Isolation, Culture, and Pathogenicity of the Bacterium Causing Phony Disease of Peach

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ABSTRACT

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Fastidious, Gram-negative, xylem-limited bacteria (XLB) were isolated from peach (*Prunus persica*) trees with symptoms of phony disease. Following inoculation by root infiltration, stunting and severe rosetting of terminal twigs occurred in 3 of 4 cultivar Lovell, 3 of 10 cultivar Halford, and 2 of 10 cultivar Nemagard peach seedlings. The XLB in situ were 0.35 × 3 μm in maximum diameter and length, were present in all inoculated peach seedlings with symptoms typical of phony disease, and were reisolated on BCYE medium 18 mo after the plants were inoculated. Results of ELISA serology with antiserum prepared against the phony peach XLB were similar with bacteria isolated from naturally diseased trees and from artificially inoculated seedlings. The serology, morphology, and ultrastructural properties of the reisolated bacteria were identical to that of XLB present in naturally infected trees and to that of the bacteria used in the original inoculations.

Additional key words: rickettsialike bacteria.

Phony disease of peach (*Prunus persica* [L.] Batsch) has caused serious and recurrent losses of trees in orchards in the southeastern USA since 1890 (8). In 1973, a "rickettsialike" bacterium was associated with the disease (7). The bacterium was limited to the xylem and was transmitted by sharpshooter leafhopper vectors (18). Pathogenicity, however, could not be tested because the bacterium could not be cultured on standard bacteriological media. Synthetic media have recently been developed for the axenic culture of several fastidious, Gram-negative bacteria (XLB) from plants, including the XLB associated with phony peach disease (3,21). Bacteria can now be cultured and then inoculated into host plants to test pathogenicity according to Koch's postulates.

Two other plant diseases etiologically similar to phony peach disease, Pierce's disease of grapevines (*Vitis vinifera* L.) and leaf scald disease of plum (*P. salicinia* Lindl), are caused by fastidious, rod-shaped, Gram-negative XLB (2,15). Similar organisms also have been associated with leaf scorch of elm (*Ulmus americana* L.) and of sycamore (*Platanus occidentalis* L.), wilt disease of periwinkle (*Vinca minor* L.), wilt of bentgrass (*Agrostis palustris* L.), and stunt of ragweed (*Ambrosia artemisifolia* L.) (4,6,10,16,17). At present, however, there are no reports on experimental proof of the bacterial etiology of phony peach disease.

This report describes the isolation of the bacterium from the xylem of peach trees affected with phony disease, the cultivation of the bacterium on an artificial medium, the development of symptoms typical of phony disease on experimentally inoculated seedlings, and the reisolation of the bacterium from diseased seedlings.

MATERIALS AND METHODS

Isolation and culture of the bacterium. Original isolates of the bacterium were obtained from 7-yr-old Dixiland peach trees growing at the Southeastern Fruit and Tree Nut Research Laboratory, U.S. Department of Agriculture, Byron, GA. Naturally infected trees showed symptoms of phony disease: shortening of internodes of terminal branches, deep green leaves, umbrella-shaped canopy profile, and undersized fruit (18). Root sections (3–5 mm in diameter) were cut into 3-cm sections that were surface sterilized five times in 0.5% sodium hypochlorite solution containing 3% ethyl alcohol for 5 min and rinsed each time in sterile distilled water. Sections were then aseptically placed in a hand vise and gently crushed and the expressed sap was blotted onto BCYE agar medium (21). The same procedure was used for reisolation of bacteria from stem and root sections of artificially inoculated peach seedlings.

Agar plates were incubated in the dark for 7 days under normal atmosphere at 21 C, then examined for development of circular, opalescent primary colonies. Colonies were examined at ×400 with a phase microscope (Carl Zeiss, Inc., New York, NY 10594) for the presence of rod-shaped cells 0.35 × 2–3 μm embedded in a matrix of filamentous strands of similar width, but of variable length. After the initial isolation and growth of bacteria from diseased peach, isolates were transferred and maintained on BCZE agar medium (15) or on BCYE.

Inoculation of test plants. The phony peach bacterium (ATCC 33489) was used as inoculum. Inoculum was grown for 15–21 days on BCYE or BCZE medium, harvested in distilled water, and diluted to a $\sim 10^7$–10^8 cells per milliliter. Plants were inoculated by root infiltration. Healthy cultivar Lovell, Halford, and Nemagard peach seedlings, 3–4 mo of age and with stem diameters of at least 4 mm, were harvested and their root systems were washed, pruned approximately 50%, and immersed in the inoculum. The plants were cut off 15 cm above the soil line, the truncated stems were connected to rubber tubing attached to a vacuum pump, and at least 1 ml of inoculum was drawn through the plants. Check plants were infiltrated with distilled water. Inoculated plants were repotted and grown in a greenhouse for 9–18 mo until symptoms developed and XLB appeared in xylem sap extracts (5). While in the greenhouse, all plants received normal care except for weekly 24- to 48-hr periods of water stress.

At 3–6 mo after the original inoculations, and subsequently at

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1- to 3-mo intervals, plants were reinoculated by stem injections. At three sites, the stems were wounded to expose xylem tissue, and a total of ~0.25 mm^3 (packed cell volume) of 21-day-old bacteria scraped from plates of BCYE agar medium was injected into the xylem with a 0.017-mm-diameter (16-gauge) hypodermic needle and syringe. The inoculation sites were then closed and sealed with Parafilm.

Lateral twig growth on inoculated and on uninoculated check peach seedlings were measured at 6-, 9-, and 18-mo intervals. Total length of all twigs on a plant were measured and the average twig length was calculated. Internodal lengths were also measured and averaged.

Previously described procedures (19) for microscopic examination of KOH extracts were used to determine the concentrations of bacteria in twig tissues of test plants.

Ten seedlings each of cultivars Lovell and Nemagard peach were inoculated with two isolates of Pierce's disease bacteria from diseased grapevines and from almond (*Prunus amygdalus* L.). Inoculations were by methods described previously (13,15). Cultivar Mission almond seedlings were similarly inoculated with an isolate of the phony peach bacterium.

Serology. Bacterial cells from primary colonies of original isolations on BCYE medium, from subcultures on BCZE medium, and from reisolations of artificially inoculated plants were harvested and antigens were prepared as described before (13). Preimmunization control serum was collected from female New Zealand white rabbits and immunization was by intravenous and intramuscular injections (21). Three rabbits were immunized by injection with the PE-11 isolate of the phony peach bacterium.

Gamma-globulin for an enzyme-linked immunosorbent assay (ELISA) was purified from antisera, the enzyme was conjugated, and test procedures conducted as described previously (11,14). Plates were counted directly in a Titertek Multiskan ELISA reader (Flow Labs., Inc., Inglewood, CA 90302) at 405 nm.

Root and stem tissue samples were prepared for ELISA by methods previously described (11). Included in the samples were peach naturally infected with phony disease, peach artificially inoculated with the phony disease bacterium, and pure cultures of the bacteria isolated from naturally diseased and from artificially inoculated peach.

Immunoglobulins for immunofluorescence were separated and conjugated from antisera, and tests were conducted as described before (21). Samples from healthy plants, from peaches naturally infected with phony disease, and from pure cell cultures of the PD bacterium, *Agrobacterium tumefaciens, Pseudomonas savastonoi, P. syringae*, pv. *syringae*, and *Bacillus subtilis* were included as controls. Treated materials were viewed with a Zeiss GFL microscope equipped with an HB-200 W mercury lamp and filter.

Morphology and ultrastructure. The presence of bacteria isolated from diseased peach on BCYE agar medium was confirmed by phase-contrast microscopy. Single-colony subcultures were made on the same medium for electron microscope (EM) studies. A drop of BCZE liquid medium containing XLB (from a 6-day-old subculture originally isolated from peach) was mixed with a drop of 2% potassium phosphotungstate in phosphate buffer (pH 7.0) on a Parafilm sheet. A collodion-coated grid was placed in contact with the mixture for 2–3 sec. Excess fluid was removed with a piece of filter paper, and immediately after the liquid on the grids had dried, were examined by a transmission electron microscope (AE 1 EM 6B) for views of single cells. Colonies were also harvested in 0.1 M phosphate-buffered saline (pH 7.0) and centrifuged at 21,000 *g* for 15 min at 4 C. The pellets were washed twice with phosphate-buffered saline and embedded in 1% sterilized Noble agar (Difco), which was then cut into small (2-mm^3) pieces in 2% glutaraldehyde solution. Specimens were prepared for electron microscopy as described by Lowe et al (9). Ultrathin sections were obtained with a diamond knife, placed on copper grids, and double-stained with uranyl acetate and lead citrate. Ultrathin sections from root tissues of inoculated peach showing symptoms of phony disease also were prepared and examined by methods previously described (15).

RESULTS

Development of symptoms on inoculated plants. Some peach seedlings showed symptoms of phony disease within 3 mo of inoculation. These plants grew at a significantly slower rate than check plants—growth of terminal twigs was reduced and internodal lengths were approximately one-half to one-third those of uninoculated plants (Table 1). Within 6 mo after inoculation, all inoculated Lovell seedlings were half the height of uninoculated checks. Average length of terminals on inoculated plants ranged from 1.3 to 4.5 cm compared to 28.0 to 31.7 cm in the checks. After 18 mo, diseased plants continued to show the characteristic stunted growth pattern (Fig. 1). Three of the 10 Halford seedlings were stunted 6 mo after inoculation. Two of the 10 Nemagard seedlings exhibited the short internodes, general stunting, and dark green foliage that are typical symptoms of the disease 8 mo after inoculation.

Bacteria were not regularly detected from twig extracts until the second year after inoculation. At 18 mo after inoculation, concentrations of at least 50 bacteria per microscope field were detected in all plants that showed symptoms (Table 1). No bacteria were detected in extracts of uninoculated plants.

Peach seedlings inoculated with Pierce's disease bacteria isolated from grape and almond did not develop symptoms after 24 mo and internodal lengths were normal. Almond seedlings inoculated with bacterial strains from peach also did not symptoms of leaf scorch or burn.

Reisolation of bacteria from inoculated plants. Bacteria identical in morphology to those used for inoculations were isolated on BCYE medium from 3 of 4 Lovell, from 1 of 10 Halford, and 2 of 10 Nemagard seedlings 18 mo after the first inoculation. All seedlings

TABLE 1. Terminal branch growth and bacterial counts of cultivar Lovell peach seedlings inoculated with pure cultures of the bacterium associated with phony disease of peach

		Interval after inoculation								
		6 mo			9 mo			18 mo		
Plant	Treatment	Avg. twig length (cm)	Internodal length (cm)	Bacterial count[a]	Avg. twig length (cm)	Internodal length (cm)	Bacterial count[a]	Avg. twig length (cm)[b]	Internodal length (cm)	Bacterial count[a]
R1	Inoculated[c]	4.5	0.5	2	8.5	0.6	5	9.2	0.5	288
R2	Inoculated	2.6	0.3	0	6.4	0.9	0	6.5	0.8	470
R3	Inoculated	2.0	0.8	1	3.0	0.5	0	3.5	0.9	56
R4	Inoculated	1.3	0.6	2	3.2	0.5	2	3.0	0.6	271
C1	Check	32.2	1.3	0	25.2	1.4	0	32.3	1.6	0
C2	Check	28.0	1.5	0	29.2	1.4	0	28.6	1.3	0
C3	Check	24.0	1.3	0	19.7	1.3	0	37.2	1.4	0
C4	Check	31.7	1.6	0	36.6	1.3	0	30.2	1.3	0

[a] Average number of bacteria per microscope field in 0.1 M KOH extracts of three twig samples per tree.
[b] Check plants pruned prior to winter dormancy period, 11 mo after inoculation.
[c] Inoculations were made in December 1980. Plants stored at 3 C for simulated winter dormancy from November 1981 to January 1982.

from which bacteria were reisolated also showed symptoms and had XLB. Similar bacteria were not isolated from inoculated peach or almond plants that remained free of symptoms or from any uninoculated plants.

Primary colonies of the reisolated bacteria grew on BCYE medium as translucent, restricted, closely appressed, circular films with distinct margins. Diameters of primary colonies ranged between 0.1 and 0.4 mm. Colonies formed after the second and subsequent passages developed as streaks or "lawns" with dense areas of dome-shaped, cream-colored growth. Bacteria were Gram-negative rods 0.35 μm in average diameter (range 0.20 to 0.38 μm) and 5 μm in maximum length (range 0.8 to 15 μm).

Serology. The ELISA reactions with antiserum prepared against the phony peach bacterium were similar with bacteria isolated from naturally diseased peach and from inoculated seedlings showing symptoms of phony disease. Purified coating gamma-globulin at a concentration of 2.0 mg/ml and a 10^{-3} dilution of enzyme conjugate were used. The $A_{405\ nm}$ values obtained for strains of the phony peach bacterium isolated in Georgia (PE-11) and in Florida (PE-005) were similar, 1.042 and 1.085, respectively (Table 2). Strains of the Pierce's disease bacterium (PD-N5 and PD-C22) were positive in ELISA but the $A_{405\ nm}$ values were approximately one-third as intense as those for the phony peach strains.

Root and stem extracts of naturally diseased peach and of inoculated, symptomatic seedlings reacted positively with antiserum against the phony bacteria. The $A_{405\ nm}$ values ranged from 0.101 to 0.820, and were significantly higher than those from healthy tissue or the buffer controls.

Fluorescence was positive when pure cultures of peach phony or Pierce's disease bacteria were tested with antisera prepared against the PE-11 isolate of the phony peach bacterium. Strong fluorescence was observed with extracts of naturally diseased or inoculated plants with symptoms of phony disease. None was observed with healthy controls or with other bacteria.

Morphology and ultrastructure. Rod-shaped bacteria of varying lengths were observed in water mounts of cells obtained from primary colonies developing on BCYE agar medium inoculated with root and stem extracts of symptomatic peach seedlings. Rods were of the same dimensions (0.2–0.35 μm × 1–5 μm) as those observed from root extracts from naturally diseased peach trees (7,12). The bacterium was Gram-negative and catalase-positive.

Numerous irregular ridges or folds were observed on cell walls of phony peach bacteria isolated from roots of inoculated seedlings (Fig. 2A). Similar detail has been observed on plum leaf scald bacteria (15) and on Pierce's disease bacteria isolated from naturally infected plants (5). The ultrastructure of phony peach bacteria was similar to that of other xylem-limited bacteria with rippled cell wall surfaces and tapered or rounded ends (Fig. 2B). Bacteria measured 0.35 × 5 μm in maximum diameter and length. Internal structure included ribosomes and DNA-like strands typical of bacteria, similar to those described in the plum leaf scald bacterium (15). Cell wall profile consisted of an outer membrane with a rippled configuration, an intermediate (peptidoglycan) layer that was electron-dense, and an inner cytoplasmic membrane (Fig. 2B). Binary fission was also observed in some cells.

The lumen of xylem elements of inoculated and symptomatic seedlings were occupied by numerous bacteria with rippled cell walls—identical to those previously reported in the xylem of naturally infected tissues (7,12).

TABLE 2. Absorbance values ($A_{405\ nm}$) from ELISA tests with extracts from healthy and diseased peach root tissues and from pure cultures of bacteria with antiserum against the phony peach (PP) bacterium

Plant material or bacterium[a]	Plants tested (no.)	ELISA (avg $A_{405\ nm}$)[b]
Root tissue		
Naturally diseased peach	5	0.548
Experimentally inoculated peach	4	0.460
Uninoculated, healthy peach	8	0.038
PPB		
Strain PE-11		1.042
Strain PE-005		1.085
Strain PE-110		1.066
Strain PD-N5		0.382
Strain PD-C22		0.370
Phosphate-buffered saline		0.014

[a] PE-11 and PE-005 designate strains isolated in Georgia and Florida, respectively, from peach trees naturally infected with PP disease; PE-110 is an isolate from a PP-infected cultivar Lovell peach seedling; PD-N5 and PD-C22 are isolates of the Pierce's disease bacterium from grape and almond, respectively.

[b] Average of three composite replicates per test. Values for diseased tissues are significantly different ($P = 0.01$) from those of healthy tissue.

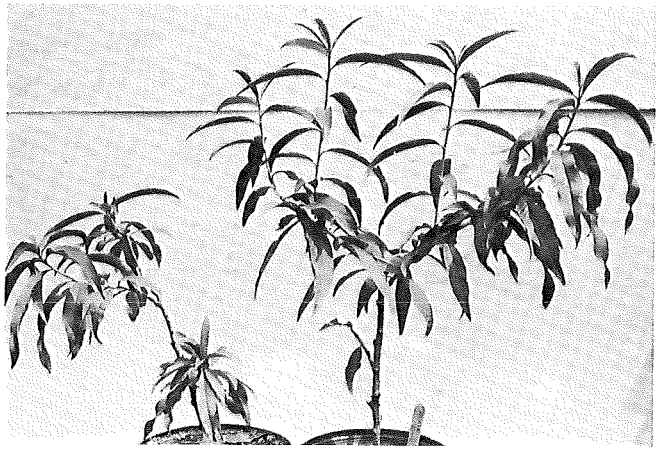

Fig. 1. Cultivar Lovell peach seedlings 18 mo after inoculation with a suspension of a pure culture of the phony peach bacterium (left) or with distilled water (right). Note severe stunting and shortened internodes of the artificially inoculated seedling.

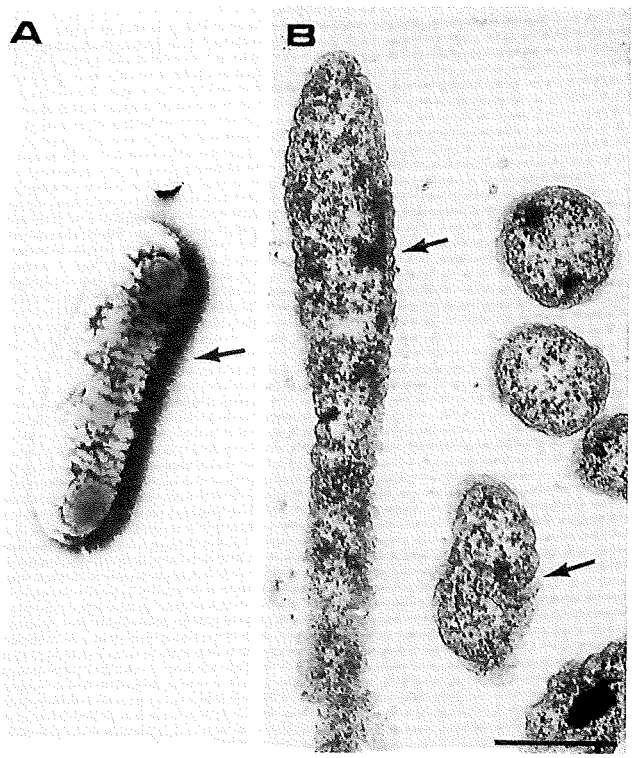

Fig. 2. Phony peach bacterium. **A,** Rod-shaped cell negatively stained with 2% potassium phosphotungstate showing typical ridges or invaginations and fimbriae (arrow) on the surface of the cell wall. **B,** Ultrathin section of a cluster of bacteria in transectional and longitudinal views with rippled cell wall configurations. Bar = 0.5 μm.

DISCUSSION

Bacteria were isolated from peach trees naturally infected with phony peach disease, and cultured on artificial medium. The bacteria were then used to artificially inoculate Lovell, Halford, and Nemagard peach seedlings. Once symptoms typical of phony disease had developed on inoculated seedlings, the same bacteria were reisolated on artificial medium from infected tissues. These bacteria were identical in morphology, ultrastructure, and serology to those present in naturally diseased peach trees and to those isolated and cultured from natural sources for the inoculation tests. Evidence of the pathogenicity of the phony peach bacterium was thus established.

The phony peach bacterium could not be isolated on standard bacteriological media, on the JD-2 medium used for isolation of the Pierce's disease bacterium, or the S-8 medium used for the elm leaf scorch organisms. Successful isolation, however, was accomplished on BCYE medium, also suitable for the plum leaf scald bacterium. The phony bacterium, therefore, may be considered to be as fastidious in growth requirements as the plum leaf scald organism. Their relationship may include reciprocal pathogenicity. The phony peach organism produces scald symptoms in plum (20), but after 2 yr peach seedlings inoculated with the plum bacteria have not yet shown symptoms of disease.

There is no published evidence of varietal differences in susceptibility to phony peach disease. Although in our tests a greater percentage of cultivar Lovell rootstocks developed disease after inoculation compared to seedlings of cultivars Halford and Nemagard, differences could be due to age of seedlings, inoculation techniques, or strain pathogenicity. Conclusions on rootstock susceptibility to phony disease will have to await controlled inoculation tests under uniform conditions.

The phony peach bacterium is entirely unrelated to the true rickettsia (1). Studies relating to the serology, ultrastructure, DNA composition, and cellular fatty acid components indicate that the organism belongs to a different taxonomic group (J. Wells and B. Raju, *unpublished*). This observation was similarly made by Raju et al with regard to the plum leaf scald bacterium (15). We, therefore, reassert the need to adopt the descriptive term "fastidious, Gram-negative, xylem-limited bacteria (XLB)" instead of "rickettsialike" in referring to this group of bacteria until their proper taxonomic position can be established.

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