The Use of Immunofluorescence for the Identification of Phony Peach Bacterium

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ABSTRACT

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Antisera were produced against rickettsialike bacteria (RLB) extracted from roots of peach (*Prunus persica*) and wild plum trees (*P. angustifolia*), respectively. The RLB recovered from peach with phony disease symptoms were stained with nonlabeled antisera (to whole cells) and counterstained with fluorescent anti-rabbit globulin conjugate. The antisera were highly specific to RLB from both plum and peach. The immunofluorescence test provides a rapid, reliable method of confirming the identification of the RLB associated with phony peach disease.

Additional key words: Prunus persica, P. angustifolia, chickasaw plum, rickettsialike bacteria.

Recent studies indicate that phony disease of peach [*Prunus persica* (L.) Batsch.] is caused by a rickettsialike bacterium (RLB) (4,7). Positive diagnosis of the disease is by grafting or vector transmission tests which require 18 to 24 mo to complete (5). Presumptive or confirmatory diagnosis of the disease is based on the presence of RLB in xylem tissues as determined by phase or electron microscopy (1). At present, there is no quick way to determine whether bacteria obtained from symptomless hosts or vectors are homologous to RLB associated with disease symptoms. This paper reports the use of immunofluorescence for a rapid and definitive identification of the bacterium associated with phony disease of peach.

MATERIALS AND METHODS

Immunofluorescence (IF) studies on plum RLB were conducted at Monticello, FL and the IF studies on peach RLB were done at Experiment, GA.

Bacterial cultures.—Bacteria used in this study were obtained from the following sources: *Xanthomonas pruni* and *X. vesicatoria* from N. Schaad, Georgia Agricultural Experiment Station, Experiment, GA; *X. dieffenbachia, Pseudomonas lachrymans, Erwinia chrysanthemi, Corynebacterium michiganense,* and *Agrobacterium tumefaciens* from J. Miller, Florida State Dept. Agriculture, Gainesville, FL; *Pseudomonas syringae* from H. English, University of California, Davis, CA; and *Bacillus subtilis* from J. H. Stuy, Florida State University, Tallahassee, FL.

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Preparation of inject-antigen.—The RLB were collected in 0.1 M KOH by vacuum infiltration (1) of roots from wild plum trees (*P. angustifolia* Marsh.) or peach trees infected with the phony peach bacterium. The suspensions were centrifuged twice in 15-ml conical tubes at 500 *g* to remove plant debris. The supernatant liquid then was spun at 3,000 *g* for 20 min. The pellet containing RLB was washed three times with 0.85% NaCl (saline). For the plum inject-antigen the cells were concentrated after the last wash by resuspending the pellet in a volume of saline to give approximately 2×10^8 cells/ml as determined by phase-contrast microscopy. For peach inject-antigen the supernatant fluids containing cells from the first low-speed centrifugation were placed in 50-ml disposable centrifuge tubes and shipped on ice to the Georgia Experiment Station. The cells were centrifuged at 7,585 *g* for 10 min and the resulting pellets containing the RLB were resuspended in saline. The RLB-free roots (as determined by phase microscopy) were used as the source of healthy peach antigen.

Antisera to fluids extracted from roots of healthy and phony-affected plum trees were produced by injecting New Zealand white rabbits. Rabbits were bled 7 days prior to injection for normal serum. Two rabbits were injected with RLB and one rabbit with antigen from healthy trees. For intravenous (IV) injections, antigen as described above was used directly. For subcutaneous (SC) injections, antigen preparations were homogenized with equal volumes of Freund's complete adjuvant (Difco) with a 3-ml syringe. The injection schedule was as follows: SC injections of 1.2, 2.0, and 2.0 ml (1:1 adjuvant) were given on days 1, 2, and 3, respectively, and 2.0 ml each on days 8, 9, and 10. A test bleeding of approximately 10 ml was obtained 7 days after the last

injection and at 30-day intervals thereafter. A booster schedule, initiated 7 mo after the last injection, was as follows: an initial SC injection containing 3.0 ml (1:1 adjuvant); 7 days later 0.5 ml IV and 5.0 ml SC; and 3 mo later 0.6 ml IV. The rabbits were bled 10 days after the last injection.

Antiserum to RLB from peach bacteria was produced by injecting New Zealand white rabbits with formalized cells of RLB as described (8). One rabbit was injected with RLB and one was injected with antigen prepared from healthy trees.

Gamma globulin to plum antigen was precipitated with ammonium sulfate (3). Protein concentration of the γ globulin was determined by the method of Lowry et al. (6). Immunoglobulins to peach were not fractionated.

Preparation of bacteria for staining.—The RLB were collected from five peach and five wild plum trees by vacuum infiltrations, processed as for inject-antigen, and stored at −20 C. The bacteria were grown on yeast extract-dextrose-$CaCO_3$ (10) or nutrient agar (Difco) slants at 30 C for 48 hr.

Three ml of saline were added to each tube and the cells were suspended with a vortex mixer. A loopful (approximately 0.005 ml) of the suspension was placed on a multiwell slide (Cel-Line Associates, Inc., Minolta, NJ 08341) and allowed to air dry. For plum antiserum staining, the smears were fixed in 95% ethanol for 3 min and allowed to air dry. For peach antiserum staining, smears were flooded with Kirkpatrick's fixative (60% ethanol, 30% chloroform, and 10% formalin), placed in a humidity chamber for 3 min, rinsed with fixative, drained, and allowed to air dry before being stained.

Staining of bacteria.—For plum IF, a drop (approximately 10 μliters) of antiserum was placed on each smear and the slide was incubated at room temperature in a dark, moist chamber for 30 min. The slides were rinsed for 10 min in FA buffer (Difco, Detroit, MI 48201). A drop of anti-rabbit globulin conjugate (Difco) was added to each smear and the slide was incubated for 30 min at room temperature in a dark, moist chamber. After they were rinsed in FA buffer for 10 min, the slides were mounted in FA buffer-saturated glycerol (1:9). For peach IF, the smears were stained and mounted as described (9). Block tests (2) were performed to determine the optimal dilution of rabbit and anti-rabbit globulin.

Microscopy.—At Monticello, FL, slides were examined with a Leitz Ortholux II microscope equipped with a fluorescence vertical illuminator. Bacteria were observed under an ×100 oil immersion objective for both phase and fluorescence microscopy. An HBO-100W ultra-high-pressure mercury lamp was used as the exciting light source. The microscope was equipped with Filter System H for wide-band blue light excitation. A K450 edge filter was used to remove background fluorescence.

At Experiment, GA stains were examined under an ×40 objective fitted to a Zeiss Orthophot microscope with an oil immersion condenser. A Zeiss HBO-200 mercury vapor lamp (direct current power supply) was used as the exciting light source. A BG-12 interference filter was used for primary filtration and a No. 47 filter as the barrier filter.

RESULTS

Block titration tests showed that for specific staining the optimal concentrations of the rabbit globulin (plum) and the anti-rabbit globulin were 1:16 and 1:32, respectively. Optimum concentrations for the antisera to peach RLB and the anti-rabbit globulin were 1:8 and 1:16, respectively. Antisera to both the phony plum and peach inject-antigens were highly specific for RLB regardless of the host source (Table 1). The fluorescence of peach or plum RLB stained with antisera to RLB from either peach or plum appeared to be equal in quality and intensity.

In this study, RLB were readily differentiated from *Xanthomonas pruni* B-100 and *Pseudomonas syringae* B-3 originating from peach. Both species are common pathogens to stone-fruits in the southeastern USA. Cross-reactions between antisera to RLB and cells of *P. lachrymans* and *B. subtilis*, respectively, were much weaker than those produced between RLB and homologous antisera and, thus, did not interfere with the accurate identification of RLB.

TABLE 1. Indirect fluorescent-antibody staining of rickettsialike bacteria (RLB) from plum and peach trees, and several other bacteria[a]

Antigen	Strain	Host or habitat	Antiserum	
			Fla #2 RLB-Plum	Ga #1 RLB-Peach
RLB		*Prunus angustifolia*	+++	+++
RLB		*Prunus persica*	+++	+++
Xanthomonas pruni	B-100	*Prunus persica*	−	−
X. dieffenbachia	077-1470	*Philodendron oxycardium*	−	ND
X. vesicatoria	B-219	*Lycopersicon esculentum*	−	−
Pseudomonas syringae	B-3	*Prunus persica*	−	−
P. lachrymans	Thayer 40	*Citrullus vulgaris*	±	+
Erwinia chrysanthemi	Ke22	*Dieffenbachia* sp.	−	−
Corynebacterium michiganense	072-1430	*Capsicum frutescens*	−	−
Agrobacterium tumefaciens	077-482	*Chrysanthemum* sp.	−	ND
Bacillus subtilis	653	soil	−	±

[a]Symbols: +++ = bright green fluorescence; ++ = green fluorescence; + = slight fluorescence; ± = cells visible; − = cells not visible; and ND = not done.

Antiserum to healthy peach and plum inject-antigen did not react with RLB from either peach or plum when used at optimal concentrations indicated by the block test. The RLB stained with only the anti-rabbit globulin conjugate did not fluoresce. When mixtures of RLB and other bacteria, as *B. subtilis* or *P. lachrymans,* were stained with antiserum to RLB from *Prunus angustifolia* and counter-stained with anti-rabbit globulin conjugate, the RLB fluoresced brightly but the other bacteria did not.

DISCUSSION

The indirect IF test provides, at present, the only rapid method of confirming the identification of the RLB from peach and plum. Strong positive-staining reactions of RLB from peach and plum with reciporcal antisera indicate that the bacteria are either identical or closely related. These results agree with earlier studies based on the transmission of phony disease from plum to peach (5). Although it is unlikely that *P. lachrymans* and *B. subtilis* would be found in *Prunus* sp., they can be readily differentiated from RLB by the quality of IF staining, cell morphology, and by their ability to grow on defined media.

Further testing of the IF technique has demonstrated that RLB contained in KOH extracts (1) and in expressed xylem sap can be stained and identified with only minor changes in protocol. Air-dried smears are fixed as described, then rinsed in water for several minutes to remove KOH and plant debris. The slides then can be processed as described or sent to a laboratory equipped for IF staining of phony peach RLB. This method eliminates the need for time-consuming transmission tests or electron microscopy to aid in the identification of phony RLB. Because of the specificity of the IF staining reaction, the test is more reliable and easier to interpret than the acidified-methanol test described by Hutchins et al. (5).

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