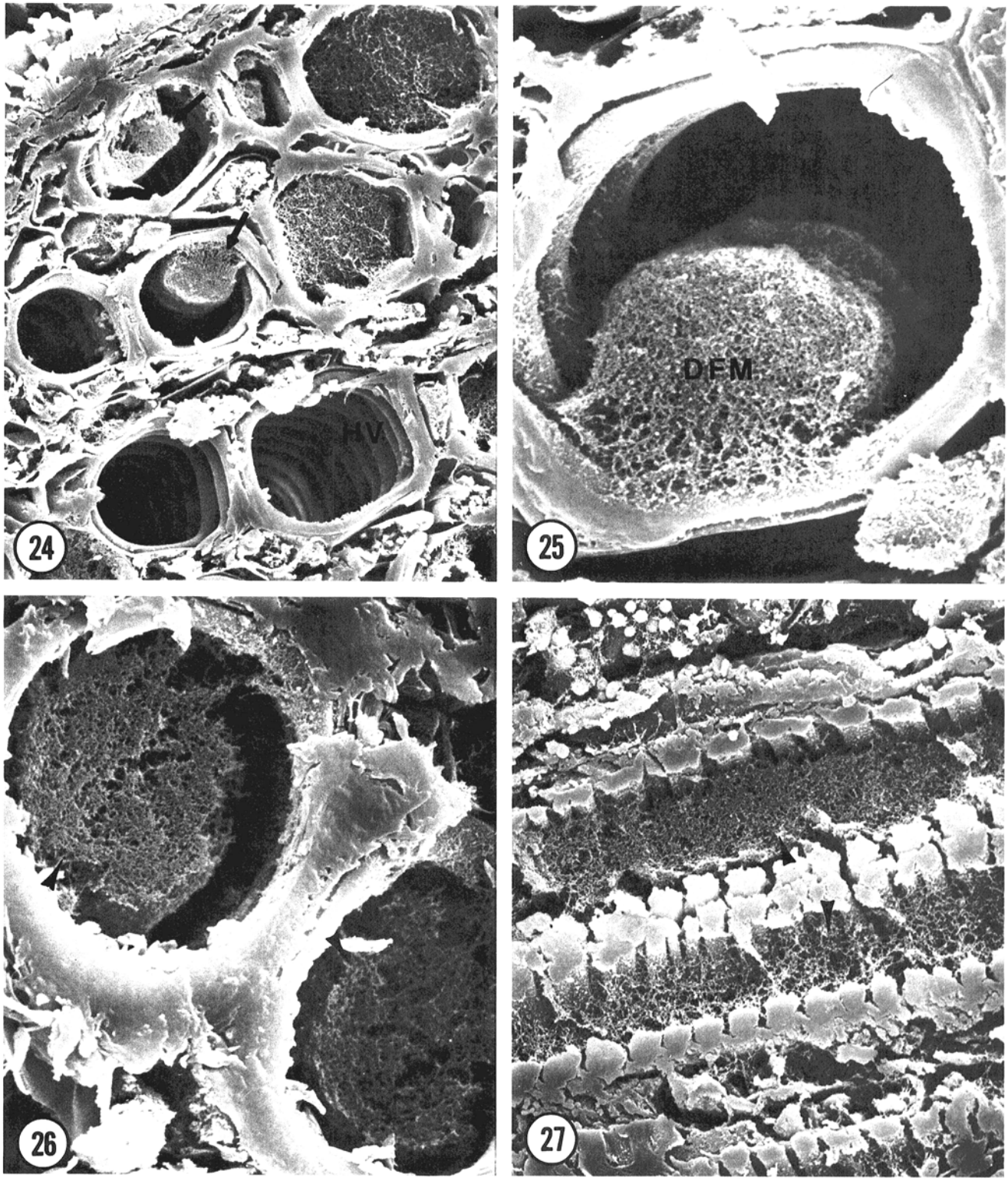
Figs. 16–19. Infected xylem vessels 48 hr after inoculation with virulent E9 isolate *Erwinia amylovora*. **16,** Three completely occluded xylem vessels. The vessel cavities contain bacteria in an extremely dense matrix. The vessels were observed 5–10 mm from the inoculation point (×2,645). **17,** The dense matrix material (DMM) contains bacteria (arrow head) that are barely perceptible (×3,174). **18,** A large number of bacterial cells (arrowheads) are embedded in a dense, gluelike matrix in the vessel lumen (×2,645). **19,** Higher magnification of bacteria (arrowheads) embedded in a dense, gluelike matrix (×5,290).

and the production of a high-molecular-weight EPS by the bacterium (1,3). The pathogen produces abundant amounts of EPS in both liquid culture and on inoculated apple fruit slices. Results of gel chromatographic and immunochemical analyses have indicated that the polysaccharide isolated from both liquid culture and fruit tissue are antigenically similar (1). It is our opinion that the observed occluding material is largely EPS produced by the bacteria in the xylem vessels in which they proliferate. Shoots treated with EPS form occluding fibrillar plugs much like those produced in vessels invaded by bacteria (Figs. 24–27). The TEM studies of Huang and Goodman (11) have shown that EPS from *E. amylovora* can occlude mature vessels and result in damage to adjacent vascular parenchyma cells. TEM observations of xylem vessel occlusion caused by EPS in Figs. 28 and 29 depict the occluding material and its effect as being nearly identical to that observed by Huang and Goodman (11).



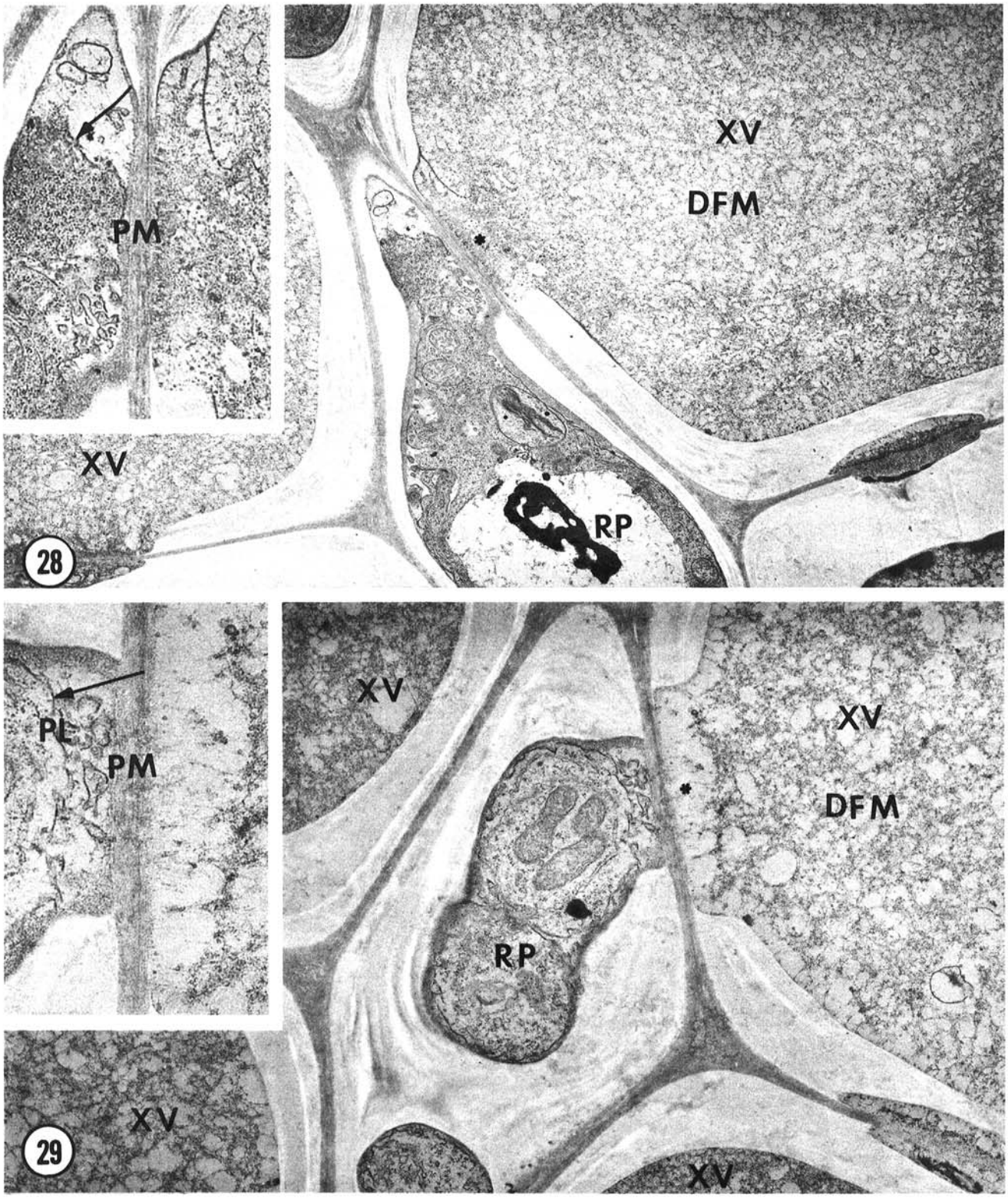
Figs. 20–23. Ooze formation at the inoculation point 48 hr after inoculation with the virulent E9 isolate of *Erwinia amylovora*. **20,** The wound created by the needle inoculation with 5×10^5 cells produced the ooze (exuded matrix material, EMM), which is adhering to the stem surface (×35). **21,** Enlargement of the previous micrograph shows the amorphous nature of the exuded matrix material (×1,150). **22,** Bacteria (arrowheads) can be seen embedded in the ooze. (×7,935). **23,** Enlarged view of bacteria (arrowheads) embedded in ooze (×10,350).

The density of the EPS that occluded xylem vessels ranged from a loose fibrillar matrix to a solid matrix. The density of the occluding EPS may be the result of internal forces within the xylem vessels, mainly the coupling of the upward conduction of water with high root pressure and high ambient relative humidity. The formation of a dense matrix and bacterial strands fostered by internal pressure in plant tissue was suggested for *E. amylovora* by Keil and van der Zwet (15) and Miles et al (17) for *X. pruni*. The density of the material appears positively correlated to the number of bacteria in the vessels. We observed an increase in EPS in vessels containing large numbers of bacteria. The loose fibrillar nature of the EPS may possibly have resulted from a partial dissolution and removal of the polysaccharide at the lower alcohol concentrations in the dehydration process.



Figs. 24–27. Xylem vessels from an apple shoot treated with the extracellular polysaccharide (EPS) from the virulent E9 isolate of *Erwinia amylovora*. **24,** Mature occluded (arrows) helical xylem vessels in shoots treated with 100 μg/ml solution of EPS (×1,610). **23,** Higher magnification revealing the nature of the dense fibrous material (DFM), which is presumed to be partially EPS (×6,325). **26,** Two xylem vessels occluded by the EPS (arrows). The EPS preserved by critical point drying in the shoot tissue had fixation characteristics that were identical to the material found occluding vessels of tissue infected by *E. amylovora* (×4,232). (See Figs. 9, 10, 12, and 14). **27,** Longitudinal section showing the plugging of vessels by EPS (arrows) in shoot tissue (×2,645).

High-molecular-weight macromolecules and bacterial polysaccharides cause wilting of specific tissues in tomato cuttings depending on the size of the macromolecule (10). Recently, Van Alfen and Allard-Turner (25) were able to quantitate the amount of dextran required to stop water conductance in various vascular tissues of alfalfa. They observed that veinlets in petioles were the most sensitive to vascular occlusion as compared to vascular tissue in the stem. The EPS of *E. amylovora* with a molecular weight of $\sim 40 \times 10^6$ daltons (1) is large enough to severely occlude xylem vessels in both the main stem of the young shoot and the petiole veinlets. Because of its large size it is probably restricted to the mature vessels of the stem and petiole and cannot penetrate the pit



Figs. 28–29. Thin sections of xylem vessels from stem tissue 4 hr after treatment with 100 μg/ml extracellular polysaccharide (EPS) of *Erwinia amylovora*. Sections were cut from the second 2-mm segment from the base of the shoot. **28,** Ray parenchyma (RP) cells between four xylem vessels (XV) revealing what appears to be the aggregation of dense fibrillar material (DFM) on the xylem vessel lumen side of the pit membrane (PM), the DFM fully occludes each lumen (the asterisk identifies the pit magnified in the inset). The parenchyma cell also shows plasmolysis (see arrow in inset) (×8,970, 7,940). **29,** Ray parenchyma cell in addition to showing fibrillar orientation of DFM near the PM, reveals intense plasmalemma (PL) vesiculation (arrow in the magnified [*] inset). Note that each of the four neighboring xylem vessel lumens is also completely filled with DFM (×3,225, 7,025).

pores of the vessels. This contention is supported by the regaining of turgor of EPS-wilted shoots when the basal centimeter is excised and the newly cut end of the shoot is placed in water. The bacterial EPS probably restricts water flow by physical blockage and also results in the plugging of pits in the walls of vessels (Figs. 9–19, 24–27), thereby restricting water flow across the walls of the annular and helical vessels in young tissue. This is particularly clear in the TEMs, (Figs. 28 and 29). The vascular bundles of young shoots (ie, tissue near the apex) are not interconnected so water from a blocked vessel cannot be transferred laterally to ones that are still functioning. The plugging of vessels would probably be irreversible since the work of Hodgson et al (10) suggests that plants cannot effectively degrade bacterial polysaccharides.

Another consideration in the formation of occluding polysaccharide plugs in vessels is the possibility that bacterial polysaccharides may combine with plant cell wall polysaccharide to form a gel similar to that shown in *X. campestris* (18). Gels formed in xylem vessels would disrupt water flow and the gel formed by an interaction between EPS and fibrils of the vessel wall would aid in the stabilization of the EPS and its localization in the xylem.

Finally, recent work in our laboratory has revealed the presence of a protein in seed, leaf, and stem tissue of apple that precipitates the extracellular polysaccharide (EPS) of *E. amylovora*. Although the role of the protein in pathogenesis is only now being studied, it too could produce a vessel plug by precipitating the EPS being produced by *E. amylovora*.

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