A Specific Fluorescent Antibody for Detection of Syringomycin in Infected Peach Tree Tissues

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ABSTRACT

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Procedures are described for producing a specific fluorescent antibody (FA) to syringomycin (SR), a toxin which is produced in vitro and in vivo by pathogenic strains of *Pseudomonas syringae*. Stem and leaf sections of peach seedlings inoculated with pathogenic strains of *P. syringae* or syringomycin, exhibited the yellow-green fluorescence of the SR-FA complex in all infected or toxin-infiltrated tissues, but not in healthy control plants. Most fluorescence was observed around the periphery of the cells and was apparent in phloem, xylem, cambium, and pith-parenchyma cells. Field samples from commercial orchards also tested positive for presence of SR by the microprecipitin test and by visualization of the FA-SR complex.

Bacterial canker disease of peach (*Prunus persica* [L.] Batsch) trees caused by *Pseudomonas syringae* van Hall is a significant factor affecting the decline or death of peach trees in the United States. Sinden and DeVay (13) showed that isolates of *P. syringae* from stone fruit trees, regardless of host preference, produce a substance in culture that is toxic to peach trees and to various fungi and bacteria. Pathogenicity of *P. syringae* is correlated with its ability to produce this toxin. DeVay et al (3) showed that partially purified preparations of the toxin, injected into young peach trees, produce disease symptoms identical to those in trees inoculated with pathogenic cultures of *P. syringae*. This phytotoxin was purified by Gross and DeVay (4) and shown to be a low molecular weight polypeptide which had been named syringomycin (SR) by Sinden (12). Extracts from diseased tissues contained compounds with the same characteristics as SR. Sinden et al (14) concluded that they were the same biologically active compound. Backman and DeVay (1) indicated that the primary action of SR appears to be a rapid, detergent-like lysis of the cell membrane.

A detailed study of SR in the host-pathogen interaction has been hindered by an inability to detect the toxin in situ. Efforts to extract the toxin from diseased tissues have been partially successful (14), but inconclusive. We have resolved this problem by producing a specific antibody to SR (11), conjugating it with fluorescein isothiocyanate (FITC) to form the fluorescent antibody (FA), which can be used diagnostically to locate the toxin in situ.

MATERIALS AND METHODS

Bacterial strains. The strains of *Pseudomonas syringae* used were 5D442, $B15^+$, and $B15^-$ (courtesy of J. E. DeVay, University of Calif., Davis) and B634 (courtesy of D. J. Weaver, U.S. Department of Agriculture, Southeastern Fruit and Tree Nut Research Station, Byron, GA). The cultures were maintained in water suspensions and nutrient broth suspensions at 4 C. *Pseudomonas mors-prunorum* f. sp. *persicae*, strain NCPPB2254, was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England.

Syringomycin (SR). Production and purification of SR was by a procedure modified from that of Gross and DeVay (4) in which 1 L still cultures of potato-dextrose broth were inoculated with *P. syringae*, strain 5D442. Following extraction with 1-butanol, the crude preparation was subjected to ultrafiltration through a PM30 membrane (Amicon Corp., Lexington, MA 02173). The SR was bound on a carboxymethyl cellulose column at pH 3 (Whatman CM52), and eluted with 0.005 N HCl at pH 2.3. The freeze-dried eluate at pH 2.3 was dissolved in 0.01 N HCl and fractionated on a Biogel P-2 column (BioRad; 1.5 × 30 cm), with 0.01 N HCl. Separation by thin-layer chromatography (TLC) was omitted. The final preparation of SR was judged to be pure on the basis of a single protein-staining band detected by discontinuous gel electrophoresis in 15% acrylamide gels at pH 4.3 and 2 ma/tube, and a single ninhydrin-reactive spot detected by TLC separation with 1-butanol:glacial acetic acid:pyridine:distilled water (15:3:10:12, v/v). Absence of carbohydrate was confirmed by spraying the thin layer plates with *p*-anisidine-HCl. Activity of the toxin extract was determined by the bioassay procedure described by Sinden et al (14) with *Geotrichum candidum* Link ex Fries strain 4139 (ATCC) as the test organism.

One-liter cultures of strains $B15^+$, $B15^-$, B634, and *P. mors-prunorum* were cultured similarly and subjected to the SR purification procedure through the butanol extraction stage. After removal of the butanol, the extract was concentrated to 50 ml for the microprecipitin test.

Antibody preparation. Two mature female New Zealand rabbits were shaved on the back and subjected to multiple subcutaneous injections of the antigen over several months. The initial antigen preparation consisted of 8 mg of purified SR dissolved in 4 ml of physiological saline (0.85%) and homogenized with 4 ml of Freund's complete adjuvant (Difco) in an ice-cooled Sorvall homogenizer until a stiff emulsion was obtained. Approximately 2 ml of this emulsion in 0.2 ml aliquots was injected into each rabbit. The rabbits were maintained on a regular diet and the injections were repeated after 3 and 4 wk. For these and all subsequent injections, Freund's incomplete adjuvant was used in place of Freund's complete adjuvant. Bleeding was via the ear vein 10 days following the third antigen injection. The antiserum titers in the rabbits were maintained by booster shots at the beginning of each month, blood was drawn 10 days later, allowed to clot, and the serum was separated by low-speed centrifugation (3,000 *g* for 10 min) and decanted into clean tubes. Serum titers were determined on each batch by the 'O' ring and the microprecipitin tests (7).

Purification of antibody. The globulin fraction was purified from the serum by repeated ammonium sulfate precipitations (final concentration, 35%) according to the method of Hebert et al (5). After the third precipitation, the resulting globulin was dissolved in 10 ml of distilled water and dialyzed against 1 L of physiological saline at pH 8 with several changes during 24 hr until the dialysate was free of NH_4^+ (detected with Nessler's reagent) and $SO_4^=$ (detected with $BaCl_2$ solution). The resulting globulin solution

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was assayed for protein by the biuret reaction (2) and adjusted to 1% protein with sterile distilled water.

Conjugation to fluorescein isothiocyanate (FITC). Fluorescent antibody (FA) was formed by methods similar to those described by Lewis et al (8) as modified by Nairn (9) and Fliermans (Savannah River Plant, Aiken, SC; *personal communication*).

Ten milliliters of the globulin solution (1% protein) were mixed with 4 ml of 0.15 M sodium phosphate bufer at pH 9. Five milligrams of FITC were dissolved in 4 ml of 0.1 M sodium phosphate buffer at pH 8 and added to the globulin solution. The resulting solution was adjusted to pH 9 with 0.1 N NaOH and the volume was adjusted to 20 ml with physiological saline. Conjugation was carried out for 20 hr at 4 C with rapid agitation on a magnetic stirrer. The FA and nonconjugated FITC were separated by column chromatography (1.5 × 20 cm column of Sephadex G-25 [Sigma Chemical Co., St. Louis, MO 63178]) and eluted with phosphate buffered saline (PBS) at pH 7.2. The FA, which was eluted by the first 30 ml through the column, was distributed in 1-ml quantities in small culture tubes and stored in the freezer at −15 C.

Serology. Antigen dilutions of 1:10 to 1:1,600 and antisera dilutions of 1:2 to 1:2,048 were employed in the microprecipitin test. Equal volumes (5 μl) of antibody or antigen were dispensed into the appropriate squares marked on a plastic petri dish.

Preparation of plant tissues containing SR. Preliminary experiments were performed on peach seedlings in which the ends of stems and leaves were immersed for 24–72 hr in 2 ml of a solution of SR containing 900 units of SR activity. Stems took up 1.5 ml of the toxin solution during the 72-hr period. Control seedlings were immersed in sterile water for a similar period. Sections of stem and leaf tissue at various distances from the point of entry of the toxin were obtained in different ways. Fresh sections of stem were cut free-hand with a razor. Frozen sections were obtained by rapid freezing of the specimens with CO_2 and then 10-μm-thick sections were cut with a cryostat (Ames™ Microtome-Cryostat II). Albumin was used to adhere the sections to the glass slides which then were stored at −15 C until stained with FA. Paraffin sections were prepared as outlined by Johansen (6).

Field samples from healthy peach trees and from those exhibiting bacterial canker were collected from commercial orchards in Edgefield County, SC during early spring of 1978. Both fresh and paraffin sections were prepared from diseased and healthy tissues. Isolates of *P. syringae* were obtained from diseased plant material collected from these orchards.

One-gram twig samples from infected trees were cut into small pieces and homogenized in 8 ml of sterile water for 2 min in a Sorvall homogenizer cooled in ice. The resulting mixture was centrifuged at 5,000 *g* for 15 min and the supernatant fluid was re-centrifuged at 11,000 *g* for 15 min to remove particulate matter. Five microliters of this second supernatant fluid and a 1:2 dilution in sterile water were mixed in the microprecipitin test with a 1:2 antibody dilution.

Detection of SR in tissues by the FA technique. Naturally infected and toxin-infiltrated plant tissue sections (containing SR) and controls (containing no SR) were stained with FA (1:2 dilution with physiological saline) for 1 hr at room temperature in the dark. Unreacted FA was removed from the slide by careful washing for 15 min with at least two changes of PBS. The specimens were mounted in a mixture of one part 0.01 M phosphate buffer (pH 7) to eight parts glycerol and covered with a coverslip. The fluorescence of the SR-FA complex was determined with a Wild M20-35280 fluorescence microscope provided with a Wild 7104 Universal lamp with an HBO 200 mercury burner. A dark-field condenser was employed together with heat-absorbing filters and the correct filters for detecting the yellow-green fluorescence of fluorescein. Photographs were on Ektachrome ASA 400 film with exposures ranging 1–15 min.

RESULTS

Introduction of the ultrafiltration step in the SR-purification procedure removed large amounts of contaminating carbohydrate. Although some of the SR passed through the membrane together with a large proportion of the carbohydrates, the majority of the SR was retained by the PM30 membrane. This indicated that the toxin either had aggregated or was associated with larger molecules which gave it an apparent molecular mass > 30,000 daltons. Purification of the SR from the material retained by the PM 30 membrane and its separation from contaminating carbohydrate and protein were achieved through the steps described in the purification scheme.

From the protein profiles of the SR extracts during various steps of the purification process (Fig. 1), the preparation of SR shown in stage 5 was considered pure on the basis of the single protein-staining band obtained by disc gel eletrophoresis. In addition, a single ninhydrin-reactive spot was obtained by TLC. This, along with the absence of reactive spots after spraying the TLC plates with *p*-anisidine HCl, indicated that no carbohydrates were present. Approximately 50 mg of pure SR was obtained from every 5-L culture.

Specific antibodies to SR were obtained from each rabbit, and although titers varied at each bleeding, 1:1,024 was obtained by the microprecipitin test, with SR purified from strain 5D442. The specificity of the antibody for SR was established from studies in which positive microprecipitin tests were obtained when the antibody was reacted with cultures of SR-producing strains of *P. syringae* (B634 and $B15^{+}$). Negative results were obtained when the antibody was reacted with cultures of strains of *P. syringae* unable to produce SR (eg, $B15^{-}$) and *P. mors-prunorum*. There appeared to be no cross reaction between FA and bacterial cell walls of *P. syringae*. No reactions were obtained when SR-containing extracts were mixed with serum from noninjected rabbits.

FA-SR complex. In a longitudinal section of a control leaf showing a leaf vein and several stomata (Fig. 2), only red fluorescence due to chlorophyll was obtained. The yellow-green fluorescence representing the FA-SR complex was visible in the SR-treated leaf (Fig. 3) and could be seen both in the vein through which the toxin was transported, and in neighboring cells in the vicinity of the stomata. External symptoms of necrosis were visible on leaves exposed to the toxin for 72 hr.

Paraffin cross sections of peach stems that had been immersed in toxin solution (Fig. 4,5) showed bright yellow-green fluorescence of the FA-SR complex, particularly at the periphery of the parenchyma cells (Fig. 5). No bright yellow-green fluorescence was visible in the controls which included peach stems and leaves immersed in sterile water prior to staining with FA, and toxin-infiltrated tissues treated with unlabeled specific antibody, which

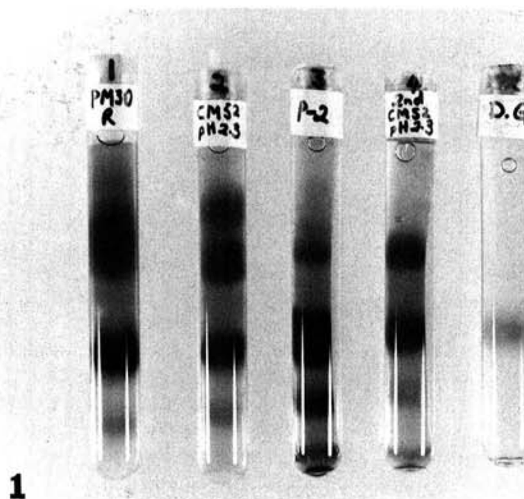

Fig. 1. Protein profiles, from disc gel electrophoresis, of the syringomycin (SR) extracts during various steps of the purification process: gel 1, The material retained by the PM30 filter; gel 2, eluate from carboxymethyl (CM)-cellulose column at pH 2.3; gel 3, eluate fraction from P-2 column exhibiting SR activity; gel 4, eluate from second CM-cellulose column at pH 2.3; and gel 5, after separation by disc gel electrophoresis and eluted from a third CM-cellulose column at pH 2.3.

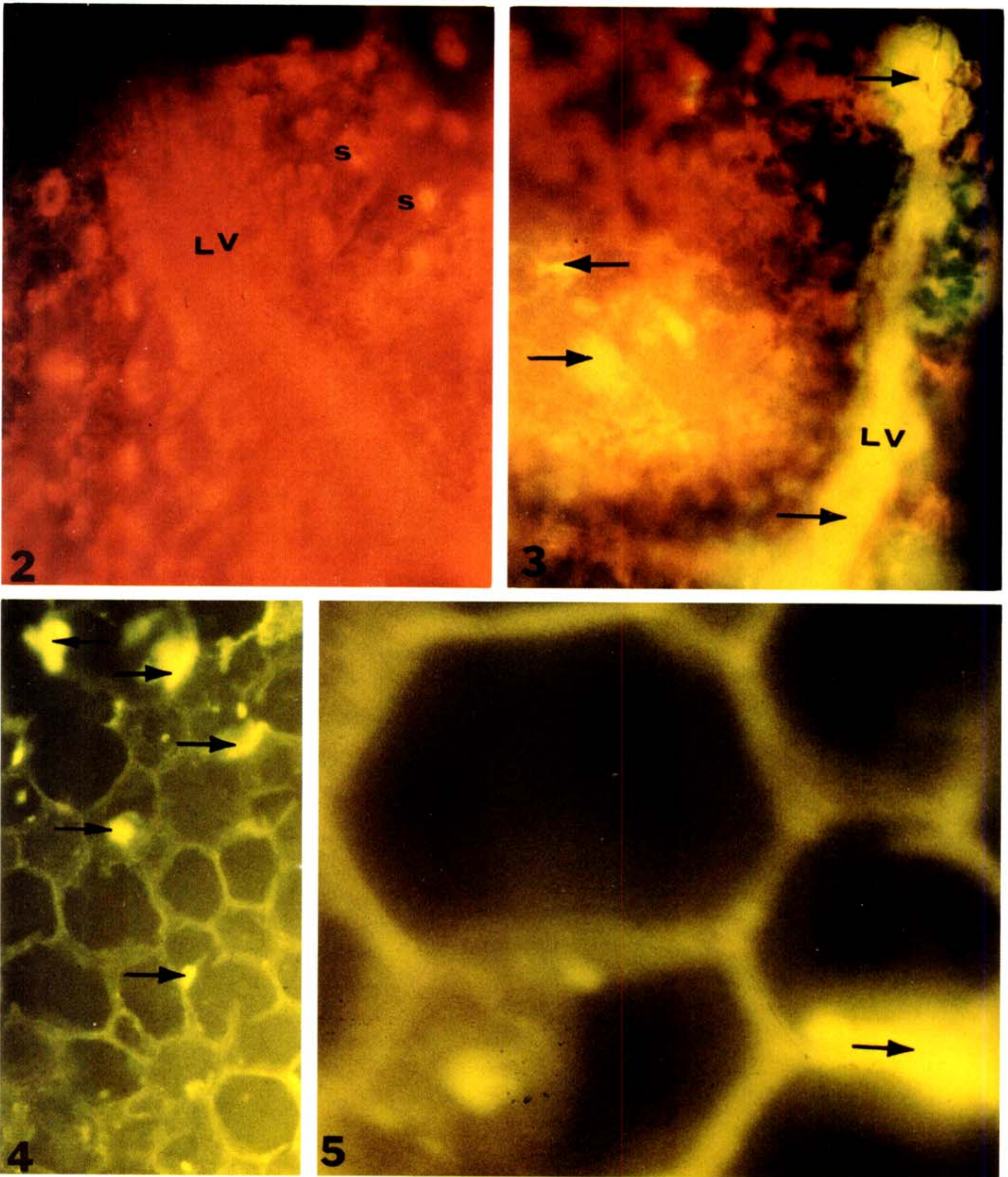

Fig. 2–5. Specific detection of syringomycin (SR) in control and *Pseudomonas syringae*-infected or SR-treated peach leaf and stem sections by a fluorescent antibody (FA) technique. The photo-micrographs are of: **2)** A longitudinal section of a fresh noninfected control leaf showing a leaf vein (LV) and stomata (S). Red fluorescence is due to chlorophyll (× 120). **3)** Longitudinal section of a fresh SR-treated leaf. Yellow-green fluorescence representing the fluorescent antibody-syringomycin (FA-SR) complex is visible in a LV and neighboring cells (→) (× 120). **4)** Paraffin-embedded cross section from the stem of a *P. syringae*-infected peach seedling. Yellow-green fluorescence due to the FA-SR complex is visible at the periphery of the cells (→) (× 120). **5)** Paraffin-embedded cross section from the stem of an infected peach seedling showing pith-parenchyma cells. Yellow-green fluorescence due to the FA-SR complex is visible at the periphery of the cell (→) (× 480).

inhibited subsequent staining with the fluorescent labeled specific antibody. We also have demonstrated that tissues infiltrated with the toxin and reacted with normal sera subsequently will stain with the fluorescent specific antibody.

Positive microprecipitin tests were obtained with extracts from infected field samples, showing that the antibody produced by SR from strain 5D442 can be used to detect SR produced by different pathogenic strains of *P. syringae* under field conditions. FA-stained sections of infected field samples also developed the characteristic yellow-green complex in parenchyma, tracheid, and phloem cells. The specificity of the reaction confirms that SR from pathogenic strains of *P. syringae* is found in infected tissues, as suggested by Sinden et al (14).

DISCUSSION

We used the FA technique successfully for detection of SR in fresh, frozen, or paraffin embedded sections of both infected and toxin-treated plant tissues. Fluorescence observed around the periphery of the cell supports the theory that SR attacks the membrane. We observed more fluorescence in fresh and frozen sections than in paraffin sections. Rough treatment of the sections may have removed some SR which was not attached to plant tissues, or denatured it so that it would no longer react with the FA. We observed fluorescence in phloem, xylem, cambium, and pith-parenchyma cells.

Although the potential of the FA technique has been realized in the field of medicine, its practice has not been as widespread in other biological fields, including the detection of bacteria in plant tissues. In 1964, Paton (10) was able to detect FA-stained *P. syringae* in the matrix of plant tissue with the fluorescence microscope. Backman and DeVay (1) also prepared antibodies to whole cells of *P. syringae*. However, in our research SR as such was purified and used as the antigen for production of the antibody. This is the first report in which the FA technique has been used for detection of bacterial toxin in infected or toxin-treated plant tissues. This increases the specificity of the reaction and provides a diagnostic tool for detecting the toxin in plant tissues. The technique should be valuable in future studies of the role of SR in the bacterial canker disease of peach and other infections.

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