Fluorescent Antibody Studies with *Eutypa armeniacae*

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ABSTRACT

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Antisera were made to both a whole cell and cell wall preparation of *Eutypa armeniacae*. Rhodamine isothiocyanate (RITC) conjugated antisera were tested for reactivity with various fungi on glass slides. Both antisera showed low specificity, but specificity was improved by cross-adsorption of the RITC-conjugated cell wall antiserum with *Phomopsis viticola*. Woody cross sections from Concord grapevines inoculated with *E. armeniacae* and also inhabited by various other fungi were stained directly with the conjugated anti-Eutypa rabbit serum. In an indirect staining procedure, sections were treated with anti-Eutypa rabbit serum then stained with RITC-labeled goat anti-rabbit gamma globulin. Both procedures specifically stained hyphae in wood sections. Hyphae stained indirectly in woody vine sections showed a much brighter fluorescence than analogous hyphae stained by the direct method. Fungi of some species that reacted strongly with the direct method on glass slides did not always react when stained indirectly in woody vine sections in which they were growing.

In histological studies of wound pathogens in which hyphae are stained in woody sections, saprophytes that would also normally invade the wounds must be excluded. When saprophytes are excluded, however, growth of the pathogen may be quite different. Exclusion of saprophytes is also difficult or impossible when studying naturally infected tissue.

The fluorescent antibody (FA) technique is a possible solution to these problems. With an FA stain specific to the fungus of interest, it can be distinguished from other fungi also present in the tissue. This would eliminate the need for sterility of the environment and allow effects of competing fungi to be evaluated.

Eutypa armeniacae, the cause of Eutypa dieback of grapevine, is a wound pathogen with a long latency period (12). Previous serological work with this organism (8,15) indicated that a specific antiserum can be produced. We describe the production of antisera to *E. armeniacae* and a technique developed to selectively stain the organism in woody sections by using these antisera.

MATERIALS AND METHODS

Antigen production. The isolate of *Eutypa armeniacae* used for antigen production was obtained from infected grape (*Vitis labrusca* L. 'Concord') wood from a pruning wound that had been inoculated with ascospores of *E. armeniacae* (from a Michigan source) about 1 yr earlier. Mycelium was grown at room temperature in potato-dextrose broth (Difco Laboratories, Detroit, MI 48201) on a rotary shaker at 80 rpm for about 1 wk. The mycelium was collected on Whatman No. 1 filter paper in a Büchner funnel, washed extensively with distilled water, frozen with liquid nitrogen, and ground to a fine powder with a mortar and pestle. Three grams of the mycelium was suspended in 25 ml 0.01 M PBS, pH 7.4 and sonicated in an ice bath for 20 min at 3.5 A with a Branson Sonifier model 5125 (Branson Instruments, Inc., Shelton, CT 06484). Microscopic inspection indicated that the mycelial fragments from this treatment had no more than one cross wall.

Two types of antigen were prepared from this material. The first, hereafter referred to as the whole-cell antigen, was produced by the sonication treatment described above. Protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA 95804), which is based on the method of Bradford (3). Lyophilized bovine gamma globulin was used as a protein standard. Protein was adjusted to 3 mg/ml with 0.01 M PBS, pH 7.4. The second antigen, hereafter referred to as the cell-wall antigen, was obtained by centrifuging the sonicated material at 4,000 rpm for 4 min at 4 C and resuspending the sonicated material in 20 ml of 0.01 M PBS, pH 7.4. This procedure was repeated until the supernatant was clear by visual inspection. Microscopic inspection of the pellet showed no cytoplasmic material within the cell walls of the mycelial fragments. The protein concentration was adjusted to 2 mg/ml as described before.

Immunization and bleeding schedule. Preimmune serum was obtained from two New Zealand white doe rabbits. Each rabbit was then injected subcutaneously with 1.5 ml of either the whole-cell or the cell-wall antigen emulsified with an equal volume of Freund's complete adjuvant (Difco). The rabbits were then injected weekly as above with 1.5 ml of the respective antigens emulsified with an equal volume of Freund's incomplete adjuvant.

When the titer had increased to within the range 1/16 to 1/64, 40 ml of blood was obtained weekly for 5–8 wk. The antiserum was stored at 4 C with a few crystals of chlorobutanol (Sigma Chemical Co., St. Louis, MO 63178) as a preservative.

Determination of titer. The antigen (whole cell or cell wall) to each homologous antiserum was diluted 1:20, 1:40, and 1:50 (v/v) with 0.01 M sodium phosphate, pH 7.2. A 0.2-ml sample of each of these preparations was mixed with an equal volume of antiserum or preimmune serum diluted with normal saline (0.85% NaCl) solution. These were then immersed in a 37 C water bath for 6 hr and refrigerated for up to 2 days. Observations were made by shaking antiserum and control serum in front of a fluorescent light.

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In a positive reaction, the particulate fraction did not homogeneously resuspend when compared with the normal serum.

Preparation of fluorescent antisera. Gamma globulin was purified from the serum by the method of Herbert et al (10); a 40% saturated ammonium sulfate mixture was used. Dialysis against normal saline followed (10). The protein concentration of the purified gamma globulin was adjusted to 10 mg/ml with normal saline. Three milligrams of RITC (United States Biochemical Corporation, Cleveland, OH 44128) was dissolved in 4 ml 0.1 M PBS (pH 8.0) on a magnetic stirrer and added to 10 ml of the gamma globulin solution to which 4 ml 0.15 M PBS (pH 9.0) had been added. The pH was then adjusted to 9.0 with NaOH. This solution was stirred slowly for 3 hr at room temperature.

The free RITC was separated from the RITC conjugated to gamma globulin on a Sephadex G-50-80 (Sigma) column equilibrated and eluted with 0.005 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl. Conjugated gamma globulin was stored in 4-ml aliquots at −20 C.

Cross-adsorption of fluorescent antibody with other fungi. To increase the specificity of the reaction, the anti-cell wall fluorescent antibody preparation was cross-adsorbed with a sonicated cell wall preparation of *Phomopsis viticola* and/or *Epicoccum nigrum* made as with the cell-wall antigen of *E. armeniacae* as described above. Various amounts of the partial cell-wall preparation were added to 4 ml of fluorescent antibody. This mixture was stirred slowly either for 6 hr or overnight and centrifuged at 500 rpm for 10 min to remove the hyphae.

Fluorescent antibody staining of fungi on glass slides. Mycelium of various fungi (Table 1) were grown as described for *E. armeniacae*, and were stored at 4 C until used. Staining was conducted by the method of Schmidt and Bankole (17). A small piece of the mycelium of the test fungus was rinsed extensively with distilled water and smeared on a glass slide. The mycelial smear was air-dried at room temperature, heat-fixed, and flooded with labeled antiserum that had been twofold serially diluted as much as possible while still retaining maximum fluorescence of *E. armeniacae*. Following incubation in a humid chamber at 37 C for 30 min, the slide was dipped in distilled water and then immersed in 1 L of normal saline solution that was stirred for 30 min, the slide was again dipped in distilled water and mounted in FA mounting fluid (Difco). As a control, each test fungus was also "stained" with the elution buffer of the Sephadex column and rinsed with saline as before.

Sectioning procedure. Two-year-old cultivar Concord grapevines were pruned above a node or branch and inoculated on the pruning wound with 500 *E. armeniacae* ascospores in 5 μl of distilled water. In some cases, the pruning wound was also inoculated with 500 macroconidia of an Australian isolate of *Fusarium lateritium* (5). Isolations to identify fungi inhabiting the canes were made from 9 to 13 mo after inoculation by aseptically transferring 10 wood chips from inside the cane near the pruning wound onto potato-dextrose agar (Difco) that had been amended with 100 g of streptomycin sulfate per milliliter. Any fungi that grew out of the wood chips were assumed to have been inhabiting the wood.

Cross sections 40 μm thick near the point of inoculation were made using a sliding microtome. These sections were stained with fluorescent antibody by using the direct method as modified from Malajczuk et al (11) or the indirect method, modified from Warnock (20).

Direct wood staining. Woody vine sections were incubated for either 30 min or 1 hr at 37 C in a humid chamber in the presence of RITC-labeled gamma globulin. Then they were dipped in distilled water and immersed for 90 min in circulating normal saline (pH 7.0) to which acetone had been added to a final concentration of 10% (v/v). The sections were then dipped in distilled water again and mounted in FA mounting fluid.

Indirect wood staining. Serum was diluted 1:50 (v/v) with normal saline. This was the greatest dilution that gave maximum fluorescence of hyphae observed in sections from a stem in which all fungi isolated were *E. armeniacae*. This diluted serum was incubated with the wood section for 25 min in a 37 C humid chamber. The sections were then placed in circulating 0.01 M PBS (pH 7.2) for 20 min at room temperature. After this rinse, the sections were incubated for 30 min in a 37 C humid chamber with goat anti-rabbit RITC (United States Biochemical) diluted 1:100 with 0.02 M PBS, pH 7.3. This was followed by a distilled water dip, then a 2-hr acetone-saline rinse as detailed before.

Observation of material. All fluorescence was observed at ×250 with a Zeiss universal microscope equipped with epifluorescence

TABLE 1. Fluorescence of fungi on slides directly stained with RITC-conjugated anti-*Eutypa armeniacae* gamma globulin[a]

Fungus	Control	Conjugate from whole-cell antigen	Conjugate from cell-wall antigen	Cell-wall conjugate cross-adsorbed with *Phomopsis viticola*
Eutypa armeniacae isolates				
Michigan grape	−[b]	+++[c]	+++	+++
California grape	−	+++	+++	+++
California apricot	−	+++	+++	++[d]
Washington grape	−	+++	+++	+++
Australia apricot	−	+++	+++	+++
Michigan grape ascospores	−	NT[d]	++	NT
Michigan grape scolecospores	−	NT	++	NT
Fungi commonly isolated from grape wood				
Alternaria sp.	−	+++	+/−	NT
Aureobasidium sp. (spores)	−	+++	+++	+++
Cytospora sp.	−	++[e]	+++	++
Epicoccum nigrum	−	+++	+++	+++
Penicillium sp.	−	−	−	NT
Phomopsis sp.[f]	−	+++	+++	+++
Phomopsis viticola	−	+++	+++	+/−
Sphaeropsis sp.	−	+++	+++	+[g]
Other fungi				
Botrytis cinerea	−	++	+	NT
Colletotrichum gloeosporioides	−	+	+	NT
Endothia parasitica	−	+++	+++	+/−
Fomes annosus	−	+++	+++	+
Fusarium lateritium	+/−	+/−	+/−	NT
Gloeosporium amphophalagum	−	+	+	NT
Guignardia bidwellii	−	−	−	NT
Hypoxylon mammatum	−	+++	+++	+
Phytophthora megasperma var. *glycinea*				
(mycelium)	−	−	−	NT
(oospores)	+++	+++	+++	NT
Poria placenta	−	+++	+++	++
Pythium ultimum	++	+++	++	++
Schizophyllum commune	−	+++	+++	++
Schlerotinia sclerotiorum	−	+++	+++	+

[a] See text for staining procedure. Observed at ×250 with a RITC-fluorescence filter set using a Zeiss universal UV microscope. Individual hyphae were rated for fluorescence in area of smear where individual hyphae were discernible. Results represent two replications of each fungus.
[b] − = no hyphal fluorescence.
[c] +++ = hyphae flouresced brightly.
[d] NT = not tested.
[e] ++ = hyphae readily visible but with dull fluorescence.
[f] Tentatively identified as *Phomopsis* sp. No beta spores were found, however.
[g] + = hyphae barely visible.

and an HBO 50W mercury-vapor lamp, a KG1 heat-absorbing filter, a BP 546/7 green interference excitation filter, an FT 580 chromatic beam splitter, and an LP 590 barrier filter.

RESULTS

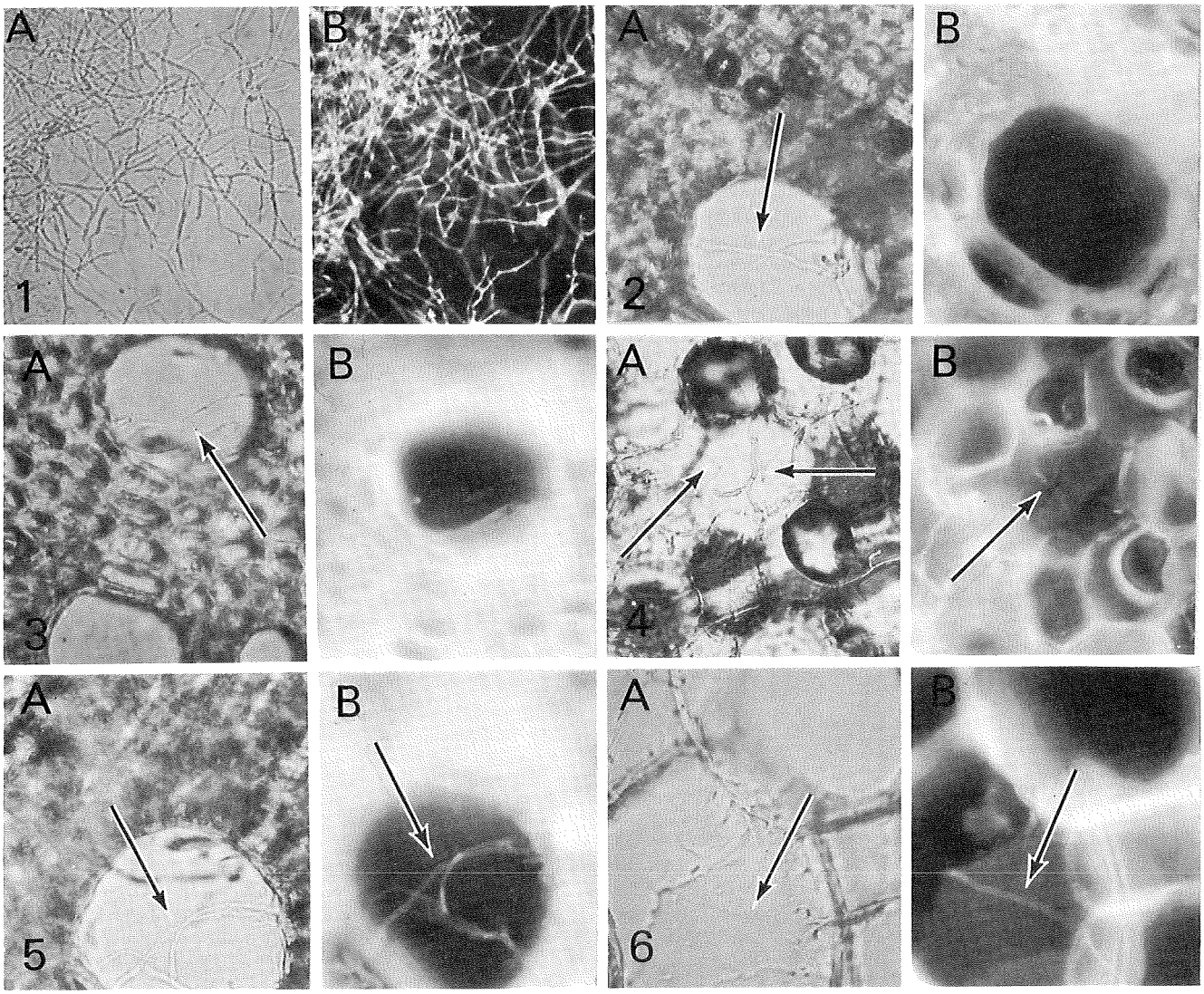
Antiserum titer. The antiserum made to the whole-cell antigen reached a titer of 1/64 four wk after the first injection and increased to 1/128 two weeks later, where it remained with subsequent bleedings. The antiserum made to the cell-wall antigen reached a titer of 1/16 seven weeks after the first injection and increased to 1/64 after 9 wk, where it remained.

Specificity of fluorescent antibody to fungi on glass slides. The reactions on glass slides of various genera and species of fungi to conjugates made with antiserum to the whole-cell antigen and cell-wall antigen as well as the cell-wall antiserum conjugate cross-adsorbed with *P. viticola*, are shown in Table 1. Both the whole-cell and cell-wall antigen antiserum conjugates showed little specificity.

The cross-adsorption procedure described previously was performed two times with *P. viticola* on the same aliquot of the anti-cell-wall conjugate, each time with a 6-hr incubation. The first and second adsorption used 0.12 and 0.15 g, respectively, of the *P. viticola* partial cell-wall preparation. Fluorescent antibody reactivity to *E. armeniacae* was not reduced by this treatment. This cross-adsorption procedure was quite successful, reducing to negligible levels reactivity of half (six of 12) of the heterologous fungi that previously reacted strongly, and moderately reducing the reactivity of another four. This cross-adsorbed FA preparation was only tested on fungi that reacted with either a rating ++ or +++ with the cell-wall conjugate.

Conjugated cell-wall serum was incubated with 0.5 g of a partial cell-wall preparation of *Epicoccum nigrum* for 6 hr. This did not succeed in reducing the reactivity of *E. nigrum* on glass slides. Conjugated cell-wall serum was also incubated overnight with 0.5 g of a partial cell-wall preparation of *E. nigrum* plus 0.5 g of a partial cell-wall preparation of *P. viticola*. Again, this did not reduce the reactivity of *E. nigrum*, although the reactivity of *P. viticola* was eliminated.

The seven fungi that reacted with either a ++ or a +++ rating to the *P. viticola* cross-adsorbed cell-wall conjugate (*Aureobasidium* sp. (spores), *Cytospora* sp., *E. nigrum*, *E. armeniacae*, *Phomopsis* sp., *Poria placenta*, and *Schizophyllum commune*) were stained with goat anti-rabbit RITC to determine if this staining was due to nonspecific binding of the conjugate to the fungus. In all cases no fluorescence was observed, indicating the reactions with the cross-adsorbed preparation were due to specific binding.



Figs. 1–6. Hyphae of *Eutypa armeniacae* viewed microscopically by **A,** transmitted light and **B,** fluorescent light (×250). **1,** Mycelium stained with RITC-conjugated whole-cell antiserum. **2,** Cross section of grape wood not stained (arrow shows hypha). **3,** Cross section of grape wood indirectly stained with rabbit normal serum (arrow shows hypha). **4,** Cross section of grape wood directly stained with RITC-conjugated cell-wall antiserum (arrows show hyphae). **5,** Cross section of grape wood indirectly stained with cell-wall antiserum (arrows show hyphae). **6,** Cross section of grape wood indirectly stained with cell-wall antiserum (arrows show hypha behind pith cell wall).

All areas of stained hyphae including the cell lumen, hyphal tips, and cell walls fluoresced with equal intensity (Fig. 1).

Staining of hyphae in wood. The RITC stain was selected instead of the more commonly used fluorescein isothiocyanate (FITC) stain because there was less autofluorescence of grape wood under the RITC fluorescence filters than under the FITC fluorescence filters.

The use of acetone in the final rinse to eliminate nonspecific binding of the labeled antisera to grape wood was quite effective and much simpler than other methods used (2,6,7,9,13). The lengthy rinse was necessary since nonspecific fluorescence of hyphae stained indirectly with preimmune serum sometimes occurred when rinsed for 1 hr.

Hyphae in unstained wood or in wood stained with preimmune serum using the indirect method sometimes showed slight fluorescence (Figs. 2 and 3).

With the direct staining procedure, some specificity of hyphal staining was observed (Fig. 4). However, the brightness of hyphal fluorescence was generally not great enough to stand out over the wood autoflourescence when some wood tissue, for example, a pith cell wall, was covering the hyphae. Hyphae in wood stained for 30 min exhibited the same intensity of fluorescence as hyphae in wood stained for 1 hr.

With the indirect staining method, hyphae that reacted strongly to the stain fluoresced in wood with much greater intensity than hyphae stained with the direct method (Fig. 5). This intensity was great enough so that the autofluorescence of the wood did not interfere with the visualization of the hyphae, even if wood tissue was in front of the hyphae (Fig. 6).

To determine if the specificity of hyphal staining in wood is the same as on slides, indirect staining was done on wood sections of stems from which only one, two, or three species of fungi were isolated. This method was limited because there were very few stems (9 of 600) that contained three or less different fungal species as determined by isolation.

The specificity of staining observed in grape wood sections generally agreed with the specificity of hyphae stained on glass slides (Table 2). In a few cases, fungi that fluoresced brightly on slides showed variable intensity of staining on wood sections. These cases are marked with a susperscript "f" in Table 2.

In the cane piece from which essentially all fungi isolated were *E. armeniacae*, hyphae were observed only in the xylem vessels and pith.

DISCUSSION

The titer achieved for both types of antisera (to whole cells and cell walls) was similar to that achieved with *E. armeniacae* in previous work as determined by gel diffusion methods (15).

The relatively low specificity of antisera to both the whole cell and cell wall antigens (Table 1) is apparently in contrast to the high specificity found with antisera made to whole cell preparations of *E. armeniacae* (8,15). A gel diffusion assay was used in the latter study, however, and fluorescent antibody staining may have different specificities than gel diffusion assays (1,4).

Specificity of the RITC-labeled antisera was not sufficient to achieve taxonomic separation of several genera of fungi. Reactivity was sometimes noted with distantly related species but not with closely related species. This can be seen most readily by noting that the basidiomycetes, *Poria placenta, Schizophyllum commune,* and *Fomes annosus*, reacted strongly to the stain whereas the ascomycetes, *Fusarium lateritium* (perfect genus: *Gibberella*) and *Guignardia bidwellii*, did not. *Eutypa armeniacae* is an ascomycete. This finding contrasts with previous serological work where a fungus was the antigen (1,16,18,19).

The conjugate from the antiserum made to the cell-wall antigen was somewhat more specific than the conjugate made from the whole-cell antigen, since *Alternaria* sp. and *Botrytis cinerea* were reduced in reactivity. The difference in specificity is rather small, though, and may just be due to variations between the individual rabbits used.

Reduced wood autofluorescence under RITC filters when compared to FITC filters was due to the RITC barrier filter eliminating all wavelengths less than 590 nm, whereas the FITC barrier filter can only eliminate wavelengths no higher than 520 nm. Therefore, the RITC system has the advantage of eliminating all autoflourescence that occurs between 520 and 590 nm. This may also explain why autoflourescence was generally undetected in unstained hyphae on slides, whereas a faint blue autofluorescence was often detected in studies where FITC was used (5,11,14,17). Craig et al (7) also attribute high autofluorescence of pea tissue under FITC filters to intense autofluorescence of protein bodies at between 490 and 530 nm, which is approximately the same emission maxima of FITC-labeled globulin (9).

When comparing reactivity of various fungal species in wood sections (Table 2) to reactivity on slides (Table 1), the only tests where the fungal reactivities were different (marked with an "f" in Table 2) were tests where certain species reacted strongly on slides but variably in wood. For example, both *Alternaria* sp. and *E. nigrum* (the only fungi found in two stems by isolation—Table 2) consistently fluoresced when stained on slides with the whole cell fluorescent antiserum but sometimes failed to fluoresce when indirectly stained in wood sections. These inconsistencies could have been due to variations in reactivity of the hyphae in the wood and may result from variability of the antigenicity of hyphae in the wood. This is consistent with some studies that demonstrate different reactivities to a fluorescent antibody stain of different segments of hyphae, with growing hyphal tips frequently showing the most reactivity (1,4,17,21).

Hyphae in wood sections colonized by various fungi can be differentially stained by a fluorescent antibody made against *Eutypa armeniacae*. Although the antisera lack complete

TABLE 2. Fluorescence of fungal hyphae in grape wood that was stained with an indirect fluorescent antibody procedure[a,b]

Stem isolation results[c]	Serum made to: Whole-cell antigen	Serum made to: Cell-wall antigen	Normal serum
Eutypa armeniacae 8 *Alternaria* sp. 1 *Phomopsis* sp. 1	+++	+++	− to +
Epicoccum nigrum 10 *Alternaria* sp. 3	− to +++[f]	− to +++	− to +
Fusarium lateritium 7	− to +	− to +	− to +
Alternaria sp. 10 *E. nigrum* 1	− to +++[f]	− to +++	− to +
Penicillium sp. 10 *F. lateritium* 2 *E. armeniacae* 1	− to +++	− to +++	− to +
F. lateritium 5 *E. nigrum* 7	NT	− to +++	− to +
Cytospora sp. 10[d]	− to +++[f]	− to ++[f]	− to +
Alternaria sp. 10 *Aureobasidium* sp. 1[e]	NT	− to +	− to +

[a] After treatment of wood sections with diluted anti-*Eutypa armeniacae* rabbit serum, the sections were stained with RITC-conjugated goat anti-rabbit gamma globulin.

[b] − = no hyphal fluorescence; + = hyphae barely visible; ++ = hyphae readily visible, but with dull fluorescence; +++ = hyphae fluoresced brightly; NT = not tested.

[c] Numbers after name of fungus denotes number of wood chips (of a total of 10) from which that fungus was isolated from a pruning stub onto PDA.

[d] Two stems were used with this isolation result.

[e] Very few hyphae could be observed in sections made from this stem.

[f] Results not explainable by specificity of fungi on slides with direct staining method (Table 1).

specificity to *E. armeniacae*, previous studies (11,19) have described the production of highly specific FA preparations to fungal antigens. Antisera specific to a fungal species could be useful in various histological investigations using the technique described.

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