Special Topics

Detection by Protein A-Gold of Antigens to *Botrytis cinerea* in Cytoplasm of Infected *Vicia faba*

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ABSTRACT

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Thin sections of leaves of *Vicia faba*, uninfected and infected with *Botrytis cinerea* were treated with two antisera, one prepared against the surface components of *B. cinerea* and the other prepared against the fimbriae of the smut fungus, *Ustilago violacea*. Then a suspension containing protein A-gold complexes was applied to the sections. A full range of serological control treatments included use of preimmune serum. Treatment with either antiserum resulted in heavy gold labeling of host cells in infected tissue but not of cells in healthy tissue. In many cases the labeled host cells were not penetrated by the fungus and were some distance from the nearest hypha. Treatment of infected host cells with preimmune serum did not result in gold labeling. Two hypotheses are proposed for these results. First, fimbrial protein, either as intact fibrils or as dissociated subunits, enters host cells well ahead of the hyphae. Second, infection by the fungus may stimulate a response by the host that includes synthesis of host proteins with antigenic regions similar to those of surface proteins of *B. cinerea* and those of fimbriae of *U. violacea*.

Additional key words: fungal antigens.

Immunocytochemistry has been extensively utilized in medical histocytochemistry but only in a very few instances in plant pathology. The technique involves the production of antibodies against specific macromolecules (antigens) and the linkage of the antibodies to protein A-gold complexes to locate the antigens. Rey (13) produced antibodies against the extracellular protease of *Nectria galligena* and demonstrated the presence of this enzyme in infected apple tissue. In another study Chamberland and co-workers (3) linked colloidal gold to chitinase or lectins to show the presence of chitin on *Fusarium* walls in root cells infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

We have attempted to show that surface antigens of *Botrytis cinerea* Pers. move into host tissue in advance of the pathogen. We used protein A-colloidal gold labeling and an antiserum specific to the surface antigens of *B. cinerea*. We also used an antiserum directed against the fimbriae of *Ustilago violacea* to detect fimbrial protein. Fimbriae in many fungi are highly conserved at least in their antigenic regions, thus allowing use of the antiserum to *Ustilago* to detect fimbriae of *B. cinerea* (9).

MATERIALS AND METHODS

Antiserum preparation. Conidia of *B. cinerea* (UWO-760) were used to inoculate complete medium (4). The resulting mat of spores and mycelia was harvested 4 days after inoculation and killed with

0.5% formalin. The mycelial suspension was centrifuged for 10 min at 1700 g and the resulting pellet resuspended in distilled water. This washing procedure was repeated three times. The washed mycelial mass was resuspended in 2 ml of Freunds complete adjuvant (Gibco) to a dense concentration (10^6 cells per milliliter).

New Zealand white rabbits (2 kg) were injected intramuscularly with the mycelial suspension and received a booster injection 2 wk after the primary injection. The rabbits were bled on the 21st day. The antisera were collected and stored at -20 C. Preimmune serum was collected before injection of the antigens. Antiserum directed against fimbriae of *U. violacea* was produced as described by Gardiner et al (8,11).

Immunofluorescence. A washed mycelial mat of *B. cinerea* was suspended in 10 ml of distilled water. The antiserum to *B. cinerea* was diluted 10-fold and 2 ml were added to 10 ml of mycelial suspension. Preimmune serum was used in the controls. The immunofluoresent technique described by Gardiner (7) was used to label the mycelium using fluorescein isothiocyanate (FITC) anti-rabbit globulin conjugate developed in goat. The samples were examined in a Zeiss photomicroscope II with number II excitation filter and 65 and 50 barrier filters.

Inoculation of plant material. Five-week-old leaves of *Vicia faba* were inoculated with conidia, 10^5-10^6/ml. The leaves were floated onto the conidial suspension with the upper epidermis immersed in the inoculum for 2–3 sec. Inoculated leaves were placed onto moist filter paper inside a petri dish. Infected leaves were harvested 12 and 16 hr after inoculation. The control plants were inoculated with distilled water.

Electron microscopy. Disks, 1 mm in diameter, were cut from infected and control leaves of *V. faba* 8 and 12 hr after inoculation with *B. cinerea*. The disks were fixed for 2 hr in 4% glutaraldehyde in cacodylate buffer at pH 6.8. This material was rinsed twice in buffer and postfixed with 2% osmium tetroxide for 1 hr. After two rinses in water, the material was stained in 5% uranyl magnesium acetate for 20 min at room temperature, dehydrated in a graded acetone series, and infiltrated with Epon-Araldite (12). Sections cut with a diamond knife on a Porter-Blum ultramicrotome (Sorvall MT-2, Norwalk, CT) were mounted on nickel grids.

Preparation of protein A-gold label. Colloidal gold particles, 15 nm, were prepared by the methods of De Mey (5) and Bendayan (1,2). Chloroauric acid (Baker) was reduced by adding 4.0 ml of 1% aqueous sodium citrate to 100 ml of a boiling solution of 0.01% chloroauric acid and cooling for 5 min until a wine red color

resulted. The colloidal gold was stable up to several months when stored at 4 C in the dark.

In preparation of the protein A-gold complex, 10 ml of the colloidal gold suspension was adjusted with potassium carbonate to pH 6.9, then added to 0.3 mg of protein A (Sigma, St. Louis, MO) in 0.2 ml of distilled water. Next the solution was centrifuged at 48,000 g (4 C) to remove the excess unbound protein A. The dark red protein A gold pellet was resuspended in 10 ml of 0.01 M phosphate buffered saline, pH 7.4, and stored at 4 C (stable for 6–8 wk).

Protein A-gold labeling. Thin sections were floated onto a saturated solution of sodium periodate for 2–3 min to remove the (preembedding) osmium textroxide (2). Sections were then washed three times in distilled water and treated with 1% ovalbumin (Sigma) for 5 min to prevent nonspecific binding. The sections were then floated on antiserum for 30 min at room temperature, washed thoroughly by passing the grid through a series of water droplets, and treated with protein A-gold solution for 30 min. This treatment was followed by further washing in droplets of distilled water and staining in 3% uranyl acetate for 20 min.

A series of serological controls, using a combination of antisera and protein A-gold as described by Bendayan (1), were performed on infected and control bean tissue sections.

The samples were viewed in a Phillips EM 200 operating at 60 kV.

Preparation for transmission electron microscopy. Mycelium of *B. cinerea* was washed three times in particle-free distilled water. A drop of fungal suspension was placed on a formvar-coated carbon-reinforced grid for 1 min. The excess fluid was drawn off and the grid stained for 30 sec with 3% ammonium molybdate (pH 7.2).

Preparation for scanning electron microscopy. Tissue was fixed in 4% glutaraldehyde and 2% osmium tetroxide, dehydrated in a graded ethanol series, and critical point dried. Leaf tissue was mounted on aluminum studs and then sputter-coated with gold-palladium. Samples were examined in a Phillips 501 scanning electron microscope at 15 and 30 kV, with a spot size of 200 μm.
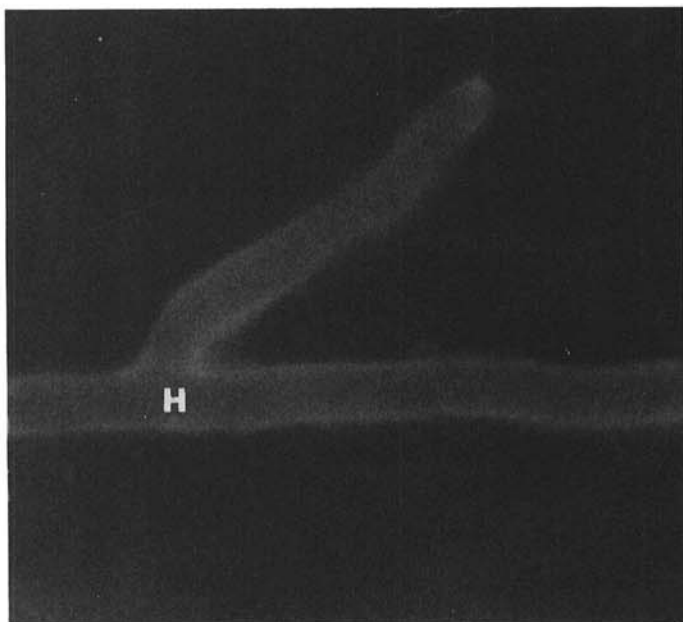

Fig. 1. Hypha (H) of *Botrytis cinerea* treated with anti-*Botrytis* antiserum followed by fluorescein isothiocyanate. Note the fluorescent halo around the mycelium. ×3,000.
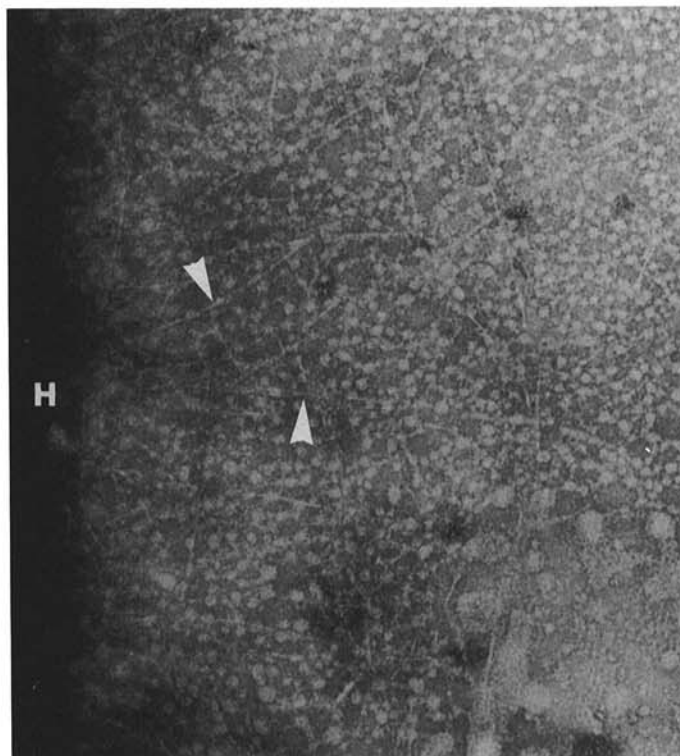

Fig. 2. Transmission electron micrograph of negatively stained (ammonium molybolate) fimbriae of *Botrytis cinerea*. Note the numerous long filamentous appendages (fimbriae) (arrows) originating at the hyphal surface. ×77,000.

RESULTS

Immunofluorescence of *B. cinerea*. The fluorescent labeling technique was used to determine if antibodies were raised against fungal antigens. Hyphae of *B. cinerea* were treated with anti-*Botrytis* antiserum followed by anti-IgG FITC and were viewed in the fluorescent microscope. The hyphal walls and the immediate external region fluoresced brightly when either anti-*Botrytis* or antifimbrial antisera were used but not when preimmune serum or water was used (Fig. 1).

Surface fibrils on *B. cinerea*. Negatively stained whole mount preparations of *B. cinerea* prepared for transmission electron microscopy revealed numerous long filamentous appendages 7.5–8.5 nm in diameter (Fig. 2). These resembled the fimbriae described on *U. violacea* and various other fungi (7). Scanning electron microscopy also showed a fibrous outer wall on the fungus and near the tip of the germ tube (Fig. 3).

Gold labeling of hyphal fragments. Sections of hyphae of *B. cinerea* were placed on grids and treated with either anti-*Botrytis* or antifimbrial antiserum and then by a protein A-gold colloidal suspension (1,2). Gold labeling of the fungal surface and cytoplasm was achieved by using either antiserum (Fig. 4). Control hyphae treated with no antiserum or with a preimmune serum were not detectably labeled.

Gold labeling of thin sections of *V. faba* leaves infected with *B. cinerea*. Thin sections of uninfected leaves of *V. faba* treated with
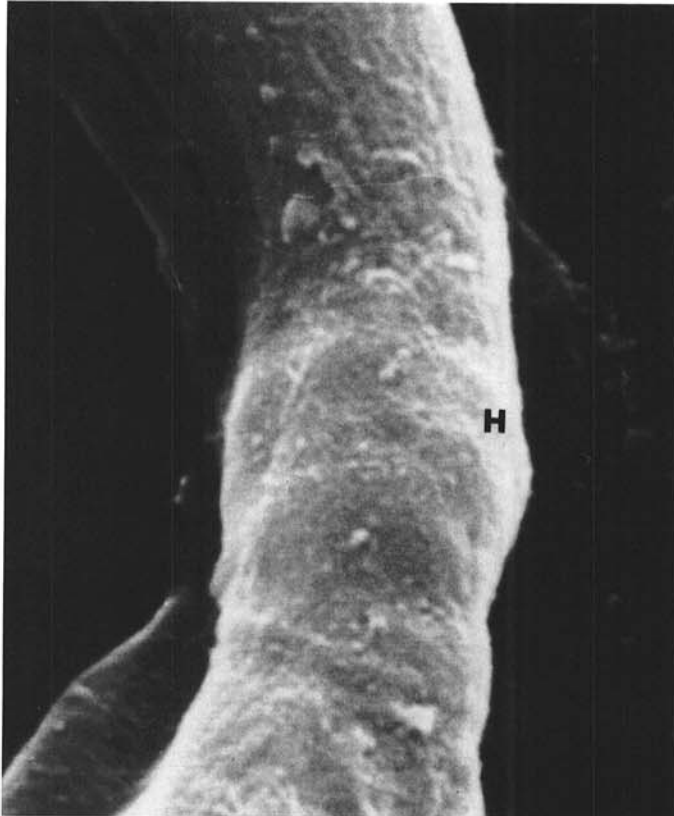

Fig. 3. Scanning electron micrograph of hypha (H) of *Botrytis cinerea*. Note the fibrous texture (fimbriae) on the hyphal surface. ×15,000.
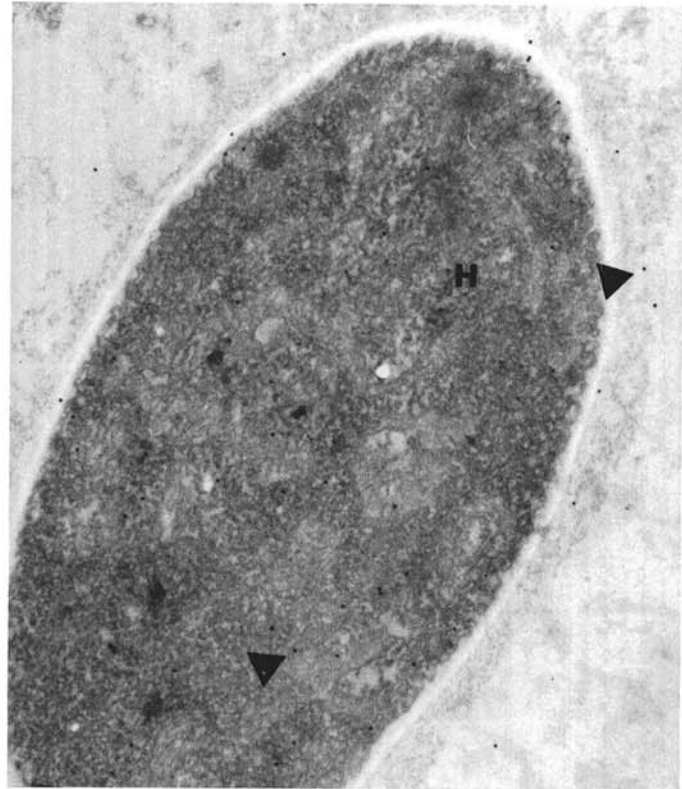

Fig. 4. Transmission electron micrograph of hyphal section of *Botrytis cinerea*. Note the electron-dense protein A-gold labeling (arrows) of the hypha (H). ×22,500.
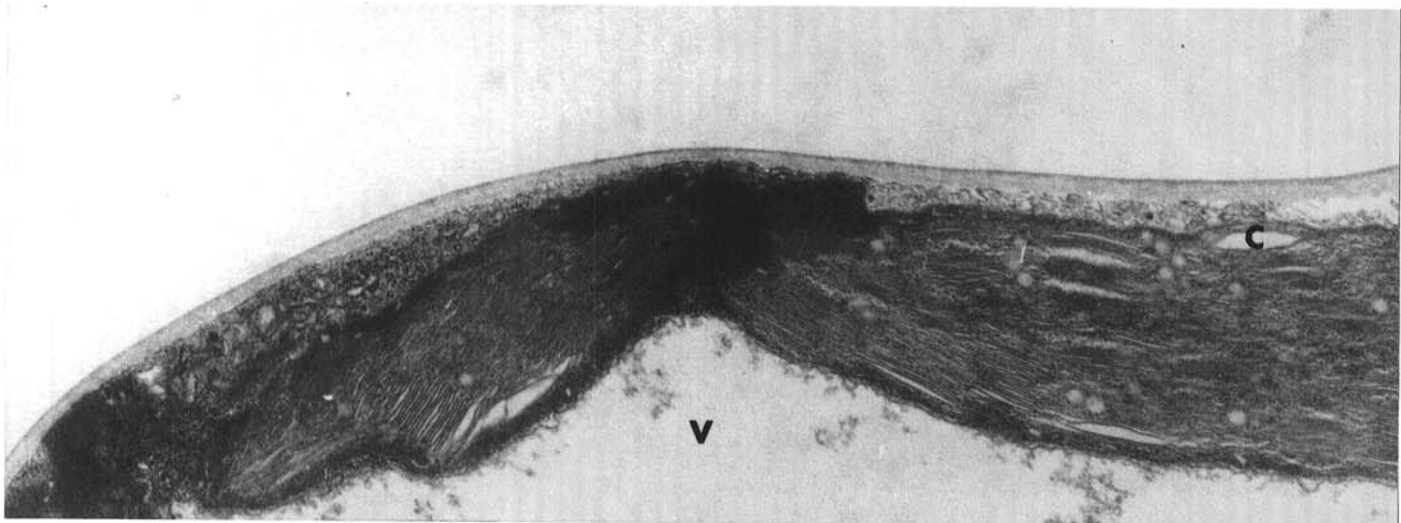

Fig. 5. Transmission electron micrograph of a section of an uninfected mesophyll cell of *Vicia faba*. Note the absence of gold labeling in the chloroplast (c) and vacuole (v). ×12,000.

anti-*Botrytis* or antifimbrial antisera and then protein A-gold showed little or no gold labeling (Fig. 5). In contrast, thin sections of host cells 12 and 16 hr after inoculation were strongly labeled whether the fungus was present in the cell or not (Figs. 6 and 7). Gold labeling in the sections showed a high amount of labeling in chloroplasts and host cytoplasm and a lesser amount in the vacuoles, mitochondria, and walls. Uninfected tissue had no more labeling than the negative control.

Serological controls treated as recommended by Bendayan (1) yielded negative results; i.e., gold labeling was absent or at uniformly low background levels. These included that of

uninfected and infected leaves of *V. faba* with protein A-gold alone, preimmune serum followed by protein A-gold, and antiserum to fimbriae of *Ustilago* followed by protein A-gold that had previously reacted with immunoglobulin or protein A followed by protein A-gold.

DISCUSSION

Our results show that antigens of the type found on the surface of *B. cinerea* are present inside host plant cells some distance from the nearest fungal hypha 8 and 12 hr after inoculation or 1 and 5 hr

Fig. 6. Transmission electron micrograph of a section of a leaf of *Vicia faba* infected with *Botrytis cinerea*, treated with anti-*Botrytis* antiserum and protein A-gold. Note the concentration of gold on the chloroplast (c) and cytoplasm (cy). v = vacuole. ×10,000.
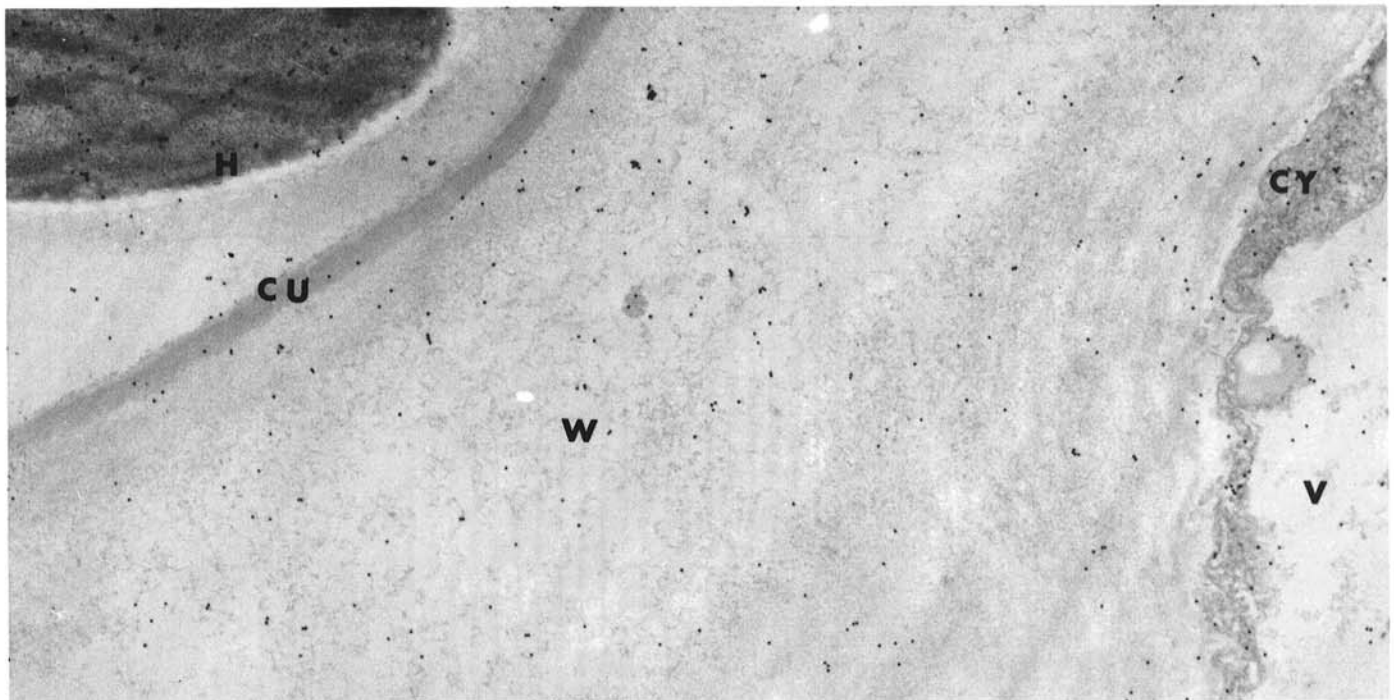

Fig. 7. Transmission electron micrograph of a hyphal section (H) of *Botrytis cinerea* and cuticle (cu), epidermal wall (w), cytoplasm (cy) and vacuole (v) of *Vicia faba*, treated with anti-*Botrytis* antiserum and protein A-gold. Note gold labeling in all tissues. ×20,000.

after fungal penetration and that no such antigens can be detected in uninfected plant tissue.

Similar results obtained with antifimbrial antiserum, however, suggest that it is the fimbrial antigen on the surface of the *Botrytis* cells that is being detected in these host cells. Although the fimbrial antiserum we used was obtained from rabbits injected with fimbriae from the unrelated fungus, *U. violacea*, we previously demonstrated that this antiserum reacts with antigens on a wide variety of fungi and that all these fungi produce surface fibrils resembling fimbriae (7–11). These studies led to the conclusion that fungi in general produce surface fibrils containing a protein that is highly conserved, at least in its antigenic region.

Our report confirms that *B. cinerea* produces surface fibrils resembling fimbriae (Fig. 3) and has surface antigens related to the fimbriae of *Ustilago* (Figs. 1 and 4). Isolation of these fibrils coupled with protein A-gold labeling after treatment with antifimbrial antiserum is being attempted to show that the observed fibrils do indeed carry the detected antigens.

A full series of immunological controls as recommended by Bendayan (1), as well as controls involving defimbriated cells (7), have established the specificity of the technique. Thus we are confident that antigens of the fimbrial type are indeed found in host cells as a consequence of infection.

Two hypotheses account for the detection of antigens in host cells not penetrated by hyphae. The first and most obvious hypothesis is that these antigens are of fungal origin. Antifimbrial antiserum of *Ustilago* binds to polymerized subunits or to the dissociated subunits (10); thus the antigens could have been derived from penetrating fimbriae or from dissociated subunits.

If the polymerized fimbriae do indeed penetrate host cells, establishing a direct physical presence of the fungus at great distances from the pathogen, then they are likely to be of great importance in the nutritional interaction between host and pathogen. Their presence in chloroplasts lends weight to this possibility. The mechanism of penetration remains unknown but could involve wall pores, plasmodesmata, or changes in wall structure brought about by infection.

If the antigens in host cells are due to dissociated subunits of fimbriae, their large molecular weight, 74,000, in species so far tested (10) would also make entry difficult unless the permeability of the wall was altered as a result of infection.

At present it is not possible to distinguish between these two alternatives; however, use of antisera specific for polymerized or depolymerized proteins could enable this to be done.

An alternative hypothesis is that infection by the fungus stimulates a response by the host that includes synthesis of host proteins with antigenic protein similar to those of fimbrial protein and antigens to *B. cinerea*. Such a response may be an attempt at resistance or it may be induced by the fungus to aid its own nutrition—perhaps a copolymerization process as envisaged by Vanderplank (14). Alternatively, there may be a combination of the two hypotheses. Common antigens have been detected in a number of plant pathogen situations (6).

These hypotheses may be tested by isolation of proteins in infected and uninfected tissues, followed by immunoglobulin analysis with antifimbrial antiserum. If the second hypothesis is correct, it may be possible to show that infected tissues produce an antigen that is not fimbrial protein.

The immunogold technique we used is a powerful tool for the localization of macromolecules in host-pathogen interactions and should be important in developing an understanding of the molecular basis of this interaction.

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