Techniques

A Specific Serological Staining Procedure for *Verticillium dahliae* in Cotton Root Tissue

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ABSTRACT

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Hyphae of *Verticillium dahliae* were detected in cotton root tissue with an indirect enzyme-linked immunoassay. A soluble protein extract of *V. dahliae* was used to prepare a specific rabbit antiserum. The reaction of this rabbit antibody to the hyphae of *V. dahliae* was detected with an alkaline phosphatase antirabbit IgG conjugant that hydrolyzed the substrate, napthol-AS-phosphate, to a product that reacted with a diazonium salt, yielding a colored precipitate outlining the fungal hyphae. The hyphae were readily observed on and in the root cortex of the host using a dissecting microscope. This technique allows for rapid determination of the presence and location of *V. dahliae* in the root systems and may be applicable to other fungal species for which a specific antiserum can be produced.

Additional key words: ELISA, fungi-specific staining, immunohistochemistry.

Histological studies of root-infecting fungi in roots grown in field soils are difficult because of the presence of diverse fungal colonies. Hyphae of most fungi appear similar when stained with conventional methods, making it difficult to identify individual species. A method by which specific fungi, e.g., *Verticillium dahliae* Kleb., could be identified on roots would permit their direct observation and facilitate ecological studies.

The inherent specificity of antibodies suggest that an immunochemical approach to the development of a selective staining procedure could be successful. Fluorescent antibody techniques have been used by previous researchers in attempts to distinguish particular fungi growing in host tissue and on other media (6,8,10,11,14,17,20). Problems inherent with these types of procedures have been a lack of specificity of the antisera, autofluorescence of tissue, and the requirement of special fluorescence detection equipment. Enzyme-linked immunosorbent assays (ELISA) have found widespread application in plant pathology; in virology, these assays are routinely used for detection and identification purposes (7,13). Their applicability to the detection of fungal pathogens in host tissues has also been reported (2,18,19). Further, histochemical techniques for localization of phosphatases, peroxidases, and other enzymes in both animal and plant tissues are known (12,16). Thus, the combination of immunochemical and histochemical techniques may be feasible for the development of a staining procedure capable of locating

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hyphae of specific fungi in host tissues.

A number of workers (5,6,8,11,14,25) have reported on the production of antisera against fungal pathogens and on the problem of obtaining high specificity. Antisera produced against *V. dahliae* using whole cells, cell walls, soluble homogenates, or conidia as antigenic sources have resulted in various degrees of specificity (5,8,25). Nachmias et al reported the production of an antiserum specific to strains of *V. dahliae* pathogenic to potatoes, using extracellular protein-lipopolysaccharide complexes as an antigenic source (19).

This paper describes a Verticillium-specific antiserum, produced with a soluble protein preparation isolated from the fungus, as well as the development of an immunoenzymatic staining procedure for visualization of fungal hyphae in plant tissue through an insoluble enzyme product.

MATERIALS AND METHODS

Antigen preparation. *V. dahliae* was isolated from infected cotton (*Gossypium hirsutum* L.) petioles on cellophane extract-pectate medium (Huisman, *unpublished*). The fungus was obtained in pure culture on potato-dextrose agar (PDA) and maintained by transfer of hyphal tips. Flasks containing 500 ml of Czapek-Dox broth amended with 0.5 g of yeast extract were inoculated with three 1-cm plugs of the fungus cut from the PDA plates with a cork borer. The flasks were incubated at room temperature on a rotary shaker for 2 wk. After the incubation period, the fungal tissue was separated from the growth media by low-speed centrifugation. The tissue was washed three times in several volumes of phosphate-buffered saline, pH 7.3 (PBS: 0.14 M NaCl, 0.0025 M $NaH_2PO_4 \cdot 2H_2O$, 0.0075 M Na_2HPO_4), then separated from the wash solution by low-speed centrifugation. The tissue was then ground in a mortar and pestle with liquid nitrogen and glass microbeads (Thomas Scientific). One volume of PBS was added to the ground fungal material and the suspension was sonicated for 15 min in an ice bath with a Model w350 Sonicator (Heat Systems–Ultrasonics Inc., Plainview, NY) at maximum setting. The suspension was centrifuged at 12,000 *g* for 15 min at 4 C. The supernatant was collected and centrifuged at 80,000 *g* at 4 C for 2 hr. One volume of saturated $(NH_4)_2SO_4$ was added to the supernatant, and proteins were precipitated overnight in an ice bath. Proteins were pelleted by centrifugation at 12,000 *g* for 15 min at 4 C, and resuspended in 4 ml of glycerol-PBS (1:1). Sodium azide was added to the protein suspensions to a final concentration of 0.01% to inhibit microbial activity. The protein suspension was stored at −10 C until needed. Protein concentration was determined with the method of Bradford (4) using the Bio-Rad protein assay (Bio-Rad Laboratories).

Antiserum preparation. For each injection, 1 ml of the soluble protein suspension (0.5 mg of protein) was dialyzed against three changes (6 hr each) of sterile PBS (500 ml). After dialysis, an emulsion consisting of 1 ml each of Freund's complete adjuvant (Difco Laboratories) and the protein suspension was prepared. The material was injected intramuscularly into the thigh of a female Dutch belted rabbit on days 1, 9, 26, and 53. Blood was collected from the marginal ear vein at weekly intervals beginning 2 wk after the final injection. The blood was allowed to clot for 1 hr at room temperature and the serum was separated by centrifugation at 12,000 *g* at room temperature. Serum was diluted with one volume of glycerol and sodium azide (0.01%) was added before storage at −10 C. The serum used in this study was collected on day 82.

Testing of serum. Serum titers were determined by indirect ELISA as described by Lommel et al (13). All ELISA tests were performed on the PR-50 EIA automatic analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH) by using polystyrene cuvette plates (Gilford) containing 50 wells. Cuvette plates were coated with soluble protein suspension diluted (1:5) with the carbonate coating buffer pH 9.6 (0.05 M Na_2CO_3). Day 0 (normal serum) and day 82 sera were diluted (1:10 to 1:6,250) with the indirect ELISA buffer (0.01 M PBS, pH 7.4, 0.05% Tween-20, 2% polyvinylpyrrolidone-10, and 0.5% bovine serum albumin). Goat antirabbit IgG conjugated with alkaline phosphatase (Miles Laboratories, Inc., Elkhart, IN) was diluted (1:5,000) with the indirect ELISA buffer.

Indirect ELISA was also used to determine serum specificity. The ELISA was the same as described above except for the preparation of the coating antigen. Tissue homogenates of conidia and hyphae of *V. dahliae* and of hyphae of a *Fusarium oxysporum* Schlecht isolate obtained from cotton roots and cotton root tissue were tested along with the soluble protein fraction from *V. dahliae*. Conidia of *V. dahliae* were separated from hyphae obtained from Czapek–Dox cultures with a 38-μm sieve. *F. oxysporum* was grown using the same culture procedure as for *V. dahliae*. Cotton root tissue was obtained from plants growing in pasteurized artificial soil in the greenhouse. All tissue preparations were washed as described above, then homogenized in an ice bath for 5 min with a Brinkman polytron after addition of one volume of PBS. Preparations were then diluted (1:10 to 1:40,000) with the carbonate coating buffer and were used in the indirect ELISA assay.

Serum specificity also was checked against tissue homogenates of 10 fungal species. The fungi included *V. dahliae*, *V. albo-atrum* Reinke & Bert., *V. tricorpus* Isaac, *V. nigrescens* Pethybr, *V. lateritium* Rabenh, *V. fungicola* Hassebr., *V. pasoliotia* Tresuhow, *F. oxysporum, F. oxysporum* f. sp. *vasinfectum* (Atk.) Sny. & Hans., and *Gliocladium* sp. Corda. The fungal homogenates were prepared as above, diluted 1:25 with coating buffer, and used in the indirect ELISA assay.

Serum absorption. The antiserum was absorbed (13) with soluble proteins extracted from two isolates of *F. oxysporum* obtained from cotton roots but nonpathogenic on cotton. Two milliliters of antiserum were incubated with 2 mg of each of the protein suspensions for 4 hr at 37 C then overnight at 4 C. The suspension was centrifuged at 12,000 *g* for 15 min, and the supernatant retained. The immunoglobulin fraction was purified from the absorbed serum in 0.02 M sodium phosphate buffer (pH 7.3) using protein A-Sepharose CL-4B affinity chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) (3). The immunoglobulin fraction was eluted with 0.1 M glycine (pH 3.0), precipitated with one volume of saturated $(NH_4)_2SO_4$, pelleted, and resuspended in 2 ml of glycerol-PBS (1:1). The absorbed immunoglobulin fraction (1:250 dilution) was compared with whole serum (1:1,000 dilution) for activity against the various proteins (25 μg per cuvette) by indirect ELISA.

Immunoenzymatic staining procedure. The immunoenzymatic staining procedure for particulate materials was based on a modification of the indirect ELISA (13) and the leukocyte (16) staining techniques. Volumes used were variable and were adjusted to always give complete submergence of the particulate sample. Sera and antirabbit IgG were diluted into indirect ELISA buffer. The materials were exposed to rabbit antiserum (1:100, 1 hr, 37 C) and then rinsed three times (5 min each) in washing solution (0.01 M PBS, pH 7.4, 0.05% Tween-20, 0.01% NaN_3). Next the materials were exposed to goat antirabbit IgG alkaline phosphatase conjugate (1:1,000, 1 hr, 37 C) and again rinsed three times in washing solution. Finally the materials were incubated in substrate solution (1–2 hr, 20 C) in the dark. The substrate solution was prepared by dissolving 2.5 g of napthol-AS-phosphate (Sigma, Chemical Co., St. Louis, MO) in 2.5 ml of *N,N*-dimethylformamide, which was added to 500 ml of 0.2 M Tris buffer (pH 9.1) and stored at 4 C in the dark until needed. Just before use, a diazo salt, Fast Blue BB salt (Sigma Chemical Co.), was added to the substrate solution (1 mg/ml). Controls were run by substituting normal (day 0) serum (1:100) for the antiserum in the above procedure.

Western blots. For western blot analysis (23,24), electrophoresis of the soluble protein extracts from *V. dahliae* and the two isolates of *F. oxysporum* was performed in a 12.5% polyacrylamide gel for 3 hr with a constant potential of 150V as described by Reynolds et al (22). After electrophoresis, the gel was incubated (1 hr, 20 C) in 200 ml of protein transfer buffer, pH 7.4, (50 mM Tris, 50 mM NaCl, 1.8 mM Na_2 EDTA, 0.01 mM dithiothreitol) containing urea (4 M), followed by incubation (3 hr, 20 C) in 300 ml of fresh protein transfer buffer with urea. The proteins were blotted by capillary transfer onto nitrocellulose sheets (Bio-Rad Laboratories)

saturated with protein transfer buffer (no urea) for 36 hr at 37 C. Blotted nitrocellulose sheets were incubated (0.5 hr, 37 C) in blocking buffer, pH 7.5, (0.05 M Tris, 0.15 M NaCl, 0.15 M Na_2 EDTA, 0.25% gelatin, 0.05% Tergitol NP40, and 2% horse serum) for 30 min at 37 C. The sheets were then subjected to the immunoenzymatic staining procedure described earlier with the following changes: antiserum, absorbed immunoglobulin, and antirabbit IgG were diluted into the above blocking buffer, incubation times were 2 hr, and washing solution consisted of PBS containing Tergitol NP-40 (0.05%). After staining, the sheets were rinsed in distilled water and allowed to dry.

Staining fungal colonies. Fungi tested included those isolated from cotton roots, along with those used in the serum specificity test. The fungi were grown for 1–2 wk on 5-cm polypropylene filters (Gelman Sciences, Inc., Montreal, Canada) lying on Czapek–Dox agar. The filters, with the fungal hyphal masses, were removed from the agar and washed in the ELISA washing solution (0.15 M NaCl, 0.1% Tween-20, 0.01% NaN_3), followed by incubation in indirect ELISA buffer for at least 5 min to prevent the immunoglobulins from binding nonspecifically to the plastic filters. The filters were cut in half and stained with the immunoenzymatic procedure: One half filter was exposed to the control staining procedure. The filters were then rinsed in distilled water and allowed to dry.

Staining colonies in root tissue. The roots were obtained from cotton plants from a field with a high population of *V. dahliae* and from greenhouse plants grown in the same soil. The roots (1–5-cm segments) were washed free of all soil and then stained as described for the polypropylene filters above. Controls were conducted using normal (day 0) serum. After staining, roots were cleared in 0.5% NaClO for 30–60 min.

Bioassay of *V. dahliae* colonies. The number of colonies of *V. dahliae* on roots have been determined (Huisman, *unpublished*). Roots from the same sources as above were plated on the cellophane extract-pectate medium, incubated at 22 C, and colonies were counted after 2 wk.

RESULTS

Serum sensitivity and specificity. An effective antiserum was produced against the protein preparations of *V. dahliae*. Dilutions of antiserum to 1:1,250 gave $A_{405} > 1$ in indirect ELISA (Fig. 1); a 1:1,000 dilution was used in subsequent assays. The serum reacted similarly with equivalent dilutions of homogenized conidia and hyphae of *V. dahliae* as with the soluble protein fraction (Fig. 2). Reactivity with homogenates of hyphae of *F. oxysporum* and cotton root tissue was minimal for all tissue dilutions (Fig. 2). In tests against homogenates of 10 fungal isolates, some cross-reactivity was detected with *V. albo-atrum*, but only minimal cross-reactions were observed with other fungi (Fig. 3).

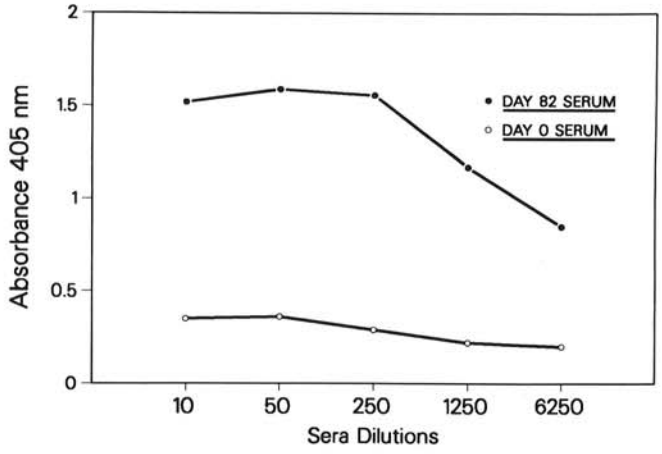

Fig. 1. Reactivity of day 82 serum with the soluble protein fraction of *Verticillium dahliae* in indirect ELISA with a 1:5 dilution of the protein solution. Each point is the mean of four replications with a maximum standard deviation of 0.07.

Both the serum and the IgG fraction from the absorbed serum were tested by western blot analysis for reactivity with soluble proteins (Fig. 4). Whole serum was reactive against a wide range of soluble proteins from *V. dahliae* and with a few high molecular weight soluble proteins obtained from the sclerotial isolate of *F. oxysporum*. The heterologous reaction was eliminated after absorption. The improvement in specificity was slight, however, as differences were not apparent by the indirect ELISA test and a fourfold decrease in serum sensitivity toward *V. dahliae* was observed (Fig. 5).

Staining of cultured fungal colonies. Mycelium of *V. dahliae* grown on polypropylene filters stained dark blue, with the blue color limited to the colony margin owing to the dark pigmentation of microsclerotia in the colony center. If pigmentation was cleared by incubating filters in 0.5% NaClO, the stain was evident throughout the colony. The NaClO did not bleach the stain. Three isolates of *V. dahliae*, two from cotton and one from tomato, stained equally well. *V. albo-atrum* also was stained by the assay. With control (day 0) serum, no staining of the hyphae of *V. dahliae* or *V. albo-atrum* occurred. Three fungi, *V. lateritium*, *Penicillium miczynski*, and *Gliocladium roseum* were lightly stained when either the day 0 or day 82 serum was used and the staining intensified with extended incubation times. The remaining fungi tested (*V. tricorpus*, *V. lecanii*, *F. oxysporum* sclerotial and

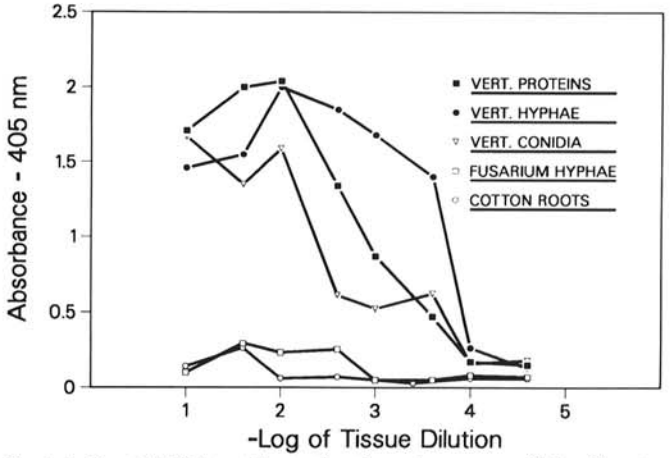

Fig. 2. Indirect ELISA test illustrating the antiserum specificity. Cuvettes were coated with homogenized tissue as indicated. Day 82 antiserum added at 1:1,000 dilution. The value for the noncoated control was 0.09 (S.D. 0.05). Each point is the mean of three replications with a maximum standard deviation of 0.17.

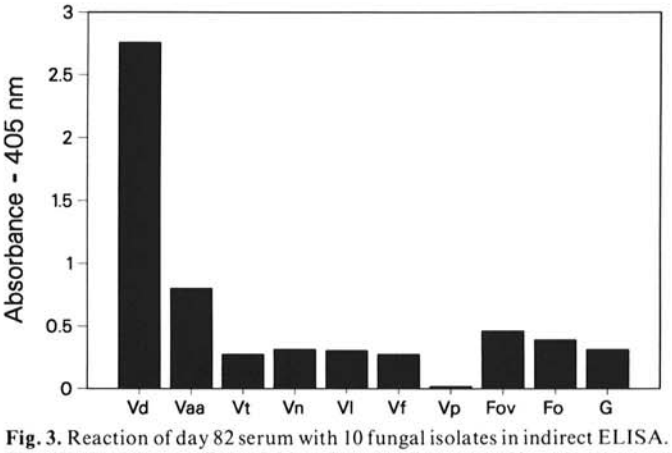

Fig. 3. Reaction of day 82 serum with 10 fungal isolates in indirect ELISA. Fungi included *Verticillium dahliae* (Vd), *V. albo-atrum* (Vaa), *V. tricorpus* (Vt), *V. nigrescens* (Vn), *V. laterution* (Vl), *V. fungicola* (Vf), *V. pasoliotia* (Vp), *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), *F. oxysporum* (Fo), and *Gliocladium* sp. (G). Cuvettes were coated with fungal homogenates diluted 1:25 and serum was diluted 1:1,000. The value for the noncoated control was 0.14 (S.D. 0.08). Each value is the mean of four replications with a maximum standard deviation of 0.12.

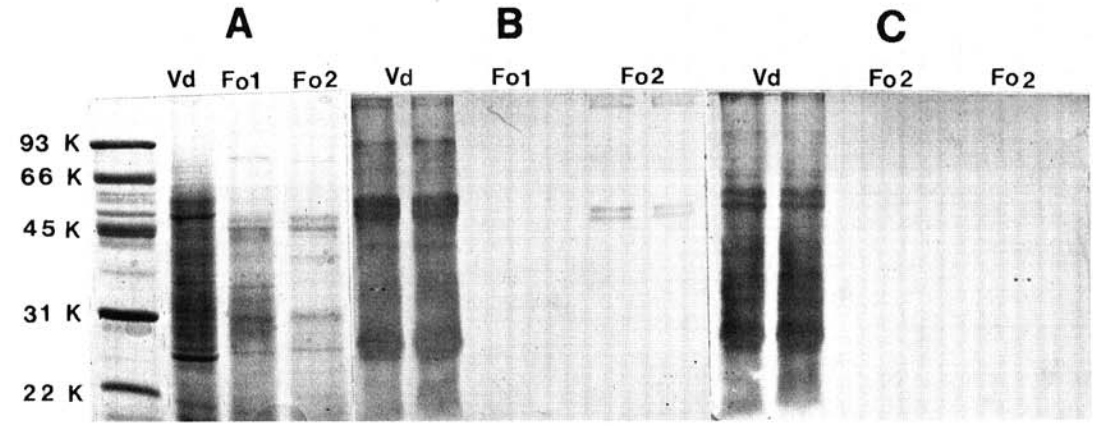

Fig. 4. Western blot analysis of whole serum (1:500) and absorbed purified IgG (1:100). **A,** Electrophoretic protein bands of *Verticillium dahliae* (Vd), sporodochial isolate of *Fusarium oxysporum* (Fo1), and sclerotial isolate of *F. oxysporum* (Fo2), on a polyacrylamide gel (12.5%) and stained with Coomassie blue. **B,** Proteins blotted on nitrocellulose and stained with the immunohistochemical procedure using whole serum. The homologous as well as a slight heterologous reaction can be observed. **C,** Proteins blotted on nitrocellulose and stained using adsorbed purified IgG.

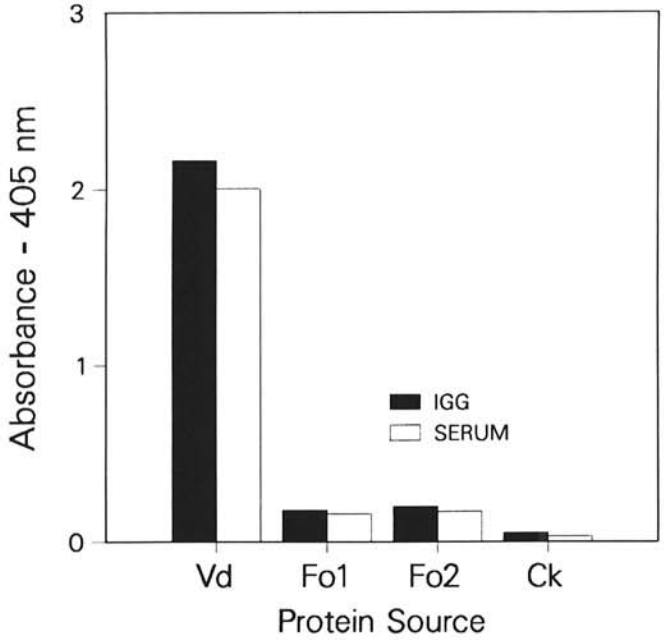

Fig. 5. Comparison of specificity of whole antiserum (1:1,000) and absorbed purified IgG (1:250) by indirect ELISA. Cuvettes were coated with buffer (CK) or 25 g of protein extracted from *Verticillium dahliae* (Vd), sporodochial isolate of *Fusarium oxysporum* (Fo1), or sclerotial isolate of *F. oxysporum* (Fo2). Each value is the mean of four replications with a maximum standard deviation of 0.09.

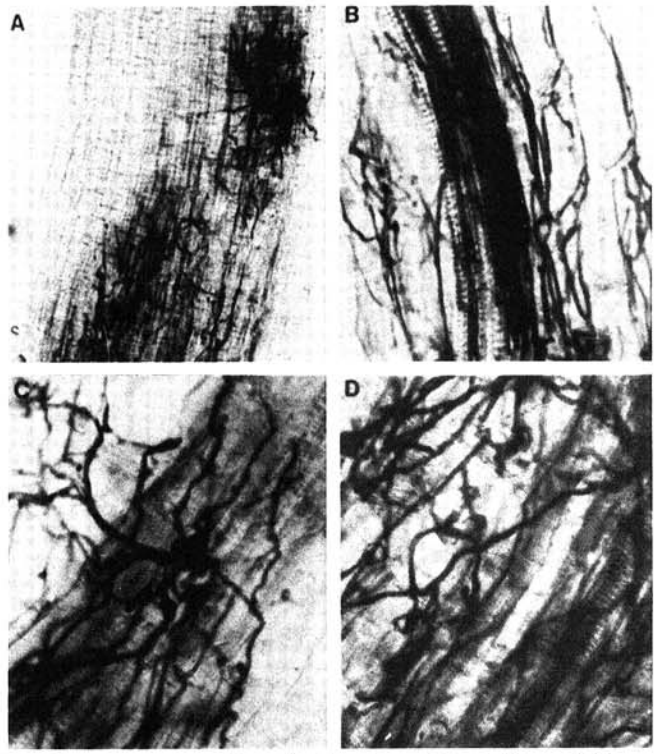

Fig. 6. Immunoenzymaticly stained hyphae of *Verticillium dahliae* on cotton roots. **A,** Two stained colonies of *V. dahliae* in close proximity on a 0.3-mm-diameter root. **B,** Stained hyphae of *V. dahliae* parallel to the stele. **C,** Stained hyphae of *V. dahliae* that had grown away from the initial colonization point on a root surface. **D,** Stained hyphae of *V. dahliae* on cortical root tissue.

sporodochial types, *F. o.* f. sp. *vasinfectum*, *Aspergillus mellus*, *Trichoderma harzianum*, *Cladosporium cladosporioids*, *Stemphyllium botryosum*, and a *Gliocladium* sp.) failed to stain with either the antiserum or the control (0 days) serum.

Observation of colonies of *V. dahliae* in root tissue. Stained hyphae of *V. dahliae* in root tissue were clearly defined and colonies could be easily observed with a dissecting microscope (Fig. 6). Hyphae could be seen both on the root surface and in the cortex of roots. Small amorphous areas of staining were occasionally visible, especially near the root apices. These stained areas were visible with both the control (day 0) serum and the day 82 serum. These areas, which were on the surface of the root tissue and did not resemble fungal hyphae, were thought to be bacterial colonies. Bacteria are known to have high endogenous alkaline Phosphatase activity (16). Hyphal staining was not observed in roots with control (day 0) serum.

When roots were removed from the staining solution, they were stained varying shades of red. This nonspecific staining occurred throughout the root tissue but was most intense in the stele region. Omission of the antiserum and antirabbit IgG incubation steps of the assay did not affect the staining. The tissue was cleared of this stain by incubation in 0.5% NaClO for 30–60 min without affecting the specifically stained hyphae. If the NaClO treatment was performed before the substrate step, no stained hyphae were observed.

Several other diazo dyes were also tested. These included Fast Yellow GC salt, Fast Red TR salt, and Fast Violet B salt. All three of these salts stained the fungal hyphae in the root tissue, but to different degrees. Fast Yellow stained the hyphae lightly, making it difficult to detect in the root tissue. Hyphae stained well with Fast Red, but the red stain in tissue caused by phenolic compounds made hyphae difficult to see before NaClO treatment. Fast Violet

was an adequate stain, but the color was not as dark as for Fast Blue BB salt.

Use of antirabbit IgG peroxidase conjugates in place of alkaline phosphatase conjugates proved unfeasible. Because of high levels of endogenous peroxidase activity in the roots, the entire tissue was turned dark brown by the peroxidase substrate.

The immunohistochemical staining assay and the bioassay gave similar colony frequencies of *V. dahliae* from plant roots (Table 1). In four trials, the number of stained colonies and the number of colonies of *V. dahliae* detected in the bioassay were closely correlated (Table 1). In three of the four experiments, colony frequency was slightly higher for the plated roots.

TABLE 1. Frequency of *Verticillium dahliae* in cotton roots as determined by staining and isolations from root tissue

Experiment[a]	Source	Colonies/100 cm root tissue (S.D.)	
		Staining	Root isolation
1	Field	26.0 (2.3)	16.4 (6.0)
2	Greenhouse	15.0 (3.4)	18.0 (10.1)
3	Field	13.8 (4.7)	26.5 (6.5)
4	Greenhouse	16.8 (7.4)	27.0 (5.8)

[a]Values for experiments 1–3 represent the mean of five replications, those for experiment 4 of four replications.

DISCUSSION

The specificity of the serum produced against *V. dahliae* was good. The day 82 serum at 1:1,000 dilution reacted with *V. dahliae* and to a lesser extent with *V. albo-atrum*. The reaction of this serum to the other fungi tested was minimal. Previous workers have reported various degrees of specificity of antisera produced against fungal antigens (1,2,5,6,8,9,14,15,17,19–21,25). Many of these antisera were prepared using tissue preparations containing fungal cell walls or cell wall materials. The sera made against antigen preparations of these types could contain antibodies made against chitin or other cell wall polysaccharides common to most fungi. Sera of this type, therefore, may not be as specific as sera made with the technique used in this study, where only soluble fungal proteins were injected.

The serum was observed to react with all tissues of the fungus that were tested. The day 82 serum at 1:1,000 dilution reacted equally well with homogenated hyphae and conidia of *V. dahliae* as with the soluble protein fraction; also, results of the western blot analysis indicate the serum reacted with a wide spectrum of the soluble protein fractions. These results indicate the serum would be likely to detect the fungus under a variety of conditions.

The western blot analysis showed the presence of common antigens between *V. dahliae* and the sclerotial type of *F. oxysporum*. Common antigens between *V. dahliae* and *F. oxysporum* f. sp. *vasinfectum* have been reported previously by Charudattan and DeVay (5). Absorption of the serum with the soluble protein extracts from both *F. oxysporum* isolates inhibited this heterologous reaction. Reaction of the serum with *F. oxysporum* was not evident in the staining of intact hyphae on polypropylene filters at the 1:100 serum dilution routinely used. From these results it was concluded that there was no benefit achieved from absorption of the serum.

The results of staining the fungal colonies on the polypropylene filters suggest that the assay was specific to *V. dahliae* and *V. albo-atrum*. *V. albo-atrum* is not found in the central valley of California, and thus poses no interference problem with the detection of *V. dahliae* in roots from plants grown in this area; however, the usefulness of this technique may be limited for the study of roots from areas where both fungi occur. *F. oxysporum* was the most abundant fungal species found on the roots of cotton grown in the experimental plot at the West Side Field Station (Gerik and Huisman, *unpublished*). Even though a weak reaction occurred with homogenized hyphae of *F. oxysporum*, no reaction was detected with the hyphal staining assays. Cultured fungal mats of three other fungal species, *Penicillium miczynski*, *Gliocladium roseum*, and *V. lateritium*, were lightly stained by the assay. Because the staining occurred both with day 82 and the control serum, the staining may have been caused by endogenous alkaline phosphatase activity in these organisms. However, these fungi apparently do not stain sufficiently in root tissue to be confused with *V. dahliae*. Further, based on the frequencies that these fungi occur on roots (Gerik and Huisman, *unpublished*), a total frequency of over 50 colonies per 100 cm of root length would have been expected if stained colonies of these fungi were easily mistaken for *V. dahliae*. Instead, the observed frequency closely correlated with that of *V. dahliae* (Table 1).

The frequency of colonies of *V. dahliae* determined by the staining procedure and the bioassay of root tissue correlated well (Table 1). The colony density observed in both assays was similar; however, the number of colonies determined by staining was usually lower than that determined by the root plating assay. This variance could result from inherent problems in distinguishing immediately adjacent colonies in the two assays. In the staining assay there may have been a slight tendency to undercount the number of colonies, since colonies often did not have clear boundaries, and two closely associated colonies may have been mistaken for one. With the bioassay there might be a slight tendency to overestimate the number of colonies. Colony counts are based on apparently individual colonies growing into the medium from root tissue. One large colony, growing from the root at two points, would appear to be two colonies. Thus, care must be taken when using both assays in a quantitative manner.

This staining assay offers many advantages over fluorescent antibody techniques. The staining assay does not require the use of a fluorescence microscope, and specimens can be viewed with a dissecting microscope. The increased field of view of a dissecting microscope allows for the screening of more specimens in less time than is required with fluorescent techniques. It may be possible to sequentially stain different fungi in the same root tissue by using different enzymes linked to the antibodies and/or different diazo dyes.

The results of this study suggest that a relatively specific fungal antiserum can be produced using a soluble protein extract. This is supported by ELISA data, western blot analysis, in vitro fungal colony staining, and close correlation between colony density as determined by staining and the bioassay. The antiserum produced in this study appears to be more specific than that of previous workers who used whole cell homogenates or cell wall preparations as immunogens (5,8,25). A specific fungal antiserum employed with an enzymatic staining technique as developed in this study should prove to be a valuable tool in the study of root-infecting fungi, for it permits their direct, selective observation in host tissue.

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