Scanning Electron Microscopy of Pierce's Disease Bacterium in Petiolar Xylem of Grape Leaves

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ABSTRACT

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Petioles from a healthy grapevine and a grapevine showing symptoms of Pierce's disease infection were examined by scanning electron microscopy. In petioles from the healthy control vine, the lumens of tracheary elements lacked bacteria and seldom had tyloses. In petioles of leaves from the diseased plant, both with and without marginal leaf burn, bacterial aggregates were found in tracheary elements, and tyloses were seen much more frequently than in control petioles. Bacteria within aggregates often were associated with small globules, and some complete plugs appeared to be comprised mainly of such globular material. The overall percentages of colonized and plugged elements in petioles of symptomless leaves from the diseased plant were less than in petioles of leaves with marginal leaf burn from the same plant, ie, 19.5 versus 22.5% colonized, and 5.8 versus 7.7% plugged. In most petioles from the diseased plant almost all of the vascular bundles contained some colonized tracheary elements. Calcium-containing crystals were found infrequently in tracheary elements of both control and diseased plants; there is no evidence that these xylar inclusions are related to Pierce's disease.

Additional key words: calcium oxalate crystals, transmission electron microscopy, X-ray energy dispersive analysis.

Pierce's disease (PD) of grapevines is caused by a rod-shaped Gram-negative bacterium that has an extremely wide host range and is transmitted by xylem-feeding suctorial insects (3,10,18,19). Although PD is limited to the New World, in certain grape-growing areas it is epidemic and of major economic importance (18). In infected plants, PD bacteria are limited to xylem tissue (3,10), and it has been suggested that marginal leaf burn (MLB) and other symptoms characteristic of the disease are caused by restricted flow of water and nutrients due to partial or complete occlusion of individual conducting elements by bacterial plugs (14). However, there is also evidence that toxins capable of inducing foliar symptoms are produced by PD bacteria (13).

In the experimental vineyard of the Mississippi State University Enology Laboratory the first grapevine to develop symptoms of PD did so in the spring of 1982. The present study describes observations made on that infected vine to verify the presence of bacteria with ultrastructural features typical of the PD bacterium and to characterize the external morphology of intraxylar bacterial aggregates. In addition, the incidence and morphology of bacterial aggregates within petioles of apparently normal leaves and obviously diseased leaves (with MLB) from the same plant are compared. Scanning electron microscopy was used because of the relative ease with which large areas, such as whole petiolar cross sections, can be examined at high resolution and with great depth of field. This report is part of a long-term study of the relationship between the occurrence of PD symptoms and the distribution of bacteria within the xylem of host plants. A preliminary report has been published (21).

MATERIALS AND METHODS

Plant specimens. A four-year-old French-American hybrid or "direct producer" grapevine, cultivar DeChaunac, which was growing in the experimental vineyard of the Mississippi State University Enology Laboratory first developed symptoms of PD in the late spring of the 1982 growing season. Typical marginal leaf scalding, delayed shoot growth, leaf mottling, wilting of apparently healthy leaves, and dwarfing of the new shoots were observed after the plant had leafed out, bloomed, and set berries. Leaves that were mature, or nearly so, were collected in October 1982 and processed for electron microscopy. In addition, leaf samples were taken from a healthy control plant growing in the same vineyard under similar conditions. The diseased vine used in this study was the first plant in the vineyard to develop symptoms of PD; later, PD symptoms appeared in other French-American hybrid vines, including the one used here as a control, and in vines of several cultivars of *Vitis vinifera*. The PD organism was subsequently isolated in pure culture from the first diseased vine, as well as from other vines showing typical PD symptoms. The method used for isolating the organism from the vineyard was that of D. L. Hopkins (8).

Light microscopy of macerated petioles. Light microscopy was used to determine the types of tracheary elements present in petiolar vascular tissue. Pieces of petioles from the control plant were soaked overnight at 55 C in a medium containing two parts of 30% hydrogen peroxide to one part of glacial acetic acid. After several rinses in distilled water, the petiolar pieces were macerated with tweezers and examined by conventional light microscopy.

Transmission electron microscopy (TEM). To confirm the presence of ultrastructural features characteristic of PD bacteria, ultrathin sections were examined by TEM. Primary fixation of pieces of a leaf with MLB was done in a mixture of 2.5% glutaraldehyde and 2% acrolein for 10 hr, immersion in 2% OsO_4 for 2 hr, and embedment in Spurr's epoxy resin. Ultrathin sections were stained sequentially with uranyl acetate and lead citrate and examined in a Siemens 101 electron microscope.

Scanning electron microscopy (SEM). Two fixation procedures were used, and each yielded satisfactory results. Cross sections cut from nine petioles (three leaves from the control plant, three leaves with MLB, and three apparently normal leaves from the same diseased plant) were fixed in 5% acrolein in 0.1 M sodium cacodylate for 5.0–5.5 hr at room temperature. Next, the specimens were rinsed in buffer, immersed in 2% OsO_4 in 0.1 M cacodylate

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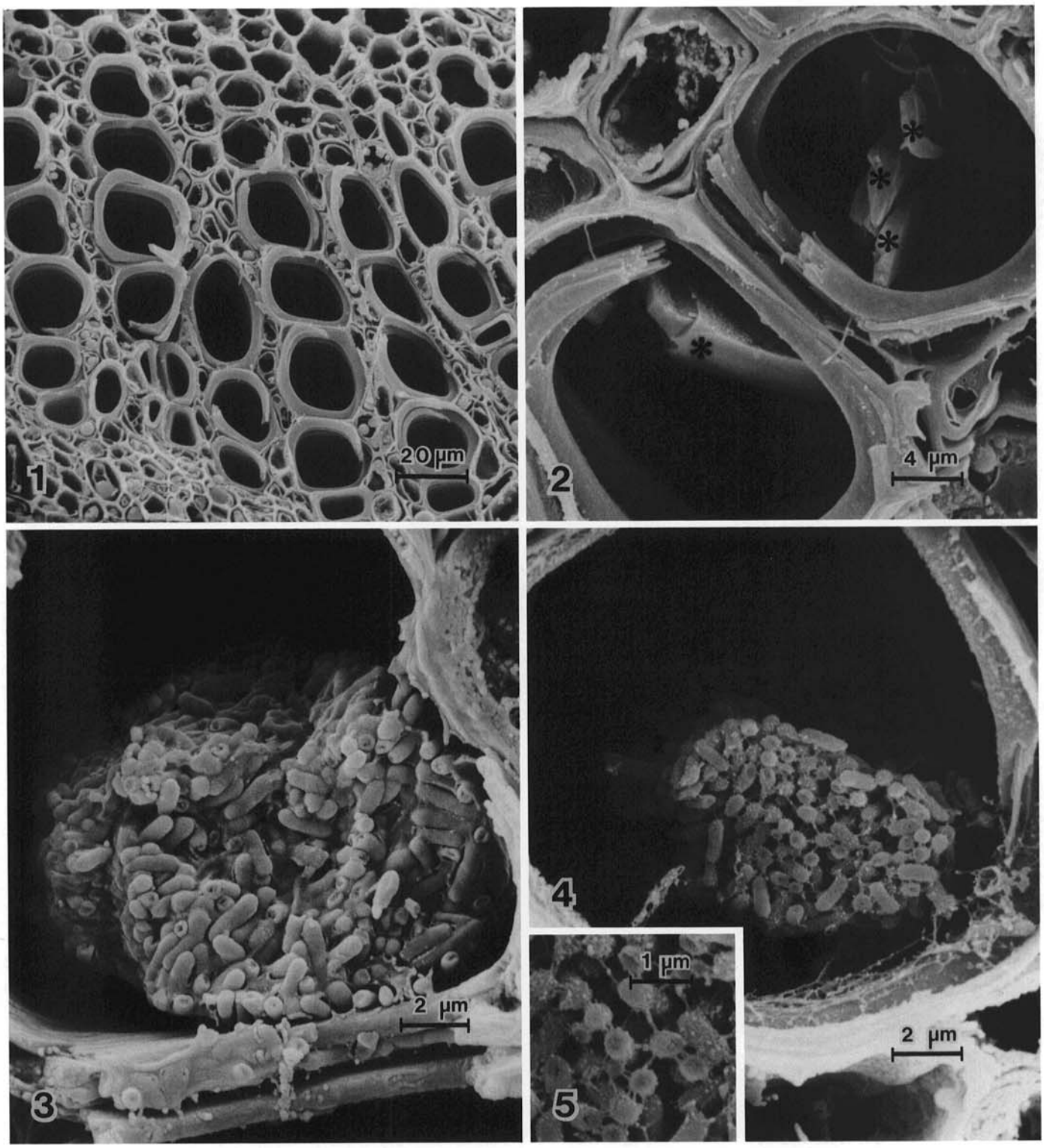
buffer for 4.5–6.0 hr, and rinsed overnight in several changes of cold buffer. Cross sections taken from nine additional matched petioles were fixed in 2.5% glutaraldehyde and 2% acrolein in 0.1 M sodium cacodylate at room temperature for 1 hr and then refrigerated overnight in the same fixative. Next, the petiolar sections were rinsed in buffer for 7–24 hr and immersed in cold 2% OsO_4 in 0.1 M sodium cacodylate for 5 hr or overnight.

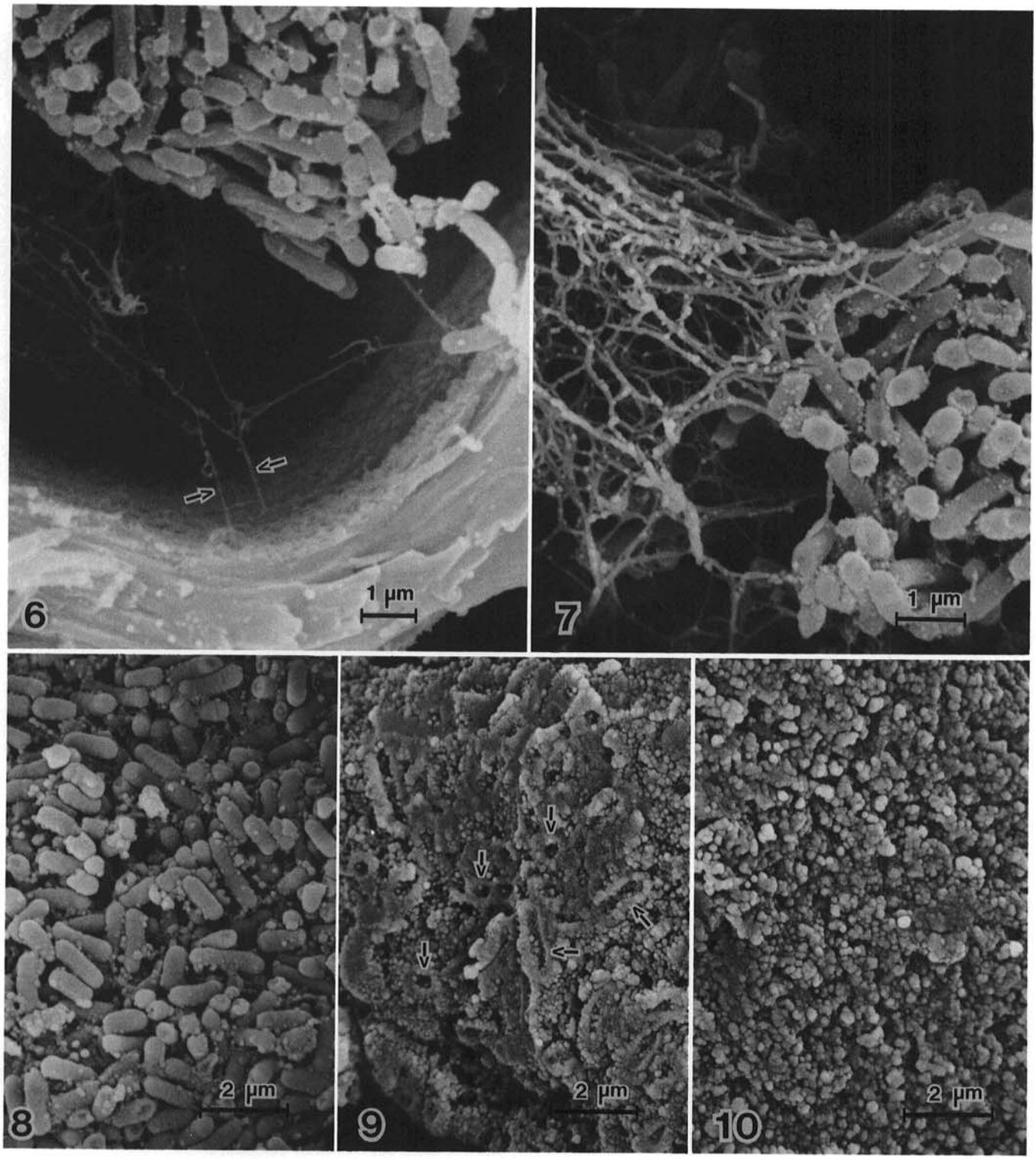
All specimens, regardless of the fixation procedure, were dehydrated in ethanol prior to freezing and fracturing in liquid nitrogen (11). During cryofractography each petiolar section was broken crosswise once. The fractured cross sections were critical-point dried in carbon dioxide, mounted on aluminum stubs with silver paste, coated with gold-palladium in a Polaron E5100 cool sputter coater, and examined in a Hitachi HHS-2R scanning



Figs. 1–5. Scanning electron micrographs of petiolar xylem of grape leaves. **1,** Typical low-magnification view of control. Note empty lumens of tracheary elements. **2,** Calcium-containing crystals in lumens of tracheary elements of control plant. Asterisks mark individual crystals found by X-ray energy dispersive spectrometry to contain calcium. **3,** Compact aggregate of Pierce's disease bacteria partially obstructs lumen of tracheary element of a diseased plant. **4,** Loosely packed bacterial aggregate in lumen of tracheary element of diseased plant. **5,** Higher magnification view of part of Fig. 4, showing the fimbriae that connect neighboring bacteria to one another.

electron microscope operated at 20 kV. The findings reported here are based on study of 321 photographs, as well as on visual observations made during 151 beam-hours of microscope use.

Eighteen petiolar cross sections were examined by SEM in detail. Observations were made of one cross section each from six petioles of the control plant, six petioles of apparently normal leaves from the diseased plant, and six petioles of leaves with MLB from the same diseased plant. All xylem tissue of each of the 18 cross sections was examined for the presence of PD bacteria. A record was kept of the percentage of tracheary elements containing bacteria, as well as the percentage of elements occluded by plugs. The tallies were made "blindly"; the person examining the petiole and doing the counts



Figs. 6–10. Figs. 6 and 7: Scanning electron micrographs of Pierce's disease bacteria in lumens of tracheary elements of petiolar xylem of grape leaves. **6,** Loosely packed bacterial aggregate and associated fibrillar material. Note that fibrils (arrows) of network appear to attach to the wall of the tracheary element. **7,** Fibrillar mass attached to bacteria in lumen of the tracheary element. **Figs. 8 to 10:** Scanning electron micrographs of material comprising plugs in tracheary elements of petiolar xylem of leaves of grapevine with Pierce's disease. **8,** Note numerous small globules among bacterial cells. **9,** Cross and longitudinal sections of bacteria (arrows) are visible within masses of globules that appear to comprise much of the plug. **10,** Note that this plug appears to be comprised mainly of globular material.

did not know to which of the three groups of petioles a specimen belonged. In addition, a map of the vascular tissue of each cross section was made, showing which vascular bundles contained bacteria. No attempt was made to map the distribution of colonized tracheary elements within individual vascular bundles.

X-ray energy dispersive analysis of crystals. Elemental analysis of crystals found in the lumen of tracheary elements was performed with a Tracor Northern 2000 X-ray Analyzer on specimens imaged with a Hitachi HHS-2R scanning electron microscope.

RESULTS

Light microscopy. Examination of macerated petioles revealed that both tracheids and vessel members were present in the vascular tissue. Since it is impossible to distinguish between the two in cross sections of petioles viewed by scanning electron microscopy, the inclusive term "tracheary element" is used in the present report (6).

Ultrastructure of PD bacterium. Transmission and scanning electron microscopy verified the presence of features known from previous studies to be characteristic of the PD bacterium and its distribution in the host (3,7,10,14). These features included: the size and rod shape of the bacterium (1.0–2.2 μm long and 0.39–0.53 μm wide, as measured on scanning electron micrographs); the ridged nature of the bacterial cell surface; the membranelike ultrastructure of the cell wall which is typical of Gram-negative cells; and the xylem-limited occurrence of the PD bacteria within the host plant.

Tracheary elements of the control plant. In petioles from the healthy control plant, the lumens of tracheary elements were typically empty and the patterns of thickenings of the secondary cell wall were clearly visible (Fig. 1). No bacteria were present, and in most petioles tyloses were seldom seen. In two of the six control petioles that were examined, a few crystals were found in the lumens of tracheary elements (Fig. 2).

Tracheary elements of a PD-affected plant. Tyloses were much more abundant in petioles from the diseased plant than in control petioles. Bacteria were found in some tracheary elements of all petioles from the diseased plant, and a variety of morphological types of bacterial aggregates occurred both in petioles of leaves with MLB and in petioles of apparently normal leaves. Sometimes the bacteria were present in small numbers, often as solitary cells or in sparse clusters attached to the wall of a tracheary element. In other instances, large numbers of bacterial cells were present and formed aggregates of various sizes and shapes. Some aggregates were compact, and the bacteria in them appeared to be stuck together by a structureless or slightly granular substance (Fig. 3). Other aggregates were loosely organized, and the outlines of individual bacterial cells were well defined (Fig. 4). In the latter kind of aggregate, fine strands, or fimbriae (16), often connected individual bacteria to neighboring cells or to the wall of the tracheary element (Fig. 5).

In a few colonized tracheary elements, sizable networks of fibrillar material were associated with bacterial aggregates (Figs. 6

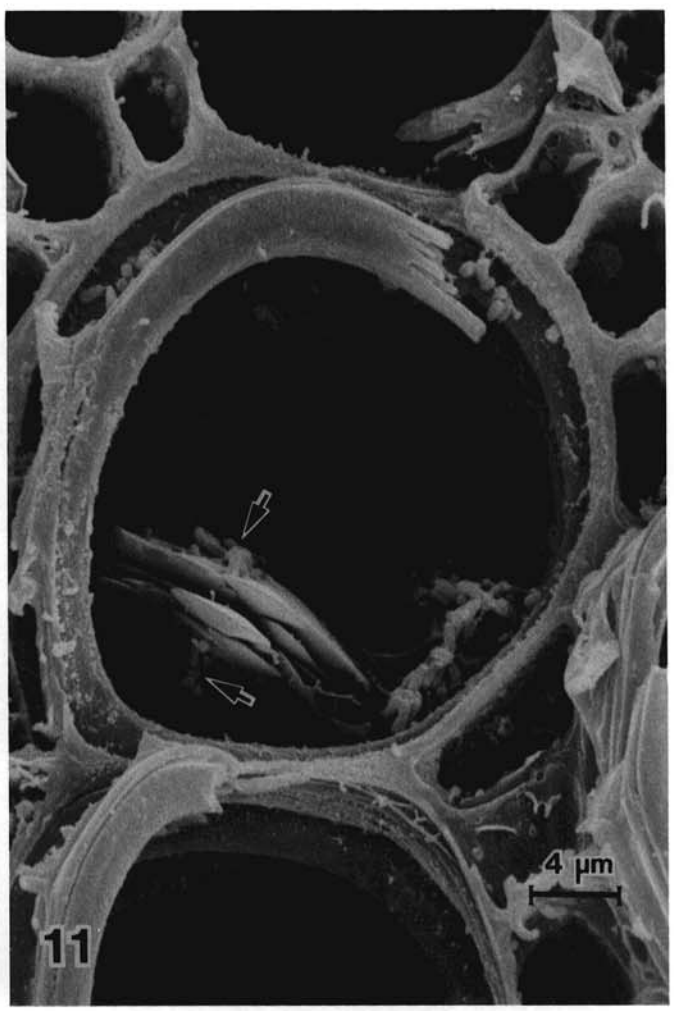


Fig. 11. Scanning electron micrograph of calcium-containing crystals in a tracheary element of petiolar xylem of grape leaf. Note the rarely seen association of Pierce's disease bacteria and crystals (arrows).

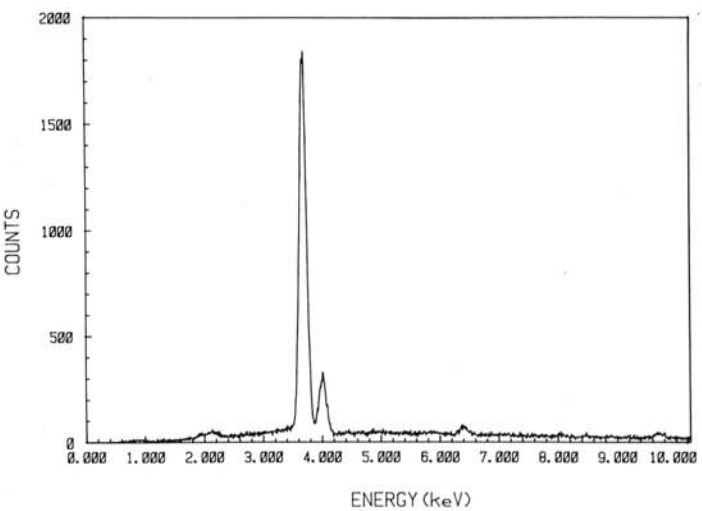


Fig. 12. X-ray spectrum (energy dispersive analysis) of crystal in tracheary element of petiolar xylem of grape leaf, showing presence of calcium. The specimen (not osmicated) was mounted on a carbon planchet with carbon paste and very lightly coated with gold. The major peaks are $Ca_{K\alpha}$ and $Ca_{K\beta}$.

TABLE 1. Percentages of colonized and completely plugged tracheary elements in petiolar xylem of 12 leaves from a grapevine with Pierce's disease

Leaf appearance	Leaf no.	Tracheary elements with bacteria (including plugs) (%)	Tracheary elements with complete plugs (%)
Normal	1	2.5	0
	2	15.9	5.4
	3	19.0	7.5
	4	24.2	4.9
	5	26.6	7.5
	6	29.8	9.0
Marginal leaf burn	1	15.8	4.1
	2	20.6	4.5
	3	20.8	9.1
	4	25.1	12.0
	5	26.6	3.7
	6	29.3	11.2

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Fig. 13. Diagrams of petiolar vascular bundles of leaves from grapevine with Pierce's disease: **A,** leaves with normal appearance; **B,** leaves with marginal leaf burn. Shading indicates that colonized tracheary elements occurred in the bundle; no bacteria were found in the xylem of unshaded bundles. The petioles are arranged according to percentage of colonized tracheary elements, with the lowest percentage on left side and highest on right (see Table 1 for exact percentages).

and 7). Typically, some fibrils of a network appeared to be attached to bacterial cells, whereas others anchored the network and its associated bacterial mass to the wall of the tracheary element.

Plugs that completely occluded the lumens of tracheary elements were found in all but one petiole. Some plugs were composed mainly of bacteria, and the outlines of individual cells were clearly visible, even though numerous small globules were also present, either attached to the surface of bacteria or filling the interstices between cells (Fig. 8). In many plugs, however, the major component of the plug was the globular material, which completely encased the bacterial cells and rendered them visible only in sectioned views (Fig. 9). In still other plugs there was little or no evidence of bacteria, and the occluding material appeared to consist almost completely of small globules (Fig. 10).

Intralumenal crystals, though rare in occurrence, were found in three of six petioles from leaves with MLB and in five of six petioles from apparently normal leaves. In only two instances were such crystals seen to be associated with bacteria (Fig. 11). X-ray energy dispersive analysis revealed that the crystals in tracheary elements contain calcium (Fig. 12).

Percentage of colonized tracheary elements. Most petioles of apparently normal leaves from the diseased plant were remarkably similar to petioles of leaves with MLB in regard to the percentage of colonized tracheary elements present (Table 1). Of a total of 3,207 elements examined in petioles from apparently normal leaves, 624 or 19.5% contained bacteria. Of 3,613 elements examined in petioles from MLB leaves, 814 or 22.5% contained bacteria. Complete plugs were found in 5.8 and 7.7% of the petiolar tracheary elements from normal and MLB leaves, respectively.

Maps of the vascular tissue also revealed no consistent difference between members of the two groups of petioles. As shown in Fig. 13, the vascular tissue of the petiole consists of a bilaterally symmetrical circle of vascular bundles, plus two small bundles outside the circle (17). In most petioles, almost all of the bundles had some colonized tracheary elements. In only one petiole, from a leaf with normal appearance, were bacteria restricted to a small part of the vascular circle (Fig. 13). This same petiole had the lowest percentage of colonized tracheary elements (2.5%).

DISCUSSION

The fimbriae, fibrillar networks, and globular material may be responsible for anchoring bacterial cells to other bacteria and/or to the wall of a tracheary element. The fimbriae are presumed to be a product of the bacterial cells and to function as attachment appendages, as do similar organelles described from many other kinds of bacteria (16). With respect to the fibrillar networks, nothing is known about the source or chemical nature of this material; the fibrils could have been produced by either bacterial or host cells. The globular material is also of unknown origin, but it is likely that it is at least partly comprised of gums secreted by the host in response to the presence of pathogenic bacteria. Gummosis is common in plants with vascular wilt diseases (4,20) and has been reported in previous studies of PD in grapevines (5,15). Although accumulations of gums in the vascular system of diseased plants undoubtedly contribute to occlusion of conducting elements, gums are also thought to restrict the movement of pathogens and thus contribute to disease resistance (20).

Tylosis is another common condition found in plants suffering from vascular wilt syndrome (20). Like gummosis, tylosis can significantly decrease vascular transport and is regarded as a beneficial host response, in that containment of invading pathogens may result. Tylose formation is well documented by light- and transmission electron microscopy in grapevines with PD (5,15). Mollenhauer and Hopkins (15) reported a higher frequency of both gums and tyloses in tolerant species of grapevines, as compared to more susceptible bunch grapevines.

The calcium-containing crystals found in the lumen of tracheary elements may be calcium oxalate. Calcium oxalate crystals are the most common kind of biomineralization in higher plants and have been described from a variety of tissues and organs of plants from numerous families (1,2,12,22). There is no evidence to suggest that the crystals, whatever their chemical composition, are related in any way to PD.

The overall percentage of colonized elements was less in apparently normal leaves compared to leaves with MLB (19.5 versus 22.5%) and the same was true for the percentage of plugs (5.8 versus 7.7%); however, comparisons of percentages from individual petioles (Table 1) reveal that in most instances it would be impossible to predict whether or not a particular leaf had MLB. An extensive light microscopic study of seasonal variations in the concentration of PD bacteria showed only small differences between petioles of symptomless leaves and leaves with marginal necrosis, but significant differences were found in leaf veins (9). Although the development of PD symptoms is positively correlated with the concentration of bacteria in the xylem of the host (9), the relative importance of physical blockage and bacterial phytotoxins in inducing foliar alterations is not known (13). Indeed, assessment of the degree of obstruction present is not a simple task for the microscopist. Total plugs, though easy to recognize and count, are

not the only physical feature that may increase resistance to flow. The amount of frictional drag can be influenced by the presence and nature of any materials found within conducting elements (20), including tyloses, bacterial clumps or mats, accumulations of gums or other secretory products, etc. Furthermore, some phytotoxins act as occlusive agents, rather than cell toxins, in that they may clog minute pores in pit membranes, either close to or far from their site of production (4,20). In such cases, the dichotomy between "toxin" and "occlusion" breaks down, for the basic effect of clogged pores or plugged tracheary elements is the same, namely an increase in resistance to flow in the vascular system of the diseased plant.

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