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Enzyme-Linked Immunosorbent Assays for Detection of Xylem-Limited Bacteria: Use of Trinder Reagent†

MARY STARNES SAUNDERS‡* AND WILLIAM J. FRENCH

University of Florida, Agricultural Research Center, Monticello, Florida 32344

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Quantitation and detection of xylem-limited bacteria with an enzyme-linked immunosorbent assay with a peroxidase conjugate is described. The use of the Trinder reagent (4-aminoantipyrine) allows the determination of extremely small quantities of peroxidase with no precipitate formation or inactivation of the enzyme by H_2O_2. Comparison of the enzyme-linked immunosorbent assay method with microscopic and histochemical tests for the presence of the phony peach disease bacterium in 9-year-old "June Gold" peach trees gave comparable results. The peroxidase conjugate with the Trinder reagent is more sensitive than the alkaline phosphatase conjugate typically used for enzyme-linked immunosorbent assay quantitation.

Phony peach disease is caused by a xylem-limited bacterium (2, 6, 10) that until recently could only be diagnosed by grafting or vector transmission tests requiring from 18 to 24 months to complete (7). An immunofluorescent technique has been used successfully for identification of the xylem-limited bacterium associated with phony peach disease (4). However, it requires expensive microscopic equipment and large amounts of antibody. An enzyme-linked immunosorbent assay (ELISA) has been used for the detection in plants of virus (1) as well as bacteria (8), including the Pierce's disease bacterium (9; B. C. Raju, A. C. Goheen, G. Nyland, S. F. Nome, D. Docampo, J. M. Wells, D. Weaver, and R. F. Lee, Phytopathology **71**:108, 1980). It has been shown to be a simple, economical method for positive identification of plant viruses or bacteria. In this paper, application of the ELISA for the positive identification of the xylem-limited bacterium in phony peach disease is described and compared with existing detection methods. The use of the Trinder reagent (4-aminoantipyrine) to enhance the sensitivity of the ELISA method is described.

MATERIALS AND METHODS

A peach orchard near Madison, Fla., was used for these studies. Two roots, 5 to 10 mm in diameter and approximately 200 mm long, were collected from each tree and tested as follows: ELISA (1), methanol test (7), microscopic examination (3), and immunofluorescence confirmation of the associated bacterium in infected trees (4). Visual symptoms (stunting, lateral branches, darker green leaves) of each tree sampled were also recorded.

Antibody-enzyme conjugation. Rabbit antiserum to phony peach bacterium was prepared as previously described (3), and the gamma globulin-enzyme conjugates were prepared by mixing 0.3 ml (1.5 mg) of alkaline phosphatase (P-4502; Sigma Chemical Co., St. Louis, Mo.) with 1 ml of gamma globulin at 1 mg/ml or by adding 10 mg of peroxidase RZ:3 (Sigma P-8375) with 1 ml of gamma globulin at 5 mg/ml. The solutions were dialyzed three times against 1 liter of half-strength phosphate-buffered saline, pH 7.4, at 4°C, followed by the addition of 0.95 ml of 2% (vol/vol) glutaraldehyde diluted in distilled water. After 4 h of incubation at 25°C on a reciprocating shaker the unbound glutaraldehyde was removed by dialysis against three 1-liter changes of phosphate-buffered saline at 4°C.

ELISA assays. The ELISA of Clark and Adams (1), with slight modification, was used. The protocol outlined in Fig. 1 was followed. Control samples of phosphate-buffered saline–0.05% Tween 20 or healthy peach extract were prepared and tested concurrently with test samples. For alkaline phosphatase, the substrate solution was prepared as follows. Stock substrate solution, consisting of 100 mg of *p*-nitrophenyl phosphate (Sigma 104), was added to 50 ml of distilled water and stored frozen in 5.0-ml samples. This stock substrate solution was diluted with 7 ml of 1.5 *M* 1-amino-2-methyl-1-propanol buffer (Sigma 221), pH 10.3, before use. For the peroxidase congugate, two substrates were used: 5-aminosalicylic acid (Sigma A-3021) and 4-aminoantipyrine (Sigma A-4382), the Trinder reagent. The stock solution for 5-aminosalicylic acid consisted of 40 mg of 5-aminosalicylic acid added to 100 ml of 0.1 M sodium phosphate buffer, pH 6.0. This solution was stored frozen in 12-ml samples. Just before use the stock solution was thawed quickly, and 0.05 ml of 3% H_2O_2 was added to the 20-ml

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‡ Present address: Center for Viticultural Science and Small Farm Development, Division of Agricultural Science, Florida A&M University, Tallahassee, FL 32307.

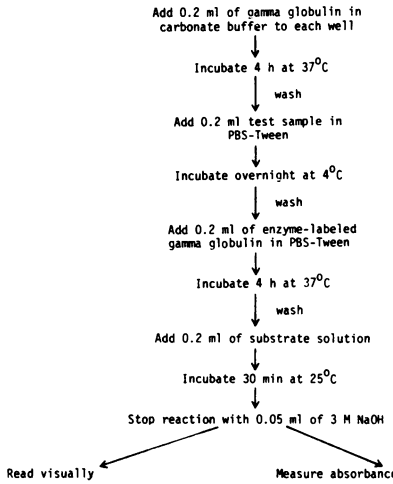

FIG. 1. Flow diagram for ELISA test. PBS-Tween, Phosphate-buffered saline–0.05% Tween 20.

samples. The stock solution for the Trinder reagent consisted of 1.0 mM 4-aminoantipyrine and 25.0 mM phenol dissolved in 0.1 M sodium phosphate buffer, pH 7.3 (5). For long-term storage this substrate solution was stored as a 5× concentrated stock solution. Just before use, 0.8 mM 3% hydrogen peroxide was added to 11 ml of the substrate solution. Absorbance values at 405 nm for the alkaline phosphatase reaction and 492 nm for the peroxidase reaction with the Trinder reagent were determined with a Bausch & Lomb Spectronic 20 spectrophotometer equipped with a microcuvette. Both reactions were also examined visually on a microtiter test reading mirror (Dynatech) against a white background. A reading was considered positive if its absorbance was twice that of the healthy peach sap control.

Sample extraction for ELISA tests. Each peach root was vacuum extracted by the method of French et al. (3) by using approximately 3 ml of filter-sterilized extracting solution consisting of phosphate-buffered saline–0.05% Tween 20 containing 2.0% ovalbumin. The two roots collected from each tree were sampled separately. Visual examination by phase-contrast microscopy of all the extracts and immunofluorescence examination (4) on representative samples was also performed. Samples were kept at 4°C if ELISA tests were to be performed the day they were prepared or stored frozen at −20°C for subsequent tests.

RESULTS AND DISCUSSION

Calibration of ELISA assay. The sensitivity of the ELISA method for detection of the phony peach bacterium in peach sap extracts was determined by using dilutions of bacteria that had been counted with a Petroff-Hauser bacteria counter and confirmed by using a Coulter counter. The presence of bacteria could be detected with dilutions of bacteria down to 10^6 bacteria per ml with the alkaline phosphatase conjugate (Fig. 2). The absorbance values of analogous healthy extracts were 0.05 or less. A value of twice the absorbance values of the control was selected for a positive result. With

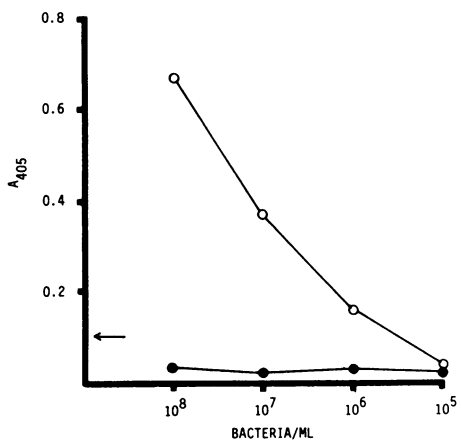

FIG. 2. ELISA absorbance values for alkaline phosphatase conjugate with phony peach bacteria antibodies against a range of concentrations of phony peach extract. Symbols: ○, absorbance at 405 nm of dilutions of infected peach sap; ●, absorbance of analogous dilutions of healthy sap. Bacteria in infected extracts were counted in a Petroff-Hauser bacteria counter. The arrow indicates the absorbance considered positive in detection of phony peach bacteria, which was arbitrarily picked at twice the concentration of healthy sap. Each point is the mean of three replicates.

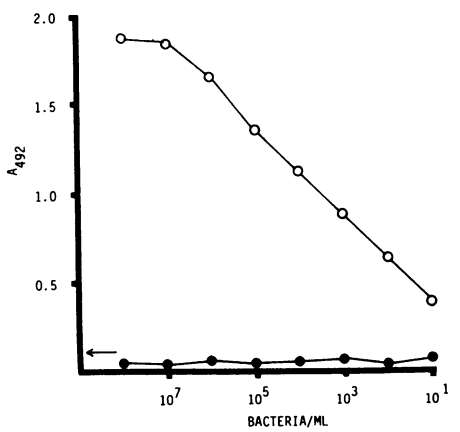

FIG. 3. ELISA absorbance values for peroxidase conjugate with phony peach bacteria antibodies against a range of concentrations of extract from diseased and health trees. Symbols: ○, absorbance at 492 nm of dilutions of infected peach sap; ●, absorbance of analogous dilutions of healthy sap. Bacteria in infected extracts were counted with a Petroff-Hauser bacteria counter. The arrow indicates the absorbance considered positive in detection of phony peach bacteria, which was arbitrarily picked at twice the concentration of healthy sap. Each point is the mean of three replicates.

the peroxidase system the sensitivity of the ELISA test was increased with the use of the Trinder reagent so that concentrations of bacteria as low as 10/ml could be detected (Fig. 3).

Comparison of ELISA, immunofluorescence, and microscopic examination of root extracts for estimating the percentage of bacteria in infected trees. The ELISA test with the alkaline phosphatase conjugate, immunofluorescence, and microscopic examination of root extracts gave comparable results. Among 32 "June Gold" peach trees examined, the following percentages of infected trees were obtained: ELISA, 25%; immunofluorescence test, 28%; microscopic analysis of roots, 28%; microscopic analysis of petioles, 54%; visual symptoms, 59%. We concluded that it is necessary to see approximately three to five bacteria per 40× field under phase contrast in the root extracts to obtain a positive ELISA value with the alkaline phosphatase system. A count of three to five bacteria per 40× field represents approximately 10^6 bacteria per ml. This value is consistent with the lowest detectable concentration as determined by dilution of known concentrations of bacteria (Fig. 2).

The ELISA test was performed with both the peroxidase-conjugated gamma globulin and the alkaline phosphatase-conjugated gamma globulin. Without exception, ELISA results were identical in both test systems. When the substrate used for the peroxidase reaction was 5-aminosalicylic acid, a partially insoluble product formed; therefore 5-aminosalicylic acid cannot be used for numerical quantitation of bacteria present in a sample, as can be done with the alkaline phosphatase system or the peroxidase system with the Trinder reagent. Visually, the peroxidase system is easier to read because of the red-brown color of the final product.

When the Trinder reagent was used as the substrate for the ELISA, bacteria could be detected at concentrations as low as 10/ml. The Trinder reagent was not used in the survey of

TABLE 1. Comparison of the methanol test, ELISA, immunofluorescence test (FA test), and microscopic and visual examination of trees for phony peach disease in "June Gold" peach trees

Tree no.	Methanol test	ELISA	FA test	Microscopic[a]	Visual[b]
34-1	Inconclusive	+	+	5	Moderate phony
34-2	Inconclusive	−	−	0	Healthy
34-7	Inconclusive	+	+	3	Healthy
34-9	+	+	+	100	Moderate phony
34-10	Inconclusive	−	−	0	Healthy
34-11	Inconclusive	+	+	4	Healthy
34-13	−	+	−	100	Light phony
34-14	−	−	−	0	Healthy
34-15	+	−	−	0	Healthy
34-16	Inconclusive	−	−	0	Healthy
34-18	Inconclusive	+	+	100	Phony
34-19	Inconclusive	−	−	0	Light phony
34-20	−	−	−	0	Healthy
34-22	Inconclusive	+	+	50	Phony
34-23	−	−	−	0	Light phony
34-24	−	−	−	0	Light phony
34-25	+	+	+	25	Phony
34-29	Inconclusive	−	−	0	Phony
34-34	Inconclusive	−	−	0	Light phony
34-35	Inconclusive	−	−	0	Phony
34-37	Inconclusive	−	−	0	Light phony
34-39	Inconclusive	−	−	0	Healthy
34-40	−	−	−	0	Healthy
34-41	Inconclusive	−	−	0	Healthy
34-42	+	−	−	0	Healthy
34-43	Inconclusive	−	−	0	Phony
34-45	Inconclusive	−	−	0	Healthy
34-47	+	−	−	0	Light phony
34-48	−	−	−	0	Light phony
34-49	Inconclusive	−	−	0	Phony
34-50	+	−	+	2	Phony
34-51	Inconclusive	−	−	0	Phony

[a] Number of bacteria per light microscope field (×40).
[b] Relative ratings for intensity of visual disease symptoms.

peach trees in 1979, but subsequent tests have shown that this reagent can successfully detect bacteria in peach trees infected with phony peach disease. With the Trinder reagent the peroxidase conjugate system for ELISA has all the advantages of the alkaline phosphatase conjugate system and the added advantage that this system is even less expensive and that the sensitivity of the ELISA system can be increased.

The ELISA has been used to detect Pierce's disease bacterium in plant tissues (9). This bacterium is xylem limited and is closely related to the bacterium that causes phony peach disease. The ELISA method used by Raju et al. (Phytopathology 71:108, 1980) necessitated concentrating the bacteria by centrifugation and sonication.

Although microscopic examination by phase-contrast methods alone does not give positive identification of the phony peach bacterium. With phase-contrast microscopy combined with the immunofluorescence technique, positive identification of the bacterium can be obtained. The ELISA technique is also capable of positive identification of the phony peach bacterium. The ELISA method is relatively simple and inexpensive when compared with the immunofluorescence technique, and positive results are easily interpreted.

Visual examination of the trees in this study indicated a higher percentage of trees with phony peach disease than was indicated by any of the analytical techniques used. Visual examination is a subjective method of determining phony peach disease. Tree decline due to other causes such as nutritional deficiencies or problems in water absorption could be misinterpreted as phony peach disease, thus increasing the number of trees indicated as positive (Table 1). The methanol test was also performed on all root samples collected. This test, which consists of incubating transverse sections of peach roots in acidified methanol and after a few minutes observing the appearance of purplish spots in a clear background of wood, was the only chemical test available for the identification of phony peach disease for approximately 40 years (7). This old method for identification of phony peach disease is unreliable, because most of the results are interpreted as inconclusive, and no distinction can be made between a tree with phony peach disease and a healthy tree (Table 1).

To obtain positive identification of phony peach disease, either the immunofluorescence technique or the ELISA should be performed. Unless a fluorescence microscope is available, the ELISA is the method of choice.

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