

## Structural and Morphological Changes Associated with the Pierce's Disease Bacterium in Bunch and Muscadine Grape Tissues

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Paper 10412 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.

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Accepted for publication 20 June 1986 (submitted for electronic processing).

### ABSTRACT

Huang, P.-Y., Milholland, R. D., and Daykin, M. E. 1986. Structural and morphological changes associated with the Pierce's disease bacterium in bunch and muscadine grape tissue. *Phytopathology* 76:1232-1238.

Bacteria with rippled, intermediate, and smooth cell walls occurred consistently in the lumen of tracheary elements of naturally infected and artificially inoculated grape leaves showing Pierce's disease (PD) symptoms from the field and greenhouse, respectively. Bacteria did not occur in tissues prepared from healthy controls. Bacteria were rod-shaped, averaging  $0.5 \times 1.5 \mu\text{m}$ . Bacteria isolated from bunch grapevines exhibiting PD symptoms also had rippled, intermediate, and smooth cell walls among the cells from a single-colony culture. Morphological change of the PD bacterium from rippled to smooth cell walls developed faster in tolerant muscadine tissues than in susceptible bunch grape tissues. An electron-dense substance was associated with smooth cell-walled bacteria but not

with bacteria with the rippled cell walls. The reason for the differential association of the electron-dense substance with bacteria with smooth and not with rippled cell-walls is not known. However, the encapsulation of smooth cell-walled bacteria by the electron-dense substance occurred more frequently in muscadine grapevines than in the bunch grapes. This substance may be an important mechanism whereby muscadine grapevines defend themselves against the PD bacterium. Based on histochemical tests, the vascular plugs associated with PD are primarily pectic substances with some gums and tannins. Therefore, the electron-dense substances surrounding the smooth cell-walled bacteria are of host origin.

Pierce's disease (PD) is caused by a gram-negative, xylem-limited bacterium measuring  $0.25\text{--}0.50 \mu\text{m}$  in diameter by  $1.0\text{--}4.0 \mu\text{m}$  in length (11). The disease was first reported on muscadine grape (*Vitis rotundifolia* Michx.) in North Carolina in 1981 (10). Since that time, we have isolated the PD bacterium from infected muscadine and bunch (*V. vinifera* L.) grapevines showing typical PD symptoms.

Bunch grapevines infected with the PD bacterium show necrosis at the leaf margin, decline in vigor, reduced yield, and they eventually die (3). In contrast, infected muscadine grapevines of the cultivar Carlos do not die and do not always show symptoms each year (1). In an attempt to identify sources of resistance to PD in *Vitis*, Mortensen et al (13) reported that of the 222 clones of bunch grapes tested, only 21 were resistant to PD, whereas 22 of the 60 muscadine clones were highly resistant to the same disease.

In earlier studies, we observed bacteria with rippled, intermediate, and smooth cell-walls in the lumen of tracheary elements of muscadine grapevines showing PD symptoms in the field (Milholland and Huang, unpublished). Leaf samples taken from the cultivar Carlos showing PD symptoms in 1979 had rippled and smooth cell-walled bacteria; however, samples taken from the same infected plant showing PD symptoms in 1982 had bacteria with only smooth cell-walls. No symptoms were observed on this same Carlos plant in 1983, 1984, or 1985; histological examinations of the symptomless leaves were not made for these 3 yr. Electron microscopy of leaf samples taken in 1982 from the susceptible bunch grape cultivar Chardonnay showing PD symptoms revealed that 90% of the bacteria in the tracheary elements had rippled cell-walls.

Gram-negative bacteria with smooth cell-walls also occur in the xylem vessels of infected periwinkle tissue along with those that have rippled cell-walls (9). Both rippled and smooth cell-walled

bacteria were observed in the process of cell division; the observed differences in morphological form of the bacteria were thought to represent different stages of growth of one organism. Progressive changes in the cell wall of the PD bacterium also were observed in naturally infected bunch grapes. These changes were interpreted to be occurring as a process of aging, or the bacteria were going through developmental stages (11). However, the possibility that the cell wall forms represent two different organisms should not be ignored (2,5).

The objectives of this study were to determine the cell-wall morphology of PD bacteria in both bunch and muscadine grapevines after inoculation with cells from an axenic culture, and to investigate the possible relationship between cell-wall morphology and plant resistance.

### MATERIALS AND METHODS

Leaf samples of muscadine grape cultivar Carlos (resistant to PD) and the bunch grape cultivar French Colombard (susceptible to PD) exhibiting PD symptoms were collected from the Horticultural Crops Research Station, Castle Hayne, NC, and Central Crops Research Station, Clayton, NC, respectively.

The PD bacterial strain used in this study was isolated from French Colombard exhibiting typical PD symptoms by expressing the sap from the petioles onto a modified JD-3 medium (6). A single colony of the PD bacterium was obtained after dilution plating and then grown on the medium at 28 C. Inoculation of French Colombard in the greenhouse with this bacterial strain resulted in typical PD disease. PD bacteria were reisolated from inoculated plants.

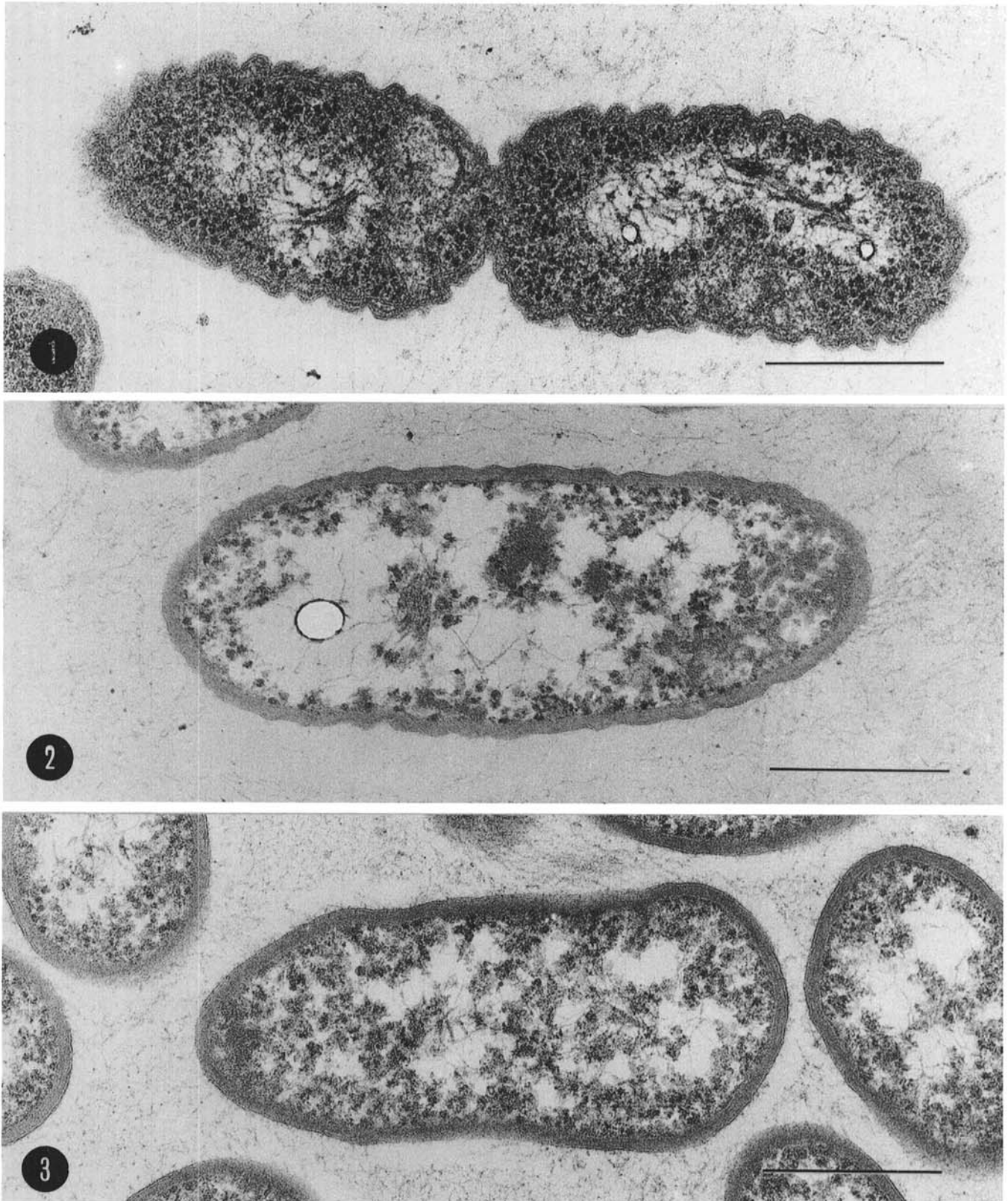
Six-week-old greenhouse-grown Carlos and French Colombard plants were inoculated at the base of the lower node by pricking the stem through 0.02-ml drops of a PD bacterial suspension of 10-day-old cultures adjusted to  $10^7$  colony-forming units per milliliter. Stems of control plants were similarly pricked through drops of sterile water. Each treatment consisted of eight plants. All plants were kept in a greenhouse at 25–30 C. Leaves of muscadine and bunch grapes collected for electron microscopy were near the inoculation point. Three samples were collected from each of the

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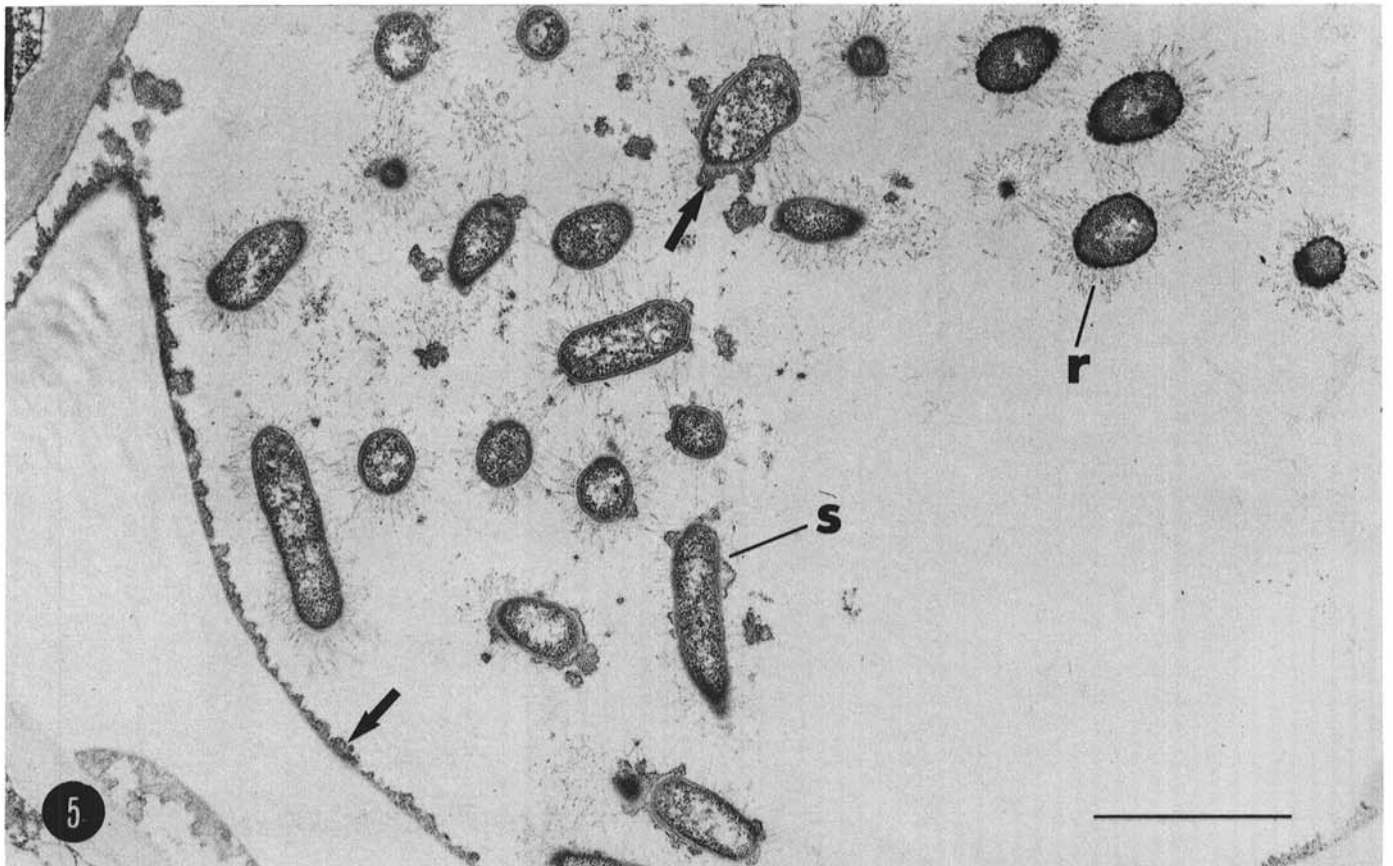
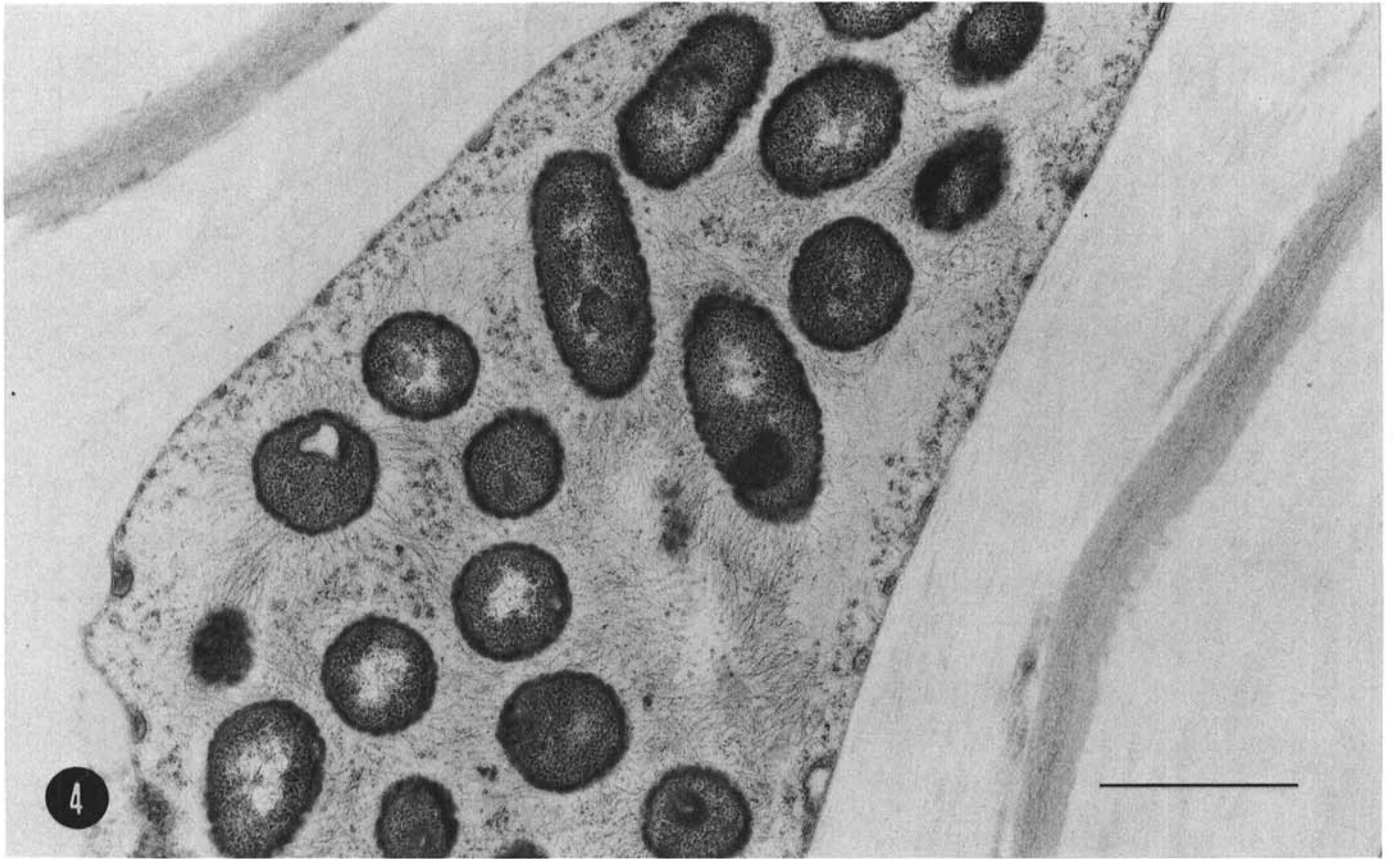
eight French Colombard plants and from each of the eight Carlos plants 2 and 3 mo after inoculation. Three samples were also collected from the control plants.

Samples of Carlos grape leaves collected from the field in 1979 and samples of Carlos and French Colombard grape leaves collected from greenhouse inoculations in 1985 were processed for electron microscopy immediately after harvesting. Leaf midveins

were cut into 1–2-mm sections and fixed with 4% glutaraldehyde in 0.066 M sodium cacodylate for 4 hr at 4 C. After washing with sucrose-cacodylate, the samples were postfixed with 1% OsO<sub>4</sub> in Millonig's buffer at 4 C for 4 hr, dehydrated, and embedded in Epon or LX-112. Pellets of 7- and 15-day-old PD bacterial cultures were prepared for transmission electron microscopy in the same manner as plant samples. Ultrathin sections were cut with a



**Figs. 1-3.** PD bacteria in lumen of tracheary elements in inoculated bunch grape. Bar = 0.4  $\mu$ m. **1,** Bacterium with rippled cell walls. **2,** Bacterium with intermediate cell wall. **3,** Bacterium with smooth cell wall is a high magnification of a portion of Fig. 13.



**Figs. 4 and 5.** PD bacteria in lumen of tracheary elements in muscadine grape showing Pierce's disease symptoms in the field. **4,** Small tracheary element filled with rippled cell wall bacteria. Note microfibrils extending from these bacteria. Bar = 1  $\mu$ m. **5,** Bacteria with smooth (s) and rippled (r) cell walls in tracheary elements. Note electron-dense substances (arrow) associated with smooth cell-walled bacteria and tracheary element walls. Bar = 2  $\mu$ m.

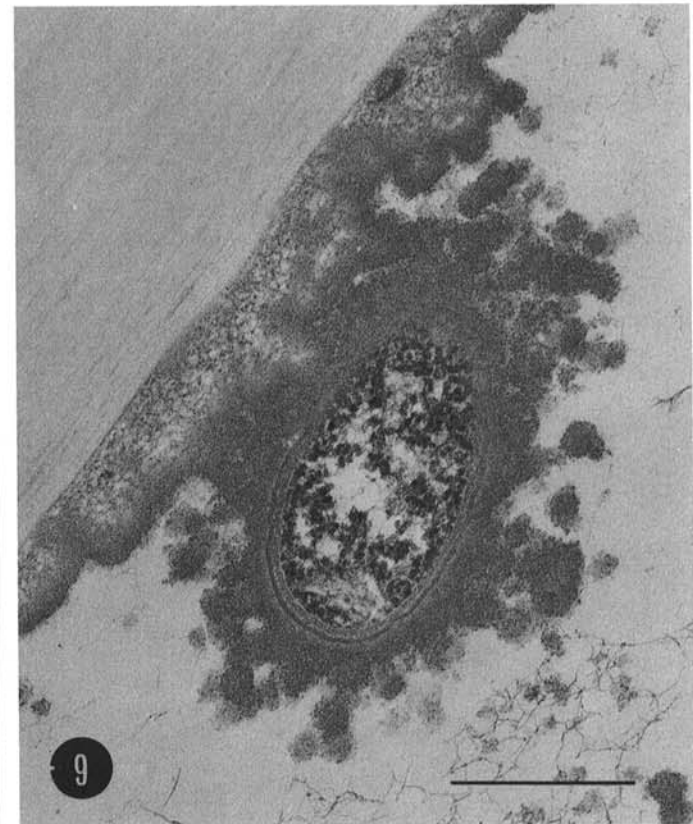
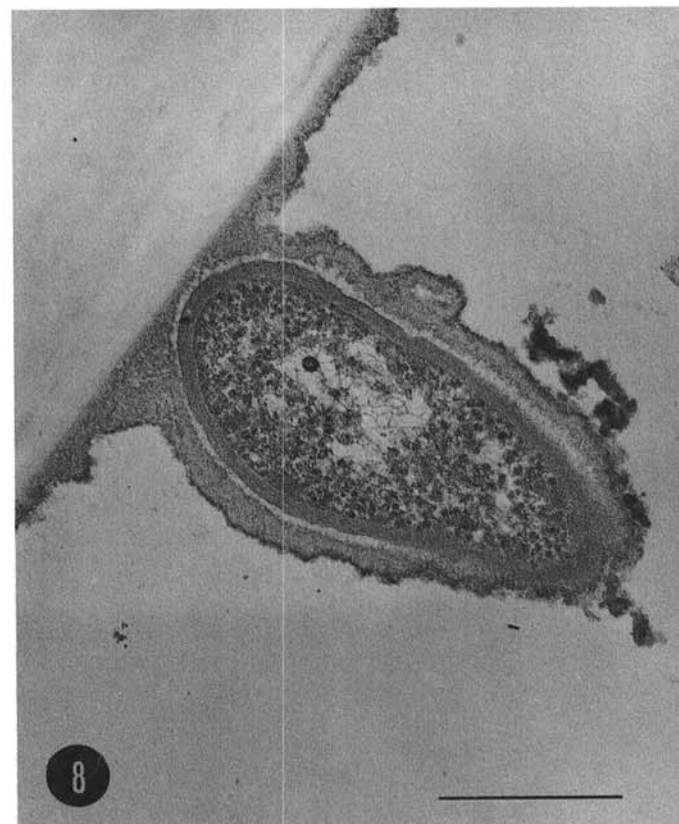
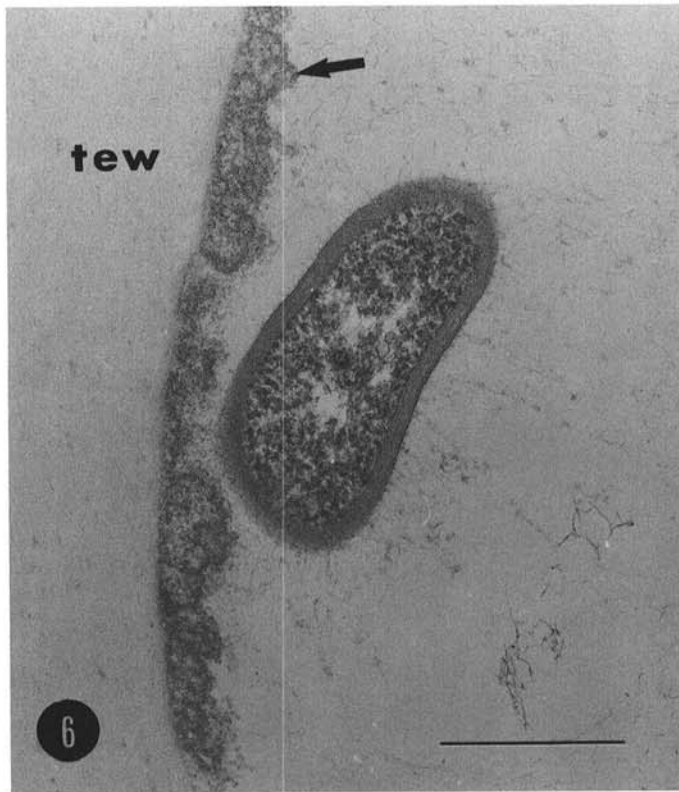
diamond knife and transferred to 300 mesh grids and sections were stained as described by Goodman et al (4). Electron micrographs were taken with a Siemens Elmiskop 1-A or JOEL 100-S electron microscope.

Serial sections of Carlos petioles known to be naturally infected with the PD bacterium were tested for pectin by the ruthenium red method and the iron absorption method, for gum with phloro-

glucinol, for tannins with ferric sulfate, and for bacteria with the Harris hematoxylin stain (8).

## RESULTS

The eight French Colombard plants inoculated with PD bacteria exhibited marginal necrosis on leaves near the inoculation



**Figs. 6-9.** PD bacteria in lumen of tracheary elements in inoculated muscadine grape grown in the greenhouse showing the association of bacteria with the electron-dense substance (arrow) originally deposited on tracheary element wall (tew). Bar = 0.4  $\mu$ m.

site after 2 mo. Systemic symptoms appeared about 3 mo after inoculation and all plants died 2 mo later. All eight inoculated Carlos plants displayed marginal necrosis on some leaves near the inoculation points after 3 mo; however, none of the plants died. All control Carlos and French Colombard plants remained healthy throughout the experiment.

Bacteria were consistently observed in the lumen of tracheary elements of leaves from naturally infected and artificially inoculated grape with PD symptoms. No bacteria were found in the tissues prepared from controls. Bacteria with rippled (Fig. 1), intermediate (Fig. 2), and smooth cell walls (Fig. 3) were observed in inoculated French Colombard plants. Bacteria were rod-shaped, 0.4–0.7  $\mu\text{m}$  in width and 1–2.3  $\mu\text{m}$  in length with an average size of  $0.5 \times 1.5 \mu\text{m}$ . Because of the rippling, the thickness of walls of rippled cells ranged from 25 to 40 nm, and that of smooth cells ranged from 35 to 43 nm and averaged 40 nm. Thickness of cell walls was obtained by measuring 100 cells of rippled and smooth walled bacteria, respectively. Cytoplasm of bacteria with rippled cell walls was generally more intensively stained than those with intermediate or smooth cell walls. Microfibrils extended from cell walls of most bacteria. Ultrastructural observations of 7-day-old bacteria grown in vitro revealed that most bacteria had rippled cell walls. Most bacteria from 15-day-old cultures, however, had smooth cell walls with uneven distribution of ribosomes and reduced electron density in the cytoplasm.

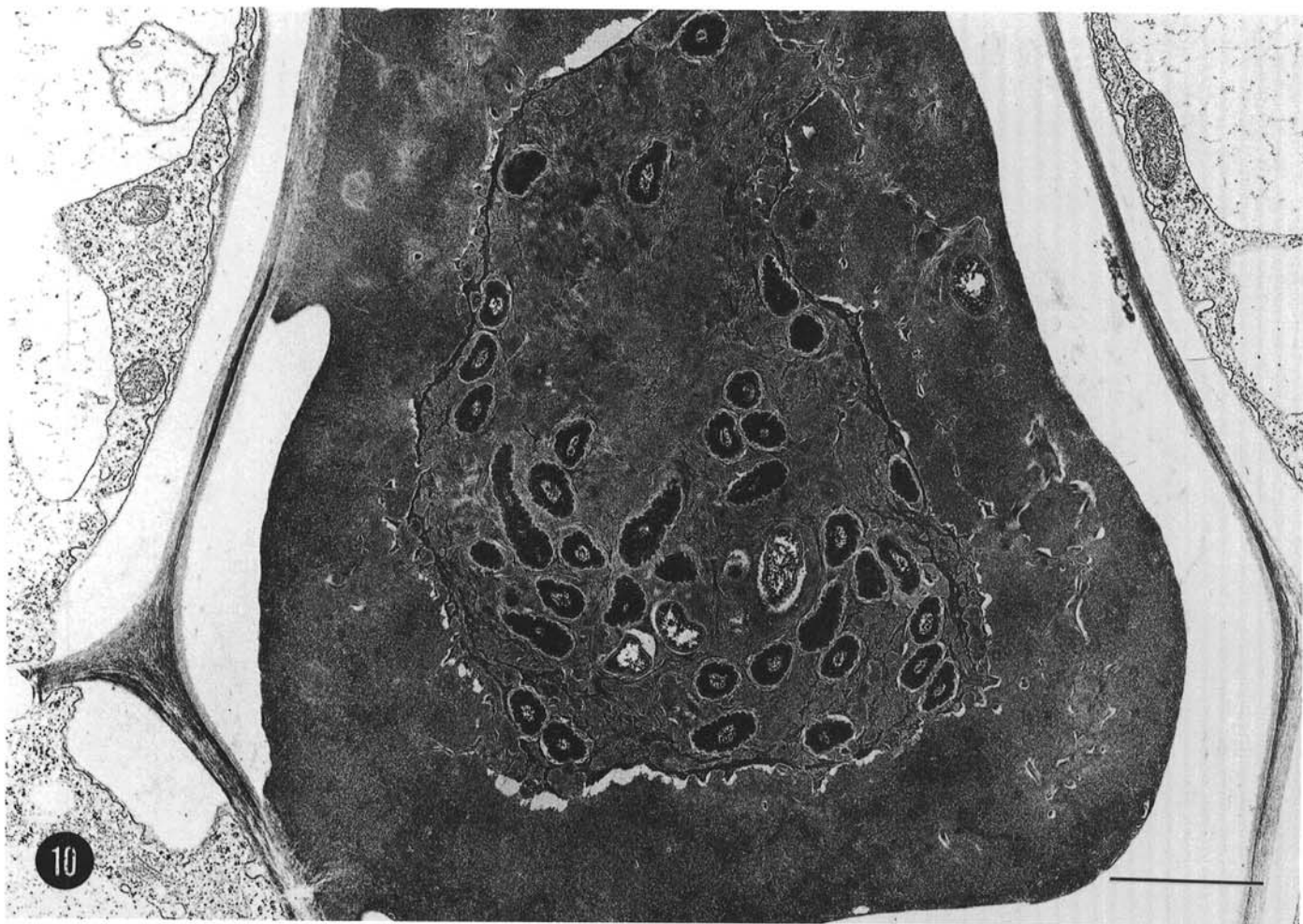
The majority of the bacteria observed in small tracheary elements of field-grown Carlos grapevines showing PD symptoms had rippled cell walls (Fig. 4), whereas those in large tracheary elements had predominantly smooth cell walls (Fig. 5). Association of bacteria with an electron-dense substance was rarely observed in the small tracheary elements, but was frequently

observed in the large tracheary elements (Fig. 5). We examined 1,405 bacteria in large elements and found that 1,124 cells (80%) had smooth walls and 281 (20%) had rippled walls. Approximately 54% of the smooth cell-walled bacteria and only 1.8% of rippled cell-walled bacteria were surrounded by the electron-dense substance.

There were no PD symptoms on leaves of Carlos plants 2 mo after inoculation and no bacteria were observed in the tracheary elements of the Carlos samples collected at this time. Leaf samples collected at 3 mo displayed mild marginal burn symptoms. We examined 975 bacteria in the tracheary elements and found 57% to have smooth cell walls, whereas the remaining 43% were rippled cell-walled. About 50% of the smooth cell-walled bacteria and 1.5% of the rippled cell-walled bacteria were surrounded by an electron-dense substance.

Electron-dense substances were observed on the inner surface of tracheary element walls of Carlos grapevines. Most bacteria with intermediate or smooth cell walls were often associated with the electron-dense substance (Fig. 6). In some instances, the electron-dense substance had separated from the inner surface of the plant cell wall and was in close proximity to the bacteria (Fig. 7). Bacteria were often completely encapsulated (Fig. 8), and after encapsulation, the electron-dense substance surrounded the bacterium (Fig. 9). At the advanced stage of encapsulation (Fig. 10), massive electron-dense substance completely filled the lumen of tracheary elements and entrapped bacteria, regardless of cell wall type.

Leaf samples of French Colombard collected 2 mo after inoculation exhibited mild marginal necrosis. Bacteria were evenly distributed in the lumens of tracheary elements (Fig. 11). The majority of bacteria had rippled walls; less than 5% had smooth



**Fig. 10.** Tracheary elements in inoculated muscadine grape grown in the greenhouse showing a mass of electron-dense substance encapsulating bacteria with all types of cell walls. Bar = 2  $\mu\text{m}$ .

walls at this stage of infection. Association of bacteria with electron-dense material occurred in the tracheary elements 3 mo after inoculation when the population of smooth cell-walled bacteria increased (Fig. 12); the association of bacteria with electron-dense material in bunch grapevines, however, was not observed as frequently as in muscadine grapevines. Tracheary elements filled with smooth-walled bacteria without the association of the electron-dense substance was observed in some cases (Fig. 13).

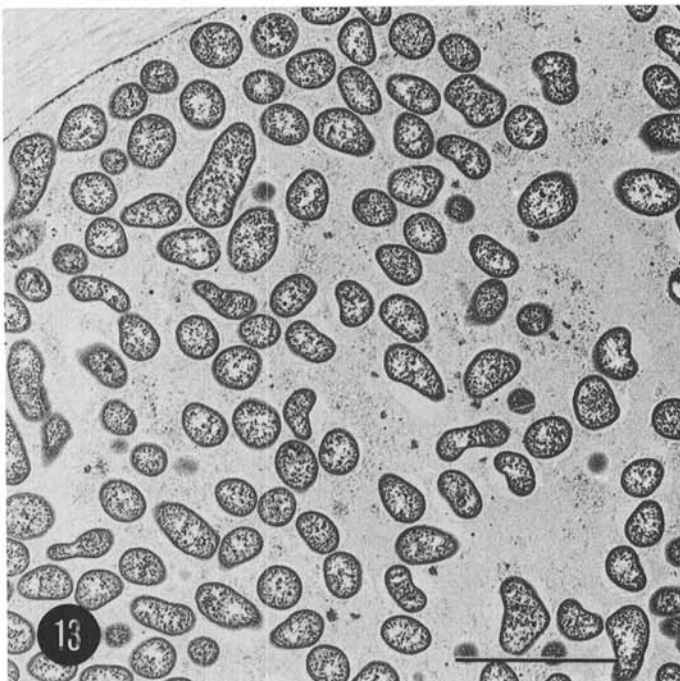
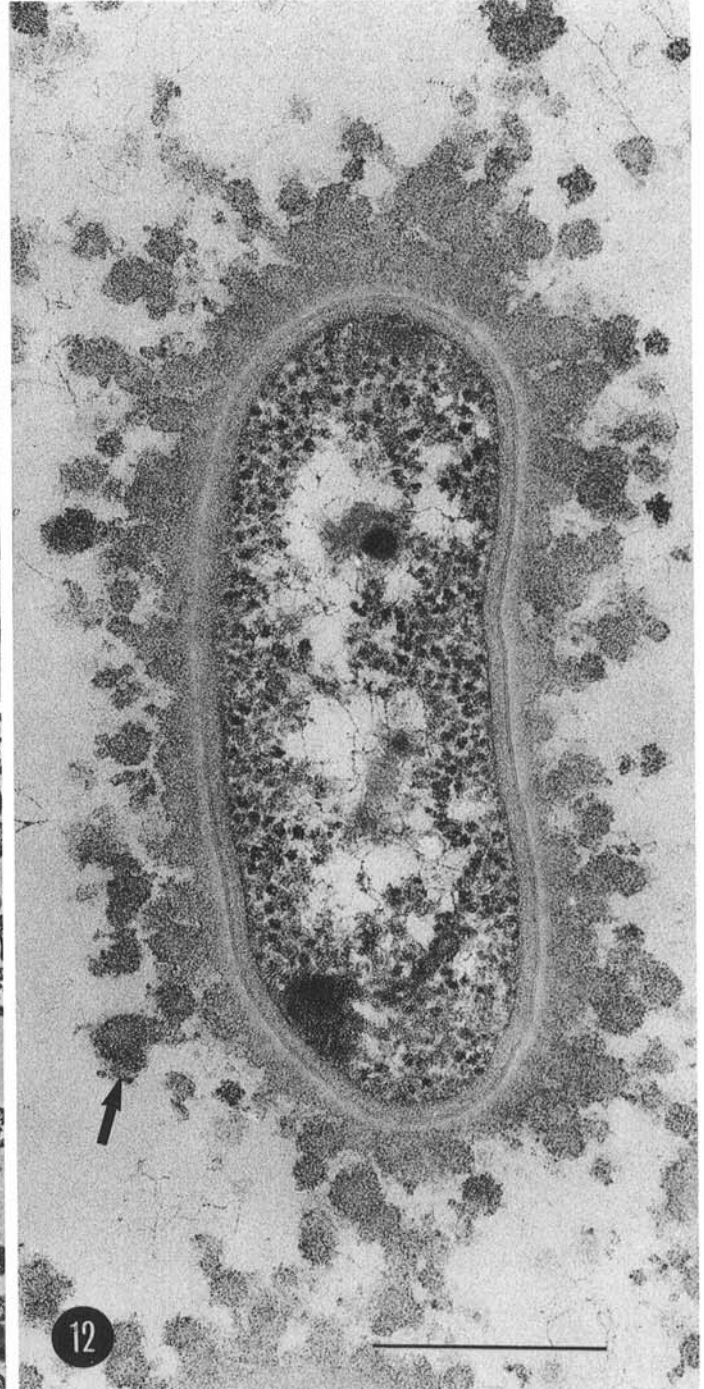
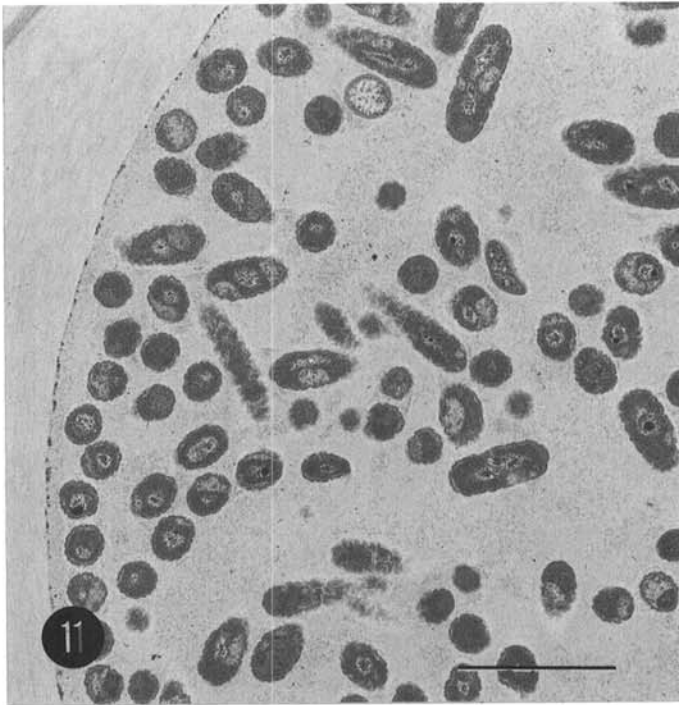
Based on histochemical analysis of serial sections, small tracheary elements stained positive for hematoxylin but rarely for ruthenium red, iron absorption, phloroglucinol, and ferric sulfate. The large tracheary elements that were plugged frequently stained positive for ruthenium red and iron absorption. Often these

elements were also positive for phloroglucinol and/or positive for tannins. A positive reaction for hematoxylin in these tracheary elements also was observed.

## DISCUSSION

The distribution patterns of PD bacteria in the grapevines and accumulation of larger quantities of electron-dense gums in tolerant muscadine than in susceptible bunch grapes are well documented (11,12). Hopkins et al (7) stated that the cell walls of PD bacteria observed in the muscadine grapevines were usually rippled. However, we found both rippled and smooth-walled bacteria in muscadine grapevines.

PD bacteria from a single-colony culture and PD bacteria from



**Figs. 11-13.** PD bacteria in lumen of tracheary elements in inoculated bunch grape grown in the greenhouse. **11,** PD bacteria evenly distributed in lumen of tracheary elements in leaves 2 mo after inoculation. Most bacteria had rippled cell walls. Bar = 2  $\mu$ m. **12,** Electron-dense substance (arrow) surrounding smooth cell-walled bacterium in lumen of tracheary elements in leaves 3 mo after inoculation. Bar = 0.4  $\mu$ m. **13,** PD bacteria in lumen of tracheary elements in leaves 3 mo after inoculation. Most bacteria had smooth cell walls but bacterial entrapment was not observed. Bar = 2  $\mu$ m.

field and greenhouse plants inoculated with an axenic culture also had rippled, intermediate, and smooth cell walls among the bacteria. This observation leads us to conclude that PD bacteria have different cell-wall morphologies.

Cytoplasm of bacteria with rippled cell walls generally stained more intense than those with intermediate and smooth cell walls. Three months after inoculation, populations of bacteria with intermediate and smooth cell walls had increased from 5 to 75%, whereas the percentage of bacteria with rippled cell walls that was prevalent at 2 mo decreased from 95 to 25% in bunch grapes inoculated with PD bacteria. These observations suggest that bacteria with intermediate or smooth cell walls are in the process of aging.

The electron-dense substance reported here is similar to the dense-staining substances thought to be gums by Mollenhauer and Hopkins (12). They reported that gums and tyloses often encapsulated the bacteria and restricted the movement of bacteria in xylem vessels, thereby playing an important role in tolerance to PD (11). We observed that about 50% of intermediate and smooth cell-walled bacteria and only 1% of rippled cell-walled bacteria were surrounded by electron-dense substances in the Carlos grapevine infected with PD bacterium. It is possible that aging bacteria are more susceptible to encapsulation by the electron-dense substance. Exception does exist in lumens of tracheary elements filled with massive electron-dense substance where bacteria were always encapsulated, regardless of their cell wall types.

The chemical nature of the electron-dense substances deposited on the inner surface of plant cell walls and surrounding the smooth cell-walled bacteria is not known. Histochemical tests revealed that small tracheary elements contained bacteria but rarely pectin, gum, or tannins. The bacteria in such elements had primarily rippled cell walls with extending microfibrils as revealed by EM. On the other hand, the large tracheary elements that were plugged, frequently contained pectin and some also contained gum and/or tannins. Bacteria were also found in these elements. Based on the histochemical tests, the electron-dense substance is of plant origin, and may be pectin, gum, and/or tannins, secreted by the host in response to the presence of the PD pathogen and responsible for bacterial encapsulation.

When PD bacteria of the same strain were introduced into the resistant and susceptible cultivars, PD bacteria multiplied more extensively in the susceptible bunch grape than in the resistant muscadine grape. Two months after inoculation, the population of smooth cell-walled bacteria were lower in the susceptible tissue. At 3 mo, when the population of smooth cell-walled bacteria increased in the susceptible tissue, encapsulation of bacteria could be found. However, susceptible cultivars such as French Colombard do not produce as much electron-dense substance to limit invasion by the PD bacteria as do the more tolerant muscadine cultivars, even in some tracheary elements that were filled with smooth cell-walled bacteria, there was no bacterial encapsulation (Fig. 13). These observations indicate that susceptible cultivars apparently do not arrest the PD bacteria in time to

prevent their systemic movement up the stem.

During the past 3 yr, PD bacteria isolated from muscadine and bunch grapes in North Carolina were frequently unable to infect muscadine grapevines and only infected a few twigs of French Colombard after subculturing on JD-3 medium over a period of 8 mo. Electron microscopy of French Colombard leaves inoculated with attenuated cultures revealed that most bacteria in the tracheary elements had smooth cell walls and were completely encapsulated by the electron-dense substance. Bacteria with rippled cell walls were rarely observed (Milholland and Huang, unpublished). These observations suggest that the amount of the electron-dense substance produced by the host is dependent on the cultivar of the grape and the virulence of the bacterium. Thus, the electron-dense substance we observed in our studies may play an important role in the incompatibility of host-parasite interactions.

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