Fluorescence Microscopy of Rapeseeds Invaded by Fungi

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ABSTRACT

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A modified acridine orange staining procedure was developed for examining sections of infected rapeseeds by fluorescence microscopy. Differentiation between seed and fungal structures was improved by prestaining with either malachite or methyl green to quench excessive fluorescence. These procedures permitted a comparison of growth and invasion patterns of *Aspergillus amstelodami, Penicillium verrucosum* var. *cyclopium,* and *Verticillium lecanii* into seeds of *Brassica campestris* 'Candle.' Variation in seed structure, such as heavy mucilage deposition, was also visualized by the modified acridine orange method. These differences in seed structure appeared to affect the timing of fungal penetration. Mycelial growth on the seed surface was most clearly differentiated by using ethidium bromide.

Additional key words: seed storage.

Rapeseed, *Brassica campestris* L. or *B. napus* L., is the most important oilseed crop in Canada, valued in the 1978/1979 crop year at $940 million U.S. (2). Seeds, stored in bulk, can spoil rapidly when the moisture content and temperature favor mold development. The predominant molds associated with stored rapeseeds in Manitoba are members of the *Aspergillus glaucus* group, *Penicillium verrucosum* var. *cyclopium* (Westling) Samson et al and *Cephalosporium acremonium* Corda, subsequently reidentified as *Verticillium lecanii* (Zimm.) Viégas (16).

Previous studies have examined the fine structure of rapeseeds (21,24,25) and the invasion of mature fungus-free rapeseeds by a member of the *A. glaucus* group, *A. amstelodami* (Mang.) Thom and Church (17). Fluorescence microscopy has been used to study the distribution of biochemical constituents in plant tissues (6,14), and the fungal invasion of leaves, stems (4,20), and seeds (12). Fluorescence techniques are more sensitive, produce higher contrast, and require less time than brightfield staining methods (6).

The objective of this work was to develop a fluorescence microscopy technique for differentiating fungal and rapeseed host tissues.

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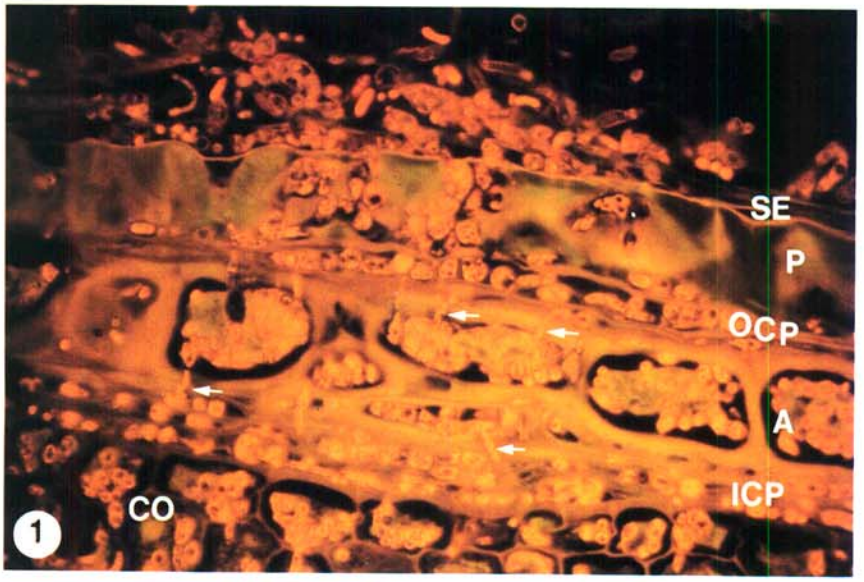
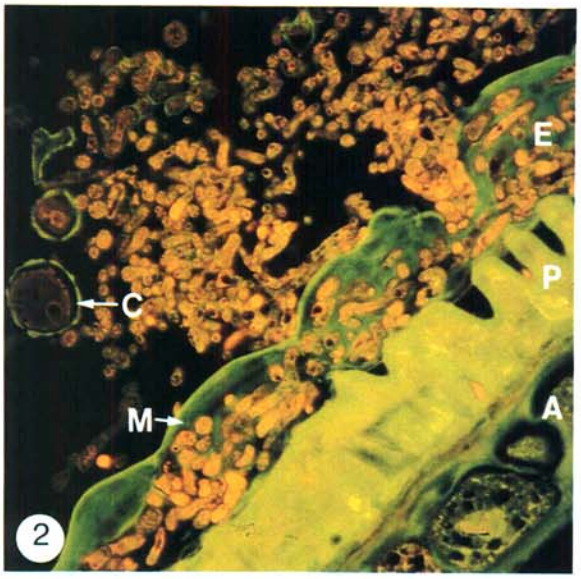
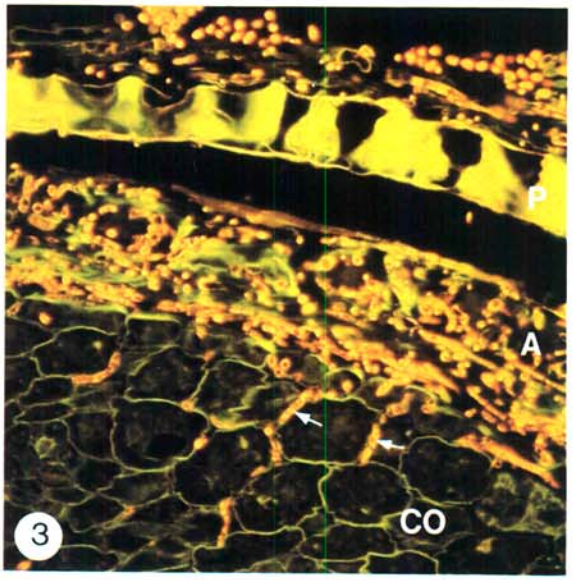
MATERIALS AND METHODS

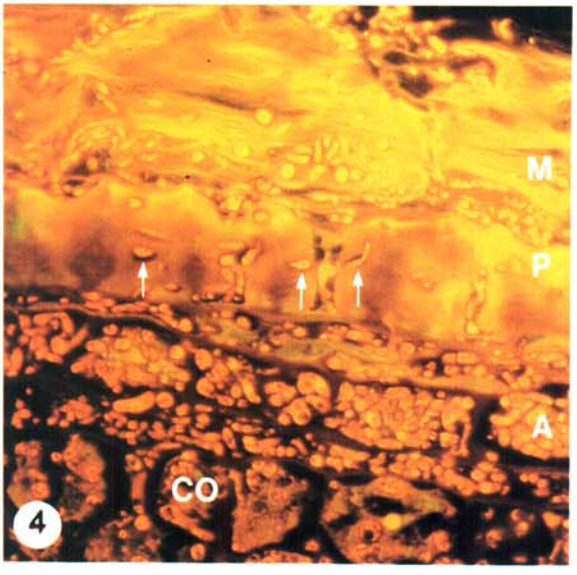
Production of fungus-free seed. Seeds of certified grade No. 1 *Brassica campestris* L. 'Candle' were sown in moist soil in 15-cm-diameter pots. The photoperiod was 16 hr (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) with day and night temperatures of 22 and 17 C, respectively. Cross pollination with camel's hair brushes was carried out several times during flowering to ensure seed set. Senescing leaves were removed to reduce seed and pod infection by preharvest fungi and watering was reduced near plant maturity. Whole pods were harvested unopened and surface sterilized by washing in 6% NaHClO for 1 min without rinsing. Seeds were plated on SFP medium (filter paper moistened with 7.5% NaCl solution) (15) and only those showing no microbial growth after 10 days were used in subsequent studies. SFP medium supports the growth of seedborne fungi while preventing seed germination. Use of the medium ensured that invading fungi were not able to enter the seeds through germination cracks. Prolonged exposure to SFP may weaken or kill the seeds, but it was considered that the advantages of the medium for evaluating fluorescence microscope studies outweighed the disadvantages.

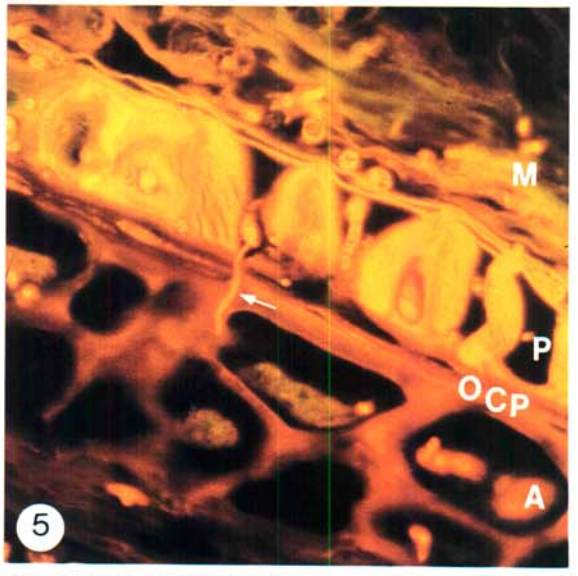
Preparation of fungal isolates and inoculation of seeds. Isolates A141 of *A. amstelodami*, P410 of *P. verrucosum*, and V2 of *V. lecanii* used in the study were obtained from rapeseeds grown in Manitoba and Saskatchewan. The three isolates were obtained from selected seeds incubated on SFP medium. Subcultures were



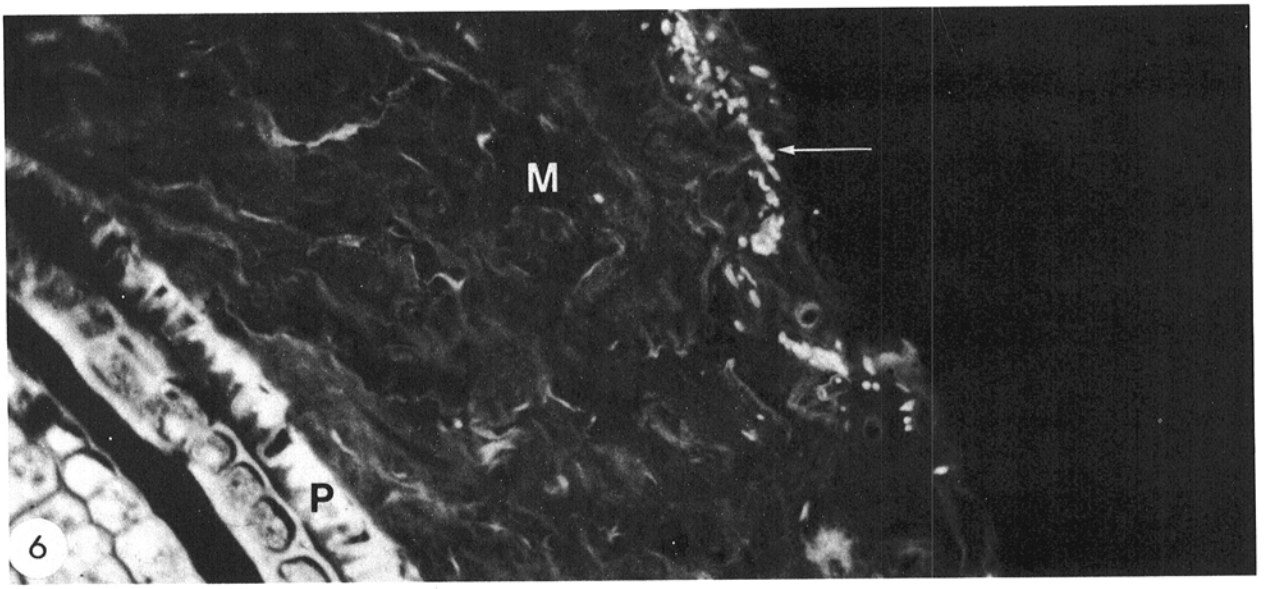


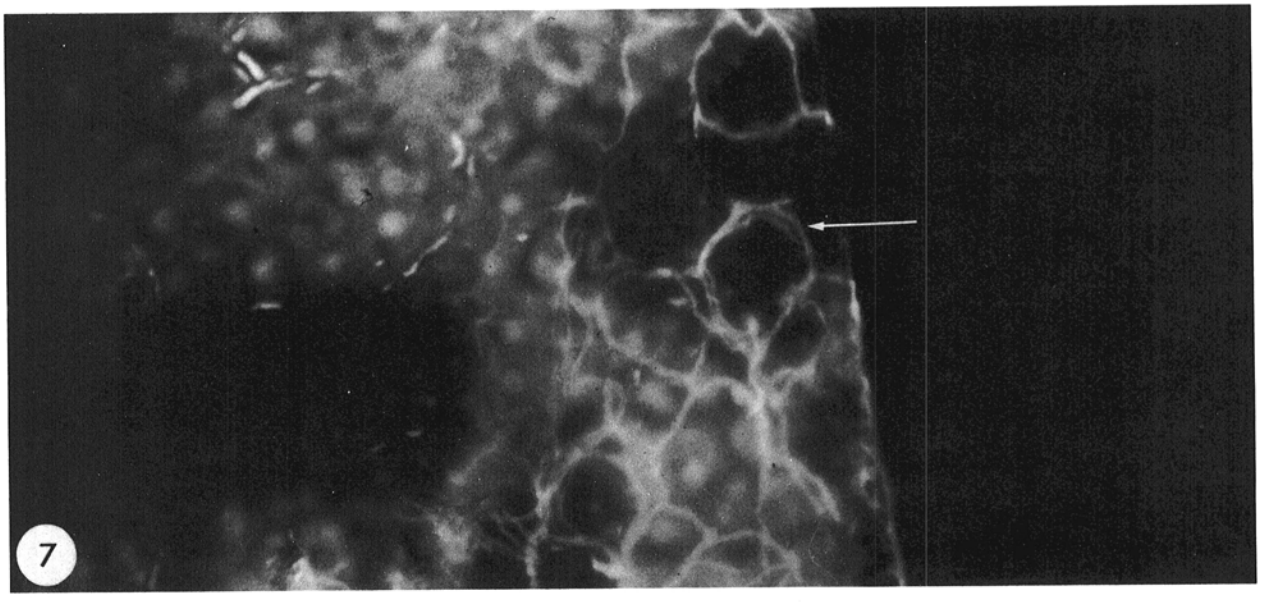


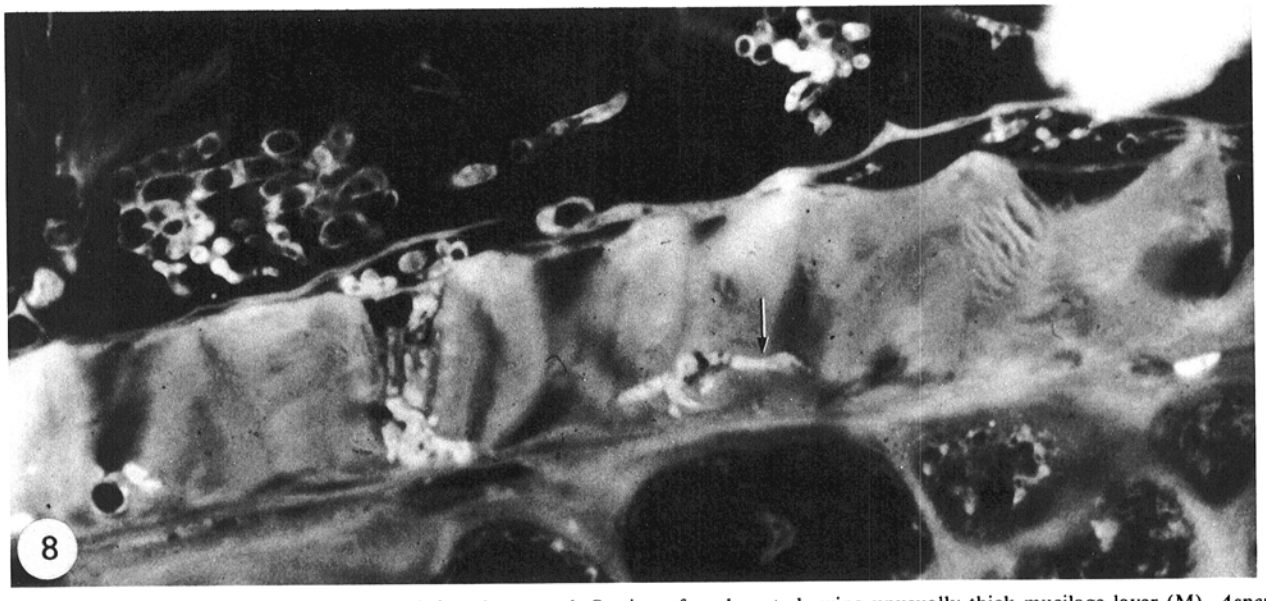




Figs. 1–5. Fluorescence microscopy of fungus-infected rapeseed sections stained with acridine orange. Seed coat layers are: epidermis (E), subepidermis (SE), palisade (P), and outer crushed parenchyma (OCP). Closely associated with the seed coat is the aleurone layer (A) and inner crushed parenchyma (ICP). Internal to the seed coat is cotyledonary tissue (CO). Mucilage (M) may or may not be present in the epidermis. **1,** *Aspergillus amstelodami* hyphae in seed tissues 3 days after inoculation. Penetrations (arrows) from outer crushed parenchyma to aleurone layer and from aleurone layer to inner crushed parenchyma are apparent. Prestained with malachite green (×910). **2,** *A. amstelodami* hyphae in seed coat 4 days after inoculation. Hyphae were abundant within the mucilage but rare in deeper layers. Conidiogenous tissue (C) showed that the fungus had completed its asexual life cycle. Prestained with malachite green (×575). **3,** *Penicillium verrucosum* hyphae in seed tissues 7 days after inoculation. Hyphae are especially abundant in the mucilage, aleurone layer, and crushed parenchyma. Growth within the cotyledon (CO) is intercellular (arrows). Prestained with malachite green (×575). **4,** *Verticillium lecanii* in all seed tissues 11 days after inoculation. The abundance of hyphae in the lower part of the mucilage and its lateral growth (arrows) through the palisade was typical. Halos were present around invading hyphae. Prestained with methyl green (×910). **5,** *A. amstelodami* hypha (arrow) penetrating aleurone cell from palisade. Prestained with methyl green (×910).







Figs. 6–8. 6, Fluorescence microscopy of a fungus-infected rapeseed. Section of seed coat showing unusually thick mucilage layer (M). *Aspergillus amstelodami* hyphae (arrow), 4 days after inoculation, were visible only at the surface of the mucilage. Stained with malachite green and acridine orange (×232). **7,** Face view of a strip of rapeseed seed coat showing surface growth of *A. amstelodami* in circular patterns (arrow). Stained with ethidium bromide (×290). **8,** Section of seed coat showing lateral growth of *Penicillium verrucosum* hyphae in the palisade (arrow). Stained with malachite green and acridine orange (×1,267).

grown on Czapek agar in tubes for 10 days at 25 C. The cultures were shaken with 5 ml of 0.6% aqueous Tween-20 (polyoxyethylene sorbitan monolaurate) for 30 sec to obtain a conidial suspension of $1.5–2.0 \times 10^6$ spores per milliliter. One microliter of suspension was added to individual fungus-free undamaged rapeseeds in petri dishes containing filter paper moistened with 2.5 ml of SFP medium. Seeds were incubated in the dark in plastic bags at 22 C and all seeds with visible fungal growth were removed for fixation after 2, 3, 4, 7, and 11 days of incubation. As a control, seed samples were incubated under similar conditions, but without fungal inoculum.

Preparation of seeds for microscopy. Incubated seeds were halved and fixed in 10% acrolein in tap water at 20 C for 4 hr. The fixative was decanted and specimens were washed twice with tap water for a total of 10 min. Specimens were placed in dry vials, dehydrated with acidified 2,2-dimethoxypropane (18), washed three times with absolute ethanol for a total of 45 min, and stored in absolute ethanol at −18 C.

Prior to being embedded, specimens were washed twice at room temperature with absolute ethanol for a total of 20 min. A mixture of 75% absolute ethanol and 25% cross-linked methacrylate (composed of 14 ml methyl methacrylate, 84 ml butyl methacrylate, 5 ml divinyl benzene, and 1 g benzolperoxide) was then added. After 2 hr of slow rotation, the mixture was replaced by a mixture of 25% absolute ethanol and 75% methacrylate. After 4 hr of rotation, the mixture was replaced by pure methacrylate. After 15 hr of rotation, the liquid was replaced by fresh methacrylate and vials were rotated for an additional 7 hr. Each specimen was placed in pure methacrylate in an oven-dried gelatin capsule. Polymerization was carried out in partial vacuum at 48 C for 24 hr. Sections 2 μm thick were cut on a LKB pyramitome fitted with a glass knife. They were placed in a drop of water on a gelatin-coated slide (9). Slides were placed on a slide warmer in a xylene-saturated atmosphere at 50 C.

Strips of seed coat from fungus-free and infected seeds were fixed for 3 min in Farmer's fixative (11), washed in water, and stained with 0.001% aqueous ethidium bromide for 1 min.

For observation of induced fluorescence, a modification of a technique by Alexander (1) was used. Sections were stained for 2 min with either 0.5% aqueous malachite green or methyl green, then counterstained for 20 min with 0.001% acridine orange in a boric acid-borax buffer, pH 8.6. Some sections were stained for 30 min with either 0.001% acridine orange or other stains (6–8,13,19,23) to explore the utility of the other stains. After washing in buffer, sections were air-dried and mounted in immersion oil (Type B, viscosity CS 1250, Cargille Labs., Cedar Grove, NJ 07009). For observation of autofluorescence, fixed seed coat strips and sections were mounted in immersion oil. Presence of lignified material was tested by staining sections with phloroglucinol (11).

Microscopy and photomicrography. Sections were examined with a Carl Zeiss model 14 microscope equipped with an IV Fl epi-fluorescence condenser, a mercury lamp, and Neofluar objectives. Stained seed coat strips and sections were observed through Zeiss filter set 07 consisting of exciter filter G 436, chromatic beam splitter FT 510, and barrier filter LP 515. For autofluorescence, filter sets 01, 07, and 13 were used. Photomicrographs were prepared by using Kodak Ektachrome 64 and 200 ASA daylight type film.

RESULTS

The seed coat of a mature uninfected rapeseed consisted of an epidermis, subepidermis, palisade, and crushed parenchyma. The aleurone and another zone of crushed parenchyma were closely associated with the seed coat. The epidermis sometimes contained layers of mucilage (24) of variable density as indicated by the intensity of stain. When the outer epidermal cell walls were broken, the mucilage layers spread into the surrounding medium and attained a thickness of up to 10 times the thickness of the palisade (Fig. 6). The phloroglucinol test indicated that the radial walls of the palisade were heavily lignified compared to the cell walls of the other tissues.

Induced fluorescence in seed coat strips. The seed surface, after staining with ethidium bromide, was seen as a network of green palisade cell walls with orange hyphae growing across or down into lumina. Initial hyphae were long and branched or clustered, with a tendency to grow in circular patterns (Fig. 7).

Induced fluorescence in seed sections. The best differentiation of fungal and seed tissue was obtained with a combination of either malachite green (Figs. 1–3) or methyl green and acridine orange (Figs. 4 and 5). With either technique, hyphae fluoresced orange and cell walls green. Of the single stains tested (6–8,13,19,23), acridine orange differentiated seed and fungal tissues, but seed tissue fluorescence was excessive. Other stains used were less satisfactory.

Autofluorescence. No marked differences in autofluorescence were observed between hyphae and seed tissues.

Invasion route. The three species of fungi traversed the seed coat tissues and entered the cotyledons. There were some variations in the occurrence of the fungi in particular tissues and in their routes and timing of invasion. In all cases, the hyphae fluoresced differentially from host tissue, which permitted following the invasion route.

Hyphae proliferated in the mucilage (Figs. 1 and 2), but sometimes were present only between the mucilage blocks. Invasion of the palisade occurred mainly in the lumina and thick radial, and to a lesser extent, lateral walls (Figs. 4 and 8). Frequently a halo of less densely staining material or a clear area was seen around the hyphae in the radial walls (Fig. 4). Once in the outer crushed parenchyma, hyphae proliferated laterally between the crushed cells (Fig. 1). Hyphae penetrating the aleurone cells were initially quite thin (Fig. 5). Sometimes hyphae in the aleurone were surrounded by a halo of less densely staining material or a clear area (Fig. 1). Hyphae in the cotyledons were both intercellular and intracellular (Figs. 3 and 4).

Variation in growth patterns. Differences in growth patterns among fungi were also revealed by using induced fluorescence. For instance, *A. amstelodami* showed rapid surface growth and could complete its life cycle without penetrating below the mucilage. In the absence of mucilage, sporulation still occurred without penetration of living aleurone cells. *V. lecanii* and *P. verrucosum* were not seen to complete their asexual life cycles before penetrating the inner seed coat layers. *P. verrucosum* showed the most rapid invasion of the physiologically active lipid-rich aleurone and cotyledon tissues. *V. lecanii*, on the other hand, exhibited slow initial growth on the seed surface, then rapidly penetrated through the entire seed coat and into the aleurone cells.

Variation in seed structure. Differences in seed structure affected the timing of fungal penetration. These were discernible by induced fluorescence. For instance, unusually dense mucilage (Fig. 6) appeared to delay penetration of cell layers beneath the epidermis.

DISCUSSION

Calcofluor White M2R (20) and Uvitex (3) are the stains most frequently used in fluorescence microscopy to differentiate plant tissue and invading fungal hyphae. Acridine orange has been used as a fluorescent stain for differentiating living and dead bacteria (22) and for examining fungi in culture (10), but has not been reported in studies of plant invasion by fungi. In the present work, only acridine orange gave a distinct color differentiation between invading hyphae and seed tissues, but this was accompanied by excessive fluorescence of the seed coat. Host tissue fluorescence was reduced by prestaining the sections with malachite green or methyl green before staining them with acridine orange, resulting in improved color differentiation. Similar quenching of excessive fluorescence with methyl green was recently obtained by Franklin and Filion (5) who used an acridine orange-methyl green combination stain to differentiate nucleoli in plant and animal cells.

The present work has determined the suitability of a fluorescent staining technique for following invasion of several fungi into rapeseeds. This technique will be used in future studies of fungal invasion of seeds under conditions more closely approximating those within storage bins.

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