Calcofluor: An Optical Brightener for Fluorescence Microscopy of Fungal Plant Parasites in Leaves

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ABSTRACT

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Leaves infected with fungal plant pathogens were fixed and cleared in lactophenol/ethanol and stained with Calcofluor White M2R New to impart fluorescence to fungal structures. The procedure did not interfere with autofluorescence of host cells that had become necrotic in response to infection and facilitated simultaneous observation of fungal development and host cell necrosis.

Additional key words: *Puccinia graminis* f. sp. *tritici*, *Melampsora lini*, *Erysiphe graminis* f. sp. *tritici*.

In histological studies of the interaction between wheat *(Triticum aestivum* L.) and stem rust (caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn.), it is desirable to visualize both necrotic host cells and fungal structures. Trypan blue or cotton blue have been used for this purpose, but have not proven practical for large numbers of observations because necrotic cells often are not well differentiated from unaffected cells. Since necrotic cells exhibit intense autofluorescence (4), they can be distinguished easily by fluorescence microscopy from weakly fluorescing, unaffected cells. However, the fungus causing the necrosis cannot be detected by this method. Reports on the use of Calcofluor [a fluorochrome of the diamino-stilbene-disulphonate type (2)] to stain fungi in culture (1, 3) and to label infection structures of *Cronartium ribicola* (5) suggested that the rust fungus could be visualized in leaf tissue after staining with this compound. The Calcofluor technique (3) was modified by controlling the hydrogen-ion cencentration of washing and staining solutions. The modified technique permitted simultaneous observations on the development of the fungus and on host cell necrosis in whole leaves of wheat.

Leaves were fixed and cleared by boiling for 1.5 min in lactophenol/ethanol (1:2, v/v) and stored overnight in this mixture at room temperature. Specimens then were washed twice for 15 min each with 50% ethanol, twice for 15 min each with 0.05 M NaOh, three times with water, and placed in 0.1 M Tris/HCl buffer, pH 8.5 for 30 min. They then were stained for 5 min with a 0.1% solution of Calcofluor White M2R New (Catalogue No. MR - 4391, American Cyanamid Co., Wayne, NJ 07470) in this buffer. This was followed by washing four times with water (10 min each) and once with 25% aqueous glycerol (30 min). The specimens were mounted in glycerol containing a trace of lactophenol as preservative and examined with a Zeiss Research Microscope equipped for fluorescence microscopy with transmitted light (XBO 150 w/Xenon high-pressure lamp; VX 150 Siemens power supply; BG 38 heat filter; BG 3 exciter filter with peak transmission between 320 and 400 nm; barrier filters 47 and 44; X10 Neofluar objective; X10 kpl ocular). In other trials the Zeiss epifluorescence equipment NXL was used and found superior for color photomicrography (HBO 50 light source; red suppression filter BG 38; exciter filter BP 390-440; chromatic beam splitter FT 460; barrier filter LP 475) because it gave a brighter field of view and because fungal structures could be distinguished more clearly in deeper layers of the host tissue. With epifluorescence equipment, germ tubes, appressoria, substomatal vesicles, hyphae, and haustorial mother cells of the stem rust fungus fluoresced a bright blue, but haustoria were not detected. Haustorial mother cells fluoresced more intensely than other fungal structures. Unaffected host cells displayed a green fluorescence, and necrotic cells were a bright orange-yellow. Staining with Calcofluor quenched some of the autofluorescence normally displayed by necrotic cells, but this occurred mainly in those cells that were not in association with the fungus. Of 574 necrotic cells detected by their autofluorescence in unstained leaves, 108 were not visible after staining with Calcofluor, but only five of these were in the proximity of fungal infections. Evidently, this quenching effect is of no importance in studies on the wheat/stem rust interaction.

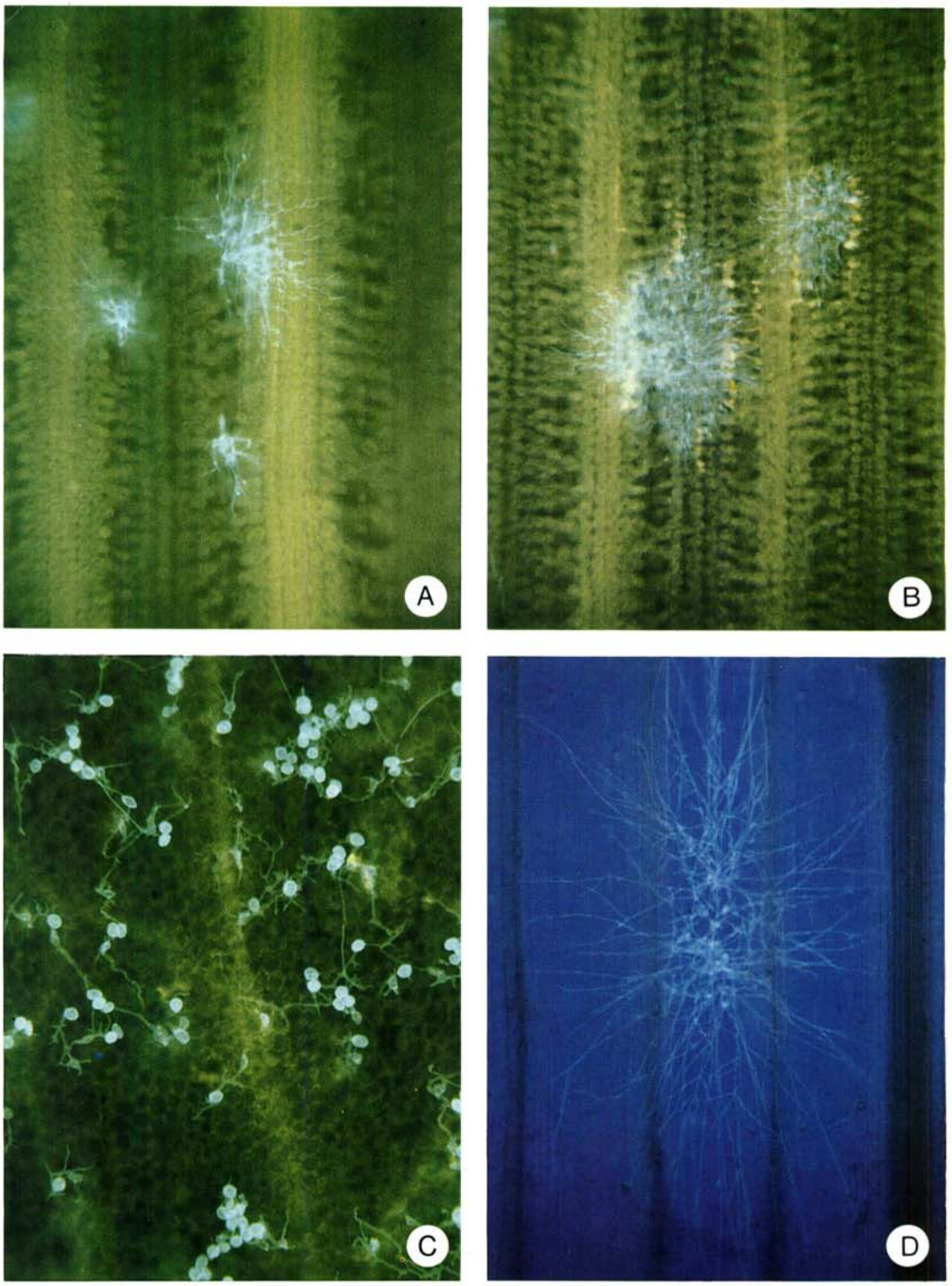

Fig. 1-(A to D). Fluorescence photomicrographs of leaf tissues infected with fungal parasites. Specimens were stained with Calcofluor to impart fluorescence to fungal structures and observed with Zeiss NXL epifluorescence equipment (A, B, C) or with Zeiss transmitted-light fluorescence equipment (D). Compatible **A**) and incompatible **B**) interactions at 19 C between wheat and *Puccinia graminis* f. sp. *tritici* shown 84 hr after inoculation. Note orange-yellow fluorescing necrotic host cells in the incompatible interaction (B) conditioned by the *Sr8* gene for resistance. **C**) Flax leaf infected with *Melampsora lini*. **D**) Wheat leaf infected with *Erysiphe graminis* f. sp. *tritici*.

Although we have used this method mostly on wheat leaves infected with stem rust (Fig. 1- A, B), preliminary trials indicated that it can be used in studies of flax rust [*Melampsora lini* (Ehrenb.) Lev.] (Fig. 1-C) and powdery mildew of wheat (*Erysiphe graminis* DC. ex Mérat f. sp. *tritici* Marchal) (Fig. 1-D). Uredospores, germ tubes, and appressoria of flax rust fluoresced brightly, but fungal structures in the host tissue appeared indistinct. Staining with Calcofluor imparted fluorescence to all structures of the mildew fungus, including haustoria. These could be seen more clearly after removal of the surface mycelium by brushing. The technique was not satisfactory on *Pyrenophora trichostoma* (Fr.) Fckl. in wheat, presumably because the fungus-associated melanin in the affected host cells absorbed much of the light required for fluorescence excitation.

LITERATURE CITED

1. DARKEN, M. A. 1961. Applications of fluorescent brighteners in biological techniques. Science 133:1704-1705.
2. HUGHES, J., and M. E. MC CULLY. 1975. The use of an optical brightener in the study of plant structure. Stain Technol. 50:319-329.
3. MAEDA, H., and N. ISHIDA. 1967. Specificity of binding of hexopyranosyl polysaccharides with fluorescence brightener. J. Biochem. (Tokyo) 62:276-278.
4. MARTE, M., and P. MONTALBINI. 1972. Microfluorescenza in foglie di fagiolo suscettibile e resistente alla ruggine. Phytopathol. Z. 75:59-73.
5. PATTON, R. F., and D. W. JOHNSON. 1970. Mode of penetration of needles of eastern white pine by Cronartium ribicola. Phytopathology 60:977-982.

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Page 500, Fig. 9 and 10: The graphs were placed over the wrong captions and should be interchanged.