Ecology and Epidemiology

Systemic Invasion of Cherry Leaves and Petioles by *Pseudomonas syringae* pv. *morsprunorum*

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ABSTRACT

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Fully expanded cherry leaves were inoculated in spring with 10^4, 10^5, or 10^6 colony-forming units (cfu) per milliliter of a virulent strain of *Pseudomonas syringae* pv. *morsprunorum*. Appearance of symptoms depended on the inoculation method. Water-soaked veins and lesions developed on leaves wounded by rubbing during inoculation. At higher inoculum concentrations, more lesions developed. Spraying pathogen suspensions onto leaves until tissue became water soaked was less effective. No lesions developed on leaves sprayed until runoff without water soaking. Systemic invasion by *P. s.* pv. *morsprunorum* of symptomless leaf and petiole tissue was confirmed by isolation of the pathogen and scanning electron microscopy (SEM). Most shoots and axillary buds of trees inoculated by applying 10^6 cfu/ml of *P. s.* pv. *morsprunorum* to leaves during wounding had died back by the next spring, implying further spread of the pathogen. SEM indicated that *P. s.* pv. *morsprunorum* applied by spraying probably gained entry through stomata and then spread intercellularly from the mesophyll through the parenchyma of the bundle sheath into the vascular system of a minor vein. Once a vein had been invaded, migration occurred to other regions in the leaf blade and petiole.

Additional key words: histopathology, *Prunus avium*.

Pseudomonas syringae pv. *morsprunorum* (Wormald) Young et al, the major causal organism of bacterial canker of sweet cherry (*Prunus avium* L.) in South Africa (23), survives throughout the growing season on the surface of symptomless leaves (25). Although epiphytic populations of *P. syringae* have received considerable attention during the past decade (11), few studies have been devoted to the internal pathology of the leaf phase of bacterial canker. We recently reported that *P. s.* pv. *morsprunorum* multiplies in the substomatal chambers of cherry leaves (22). Masses of bacterial cells seen on the surface of leaves

were presumed to have emerged from stomata. The present investigation considers systemic migration of *P. s.* pv. *morsprunorum* from inoculated leaves through petioles into stems of cherry trees.

MATERIALS AND METHODS

Inoculum. Virulent strain 428 of *P. s.* pv. *morsprunorum* isolated in South Africa from a Black Tartarian cherry tree was used. Overnight growth on slants of nutrient-yeast extract-glycerol agar (26) incubated at 26 C was suspended in sterile distilled water and adjusted turbidimetrically to 1.5×10^8 cells per milliliter. Further dilutions were made to obtain suspensions containing 10^4, 10^5, and 10^6 colony-forming units (cfu) per milliliter. These values were at the same time confirmed by dilution plating. Suspensions were used immediately.

Inoculation of test trees. Leaves on vigorous shoots (20–25 cm) of the current season's growth on 3-yr-old potted Black Tartarian cherry trees were inoculated in a greenhouse in spring (September). Trees were covered with plastic bags for 48-hr periods before and after inoculation. To follow later disease development, some trees were moved to field conditions 10 days after being inoculated.

All three inoculum concentrations were included in three methods used to inoculate leaves. In the first method, the youngest fully unfolded leaf was covered with a sheet of aluminum foil to expose a 12-mm-diameter circular area on the abaxial surface. Inoculum or water (control) was gently sprayed onto this area with an atomizer until runoff. Care was taken to ensure that tissue did not become water soaked. The second method resembled the first, but the spray application was continued until tissue of the exposed area became water soaked. In the third method, a 10-μl drop of suspension or water was placed in the central area of the abaxial surface, alongside the main vein. The two halves of the leaf blade were pressed together and carefully rubbed between the fingers. Each inoculation method at a given inoculum concentration was applied to a separate leaf on different shoots of 40 individual trees.

Isolation of bacteria from inoculated trees. Shoots with treated leaves were removed 10 days after inoculation. A 2-cm segment of leaf, extending across the width of the lamina, with the site of inoculation at the center, was removed aseptically. The remaining two portions of leaf blade and the detached petiole composed the other three segments. Corresponding segments were removed from control leaves.

Each segment was surface-disinfested with 70% ethanol, cut into smaller pieces, and shaken vigorously for 1 min in a test tube containing 10 ml of sterile distilled water. After 1 to 2 hr, 10-fold dilutions of the suspension were plated onto King's medium B (15) and Difco nutrient agar supplemented with 5% sucrose (18). Plates were examined after 3 days' incubation at 26 C. The identity of representative bacterial isolates with colonies resembling those of *P. s.* pv. *morsprunorum* was verified by the oxidase test with Difco oxidase-differentiation disks, GATTa tests (17), and the hypersensitive reaction on tobacco leaves (16).

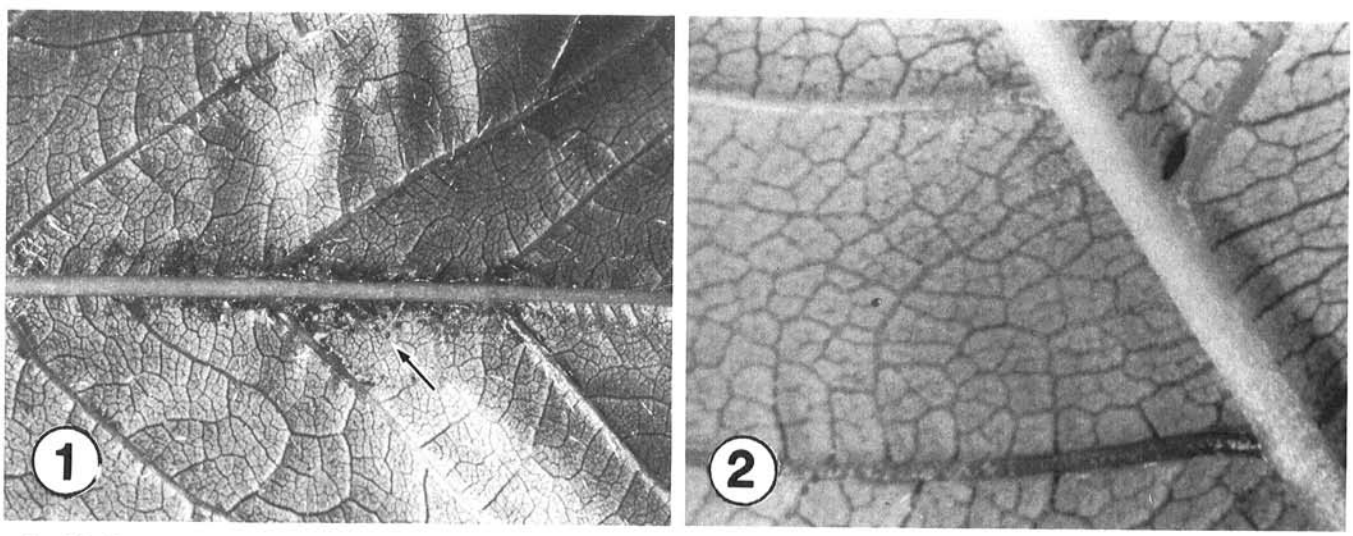
Scanning electron microscopy (SEM). Shoots to be used for SEM studies were also removed from potted trees 10 days after inoculation. Inoculated leaves (sprayed with 10^6 cfu/ml of *P. s.* pv. *morsprunorum* until water soaked) from five shoots and control leaves from two shoots were segmented as described in the previous section. Squares of tissue (1 cm^2) cut from each individual segment were serially sectioned into 1-mm × 1-cm pieces with a new razor blade. Sections were fixed in a 5% glutaraldehyde solution, followed by acetone dehydration (6), then dried in a critical point drier under CO_2, followed by gold-coating as described previously (22). Leaf surfaces, cross sections through leaf blades, and cross and longitudinal sections of petioles were examined with an ISI 100-A SEM (International Scientific Instruments, Santa Clara, CA).

The presence of *P. s.* pv. *morsprunorum* in preparations was checked by identifying bacteria isolated from leaf and petiole tissue bordering tissue selected for SEM. The pathogen was usually isolated in pure culture from all inoculated leaves and their petioles but never from control tissues.

RESULTS

Symptom development. The appearance of symptoms on leaves for the first 14 days after inoculation depended on the method of inoculation and the inoculum concentration. Symptoms were most pronounced and appeared sooner at the highest inoculum concentration (10^6 cfu/ml) when *P. s.* pv. *morsprunorum* was introduced through wounds made by rubbing leaves together. Spraying the pathogen onto leaves until tissue became water soaked was less effective. No lesions developed on leaves sprayed until runoff with any of the three inoculum concentrations. None of the control leaves or their shoots developed disease symptoms at any stage of the investigation.

At an inoculum level of 10^6 cfu/ml, veins and adjacent mesophyll tissue of wounded leaves were water soaked after 3 days (Fig. 1). Exposed leaf areas that had been sprayed until water soaked retained this condition for only 1 hr. Small, circular, or angular water soaked spots, mostly confluent, appeared 3–5 days after inoculation. The spots did not enlarge, but underlying mesophyll cells collapsed, eventually resulting in dry, necrotic lesions, frequently surrounded by yellow halos. Shotholes appeared later when necrotic tissue fell out. Lesions were more

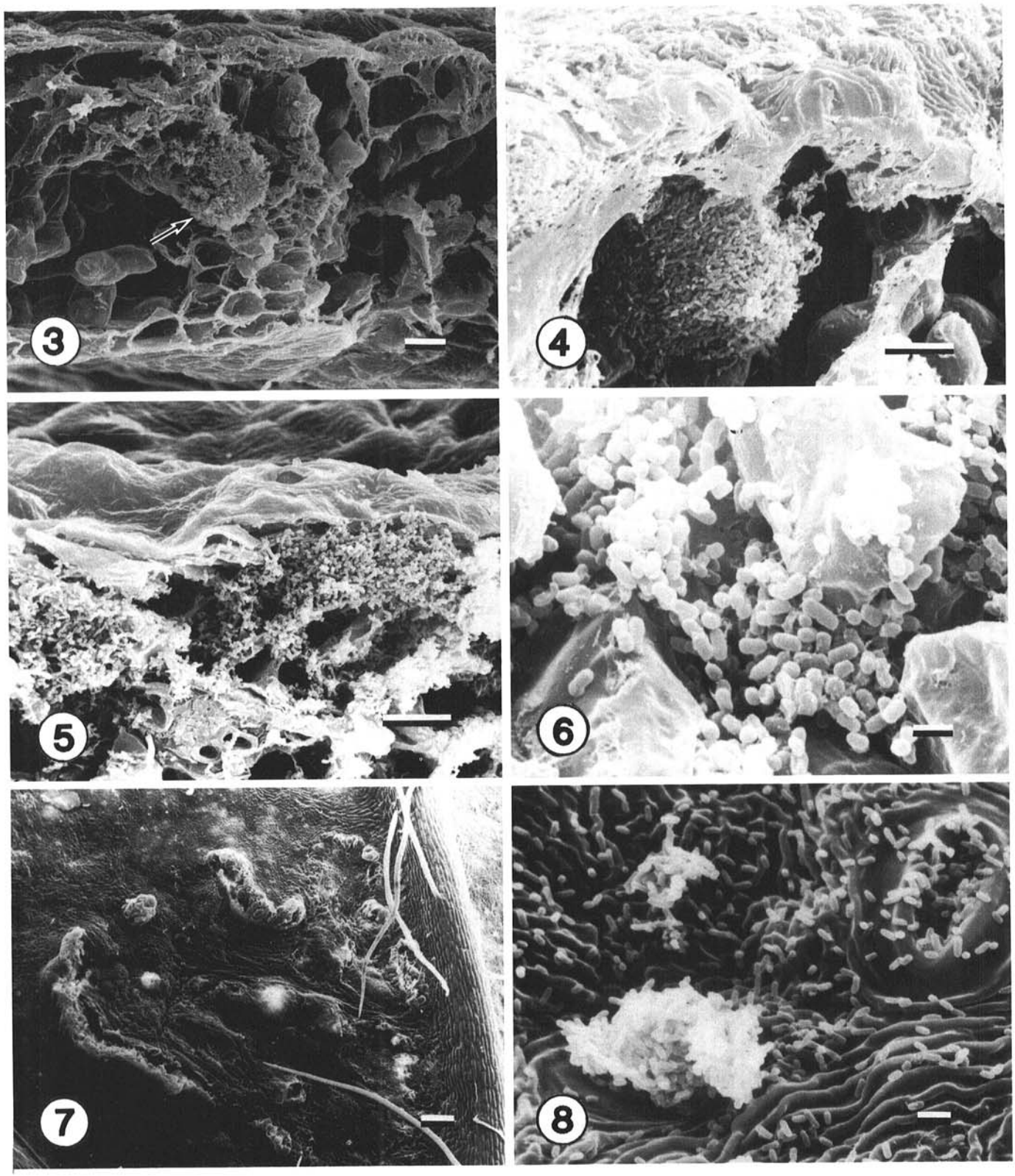


Figs. 1 and 2. Water-soaked veins caused by *Pseudomonas syringae* pv. *morsprunorum* on cherry leaves. **1,** Three days after wound inoculation. Lesions (arrow) are also evident in the leaf lamina. **2,** First symptoms consisting of water-soaked veins on a new leaf that had developed from an axillary bud of a leaf sprayed during the previous spring with 10^6 cfu/ml of the pathogen.

evenly distributed over areas that had originally been sprayed until tissue became water soaked, but symptoms on veins were less pronounced than on wounded, inoculated leaves.

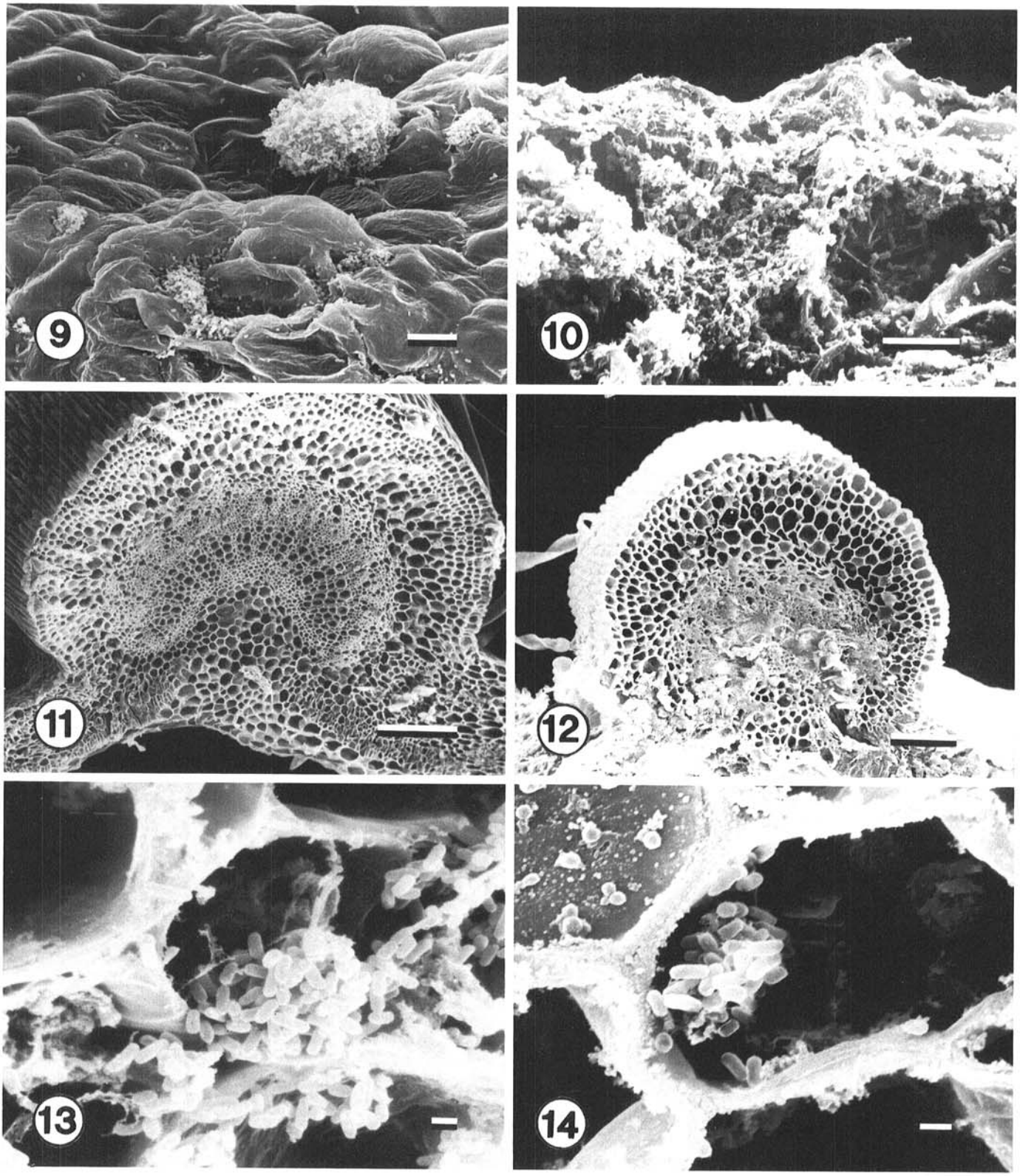
Leaves sprayed until water soaked developed fewer spots with 10^5 than with 10^6 cfu/ml. At 10^4 cfu/ml, a few small necrotic lesions developed within 14 days of inoculation on the wounded leaves only. Symptom development during this period was confined to the area infiltrated, irrespective of the method of inoculation.

Symptoms on inoculated trees were prominent shortly before bud swell the following spring. Shoots of trees inoculated by



Figs. 3–8. Scanning electron micrographs of cherry leaves inoculated with *Pseudomonas syringae* pv. *morsprunorum*. Scale bars = 10 μm (3–5), 1 μm (6), 500 μm (7), and 2 μm (8). **3,** Cross section showing bacteria (arrow) in minor vein away from the site of inoculation in a symptomless area of the leaf blade. **4–6,** Cross sections of symptomless area within the inoculation site. Note masses of bacteria in substomatal chamber (4) and at low (5) and high magnification (6) in the spongy parenchyma of the mesophyll. **7,** Surface view of lesion next to main vein. Note ruptured and collapsed areas. **8,** Individual bacteria and clumps of bacteria associated with stomata within the lesion area.

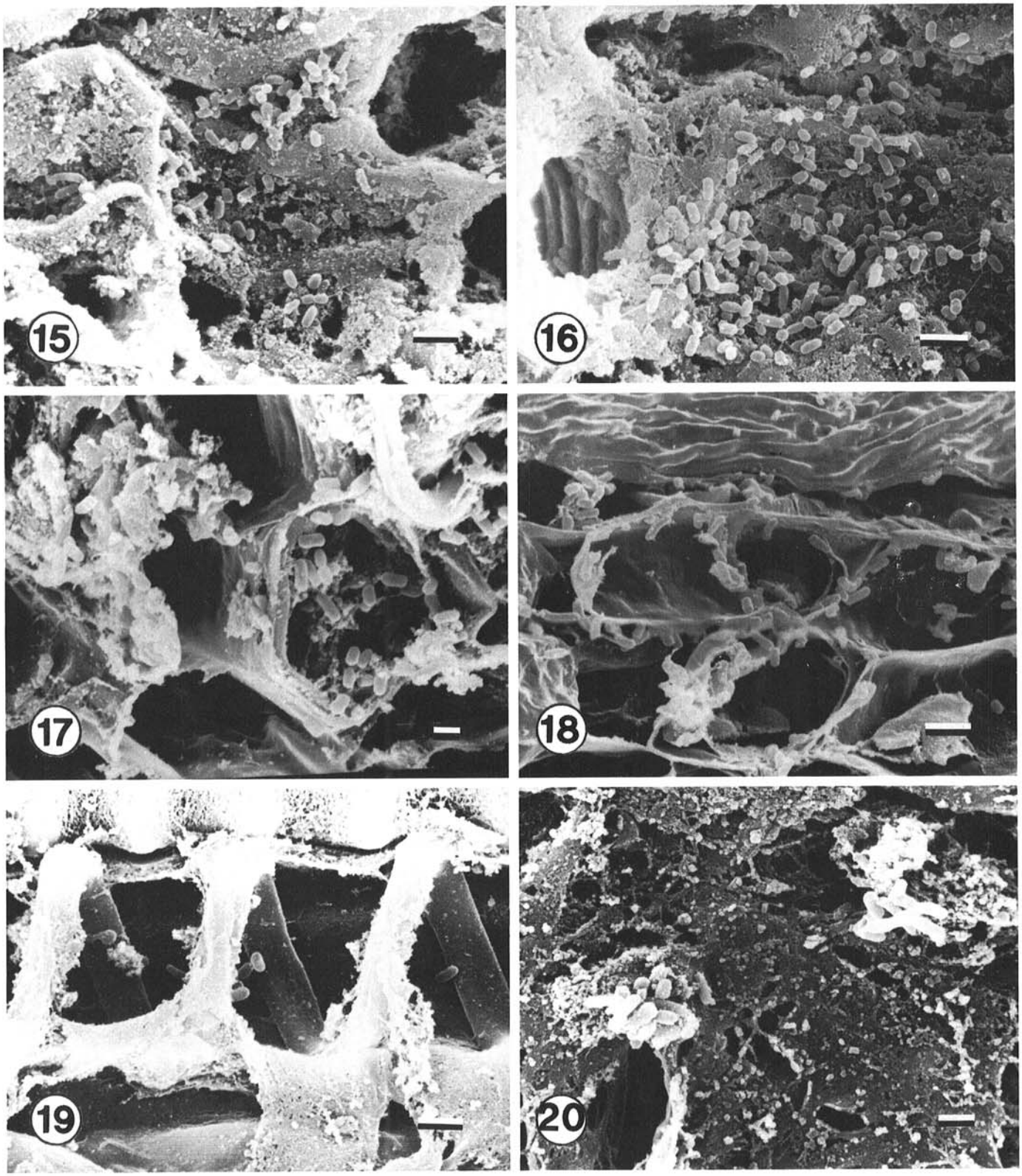
applying 10^6 cfu/ml to leaves and then wounded had cankers and were dark red. Most buds on these shoots were dead, and small drops of gum exuded from the scales. Symptoms were often absent from trees where leaves had been sprayed until runoff or water soaked with 10^5 and 10^6 cfu/ml. When symptoms did appear, all buds on a shoot, or only the axillary bud of the leaf originally inoculated together with one or two nearby buds, died. Cankers originating from diseased buds frequently girdled and often extended along the entire length of shoots. No symptoms developed on shoots or buds of trees inoculated by spraying 10^4



Figs. 9–14. Scanning electron micrographs of cherry leaf tissue invaded by *Pseudomonas syringae* pv. *morsprunorum* (9, 10, and 12–14) and uninfected tissue (11). Scale bars = 10 μm (9), 2 μm (10), 100 μm (11 and 12), and 1 μm (13 and 14). **9,** Surface view of lesion with collapsed epidermal and stomatal guard cells. **10,** Cross section through lesion in Fig. 9 showing masses of bacteria embedded in fibrous material. **11,** Cross section of main vein of an uninfected leaf revealing the vascular system and parenchyma and collenchyma of the cortex. **12 and 13,** Invaded leaf with occluded main vein (12) containing numerous bacteria in the parenchyma (13). **14,** Bacteria in the cortical collenchyma of the main vein.

cfu/ml onto leaf areas. However, water soaked veins (Fig. 2) were seen on some new leaves developing from axillary buds of leaves originally treated with suspensions containing 10^5 or 10^6 cfu/ml.

Isolation of pathogen. *P. s.* pv. *morsprunorum* was isolated from all segments of leaves inoculated with 10^6 cfu/ml, irrespective of the method of application. However, on leaves sprayed until runoff with 10^4 or 10^5 cfu/ml, the pathogen was recovered only from the segments that included the site of inoculation. The pathogen was isolated from all segments of leaves inoculated at these two levels either by spraying until tissue became water soaked or by rubbing to ensure wounding. *P. s.* pv. *morsprunorum* was not isolated from any of the control segments.



Figs. 15–20. Scanning electron micrographs of cherry leaf (15–17) and petiole (18–20) tissue invaded by *Pseudomonas syringae* pv. *morsprunorum*. Scale bars = 2 μm (15, 16, and 18) and 1 μm (17, 19, and 20). **15 and 16,** Advanced stage of pathogenesis with bacteria embedded in dense granular material present in occluded xylem vessels. **17,** Early stage of invasion of xylem vessels. Granular material not yet prominent. **18–20,** Bacteria in the cortex (18) and helical xylem vessels (19) and in phloem tissue (20).

Scanning electron microscopy. Preliminary observations by SEM indicated that the general pattern of tissue colonization by *P. s.* pv. *morsprunorum* was not influenced by the leaf inoculation method or the inoculum concentration. However, invasion was accelerated by wounding leaves or allowing tissue to become water soaked during application of inoculum containing higher levels of the pathogen. Bacteria were not seen in specimens prepared from control leaves treated with water.

Bacteria were observed in tissue removed from the distal and proximal parts of the leaf at a distance of 3 cm or more from the site of inoculation, irrespective of the method of application or of the inoculum level. In these areas, which showed no external symptoms, bacteria occurred most commonly in veins of the leaf blade (Fig. 3) and infrequently in the intercellular spaces of the mesophyll. However, in symptomless tissue at the inoculation site, masses of bacteria commonly occurred in the substomatal cavities (Fig. 4) and in the intercellular spaces of the mesophyll (Figs. 5 and 6). The high intercellular populations were also present in a narrow band (1 to 2 mm) of tissue bordering the site of inoculation.

Figure 7 shows the collapsed and disrupted surface layer of a lesion situated next to the main vein. Most cells within lesions lost their turgor and became flattened. Individual bacteria were scattered over the surface of lesions, but clumps of cells were mainly associated with stomata (Figs. 8 and 9). A section through the lesion area shown in Figure 9 revealed masses of bacteria embedded in fibrous material (Fig. 10).

A cross section of the main vein (Fig. 11) of a control leaf indicated that the lumina of xylem elements were invariably clear. In contrast, in an inoculated leaf, occlusions occurred in the vascular bundles of veins in contact with the advancing margin of colonized mesophyll tissue (Fig. 12). In these specimens bacteria were seen in the parenchyma (Fig. 13) and collenchyma (Fig. 14) of the cortex and even more frequently in the phloem parenchyma and phloem (Fig. 15) and xylem (Figs. 16 and 17). Infected veins contained granular material (Figs. 15 and 16) and seemed to be occluded. However, these veins showed no evidence of deterioration. Only solitary cells or small clumps of the pathogen associated with granular material (Fig. 17) were observed in the veins away from the occluded areas. Although less abundant than in the main vein of infected leaves, bacteria also occurred in the cortex (Fig. 18), xylem (Fig. 19), and phloem (Fig. 20) of petioles.

DISCUSSION

Necrotic lesions developed within 14 days on cherry leaves inoculated with *P. s.* pv. *morsprunorum*, provided certain conditions had been met. Extensive damage, characterized by the appearance of irregular lesions, occurred on leaves wounded during application of the highest inoculum concentration. However, a wound was not a prerequisite for infection. The development of smaller, more regular spots on leaf areas inoculated by spraying implied entry of the pathogen through stomata.

Systemic spread of *P. s.* pv. *morsprunorum* was confirmed by its isolation from symptomless leaf and petiole tissue away from the site of inoculation. This agrees with reports on migration of *Xanthomonas campestris* pv. *pruni* in plum tissue (8), *Erwinia amylovora* in apple shoots (10,19,30,31), and *Corynebacterium flaccumfaciens* subsp. *poinsettiae* in poinsettia leaves (2). In our investigation, expression of disease symptoms during the following spring indicated that the pathogen had spread to stems and axillary buds. Establishment of *P. s.* pv. *morsprunorum* in tissue of symptomless cherry leaves and petioles during summer is therefore of considerable ecological significance.

SEM supports our previous proposal (22) that *P. s.* pv. *morsprunorum* infects cherry leaves through stomata. Large masses of bacteria were seen in the substomatal chambers (Fig. 4). From there, the pathogen probably spread intercellularly through the mesophyll (Figs. 5 and 6). We suspect that lesions were formed when the physical pressure exerted by the expanding masses of bacteria in the intercellular spaces (13,27–29) ruptured parenchyma cells. Significantly, invasion was initially limited to the mesophyll below the inoculated zone, but the pathogen spread to other areas once a vein had been invaded. Bacteria presumably progressed intercellularly from the mesophyll through the parenchyma of the bundle sheath into the vascular system of the minor vein (Fig. 5) and from there to the main vein. Bacteria were first present in symptomless leaves in the phloem and phloem parenchyma of veins near the site of inoculation before they spread to other tissue. This agrees with a report that *C. f.* subsp. *poinsettiae* enters the phloem of host leaves intercellularly during the initial stages of pathogenesis (2). *P. s.* pv. *morsprunorum* also occurred in xylem tissue of cherry leaves (Figs. 15–17) as invasion progressed. It is unlikely that the pathogen was transported passively through the xylem. Although single bacterial cells occurred commonly in vessels, large clumps resembling microcolonies were often seen away from the site of inoculation (Fig. 17). How the pathogen entered the xylem lumen is uncertain, since vascular bundles showed no signs of deterioration. Nelson and Dickey (20) speculated that *P. s.* pv. *caryophylli* moves from an infected vessel member of carnation to adjacent xylem parenchyma through the pit membrane, via the plasmodesmata, to initiate the development of bacterial pockets. Entry of *P. s.* pv. *morsprunorum* into the lumen of xylem vessels might occur readily if pit membranes are ruptured or deteriorated. Alternatively, pressure exerted by bacterial masses on the pit membrane could increase the diameter of plasmodesmata. Veins appear to be of major importance in the systemic spread and nutrition of *P. s.* pv. *morsprunorum* in cherry leaves. However, the presence of sparse populations of the pathogen in adjacent tissue (Figs. 13 and 14) indicated that some lateral spread did occur from the vascular system.

Granular material in veins could comprise gums or gels secreted by the host in response to the pathogen (33). We suspect that fibrillar material was secreted by the pathogen rather than the host. This agrees with a recent SEM study of Pierce's disease of grapevines (32).

In England *P. s.* pv. *morsprunorum* washed from leaves by rain water invades cherry trees in autumn through leaf scars (3–5). The pathogen is drawn into xylem vessels of the leaf traces by negative pressure and eventually migrates into medullary rays and other living tissue. This type of infection occurs more readily if premature leaf fall exposes a scar without a fully developed protective layer (5). We question the importance of leaf scar infection in South Africa. Heavy winds in fall are rare in the local deciduous fruit growing areas, and chemical sprays applied during this period do not reduce later disease development (I. M. M. Roos, *unpublished*).

In general, the histopathology of bacterial canker of sweet cherry resembles that of some other bacterial plant diseases (2,10,12,19,30,31). The presence of *P. s.* pv. *morsprunorum* in symptomless cherry twigs agrees with reports on detection of *P. s.* pv. *syringae* in peach (9) and plum (21,24) trees. Other workers have also isolated pathogenic bacteria from apparently healthy tissue (1,7,14,19). These and other local considerations, including forcible extrusion of masses of *P. s.* pv. *morsprunorum* cells through stomata (Fig. 8), epiphytic growth of the pathogen on leaves (25), and survival in buds (24), complicate efforts to control bacterial canker of cherry.

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