Immunofluorescent Detection of Xylem-Limited Bacteria In Situ

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Florida Agricultural Experiment Stations Journal Series Paper 3462.

Appreciation is expressed to Dr. L. Claflin for antiserum to *Corynebacterium michiganense* and to the USDA, SEA-AR, Horticultural Research Laboratory, Orlando, for use of the cryostat.

Accepted for publication 23 April 1982.

ABSTRACT

Brlansky, R. H., Lee, R. F., Timmer, L. W., Purcifull, D. E., and Raju, B. C. 1982. Immunofluorescent detection of xylem-limited bacteria in situ. Phytopathology 72:1444-1448.

Xylem-limited bacteria (XLB) were detected in situ in sectioned material using gamma globulins (IgG) specific to Pierce's disease bacterium (PDB) or the ratoon stunting disease bacterium (RSDB) and conjugated to tetramethylrhodamine isothiocyanate (TRITC). Longitudinal and transverse cryostat-cut sections, 30–40 μm thick, were prepared from plants affected by Pierce's disease of grape, periwinkle wilt, plum leaf scald, a disease of ragweed, and sugarcane ratoon stunting disease. Sections were immersed in TRITC-labeled IgG for 1 hr at room temperature, rinsed in phosphate-buffered saline, mounted, and viewed with a fluorescent microscope at 560–590 nm. Orange-red fluorescing bacteria were readily observed in and adhering to xylem vessels of diseased plants affected with Pierce's disease, periwinkle wilt, plum leaf scald, and diseased ragweed when incubated in TRITC-labeled IgG to PDB. Fluorescing bacteria were observed in sugarcane with ratoon stunting disease when incubated in TRITC-labeled RSDB IgG. No fluorescing bacteria were observed in sections of infected plants immersed in normal serum or in IgG specific for *Corynebacterium michiganense* labeled with TRITC or in healthy plants incubated in PDB or RSDB specific IgG. Fluorescein isothiocyanate-labeled antiserum was not useful since xylem vessels autofluoresced at the same wavelength as fluorescein.

Recently, several fastidious, Gram-negative, xylem-limited bacteria (XLB), often referred to as rickettsialike bacteria (RLB), have been discovered in plants (9). Pierce's disease of grapes (PD), almond leaf scorch (ALS), and alfalfa dwarf (AD) are caused by the same Gram-negative, fastidious bacterium (4). Similar bacteria have been associated with many vascular diseases such as phony peach (10), plum leaf scald (13), periwinkle wilt (15), and elm leaf scorch (8). Recently the bacteria associated with phony peach and plum leaf scald have been cultured (2,17,18), and these diseases are caused by the same organism (19). A Gram-positive coryneform bacterium also has been consistently isolated and cultured from the xylem of sugarcane with ratoon stunting disease (RSD) (3).

Detection of these bacteria in plant tissues is often difficult. Direct (14) or indirect (6) immunofluorescent labeling of the bacteria in xylem extracts obtained by squeezing (14) or by vacuum extraction (5) have been useful for detection. Direct or indirect labeling of these bacteria in tissue sections has not been reported.

The purpose of this study was to develop a method of labeling XLB in situ with fluorescent labeled antibodies in order to rapidly detect and identify these bacteria in xylem.

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0031-949X/82/11144405/$03.00/0

MATERIALS AND METHODS

Bacterial isolates and plant material. Two isolates of the Pierce's disease bacterium (PDB), a Florida isolate from grape (LH-2) and a California isolate from alfalfa (AD-12), and a Louisiana isolate of the ratoon stunting disease bacterium (RSDB) were used to prepare antisera. Pierce's disease affected grape (*Vitis vinifera* L., 'Chardonnay'), periwinkle wilt affected periwinkle (*Catharanthus roseus* L. (G. Don)), plum leaf scald affected plum (*Prunus cerasifera* Ehrh. 'Myrobalan'), ragweed (*Ambrosia artemesiifolia* L.) affected by an unknown XLB, sugarcane (*Saccharum officinarum* L. 'L62-96') affected by RSD, and healthy control plants were used in this study to obtain cryostat sections.

Culture. Pierce's disease bacterium isolates LH-2 and AD-12 were cultured on agar plates and slopes of Davis' PD-2 medium (4). Agar slopes were used to inoculate 50 ml of PD-2 broth in shake cultures that were incubated at 23 C. Shake cultures were harvested after 10–14 days.

The ratoon stunting disease bacterium was isolated from the internodal xylem squeezings of sugarcane with RSD on BCZE medium (17). BCZE medium is a modification of the BCYE medium (18) used for the isolation of the bacteria associated with plum leaf scald and phony peach diseases. BCZE medium consists

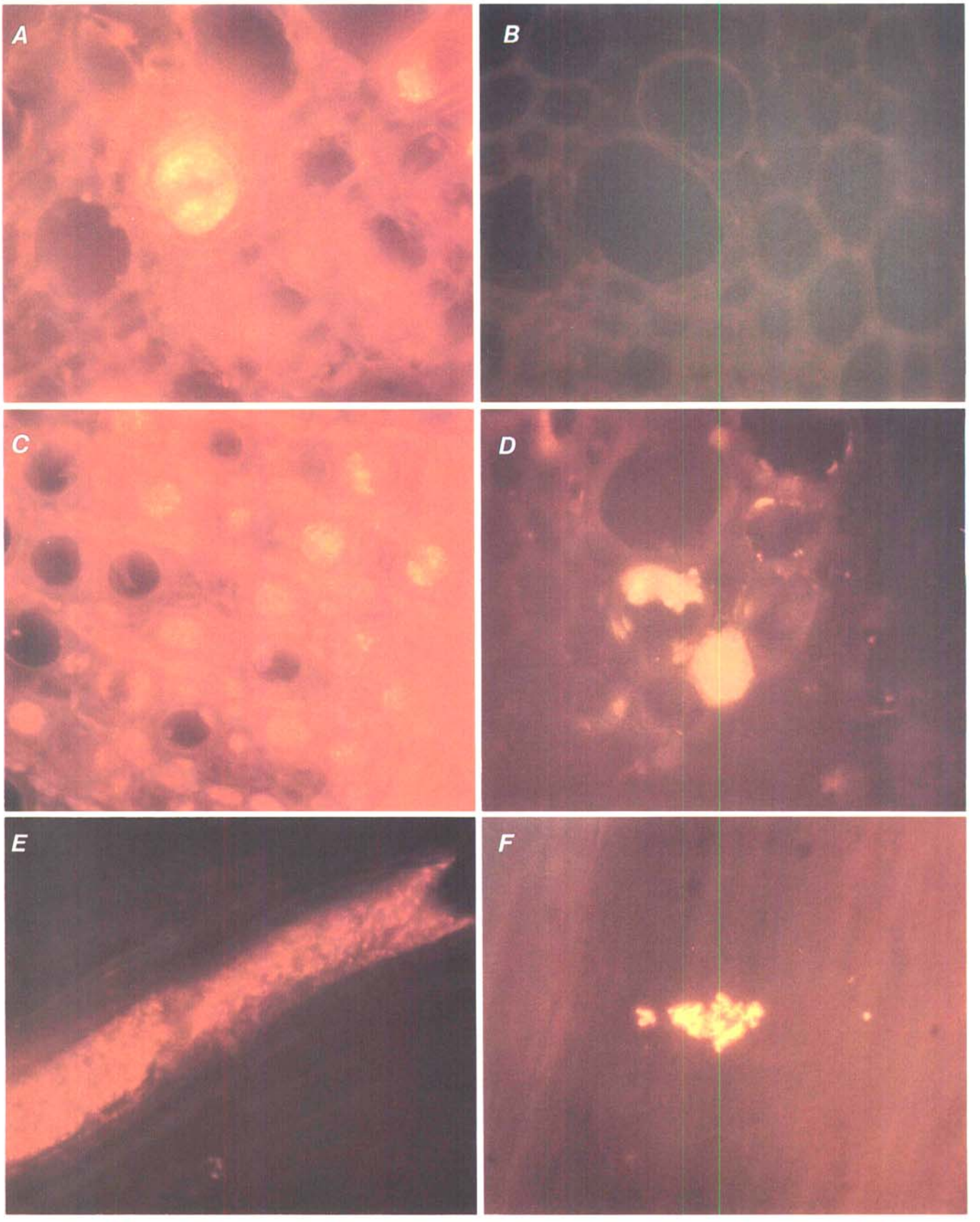

Fig. 1. Bacteria detected in the xylem vessels of plants by using tetramethylrhodamine isothiocyanate (TRITC)-labeled antisera. **A,** Pierce's disease bacteria (PDB) in grape xylem. **B,** Healthy grape xylem treated with TRITC-labeled PDB antiserum. **C,** Fluorescing bacteria in xylem vessels of plum twigs with plum leaf scald disease. **D,** Bacteria in xylem vessels of periwinkle stems with periwinkle wilt disease. **E,** Bacteria in the xylem of ragweed affected with an undescribed disease. **F,** Sugarcane xylem containing the sugarcane ratoon stunting disease bacterium. A–F (×495).

of Bacto agar (Difco Laboratories, Detroit, MI 48232), 17.0 g; yeast extract (Difco Laboratories), 10.0 g; KH_2PO_4 (Sigma Chemical Co., St. Louis, MO 63178), 1.0 g; $MgSO_4$, 0.2 g; KH_2PO_4 (Sigma Chemical Co.), 1.0 g; soluble potato starch, 2.0 g; glucose, 4.0 g; and distilled H_2O, 980 ml. The medium was adjusted to a pH of 6.6 with either 1 N NaOH or 1N HCl and autoclaved for 20 min at 121 C. Ten milliliters of a freshly prepared 2.5% ferric pyrophosphate solution and 10 ml of a 5% L-cysteine solution in distilled water were passed through a 0.2-μm Millipore filter and were added to the cooled (50 C) medium. The bacteria isolated were identical in size, structure, and growth characteristics as those isolated by Davis et al (3). Bacteria from plates were transferred to BCZE broth media and shake cultured for 14 days at 28 C.

Antisera production. Antisera were prepared to the AD-12 and LH-2 isolates of PDB. Bacteria from shake cultures were centrifuged at 20,000 *g* for 15 min, washed three times in 15 mM sodium phosphate buffered saline (PBS), pH 7.4, and resuspended in sterile distilled water. One-milliliter samples of 10^8 cells per milliliter (determined with a Batch Counting Chamber, Catalogue No. 900, Hausser Scientific, Blue Bell, PA 19422) were lyophilized for storage.

The lyophilized PDB were resuspended in half the original volume using sterile distilled water and an equal volume of Freund's incomplete adjuvant added. Three injections were made at weekly intervals to female New Zealand white rabbits. Each time 0.15 ml was injected into the foot pad (or toe pad) and 1.5 ml intramuscularly. The rabbits were bled from an ear vein at weekly intervals following the final injection and the serum separated from the blood collected 14 days after the last injection was used. Gamma globulin (IgG) was separated from the whole serum using either the sodium sulfate procedure of Kekwick (12) or the protein A-Sepharose affinity chromatography procedure of Miller and Stone (16). Both procedures gave good separation of the IgG fraction. The endpoint dilution in Ouchterlony double diffusion tests with 10^9 bacteria per milliliter was 256 for isolate LH-2 and 128 for isolate AD-12.

Antiserum was prepared to the Louisiana isolate of RSDB. Bacteria from broth cultures were centrifuged at 20,000 *g* for 15 min, washed five times in PBS, and resuspended in one-half the original volume. Ratoon stunting disease bacteria (10^9 bacteria per milliliter) were injected intravenously by several injections into New Zealand white rabbits with increasing dosages of 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml at 3-day intervals followed by two dosages of 2.0 and 2.25 ml, each mixed with an equal volume of Freund's incomplete adjuvant at 2-day intervals. Rabbits were bled from an ear vein 1 wk after the final injection, the serum was separated, and the IgG fraction was separated from the serum by the method of Kekwick (12). The endpoint dilution with RSDB (10^9 bacteria/ml) in Ouchterlony double diffusion tests was 512.

Conjugation of antisera. The IgG to PDB (LH-2 and AD-12 isolates) and RSDB was conjugated with either fluorescein isothiocyanate (FITC) (Miles Laboratories, Elkhart, IN 46515), tetramethylrhodamine isothiocyanate (TRITC), or substituted rhodamine isothiocyanate (XRITC) (Research Organics, Inc., Cleveland, OH 44125). Gamma globulins were conjugated to FITC by the method of Blakeslee et al (1). For conjugation with TRITC or XRITC, sodium-sulfate fractionated IgG (1 mg/ml) was dialyzed against 0.05 M borate buffer, pH 9.3, containing 0.4 M NaCl. After equilibration, the dialysis tubing containing the antiserum was transferred to 0.05 M borate buffer, pH 9.3, containing 3 mg of either TRITC or XRITC in 0.2 ml dimethylsulfoxide for each 100 ml volume of buffer as recommended by Research Organics Inc., Cleveland, OH 44125 in their Bulletin 95. After dialysis for 17 hr at 4 C, the unconjugated fluorochromes were separated from IgG conjugates by chromatography on a Sephadex G-50 column (1.5 × 45 cm) equilibrated with 15 mM sodium phosphate buffer, pH 7.4, which contained 0.85% sodium chloride and 0.02% sodium azide (PBS). Column fractions were monitored spectrophotometrically at 280 nm for protein, 495 nm for FITC, 560 nm for TRITC and 580 nm for XRITC.

Specificity of the fluorescent-labeled PDB and RSDB IgG was tested using the direct immunofluorescent method as described (14). Cultured bacteria tested with both PDB and RSDB antisera included *Xanthomonas pruni* (obtained from C. I. Kado, Department of Plant Pathology, University of California, Davis 95166), *Corynebacterium michiganense* (obtained from L. Claflin, Department of Plant Pathology, Kansas State University, Manhattan 66506), PDB isolate LH-2, PDB isolate AD-12, and RSDB (Louisiana isolate). Xylem squeezings from plants affected by periwinkle wilt, plum leaf scald, and a disease of ragweed also were tested.

Bacterial smears from agar plates or squeezings from plants were spread on slides coated with 1.5% gelatin, containing 0.02% merthiolate in distilled water and dried. Slides were washed for 2 min in PBS and dried. Fluorescent-labeled IgG diluted 1:10 was placed on the dried bacterial smear and incubated for 45 min at 37 C in a humidity chamber. Slides were washed for 2 min in PBS, air-dried, and mounted in Aqua Mount (Lerner Laboratories, New Haven, CT 06513).

Observations were made with a Leitz Dialux fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ 07647) by using a 50V mercury lamp, and viewed in the 560–590 nm range for TRITC (Leitz filter block N2) and in the 380–420 nm range (Leitz filter block H2) for FITC.

Cryostat sections were cut from infected and healthy plant material by using a Harris model WRC cryostat (Harris Manufacturing, Inc., North Billerica, MA 01862). Longitudinal and transverse sections 30–40 μm thick were immersed in labeled PDB IgG, labeled RSDB IgG antiserum, labeled normal IgG, or in labeled IgG specific for *C. michiganense*. Sections were incubated in labeled IgG (diluted in PBS buffer to 0.03 absorbance units at 280 nm) for 1 hr at room temperature or at 37 C for 30 min. Sections were floated in PBS for 10–20 min, mounted on microscope slides in Aqua Mount, and observed as described for bacterial smears. Photographs were taken with Kodak Ektachrome EL 400 color film and/or Kodak Technical Pan 2415 black-and-white film.

In order to show the presence of bacteria in the test material alternate sections were prepared for scanning (SEM) and transmission (TEM) electron microscopy. Sections were fixed at 4 C in 3% glutaraldehyde in 0.06 M potassium phosphate buffer, pH 6.8, for 12 hr, washed three times at 20-min intervals each in buffer and dehydrated in a 30–100% acetone series. Samples for SEM were transferred to an acetone:freon (2:1, 1:2) series for 20 min each and then into two changes of 100% freon and critical-point dried in a Bomar Critical Point Drier (The Bomar Co., Tacoma, WA 98401). The specimens were mounted on SEM stubs, sputter coated with 100 Å gold-palladium, and viewed in a JEOL JSM35 scanning electron microscope. For TEM, specimens were placed in Spurr's medium:acetone (1:1) for 12 hr, transferred to a 2:1 mixture for 12 hr, and then in 100% Spurr's medium for 12 hr. The specimens were then embedded in Spurr's and polymerized at 70 C. Ultrathin sections were made on a Huxley LKB ultramicrotome (LKB Instruments, Inc., Rockville, MD 20352), stained with uranyl acetate and lead citrate, and viewed with a Philips 201 transmission electron microscope.

RESULTS

Antisera conjugated readily with either FITC or TRITC. The $A_{280\,nm}/A_{495\,nm}$ ratios for FITC conjugated antisera ranged from 0.7 to 1.0 while the $A_{280\,nm}/A_{560\,nm}$ ratios for TRITC conjugates ranged from 1.8 to 2.4. Attempts to conjugate XRITC to antisera were unsuccessful using two different lots of XRITC. The $A_{280\,nm}/A_{580\,nm}$ ratios of XRITC conjugated antisera ranged from 18.0 to 26.3, indicating very poor binding of XRITC to the antisera. Spectral scans of XRITC in buffer showed additional absorbances at 545, 380, and 270 nm.

In specificity tests, smears of PDB isolates LH-2 and AD-12 fluoresced when incubated with either TRITC or FITC labeled PDB IgG (LH-2 or AD-12). Smears of ratoon stunting disease bacterium fluoresced only when incubated with labeled RSDB IgG. No fluorescent reactions were found when either *C. michiganense* or *X. pruni* smears were incubated with either PDB

IgG or RSDB IgG. Xylem squeezings from the affected grape, periwinkle, ragweed, and plum contained fluorescing bacteria only when incubated with PDB IgG labeled with FITC or TRITC.

Orange-red fluorescing bacteria were observed in xylem vessels of XLB infected plants incubated in TRITC-labeled IgG (AD-12 and LH-2) (Fig. 1A–E) and ratoon stunt diseased sugarcane incubated in RSDB IgG (Fig. 1F). Fluorescing bacteria were seen in vessel lumina and adhering to the vessel walls in longitudinal and transverse sections (Fig. 1). Large masses of bacteria were often seen streaming out of the xylem vessels. In RSD-infected plants fluorescing bacteria were seen as microcolonies of tightly aggregated cells that appeared to be branching (Fig. 1F). Using this in situ immunofluorescence bacteria were found in all of the infected plants tested, but not in any healthy plants. Scanning (Fig. 2A) and transmission (Fig. 2B) electron microscopy from the same sectioned samples showed the presence of bacteria in the xylem of infected plants. No fluorescing bacteria were observed in infected plant sections incubated in labeled normal IgG or in labeled IgG specific for *C. michiganense*. However, an autofluorescing material, believed to be some type of plugging material, was found in periwinkle wilt affected plants (Fig. 3A). Similar occlusions were not found in the vessels of healthy periwinkle. This material fluoresced at both ultraviolet ranges. TRITC-fluorescing bacteria were seen apart from occlusions as small orange-red points mixed with the plugging material. In the scanning electron microscope the plugging material appeared to totally block some vessels (Fig. 3B).

Fluorescing bacteria could not be identified in vessels labeled with FITC-antisera. In both infected and healthy plants, xylem vessels autofluoresced in the 380–420 nm range. We could not distinguish autofluorescence of unlabeled vessels in healthy or

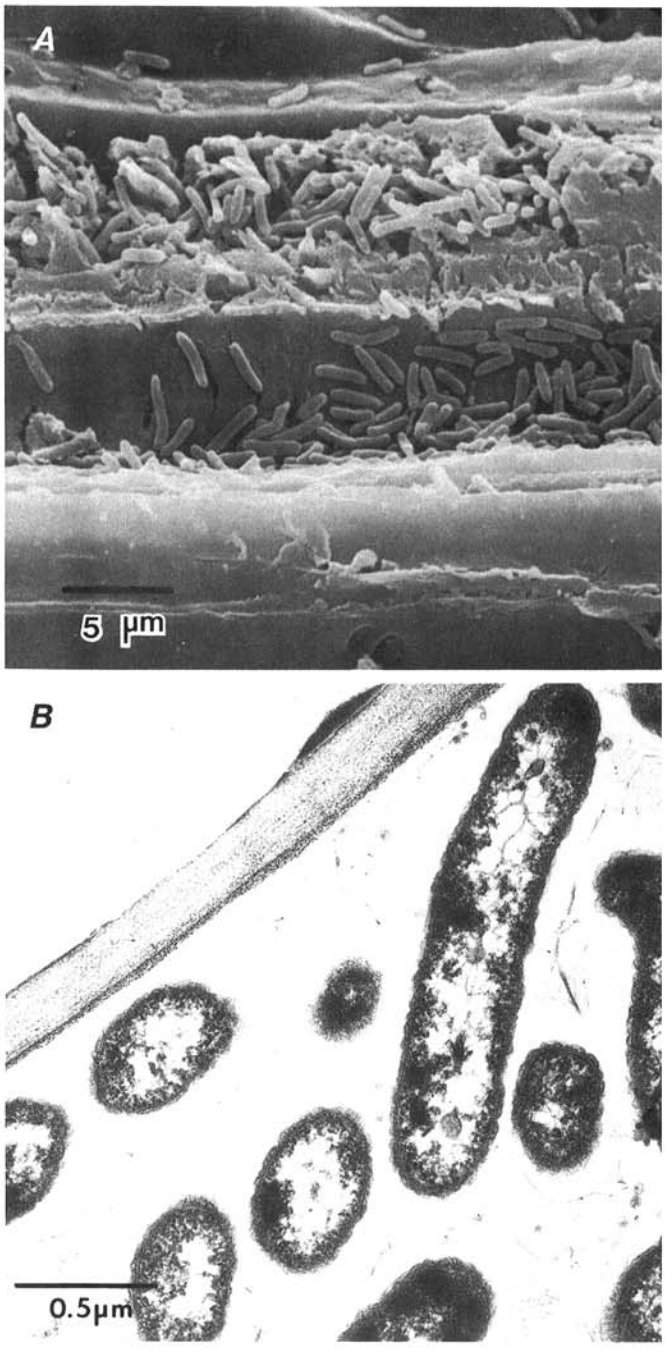

Fig. 2. Electron microscopy of periwinkle and ragweed xylem vessels. **A,** Scanning electron micrograph of a xylem vessel of periwinkle wilt-affected periwinkle showing an abundance of bacteria (×3,000). **B,** Transmission electron micrograph of bacteria in a xylem vessel of affected ragweed (×38,360).

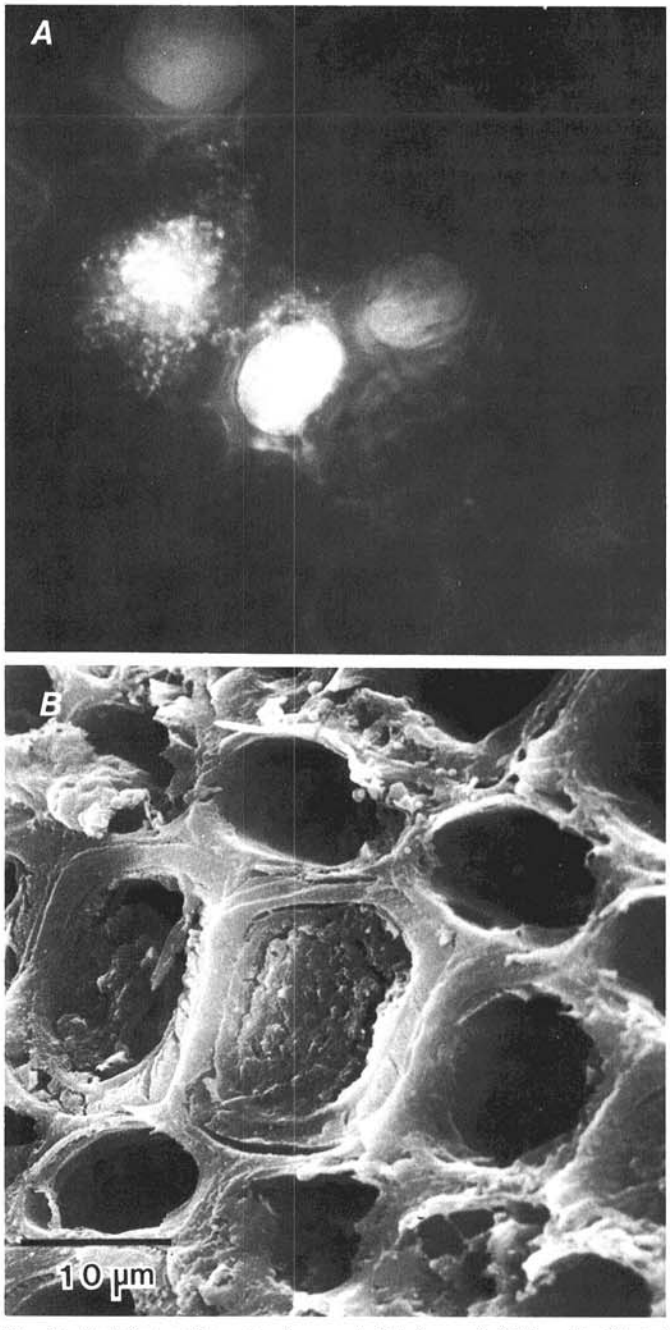

Fig. 3. Autofluorescing plugging material in periwinkle wilt-affected *Catharanthus roseus* xylem. **A,** Autofluorescing material as seen with light microscopy (×495). **B,** Scanning electron micrograph of plugging material (×2,000).

diseased tissue from FITC-labeled bacteria within vessels. Fluorescing bacteria occasionally could be seen in sections if they were removed from the autofluorescing xylem.

DISCUSSION

The results of this study demonstrate the usefulness of TRITC-labeled antisera for in situ detection of xylem-limited bacteria. TRITC was superior as a fluorescent label because there was less autofluorescence of the xylem vessels in the 560–590 nm wavelength range. Direct or indirect labeling of xylem-limited bacteria in tissue sections has not been reported. Hapner and Hapner (7) showed that rhodamine was superior to fluorescein as a fluorochrome for use in immunohistofluorescent studies of plant tissues. Less autofluorescence occurred when plant tissues were illuminated in the rhodamine range (546–590 nm) than in the fluorescein range (380–420 nm). Jeffree et al (11) used rhodamine and fluorescein as indirect labels to determine the distribution of lectin in cells of *Datura stramonium*.

XRITC, which has been reported to be a high emitter of a red fluorescence, was not useful in this study because it could not be conjugated. This probably is evidence of the unstable nature of the compound; there were additional absorbance peaks at 545, 380, and 270 nm.

The TRITC procedure is useful in direct detection of bacteria in the xylem vessels of affected plants. Bacteria present in vessels where specific antibodies can readily penetrate were easily labeled. Transverse and longitudinal cryostat sections were excellent for this type of labeling. Labeled bacteria in longitudinal sections could be identified in specific vessels and in particular locations within a xylem vessel. Bacteria in transverse sections could be seen in large masses within vessels and as individual bacteria adhering to vessel walls. Free-hand sections cut with a razor blade were also successfully prepared. The thinner sections allowed better penetration of specific antibodies and better viewing of bacterial fluorescence. This technique could be very useful in locating bacteria in various areas of plants, screening tissue for the presence of bacteria prior to preparation for electron microscopy, and in following the movement of bacteria within a plant.

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