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Axenic Culture of the Bacteria Associated with Phony Disease of Peach and Plum Leaf Scald

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Abstract. Bacteria associated with phony disease of peach (PDP) and plum leaf scald (PLS) were consistently isolated from diseased trees but not from healthy trees. Colonies of the bacteria grew slowly on PW agar, reaching 0.2 mm to 0.7 mm in diameter in 2 to 3 weeks. The bacteria did not grow on nutrient agar or other general-purpose media. Cells of the bacteria were 0.3 μm to 0.4 μm in width and 2.6 μm to 20.0 μm in length. The topography of the cell walls revealed numerous ridges and furrows. Cells extracted from diseased plants and those from culture gave a strong fluorescence when stained with immunoglobulin G to cells and purified membranes of the bacteria extracted from peach and plum in earlier studies. Immunoglobulin G to cells of the Pierce's disease bacterium from culture also reacted with the bacteria. No discernible differences were observed between strains associated with PDP and PLS in the United States and PLS in Brazil.

Phony disease of peach (PDP) occurs extensively in the southeastern United States [20]. Plum leaf scald (PLS) was first reported in Argentina [9] but has more recently been reported in Brazil, Paraguay, and the southeastern United States [11,12,26]. Serious economic losses occur in peach and plum production because of these diseases.

Xylem-limited bacteria often referred to as "rickettsia-like" were found to be closely associated with PDP [19,30], PLS [11,12,23,26], and other plant diseases [14,16,18,27,28]. These small, rod-shaped bacteria form a distinct group based on morphology, ultrastructure, serology, and other characteristics [7,17]. Only the xylem-limited bacterium associated with Pierce's disease, almond leaf scorch, and alfalfa dwarf disease has been isolated in axenic culture and shown to be pathogenic [3,6,33]. Virtually nothing is known about the pathologic and taxonomic relationships of the xylem-limited bacteria, largely because such studies have been hindered by the inability to isolate most of the bacteria in axenic culture.

Recently, periwinkle wilt (PW) medium was developed for the axenic culture of the xylem-limited bacterium associated with periwinkle wilt disease [M.J. Davis, unpublished data; 27]. In this report, we describe the isolation in axenic culture on the PW medium of the bacteria associated with PDP and PLS.

Materials and Methods

Media. PW agar medium consisted of deionized, distilled water, 1,000 ml; Phytone peptone (BBL, Cockeysville, Maryland), 4.0 g; Trypticase peptone (BBL), 1.0 g; K_2HPO_4, 1.2 g; hemin chloride stock (0.1% bovine hemin chloride [Sigma Chemical Co., St. Louis, Missouri] in 0.05 N NaOH) 10 ml; KH_2PO_4, 1.0 g; granulated agar (BBL), 12.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; phenol red stock (0.2% phenol red in distilled water), 10 ml; glutamine stock (8.0% glutamine-free base, Sigma; in distilled water), 50 ml; and bovine serum albumin fraction-five (BSA) stock (20% bovine albumin, Sigma, no. A4503; in distilled water), 30 ml. All ingredients except BSA and glutamine were added, mixed, and dissolved in the order given. The basal medium was autoclaved for 15 min. The glutamine stock was gently heated to dissolve the glutamine, and both the glutamine and BSA stocks were filter-sterilized (0.2-μm membrane filter). The filter-sterilized stocks were added to the autoclaved basal medium at 50°C.

PW broth medium was prepared as described for the agar medium above, except that the agar was omitted. The PD2 medium for the Pierce's disease bacterium was made as originally described [4]. The PD3 medium was prepared by substituting soluble potato starch (Sigma) at 2 g/liter for the BSA in the PD2 medium, thus making the medium completely autoclavable. The SC medium for the ratoon stunting disease bacterium was prepared as originally described [2].

Culture conditions. All cultures were incubated aerobically at 28°C and subcultured every 7 to 14 days. Each broth culture was initiated by inoculating 50 ml of medium in a 250-ml Erlenmeyer flask with a 1-cm^2 agar plug from a heavily streaked plate.

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Subsequent subcultures were made by transferring 1 ml of the broth culture to 50 ml of fresh medium. Broth cultures were incubated on a gyratory shaker with a 2.5-cm stroke at 200 rpm.

Strains and isolation techniques. During preliminary evaluations of media, inocula were obtained by triturating 15 to 20 surface-sterilized [6] petioles from greenhouse-grown peach trees with symptoms of phony disease of peach. The petioles were triturated in 5 ml of 0.01 M potassium phosphate buffer, pH 6.9, using a mortar and pestle. Hundredfold serial dilutions to $1:10^8$ of the triturate were plated in 0.01-ml amounts onto each test medium. Inocula for attempting to isolate the phony disease of peach (PDP) and plum leaf scald (PLS) bacteria from field-collected material were obtained from surface-sterilized [6] stem sections, root sections, and leaf petioles. Xylem sap containing the bacteria was expressed from the sample using alcohol-flamed pliers, removed from the cut surface with an inoculation loop, and streaked onto culture media. Pierce's disease strains VF5 and VP4 were isolated and maintained on the PD2 medium as described [3,4].

Plant materials. Peach inocula for preliminary evaluations of media formulations were obtained from petioles of "Nemaguard" peach trees infected with phony disease of peach. Two 3-year-old trees, inoculated 2 years earlier with PDP-infected root grafts, were maintained in a greenhouse and sampled as needed. Thereafter, inoculum was obtained from five naturally infected seven-year-old "June Gold" trees on "Nemaguard" rootstocks grown in commercial orchards in Madison County, Florida. Healthy trees and PDP-infected trees were selected on the basis of symptomatology and on the presence or absence of rod-shaped bacteria, which were visible by phase-contrast microscopy in sap expressed from root samples. Isolations were attempted in January 1981.

Samples from plum in the United States were obtained from five-year-old plum trees at the Agricultural Research Center, Monticello, Florida. The cultivars used were one "Marianna", one *Prunus cerasifera* × *P. munsoniana*, and three "Santa Rosa". Controls were from symptomless trees of the same cultivars grown in screen houses. Attempts were made to culture the bacteria from petioles in October and from stems in November 1980.

Stem samples from plum in South America were collected in December 1980 from five- to eight-year-old trees near Curitiba, Paraná, and Pôrto Alegre and Cascata, Rio Grande do Sul, Brazil. Cultivars used in the isolations were "Santa Rosa", "Ozark Premier", "Pluma 7", and "The First". Ten PLS-infected trees and two healthy trees were sampled. Microscopic confirmation of PLS infection and isolation attempts were made in the laboratory of Alberto Feliciano, EMBRAPA, UEPAE, Cascata, Rio Grande do Sul.

Electron microscopy. PDP and PLS bacteria were transferred from colonies on PW agar with a loop to a drop of 3.0% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0, on a Formvar film-coated copper specimen grid. After one min, the glutaraldehyde solution was removed with filter paper, and a drop of 1.0% aqueous potassium phosphotungstate, pH 6.5, was placed on the coated grid for 30 s, then removed with filter paper. The bacteria were then examined with a transmission electron microscope.

Serology. Indirect immunofluorescence was performed using immunoglobulin G (IgG) to whole cells and to purified membranes and using FITC-conjugated anti-rabbit IgG (GIBCO, Madison, Wisconsin). Cells of the PLS and PDP bacteria were extracted from roots or stems of infected trees by vacuum infiltration [10] using 0.05 M potassium phosphate buffer, pH 7.2, 0.85% NaCl (PBS). The extracted cells were harvested by centrifugation at 12,000 × *g* for 20 min. Cultured cells were obtained from PW agar by removing colonies with a loop and suspending in PBS. Antisera to formalinized cells [31] of the PDP and PLS bacteria and to purified membranes [24] of the PLS bacterium were made with host-extracted cells purified by Renografin density gradient centrifugation [25]. Antiserum to whole cells of the Pierce's disease bacterium was obtained as described elsewhere [5]. IgG was obtained from each antiserum by Sephadex A-500 column chromatography [8]. A block test [15] was used to determine the optimum dilutions of IgG and anti-rabbit IgG. Staining was performed as described [31], and slides were examined with a Zeiss standard incident-light fluorescence microscope. Bacteria were observed under a Planapo 100× oil-immersion objective.

Results

Isolation and growth in culture. The xylem-limited bacteria associated with phony disease of peach (PDP) and plum leaf scald (PLS) were isolated on PW agar medium. Colonies of the bacteria were readily visible using a dissecting microscope (30×) after 10 days of incubation. Colonies of the PDP and PLS bacteria were indistinguishable (Fig. 1). The colonies were opalescent white and reached 0.2 to 0.7 mm in diameter in 2 to 3 weeks. The colony morphologies ranged from small, circular, convex colonies with entire margins to larger, circular, umbonate-to-flat colonies with undulate margins. The surface on the convex portion of the colonies was smooth, whereas the surface on the lesser elevated portion of the colonies was rough.

The PDP and PLS bacteria were readily subcultured by streaking onto PW agar plates and by broth inoculation. Broth cultures became only faintly turbid after 7–14 days incubation. Frequently, visible aggregations of cells adhered to the glass at the surface of the medium. Even though no appreciable degree of turbidity was apparent, populations developed in excess of 10^9 colony-forming units/ml. An alkaline pH shift was indicated by the medium turning darker red owing to the phenol red pH indicator. The PDP and PLS bacteria differed in culture from the Pierce's disease bacterium. Strains of the Pierce's disease bacterium grew more rapidly, forming colonies on the PW, PD2, PD3, and SC media that were visible to the unaided eye within 7 days. The PDP and PLS bacteria grew on the PW medium only and grew more slowly. The PDP, PLS, and Pierce's disease bacteria did not grow on nutrient agar or other general-purpose agar.

Cell morphology. Electron micrographs (Fig. 2) showed that cells of PDP and PLS bacteria were narrow and rod-shaped, measuring 0.3–0.4 μm wide and 2.6–20.0 μm long; they appeared to divide by binary fission. The cell-wall topography showed numerous ridges and furrows.

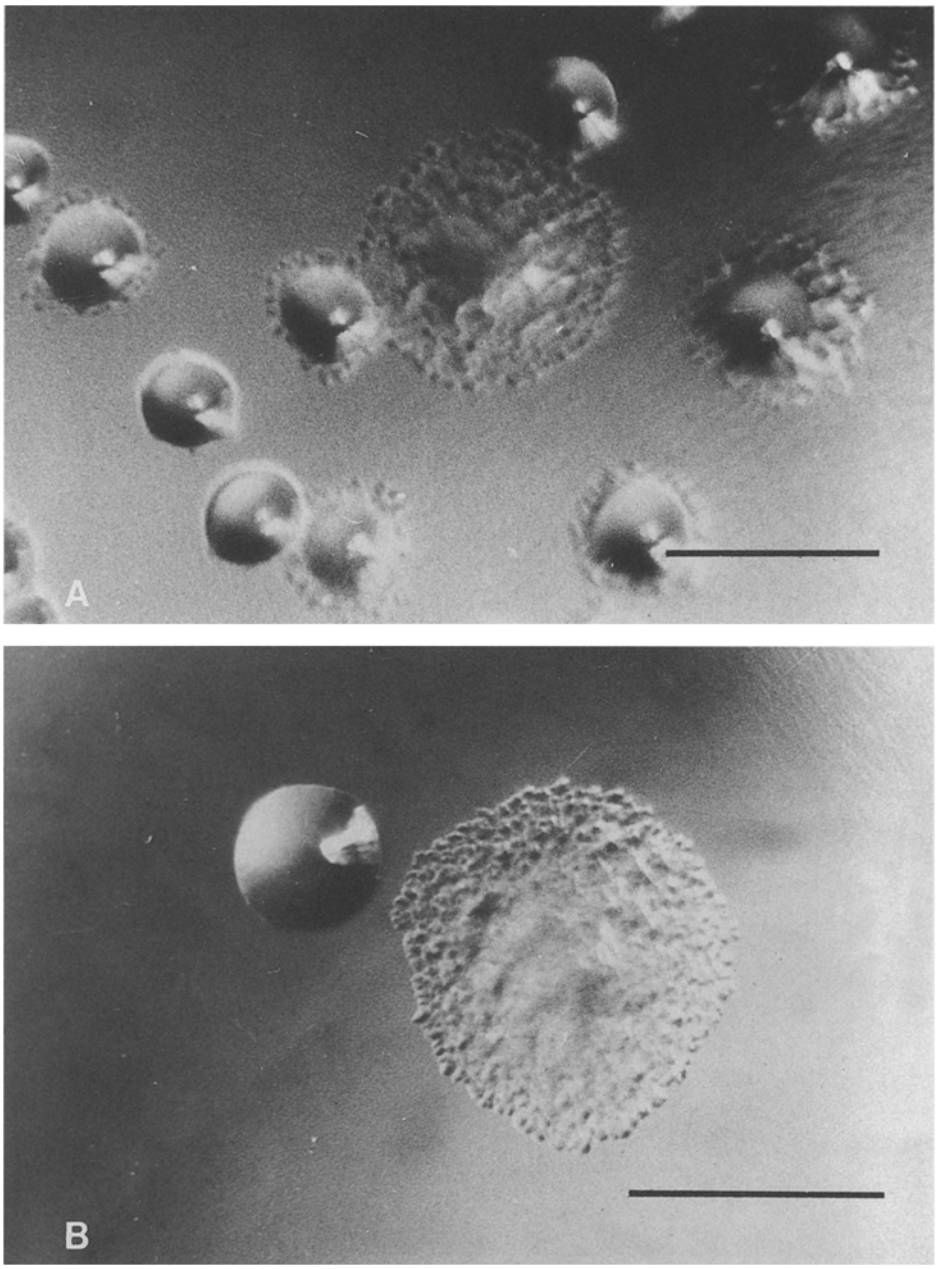

Fig. 1. Colonies on PW agar three weeks after inoculation with 10^8 dilutions of PW broth cultures. The colonies are of (A) phony disease of peach bacterium and (B) plum leaf scald bacterium as seen by reflected light through a dissecting microscope (bar = 0.05 mm).

Association with PDP and PLS. In the United States, the bacteria were consistently isolated from PDP-infected peach root samples (22 successful isolations from 24 attempts) and were not isolated from healthy peach roots (0/20). The bacteria also were consistently isolated from petioles (27/32) and stems (24/32) of PLS-infected plum trees and were not isolated from petioles (0/20) or stems (0/20) of healthy plum trees. Similarly, in Brazil, the bacteria were readily isolated from PLS-infected stems (75/83) when the samples were processed within 24 h. Material that had been collected in the rain and processed 48 h later yielded only 4/30 successful isolations, owing to competition from fast-growing contaminants. Again, the bacteria were not isolated from healthy trees. Growth rates and colony appearances did not differ discernibly between strains from Brazil and Florida.

Serology. Both cells of the PDP and PLS bacteria extracted from infected plants and cells from culture gave a strong fluorescence when reacted with IgG to cells of the PDP and PLS bacteria or with IgG to membranes of PLS organisms extracted from host plants (Table 1). Cells of PDP and PLS bacteria failed to fluoresce when reacted with IgG to cells of *Xanthomonas campestris*, and reciprocal tests were also negative (Table 1). PLS cells from the United States and Brazil gave a strong fluorescence when reacted with IgG to PLS cells extracted from plum in the United States. PDP and PLS cells grown in culture also gave a strong fluorescence

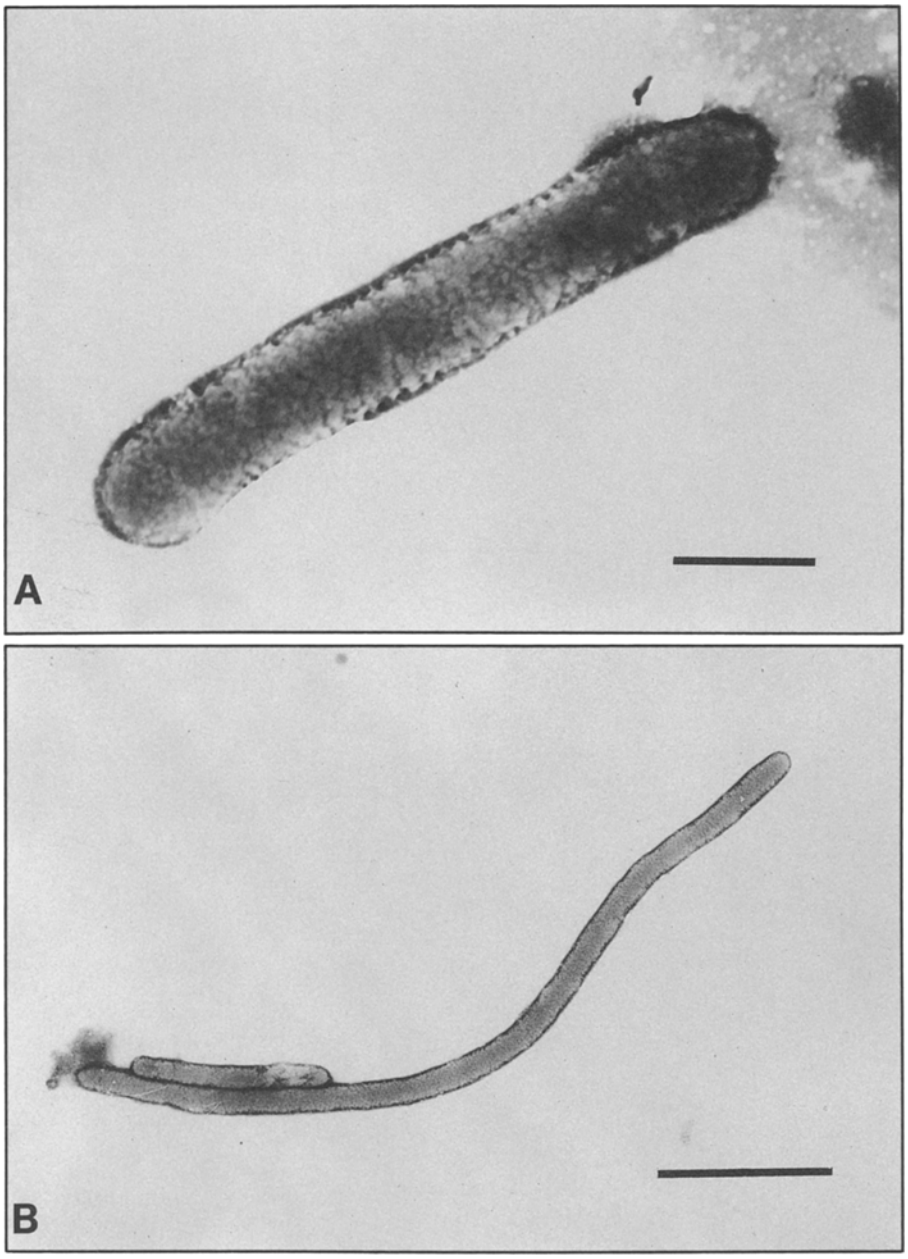

Fig. 2. Transmission electron photomicrograph of negatively stained cells of the plum leaf scald (PLS) bacterium for PW agar. The (A) single cell shows the ridged and furrowed topography of the cell wall (bar = 0.05 μm), and (B) paired cells demonstrate the variability of cell length seen in culture; the shorter cell is approximately the size of cells extracted from plum with PLS (bar = 2.5 μm).

when stained with IgG to cells of the Pierce's disease bacterium grown in culture. No discernible differences in staining with IgG to the Pierce's disease bacterium were observed for PDP and PLS cells obtained from single-colony isolates from 12 convex and 12 umbonate colony types.

Discussion

The xylem-limited bacteria associated with phony disease of peach (PDP) and plum leaf scald (PLS) were isolated from diseased trees and maintained in axenic culture on PW medium. Features used to identify the bacteria were: (i) consistent isolation from diseased trees but not from healthy trees, (ii) slow growth rate, (iii) colony characteristics, (iv) cultural requirements, (v) cell morphology, and (vi) immunofluorescent staining. Based on these features, the PLS bacteria from the United States and Brazil and the PDP bacteria from the United States appeared to be identical. However, taxonomic studies are needed to characterize these and other xylem-limited bacteria and to determine whether any relationship exists between the xylem-limited bacteria and known taxa. The present and other serological studies, mostly using the fluorescent-antibody technique, have shown that in all cases thus far examined the xylem-limited bacteria are related and indistinguishable, but no serological relationship has been established with any other bacteria [5,13,16,29,35]. Future serological studies using more qualitative techniques such as Ouchter-

Table 1. Indirect fluorescent-antibody staining of plum leaf scald (PLS) and phony disease of peach (PDP) cells from infected plants and agar culture.

Cells	Origin	Immunoglobulin G to: PLS cells[a]	PLS membranes[a]	PDP cells[a]	*Xanthomonas campestris* cells
PLS	plant	++[b]	+++	++	–
PLS	culture	++	++	+++	–
PDP	plant	++	+++	+++	–
PDP	culture	+++	+++	+++	–
Xanthomonas campestris	culture	–	–	–	+++

[a] Cells extracted from diseased trees by Renografin density gradient centrifugation [25].
[b] Symbols: – = no fluorescence (cells not visible), ++ = green fluorescence, +++ = bright green fluorescence.

lony gel double-diffusion and more specific antibodies such as IgG to ribosomes, purified membranes, and membrane proteins might distinguish among different xylem-limited bacteria [32].

Colonies of PDP and PLS bacteria were small and developed slowly on PW medium, whereas colonies of Pierce's disease bacteria grew much more rapidly. The PW medium was derived from the PD2 medium developed for the Pierce's disease bacterium [4]. The PW medium differs from the PD2 medium in that it contains glutamine instead of citrate and succinate and in that the concentrations of some of the other ingredients differ. Although the Pierce's disease, PDP, and PLS bacteria are similar in many respects, the Pierce's disease bacterium appears to be the least nutritionally fastidious, growing well on all the media used except general-purpose media, whereas the PDP and PLS bacteria grew on the PW medium only. The PDP and PLS bacteria from culture morphologically resembled previous descriptions [10,11] of the bacteria extracted from host tissue, except that the cell lengths were sometimes much longer. Longer cells appeared to become more frequent as the colonies aged. A similar phenomenon was observed with the Pierce's disease bacterium until improvements in the culture medium were made [1].

The pathogenicity of the PDP and PLS bacteria has yet to be determined. An etiological relationship between PDP and PLS is suspected but not clearly established. Reciprocal graft transmissions between peach and plum resulted in PDP symptoms developing in peach and PLS symptoms developing in plum [12; W.J. French, unpublished data]. Both diseases affect other *Prunus* species to various degrees [21,22,23], and wild plum has long been known as an important symptomless carrier of PDP [21]. However, further comparative transmission studies are needed to determine relationships between PDP and PLS in host range and symptomatology. PDP characteristically takes 18 or more months for symptom development, and the incubation period for PLS symptom development is likely to be between 6 and 18 months, based on field observations. Techniques for inoculating trees with the bacteria from culture, including possibly those used for studying almond leaf scorch [6], will have to be examined. Considering the consistent isolation of the PDP and PLS bacteria from diseased trees but not from healthy trees, the serological relationship between cells growing in culture and cells in diseased trees, and the relatedness of the bacteria to the Pierce's disease bacterium (a known plant pathogen), the probability is high that the bacteria growing in culture are the organisms inciting PDP and PLS.

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Addendum

After this report was submitted and accepted for publication, a recent independent report [34] presenting similar results was brought to our attention. Although the medium formulations used in the two studies are almost totally different, essential factors for in vitro growth of the bacteria must be provided by both media. A thorough comparison of the formulations might help explain the fastidious nature of these bacteria and lead to the development of even better media.