The Use of Fluorescent Antibodies for Detection of Pierce's Disease Bacteria in Grapevines and Insect Vectors

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ABSTRACT

An antiserum was produced against a pure culture of the bacterium that causes Pierce's disease (PD) in grapevines. Immunoglobulins were conjugated with fluorescein isothiocyanate and used for immune labeling of bacteria released from vascular tissue of PD-infected grapevine leaves and from infective leafhopper vectors. Evidence for specificity of the staining reaction is presented together with descriptions of the methods used.

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The current means of positively diagnosing Pierce's disease (PD) of grapevines is by transmission to indicato. plants, either by grafting or with insect vectors. The first external symptoms on an indicator plant such as *Vitis vinifera* L. 'Emperor' appear about 55 days after inoculation. The first internal (anatomical) symptoms of infection have been observed in tissues collected 24 days after inoculation by vector feeding (5). The recent discovery that a gram-positive bacterium is the cause of PD (1) opened the way for development of alternative, more rapid approaches to diagnosis. This paper reports the use of the fluorescent antibody (FA) method of Coons (2) for rapid and definite diagnosis of diseased vines. The method was also found useful for quickly determining whether insect vectors were carrying the organism.

MATERIALS AND METHODS.—The bacterial culture, originally isolated from infective leafhoppers *(Draeculcephala minerva* Ball), was maintained on 523 agar medium (6) at 29 ± 1 C. Bacterial immunogens for immunizing rabbits were prepared by centrifuging a 24-hour-old 523 broth culture. The pelleted cells were washed twice in saline and then subjected to sonication with a Biosonik III (Bronwill Scientific Co.) sonicator in 0.01 M phosphate buffer (PB) at pH 7.2. Antisera were produced in female New Zealand white rabbits weighing 2.27-4.09 kg (5-9 lb). Normal sera were obtained before immunization. A preparation containing approximately 100 μg protein/ml was used for immunization as follows: Starting with 0.25 ml, daily intravenous injections were given, increasing the dosage by 0.25 ml/day up to 2.0 ml, an amount which was given on the 8th, 9th, 10th, and 14th days. Also, 0.5 ml of bacterial homogenate was emulsified with an equal volume of Freund's complete adjuvant (Difco) and administered intramuscularly each day. Triturate from approximately 2×10^{11} bacterial cells was injected per rabbit. After 18 days from the initiation of immunization, sera from the two animals exhibited homologous precipitin titers of 1:1024 and 1:2048, respectively.

Immunoglobulins were separated from bulk sera with a DEAE Sephadex A-50 column as recommended by Dedmon et al. (3). The protein concentration was monitored by optical density (OD) at 280 nm. Antibacterial immune γ-globulins were conjugated with fluorescein isothiocyanate (FITC) through dialysis membranes (3). For separation of free FITC and highly charged protein from conjugated antibody, a DEAE-Sephadex A-50 column covered with a layer of coarse G-25 Sephadex was used (7). Characteristics of conjugates were measured by OD at 280, 490, and 496 nm utilizing a 3.5 μg/ml FITC solution as standard. The concentration of bound FITC-protein was 911 μg/ml. The molar dye/globulin ratio was 2.52; and the conjugate had the highest staining dilution at 1:8.

To establish immunological specificity of the antibody conjugate, bacteria grown in artificial media were prepared for staining as follows: one drop (approximately 0.01 ml) of a liquid culture was placed on a protein-coated (1.5% Difco gelatin solution containing 0.02% colorless merthiolate) glass slide, and allowed to dry on a hot plate at 40 C. The slide was washed in PB for 1-2 minutes, air-dried, stained for 30 minutes in a humid chamber at 37 C with a 1:8 dilution of FA, and again washed with PB. The stained bacteria were then mounted in glycerol-PB (9:1, v/v) under a coverslip and examined with a Zeiss GFL research microscope equipped with a HB 200 W mercury

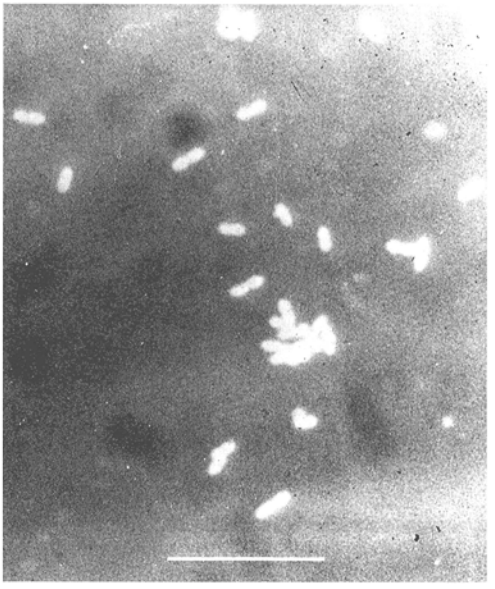

Fig. 1. Fluorescence micrograph of the bacterial pathogen which causes Pierce's disease of grapevine. Grape leaf midribs were sectioned, treated with KOH to cause exudation of the bacteria from vascular tissues, dried, and treated with specific immunoglobulins which had been conjugated with fluorescein isothiocyanate. Labeled cells were characterized by a yellow-green fluorescence when viewed under light filtered 400-650 nm. Scale bar is 5 μm.

vapor lamp and filters for observing fluorescence in the range of 400-650 nm. The controls used to establish specificity were: (i) a pure culture of *Corynebacterium michiganense* treated with labeled antibody prepared against PD-bacteria; (ii) PD-bacteria stained with fluorescent antibody specific for potato virus X (PVX); and (iii) blocking of labeled antibody prepared against PD bacteria with unlabeled antibody to PD bacteria. No fluorescent staining was observed in any of the three controls, whereas PD bacteria stained with homologous FA exhibited characteristic apple-green fluorescence under ultraviolet (UV) illumination.

Bacteria from diseased grapevines were prepared for FA staining. Midribs from leaves showing PD symptoms (experimentally inoculated) were excised, and the veinal tissue was transversely hand-sectioned (0.1-0.2 mm thick) with a razor blade. The tissue slices were mounted in a drop of sterile distilled water on a protein-coated glass slide, and then flooded with 0.1 molar KOH (4). Following treatment of the tissue sections with KOH, a standard Zeiss RA research microscope equipped with phase-contrast optics was used to observe bacteria being released into the mounting medium. After the bacteria were released, the glass slide was placed without washing on a warming table to dry. The tissue sections were then removed leaving only the bacteria adhering to the slide. The latter were then stained with fluorescent antibody for 30 minutes at 37 C, washed in PD, and mounted in buffered glycerol for observation with the fluorescence microscope.

RESULTS.—Brightly fluorescing bacterial cells, single or in groups, were observed close to where the tissue sections had been placed on the slide (Fig. 1). Bacteria similarly extracted from PD-infected tissue, and stained with FA specific for PVX, did not become stained.

Specifically stained bacteria could also be found in excreta from infective leafhoppers. Noninfectious leafhoppers were allowed to feed on healthy grapevines and then were transferred to plants with Pierce's disease. Excreta were collected with a glass rod held to the anus of the insect. Collections were made from the same insect after it had fed on the healthy and diseased vines, respectively. The excreta were placed on a protein-coated glass slide, dried on a hot plate, washed in PB, stained with fluorescent antibody for 30 minutes, and washed with PB. Single, and groups of, yellow-green-fluorescing cells were observed with the fluorescence microscope on the slide smear of excreta from leafhoppers which had fed on diseased plants. No fluorescing bacteria were observed on smears prepared with excreta from leafhoppers which had only fed on the healthy grapevines.

CONCLUSIONS.—The technique described herein proved to be a very specific, simple, and rapid diagnostic method to identify Pierce's disease bacteria in infected plants and in contaminated insect vectors. The reliability of the FA method for detecting the bacteria in PD-infected plants was tested further on more than twenty diseased and healthy vines and gave consistent results (100% efficiency). We believe that this technique can be very useful for detecting diseased plants in vineyards and should serve as an aid to breeding for resistance and facilitate the screening of antibiotics which may have possible use in therapy.

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