Techniques

High Contrast Resolution of the Mycelia of Pathogenic Fungi in Corn Tissue After Staining with Calcofluor and Destaining with Cellulase

A. T. Trese and D. C. Loschke

Department of Plant Pathology, University of Missouri, Columbia 65211, and Department of Plant Pathology, University of Florida, Gainesville 32611, respectively.
Florida Agriculture Experiment Station, Journal Series 9814.
Accepted for publication 29 August 1989 (submitted for electronic processing).

ABSTRACT

Trese, A. T., and Loschke, D. C. 1990. High contrast resolution of the mycelia of pathogenic fungi in corn tissue after staining with Calcofluor and destaining with cellulase. Phytopathology 80:196-200.

We have improved on a novel staining technique to monitor fungal growth in corn inoculated with *Exserohilum turcicum, Bipolaris maydis*, or *Bipolaris zeicola*. Infected leaf pieces are overstained with Calcofluor until both host cells and fungal mycelia fluoresce brightly. This is followed by selective destaining of the plant cell walls with the enzyme cellulase. The resulting high contrast resolution of fungal mycelia allows thorough examination of infection sites in all three host-pathogen combinations. Using this method, we measured fungal growth in monogenically resistant corn isolines inoculated with two races of *E. turcicum*. Fungal growth in compatible and incompatible interactions is similar 3 days after infection, indicating that resistance is not yet cytologically apparent.

Northern corn leaf blight is caused by the Ascomycete, *Exserohilum turcicum* (Pass.) Leonard & Suggs (teleomorph, *Setosphaeria turcica*). Control of the disease is usually achieved by resistance genes in the host (7). Hooker (3) has described both polygenic and monogenic resistance. Several dominant, monogenic alleles for resistance have been identified, including *Ht*1 and *Ht*2. Corresponding races of the pathogen have also been identified. Race 1 produces a compatible interaction only on hosts lacking both of the resistance genes; race 2 can overcome gene *Ht*1 but not *Ht*2. The virulence phenotype of race 2 is conditioned by a single gene (4). Thus, the *Zea mays–E. turcicum* interaction appears to fit the gene-for-gene model (1).

Hilu and Hooker (2) described the phenotype of the incompatible interactions as "chlorotic lesions" rather than the "necrotic lesions" of the compatible interaction. Chlorotic lesion

© 1990 The American Phytopathological Society

reactions can be distinguished by accelerated lesion development, appearing 1 or 2 days earlier. The developing lesions produce a chlorotic halo and have reduced necrosis and distinctly limited sporulation. After examining infected tissue, 6–8 days after inoculation, Hilu and Hooker (2) concluded that fungal growth in resistant plants is largely limited to a slow spread through mesophyll tissue. Xylem tissue is penetrated but growth there is minimal. In compatible interactions, hyphae quickly penetrate xylem vessels and spread rapidly through them, growing into the mesophyll tissue only later, as the lesions become necrotic.

Before molecular characterization of the resistance response, we were interested in defining the initial chronological divergence between infections in compatible versus incompatible interactions. To accomplish this we needed to examine many infection sites, determine the amount of fungal growth present, and identify the type of host tissue infected. Use of sectioned material was not practical, so we evaluated a whole mount staining technique that uses either aniline blue or a mixture of methylene blue and acid fuchsin (2). This method gave very poor results, revealing only a small fraction of the hyphae present inside vascular bundles. Therefore, we developed a new method that relies on staining both fungal and host cells with Calcofluor (6), followed by selective destaining of the host cells with purified cellulase. Although the technique requires a number of steps, the result is high contrast resolution of all fungal structures in the host, allowing detailed study of each infection site.

In this paper we describe the method and give results from our study of infection sites in the corn–*E. turcicum* interaction, where we have compared fungal growth in compatible versus incompatible interactions. We also include results of using the technique to examine *Bipolaris maydis* and *B. zeicola* infection sites in corn.

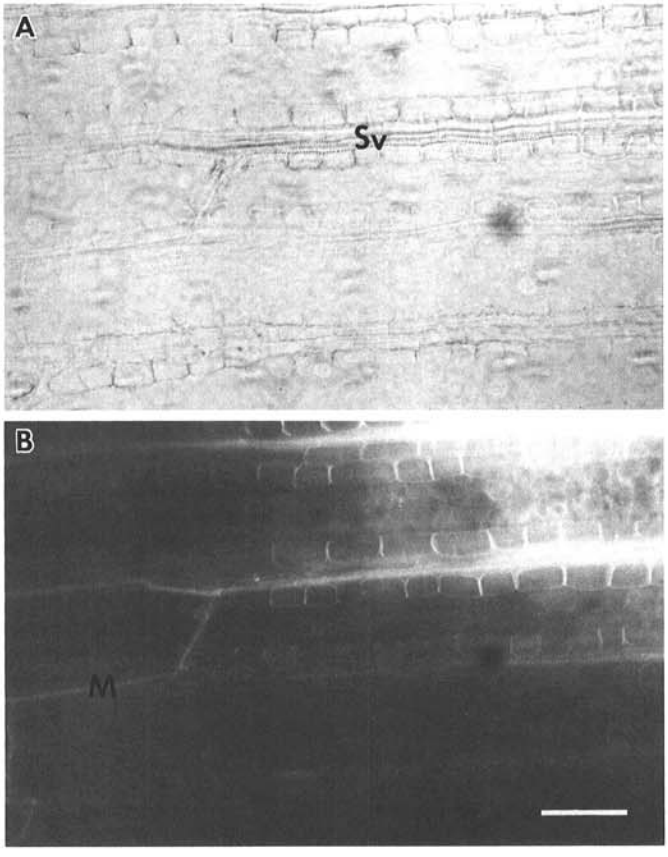

Fig. 1. Partial destaining of *Exserohilim turcicum*-infected corn tissue. **A,** Transmitted white light. **B,** Same area, illuminated by fluorescent light. Destaining is complete at the lower left but incomplete at the upper right. Sv = small vein, M = mycelium, bar = 100 μm.

MATERIALS AND METHODS

Corn genotypes used in this study were congenic lines of MO17 homozygous for paired resistance genes *ht1ht*/*ht2ht2*, *Ht1Ht*/*ht2ht2*, *ht1ht*/*Ht2Ht2*, and congenic lines of A632 homozygous for *ht1ht1*/*ht2ht2*, *Ht1Ht1*/*ht2ht2*. Hybrid M14 × W23 was used for studies with *B. zeicola* and *B. maydis*.

Isolates of *E. turcicum* were race 1 and race 2, obtained from Dave Smith, DeKalb-Pfizer Genetics, 3100 Sycamore Rd., DeKalb, IL. Spores were produced by inoculation of a susceptible line, Silver Queen sweet corn (Johnson and Faris, Gainesville, FL), and spores from lesions were collected with a small, mouth-operated vacuum collector. Spores were stored dry at −20 C until use. Preliminary inoculations were done to confirm the expected compatible and incompatible reaction types with the test corn genotypes. Spores of *B. maydis*, race 0, and *B. zeicola*, race 3, were obtained from Dr. Oscar Calvert, University of Missouri-Columbia.

Spore suspensions of *E. turcicum* were made to 20,000/ml in 0.05% (v/v) Tween 20 in water. Young corn seedlings at the five- to six-leaf stage were inoculated by atomizing with the spore suspension until runoff. The plants were then kept in a dew chamber in the dark for 24 hr at 24 C before being moved to the greenhouse. MO17 isolines were inoculated with race 2, and the A632 isolines with race 1. The experiment was repeated once.

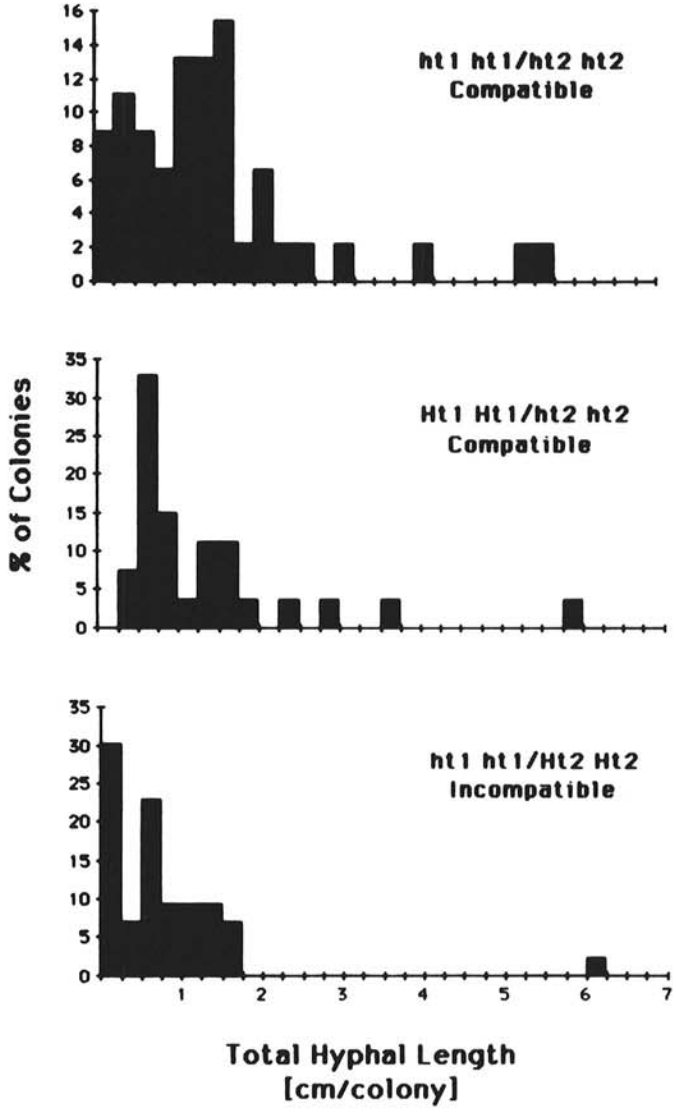

Fig. 2. Frequency distribution of colony sizes 3 days after inoculation of MO17 isolines with *Exserohilum turcicum* race 2. Upper: $N = 45$, center: $N = 27$, lower: $N = 43$.

Similar inoculation procedures were used with *B. zeicola* and *B. maydis*.

Leaf tissue samples were taken after 3 and 5 days from *E. turcicum*-inoculated plants, and after 7 days from plants inoculated with *B. zeicola* or *B. maydis*. Leaf pieces approximately 1 × 3 cm were selected from the centers of leaves with well-separated infection sites. The leaf tissue was first cleared overnight in methanol:chloroform (3:1, v/v), rinsed well in methanol to remove chloroform, and then gradually rehydrated to water in a graded methanol:H_2O series. At this step, the leaf pieces could be stored for several months in water at 4 C. Thorough staining was achieved by immersion in 0.01% (w/v) Calcofluor (Cellufluor, Polysciences) in 0.1 M Tris-HCl buffer at pH 8.0 for at least 6 hr. After staining, leaf pieces were rinsed in Tris buffer for 2 hr and then autoclaved for 8 min in beakers containing 0.5 N KOH. Boiling in KOH was not sufficient. During autoclaving the leaf pieces were held submerged by separately anchoring one corner of each to the beaker bottom with a glass slide. After autoclaving, a piece of polypropylene mesh was used to gently transfer the now softened leaf pieces to fresh Tris buffer, where they were rinsed for 30 min, with periodic gentle swirling.

The tissue for most of this study was next embedded in a thin layer of 0.5% (w/v) agarose before treatment with cellulase. However, a later improvement consists of substituting polyacrylamide for the agarose. In this case, the tissue was transferred, after rinsing, to a solution of acrylamide, (3.8% acrylamide, 0.11% bis acrylamide, w/v), in 0.125 M Tris buffer, pH 6.8. After equilibration for 1 hr, ammonium persulfate and Temed were added to 50 mg/L and 0.5 ml/L, respectively, and the tissue gently transferred to the rough surface of a frosted glass slide. Several drops of the acrylamide solution were added to cover the leaf piece, and then a siliconized cover slip was placed on the solution. After polymerization was complete, the cover slip was removed and the slide submerged in 0.25% (w/v) cellulase (Cooper-Worthington) in 0.5% (w/v) MES buffer, pH 5.0. Digestion was allowed to proceed at 37 C for 12 hr or more, after which the enzyme solution was withdrawn and the cover slip replaced.

Slides were examined with a Zeiss epifluorescence microscope equipped with an HBO mercury lamp, emission filter 390-440, and barrier filter LP-475. To determine the amount of mycelial growth in individual infection sites of *E. turcicum*, the foci were viewed on a television monitor. A wheel type map reader was used to make a linear trace of each mycelial strand, and a measure of total hyphal length was calculated. Photomicrographs were made with Kodak Tri-X and Tech-Pan black and white film, and Ecktachrome, daylight balanced, color print film.

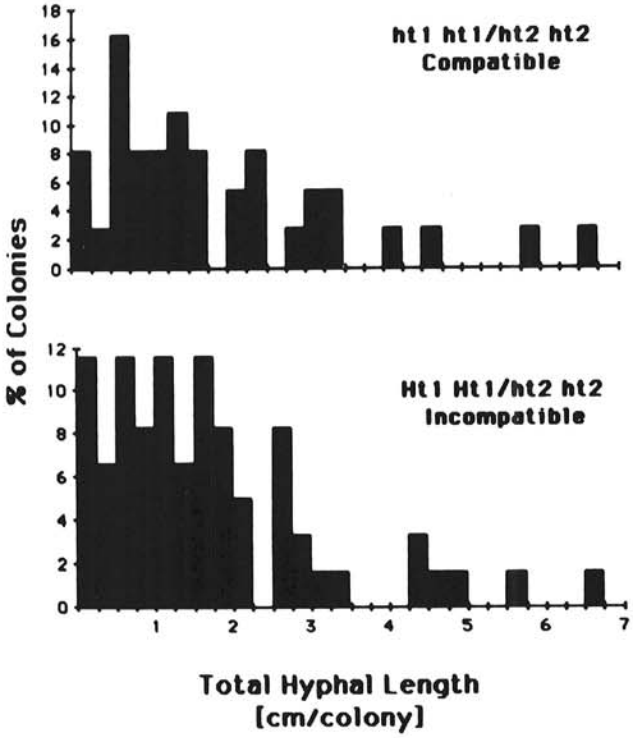

Fig. 3. Frequency distribution of colony sizes 3 days after inoculation of A632 isolines with *Exserohilum turcicum* race 1. Upper: $N = 32$, lower: $N = 60$.

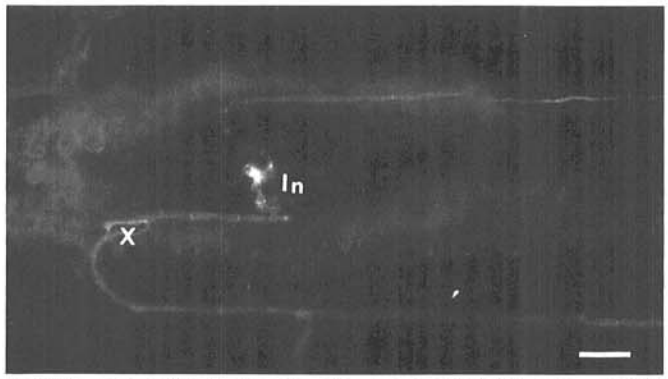

Fig. 4. Three-day-old infection site of an incompatible interaction, MO17 *ht1ht1/Ht2Ht2* × race 2. A small amount of mycelial growth is present in the mesophyll tissue at the site of infection (In), but the majority of growth is in the xylem vessels (X). Hazy circle of background fluorescence is yellow autofluorescence of the host tissue. Bar = 50 μm.

RESULTS AND DISCUSSION

Our goal was to examine the infection process in resistant and susceptible corn isolines inoculated with different races of *E. turcicum*. Because the whole mount staining method of Hilu and Hooker (2) gave unacceptable results, we evaluated a published staining technique using Calcofluor (6). Calcofluor staining

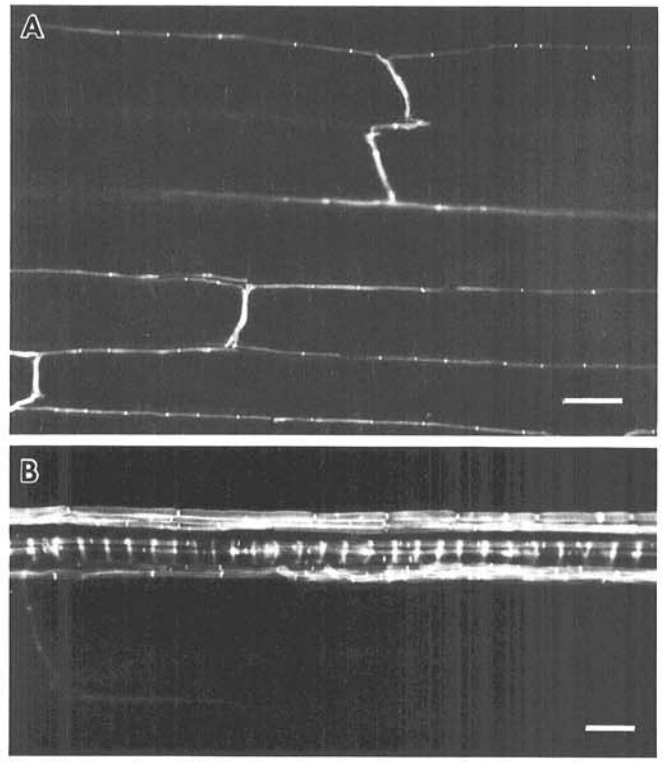

Fig. 5. Three-day-old infection site of an incompatible interaction, MO17 *ht1ht1/Ht2Ht2* × race 1. **A,** Infected area where growth is limited to small veins. Bar = 100 μm. **B,** Infected major vein, showing large mycelial strands crowding xylem vessels, and branching into minor veins. Bar = 50 μm.

techniques used to study disease interactions generally rely on the inherent preference of Calcofluor for staining fungal cell walls relative to plant cell walls, which occurs in some host-pathogen combinations (6). However, in the corn–*E. turcicum* interaction, the plant cell walls stained too rapidly, obscuring any stained fungal structures.

We consequently developed a method that includes thorough staining of all the tissue with Calcofluor, followed by selective destaining of the plant cell walls with purified cellulase. The tissue was first treated with a hot alkali solution to partially dissolve and loosen the cuticle, allowing greater penetration of the enzyme, and to solublize at least some of the plant pectin. During cellulase digestion, the tissue was embedded in a polyacrylamide matrix to maintain tissue integrity. The original spacing and parallel orientation of the vascular bundles was maintained during the digestion process, indicating that the integral arrangement of the plant and fungal tissues remained undisturbed (Fig. 1).

We were unable to quantify mycelial growth in different isoline × race combinations at 5 days postinoculation. Many of the infection sites had expanded until they overlapped into neighboring foci, making it impossible to measure each site independently. However, we were able to make such measurements of infection sites at 3 days after inoculation. Frequency diagrams show the distribution of colony sizes, in each of five isoline × race combinations (Figs. 2 and 3) after 3 days. Fungal colony development is similar in compatible and incompatible interactions after 3 days, indicating that resistance was not apparent cytologically. In all of the *E. turcicum* infection sites examined, there was a small amount of mycelial growth in the mesophyll tissue at the point of infection, but all further fungal development occurred in the vascular tissue (Fig. 4), where growth was usually abundant. Our work contrasts with that of Hooker (3), which describes the monogenically determined resistant reaction as one in which "hyphae rarely penetrate the xylem and lesion enlargement is limited to slowly growing hyphae in the mesophyll tissue." This conclusion was based on studies of an inbred line as the resistant host and a distantly related, susceptible, F1 hybrid. The difference in results may be due to the fact that we used congenic lines for comparison rather than less related genotypes, as used by Hooker.

In all combinations there was substantial variation in total hyphal length per colony because there were two types of infection sites. In some, only small veins were colonized, and mycelial growth was usually single-stranded (Fig. 5A). In others, both small veins and large vascular bundles were colonized and fungal growth was often four or more strands abreast, mycelia were larger, and total linear growth was usually greatly increased (Fig. 5B). Large vascular bundles were colonized in approximately 16% of the infection sites. It is possible that only these infection sites would have eventually developed into mature lesions. A future study in which a lower inoculum density is used should reveal at what time the differences between resistance and susceptibility can be observed as a diverging pattern of fungal growth.

This technique was also successful for high contrast visualization of *B. maydis* and *B. zeicola* mycelia in 7-day-old infection sites. The spore, germ tube, appressoria, and spreading hyphae within the host tissue could be seen in an infection site of *B. maydis* (Fig. 6A). Both *B. maydis* and *B. zeicola* had two distinct types of mycelia, as previously described for *B. maydis* by Wheeler (8). One was a thin spreading form in the mesophyll and epidermal layers (Figs. 6B and 7A), and a second as a thick, flattened form growing near the surface, apparently forming a

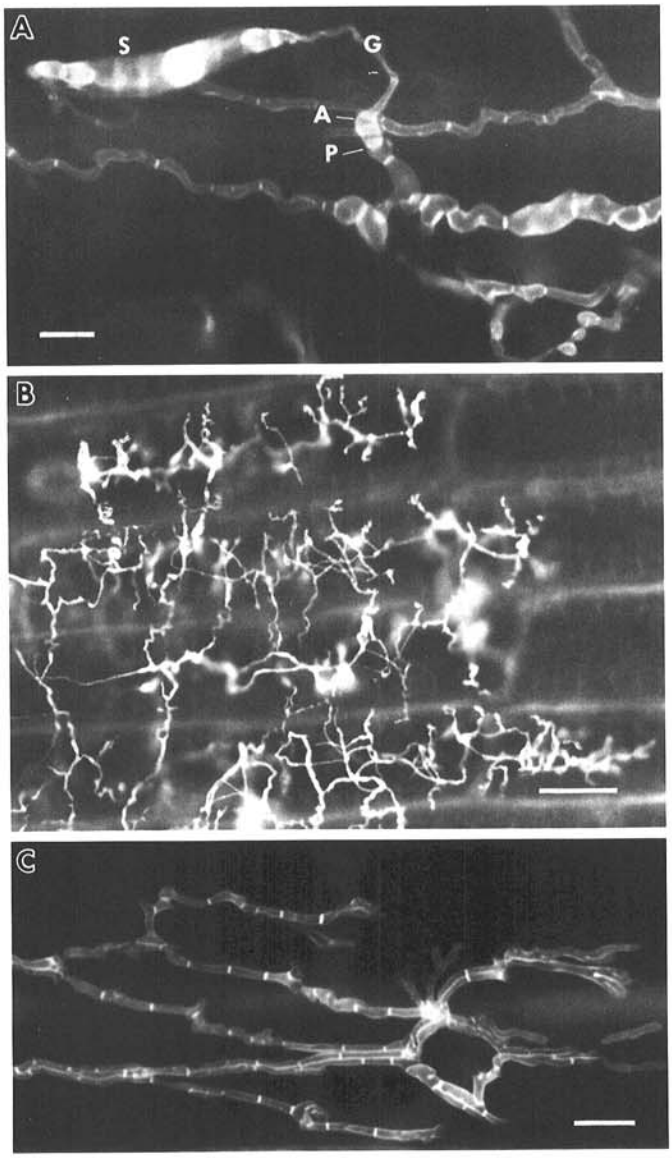

Fig. 6. *Bipolaris maydis* infection sites in compatible corn hybrid M14 × W23, 7 days after inoculation. **A,** Germinated spore (S), germ tube (G), appressorium (A), and site of penetration (P). Bar = 20 μm. **B,** Highly branched mycelia ramifying through leaf tissue. Bar = 100 μm. **C,** Thick, flattened mycelia at the leaf surface. Bar = 50 μm.

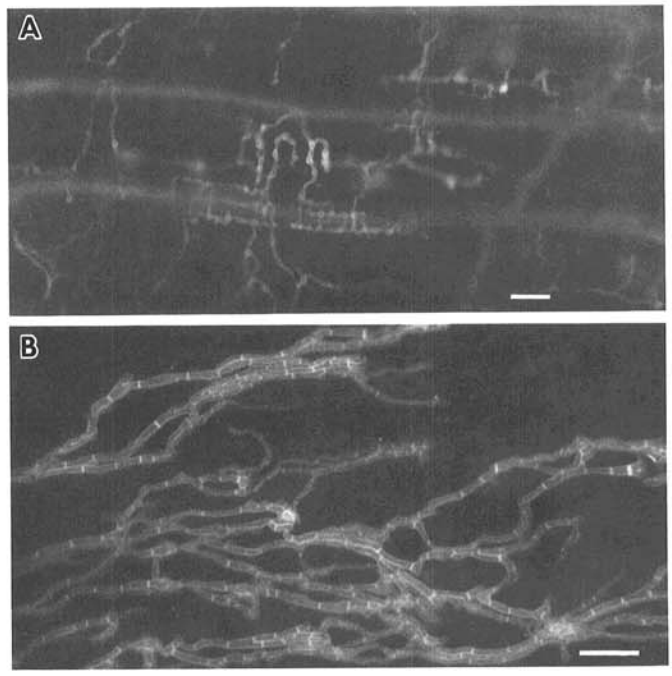

Fig. 7. *Bipolaris zeicola* infection site in compatible corn hybrid M14 × W23, 7 days after inoculation. **A,** Branched hyphae ramifying through leaf tissue. Bar = 50 μm. **B,** Larger, flattened mycelia growing at the leaf surface. Bar = 50 μm. These black and white prints were made from color negatives, hence the reduced contrast.

thallus layer (Figs. 6C and 7B). Because the hyphal growth pattern in these interactions was very branched and spreading, it would not be possible to measure total hyphal length, but colony diameters could be determined easily.

This technique has been applied to only the three corn pathogens described, yet it should be generally useful in other combinations where the pathogen has chitinous cell walls or other cellulase resistant polymers that stain well with Calclfluor. Even though the technique requires several steps, the results are far superior to simpler staining methods (2) and provides more information with much less effort than would a comparable study based on sectioned material.

LITERATURE CITED

1. Flor, H. H. 1955. Host parasite interaction in flax rust—its genetics and other implications. Phytopathology 45:680-685.
2. Hilu, H. M., and Hooker, A. L. 1964. Host-pathogen relationship of *Helminthosporium turcicum* in resistant and susceptible corn seedlings. Phytopathology 54:570-575.
3. Hooker, A. L. 1975. *Helminthosporium turcicum* as a pathogen of corn. Rep. Tottori Mycol. Inst. (Jpn.) 12:115-125.
4. Lim, H. M., Kinsey, J. G., and Hooker, A. L. 1974. Inheritance of virulence in *Helminthosporium turcicum* to monogenic resistant corn. Phytopathology 64:1150-1151.
5. Maeda, M., and Ishida, N. 1967. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J. Biochem. (Tokyo) 62:276-278.
6. Rohringer, R., Kim, W. K., Samborski, D. J., and Howes, N. K. 1977. Calcofluor: An optical brightener for fluorescence microscopy of fungal plant parasites in leaves. Phytopathology 67:808-810.
7. Shurtleff, M. C., ed. 1980. Compendium of Corn Diseases. The American Phytopathological Society, St. Paul, MN.
8. Wheeler, H. 1977. Ultrastructure of penetration by *Helminthosporium maydis*. Physiol. Plant Pathol. 11:171-178.