Use of Dual-Stain Fluorescence Microscopy to Observe Antagonism of *Pyrenophora tritici-repentis* by *Limonomyces roseipellis* in Wheat Straw

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ABSTRACT

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A procedure was developed for differential fluorescence staining of the wheat tan spot pathogen, *Pyrenophora tritici-repentis*, and its antagonist, *Limonomyces roseipellis*. *P. tritici-repentis* was stained specifically by indirect immunofluorescence with the green fluorochrome FITC, and both fungi were stained red with a nonspecific lectin-conjugated fluorochrome (TRITC). Toluidine blue-O was used as a counterstain for observation of TRITC staining. In dual-stained sections of autoclaved wheat straw that was colonized by the two fungi, *L. roseipellis* was observed to grow in close association with and to penetrate hyphae of *P. tritici-repentis*.

Additional keyword: Triticum aestivum.

Wheat (*Triticum aestivum* L.) grown under continuous, reduced-tillage culture is exposed to diseases, the causal agents of which persist in infested crop residue. One such residue-borne pathogen is *Pyrenophora tritici-repentis* (Died.) Drechs., an ascomycete that causes tan spot on winter wheat in the central United States (6,17), as well as in several other wheat-growing regions of the world (14,19).

Pseudothecia of *P. tritici-repentis* are produced during saprophytic growth of the pathogen in infested straw following harvest; ascospores constitute primary inoculum for tan spot epidemics. Disease severity is correlated with the amount of primary inoculum present in the field (1,15). If the development of primary inoculum of *P. tritici-repentis* in residue could be suppressed, one could reduce losses from tan spot while retaining the soil-conservation benefits of reduced-tillage wheat culture. One potential strategy for reducing inoculum production is biological control by the action of straw-colonizing antagonists.

Limonomyces roseipellis Stalpers & Loerakker is a basidiomycete that suppresses production of pseudothecia by *P. tritici-repentis* in infested wheat tissue (10; Pfender, Sharma, and Zhang, *unpublished*). In an effort to understand the mechanisms by which this antagonist interacts with *P. tritici-repentis*, the staining technique reported herein was developed and used to visualize the hyphae of these two fungi as they interact in straw tissue. Studying the spatial relationships and interaction of these fungi in plant tissue should provide information on possible spatial partitioning of the resource between the fungi and also show the nature of their interaction under spatial constraints and nutritional conditions similar to those in the natural habitat.

Visualization of fungal hyphae in plant tissue, sometimes difficult with conventional transmitted-light microscopy, is often improved by the use of fluorochromes and fluorescence microscopy. For example, fluorochrome-conjugated wheat germ agglutinin (a lectin) aids visualization of fungal hyphae in plants by its preferential binding to the chitin residues in fungal walls (8). Calcofluor, which binds to polysaccharides in fungal walls and fluoresces under ultraviolet light, has recently been used to view hyphae of *Bipolaris* spp. in whole-mounts of maize tissue (20). A common problem with fluorescence staining of fungi in plant tissue is host autofluorescence, which may obscure the fungal hyphae. Trese and Loschke (20) overcame this problem by the use of enzymatic degradation of stained host cellulose. The problem is largely avoided by the use of the fluorochrome fluorescein; under the appropriate excitation wavelengths, fluorescein appears yellow-green, whereas host autofluorescence, chiefly of lignin, appears yellow. In observing the fluorochrome tetraethylrhodamine, autofluorescence of lignified host tissue is a severe problem.

To view the mycelial interaction of two fungi microscopically, it is necessary to distinguish the fungi from one another. Because hyphae of most fungi are generally similar in appearance, antibody-based techniques have been developed to specifically stain selected fungi, and thus distinguish them from other fungi, in situ. An indirect-antibody method is generally used in which the antibody is allowed to bind to the hyphae and is then visualized by means of a chromogenic enzyme system (4,22) or a fluorochrome (2,3).

Our goal was to observe growth of *P. tritici-repentis* and *L. roseipellis* in wheat straw tissue and to microscopically distinguish their hyphae from one another as they interact. For this purpose, we developed a technique for dual fluorescence staining. An antibody-based FITC (fluorescein isothiocyanate) stain was used to visualize *P. tritici-repentis*, a lectin-conjugated TRITC (tetraethylrhodamine isothiocyanate) stain was used for *L. roseipellis*, and a counterstain was used to overcome autofluorescence problems in viewing the TRITC-stained hyphae.

MATERIALS AND METHODS

Cultures. Isolate 6R180 of *P. tritici-repentis*, isolated from diseased wheat in Kansas, was used in the study. Isolate 3T163 of *L. roseipellis*, which is antagonistic to *P. tritici-repentis* (10,12), was isolated from reduced-tillage wheat residue in Kansas. The cultures were maintained on one-fourth strength potato-dextrose agar at 4 C; the isolate of *P. tritici-repentis* was renewed at 6-mo intervals by reisolation from inoculated, symptomatic wheat plants.

Antibody preparation. Mycelium of *P. tritici-repentis* was grown for 9 days in the dark at 22 ± 2 C on washed, autoclaved cellophane (type 215 PUT80, Flexel Inc., Atlanta, GA) placed on the surface of dilute carrot juice agar (CJA; 15 g of agar and filtered broth from 17.5 g of autoclaved carrots per liter). The mycelium was scraped from the cellophane and dried under a stream of sterile air, frozen in liquid nitrogen, and ground to

a fine powder in a mortar and pestle. The powder was then suspended in sterile phosphate buffer (0.01 M, pH 7.6) and pelleted by centrifugation, treated for 30 min with formalin acetic acid (FAA; 50 ml of 95% ethanol, 5 ml of glacial acetic acid, 10 ml of 38% formaldehyde, and 35 ml of distilled water) diluted 1:9 with phosphate buffer, and rinsed repeatedly by centrifugation in sterile phosphate buffer. After resuspending in buffer, the mycelial preparation was sonicated at 100 W with a Braun Sonic Model 1510 (Fisher Scientific, Pittsburgh, PA) for 30 10-sec intervals with 40 sec of cooling on ice between intervals.

The mycelial suspension (approximately 10 mg dry weight per milliliter of buffer) was mixed 1:1 with Freund's complete adjuvant, then injected into each of three white leghorn chickens. Chickens were chosen as the animal for antibody production in order to obtain relatively large amounts of antibody. Multiple injections, totaling 1 ml per bird, were made subcutaneously in the neck and intramuscularly in the breast. A booster injection (in which incomplete adjuvant replaced complete adjuvant) was made 1 wk later; multiple injections totaling 1 ml were made intramuscularly in the breast of each bird. Eggs were collected daily and stored under refrigeration. Antibodies were extracted from the yolks of eggs that had been produced 34–36, 44–46, 54–56, and 64–66 days after the first injection. Preimmune eggs were collected the day before the first injection.

Immunoglobulins were extracted from egg yolks by the method of Polson et al (13), as modified by Young (21). The yolk was mixed with 2× its volume of 0.01 M chilled phosphate-buffered saline (PBS), pH 7.4 (8 g of NaCl, 0.2 of g of KH_2PO_4, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KCl per liter), and 50% polyethylene glycol solution (PEG 10,000; Sigma P6667, Sigma Chemical Co., St. Louis, MO) was added to bring the PEG concentration to 3.75% (w/v). After centrifugation at 10,000 *g* for 20 min at 4 C, the pelleted albumins were discarded. The supernatant was then filtered through cotton wool, and 50% PEG 10,000 was added to bring the concentration to 12% PEG. The antibody was then pelleted by centrifugation at 10,000 *g* for 20 min at 4 C, the pellet was resuspended in a volume of PBS equal to half of the original yolk volume, and the resulting solution was dialyzed against sodium phosphate buffer (0.1 M, pH 8.0) containing 0.02% sodium azide. To purify the immunoglobulin preparation, it was first precipitated by mixing with an equal volume of 60% ammonium sulfate and centrifuging at 10,000 *g* for 20 min. This pellet was resuspended and dialyzed against 25 mM phosphate buffer (pH 8.0) containing sodium azide. The antibody was further purified by passing it through a DEAE cellulose column in the 25 mM buffer and collecting 2-ml fractions. Absorbance at 280 nm was measured, and fractions were pooled to produce early-, mid-, and late-peak pools of eluate.

Fractions were tested by indirect staining of the homologous (*P. tritici-repentis*) and heterologous (*L. roseipellis*) hyphae. The fungi had been grown on the surface of plastic petri dishes by placing a colonized plug of dilute CJA in the bottom of an empty dish and affixing water-saturated filter paper to the inside of the lid to maintain high humidity. After several days of growth, the agar plug was removed, the hyphae were treated with FAA, and the indirect fluorescent antibody stain was applied. Antibody preparation from the preimmune eggs was compared with that from eggs of various postinjection times.

The antibody preparation was absorbed with a heterologous fungus to reduce nonspecific staining. Because *L. roseipellis* produces copious amounts of extracellular slime in culture, it was unsatisfactory as an absorbant. Therefore, we used *Laetisaria arvalis* Burdsall, a basidiomycete from the same family as *L. roseipellis*. Mycelium of *L. arvalis* was grown for several days on cellophane over water agar, collected, air-dried, and ground in liquid nitrogen. It was treated with FAA and thoroughly rinsed before use as the antibody absorbant.

Preparation of colonized straw. Wheat (cultivar TAM 105) was grown to maturity (senescence) in a greenhouse, then the straw was harvested and stored until use. To colonize the straw with the two fungi of interest, the sheath was first removed from pieces of internode straw 50 mm long. These straw culms were autoclaved and placed on the surface of moist vermiculite (45 cm^3 vermiculite autoclaved with 40 ml of water) in petri dishes. Straws were then inoculated with the fungi. Inoculum consisted of a 2- × 2-mm piece of straw that had been autoclaved and placed for 2 days on an actively-growing culture of the appropriate fungus on dilute CJA medium. The 50-mm-long straws were inoculated at one end with *P. tritici-repentis* or with *L. roseipellis*. Some straws were inoculated with both fungi, one at either end. The inoculated straws were incubated on the moist vermiculite at 24 ± 2 C, and randomly chosen straws were taken daily for 11 days. After sampling, each straw was sliced longitudinally and the half that had been resting on the vermiculite surface was discarded. The remaining half-cylinder of the straw culm was then again split longitudinally to produce two strips, each of which was cut into 5-mm-long segments. The segments from one strip were sequentially plated on agar medium to determine the location of each fungus at the time of sampling, and the corresponding segments from the other strip were fixed, embedded, and stained.

Staining procedure. Tissue pieces were placed in FAA for several days then rinsed and embedded through a graded series of the water-soluble wax polyethylene glycol (16). The tissue was sectioned paradermally at 16-μm thickness. Individual sections were placed in small dishes containing phosphate buffer with 0.02% sodium azide and left overnight to remove the embedding wax.

Indirect fluorescent-antibody staining of *P. tritici-repentis* in the straw sections was carried out in the small dishes, according to the following schedule. Normal goat serum (Sigma, St. Louis, MO) was applied for 30 min to block nonspecific immunoglobulin G (IgG) binding. After rinsing several times with PBS, antibody (diluted 1:250 in PBS) was applied for 2 hr, then rinsed with PBS. FITC-conjugated anti-chicken IgG (Sigma, St. Louis), diluted 1:20 in PBS, was then added. After 1 hr, the sections were rinsed.

The nonspecific fluorescent stain and counterstain were then applied to stain all of the hyphae present (both *L. roseipellis* and *P. tritici-repentis*). Tissue sections were first rinsed in 0.02 M sodium benzoate buffer at pH 4.4 (0.5 g of benzoic acid and 0.58 g of sodium benzoate per liter distilled water), then the counterstain, toluidine blue-O (C.I. 52040; 0.5 mg/ml in sodium benzoate buffer), was applied for 10 min and rinsed with the benzoate buffer. These buffer and stain solutions were made fresh immediately before each use. Sections were then rinsed in PBS and treated for 1 hr with TRITC-conjugated wheat germ agglutinin (WGA-TRITC; Vector Labs, Burlingame, CA) diluted 1:400 in PBS (8). Sections were then thoroughly rinsed in PBS and mounted in modified Farrants medium (5). Farrants medium was made by dissolving 40 g of gum arabic in 80 ml of distilled water plus 40 ml of glycerol, filtering to remove debris particles, adjusting the pH to 9.5 with NaOH, and adding 0.02% sodium azide.

Stained tissue was examined with a Zeiss epifluorescence microscope equipped with two filter sets housed in a sliding carrier so that the tissue could be alternately viewed under two fluorescence conditions. For the FITC fluorescence, we used a BP450-490 emission filter and an LP520 barrier filter (Zeiss filter combination 48-77-09); for the TRITC fluorescence, we used BP546/12 and LP590 emission and barrier filters, respectively (filter combination 48-77-15).

Photographs were taken at ×250 and ×400. FITC fluorescence was photographed at 25–40 and 10–20 sec using Ektachrome 160 tungsten color film and Kodak T-Max 100 black and white film, respectively. For TRITC fluorescence, slightly shorter exposure times were used.

RESULTS AND DISCUSSION

Antibody and immunofluorescence staining. In staining tests of hyphae grown on the surface of plastic petri dishes, use of the immunoglobulin from preimmune eggs resulted in very faint staining of the fungal hyphae. Antibody from all three chickens injected with the mycelial suspension of *P. tritici-repentis*

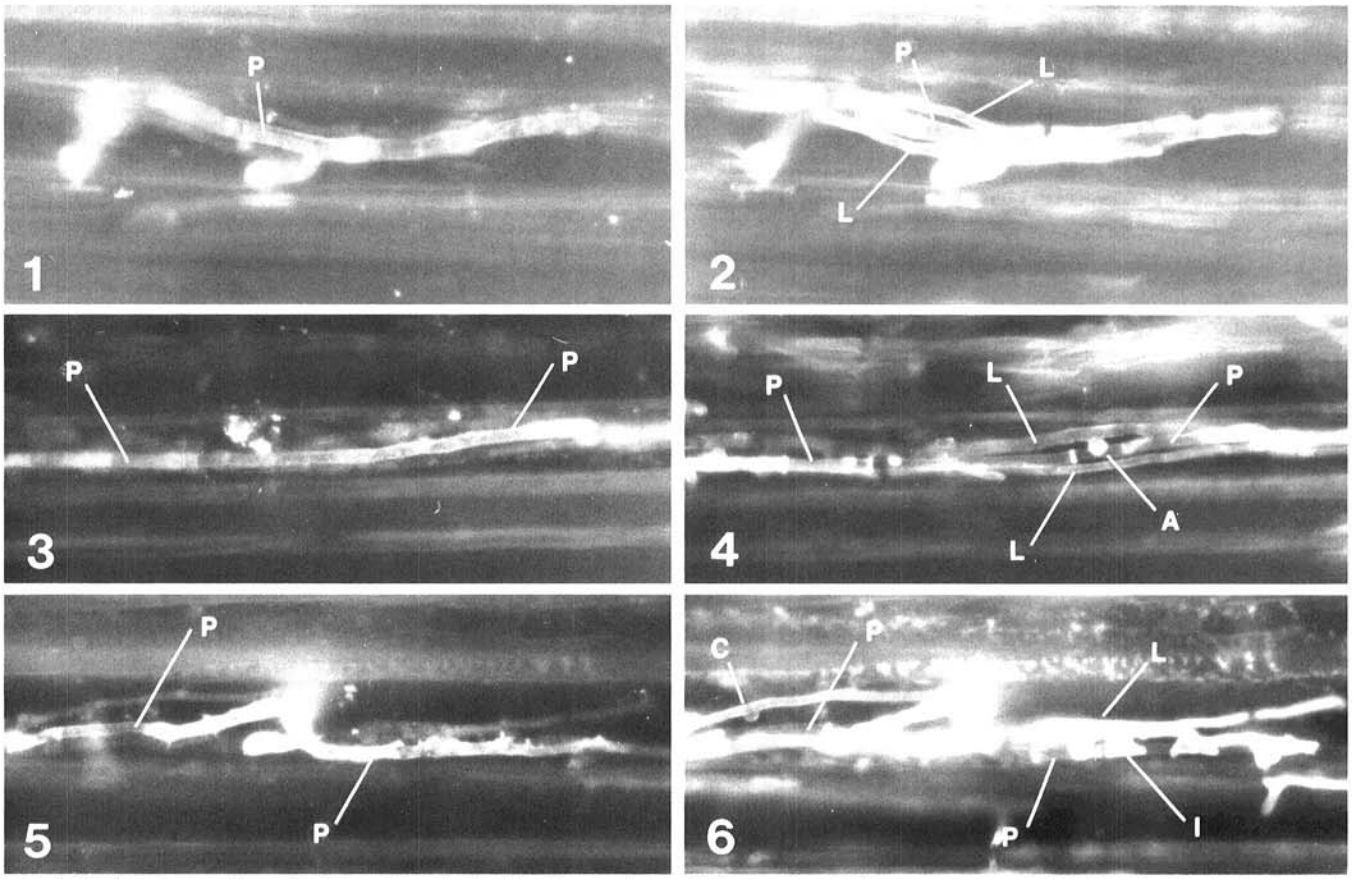
produced antibodies to the fungus, as indicated by staining of the hyphae. Staining intensity and specificity of antibody from one chicken was much better than that of the other two, and antibodies from this chicken were used exclusively for staining. Eggs with the best antibodies (brightest staining of *P. tritici-repentis* and least staining of *L. roseipellis*) were those collected approximately 45 days after the first injection; antibody from earlier-produced eggs stained *P. tritici-repentis* less brightly, and those from later dates stained *L. roseipellis* slightly more intensely.

The antibody was not highly specific for *P. tritici-repentis*; there was some cross-reactivity to *L. roseipellis*. After antibody absorption with *L. arvalis*, the antibody no longer reacted with *L. roseipellis*. However, several other fungi that are common to wheat straw, such as *Septoria nodorum* Berkeley and *Cladosporium* Link:Fr. spp., still reacted with the antibody. Therefore, we could not use this antibody to distinguish *P. tritici-repentis* from other, unknown fungi in naturally infested straw. Nonetheless, this particular polyclonal antibody was useful to observe growth and interaction of *P. tritici-repentis* with *L. roseipellis* in artifically inoculated plant tissue. In other work (Pfender, King, and Rabe, *unpublished*) we found that antibody produced in rabbits was similarly nonspecific.

General fluorescent stain and counterstain. *L. roseipellis* was stained well by WGA-TRITC. The lectin also reacted with *P. tritici-repentis*, as expected, but was somewhat inconsistent. Melanized hyphae of the latter fungus did not stain well, perhaps because the chitin residues were protected by other wall constituents. In plant tissue, the use of a counterstain for the WGA-TRITC was mandatory, because the plant tissue autofluoresced strongly under the 546-nm excitation wavelength and was indistinguishable from the stained hyphae. Of several possible counterstains we tested, toluidine blue-O was the only one that was satisfactory. This dye was earlier reported to bind to lignin in plant tissues (9) and had been used as a counterstain for ultraviolet-stimulated fluorescence staining with aniline blue (18). We found that it rendered the plant tissue a deep red, contrasting with the brighter, yellow-red of the TRITC-stained hyphae. Although toluidine blue-O is easily lost from plant tissue in alcohols and organic solvents, it remained in the tissue with the water-based staining and mounting method reported here. It also tended to diffuse from the stained tissue and obscure the sections if they were placed directly into the Farrants mounting medium. This problem was alleviated by placing the sections briefly in some of the Farrants medium, then transferring them to fresh mounting medium on slides.

Dual-staining. *P. tritici-repentis* and *L. roseipellis* were distinguished from one another in sectioned wheat straw tissue by the staining procedure described. *P. tritici-repentis* stained green and also, erratically, red. *L. roseipellis* consistently stained red and never stained green. Thus, hyphae of *L. roseipellis* were visible with the TRITC-selective epifluorescence filter but disappeared under the FITC-selective filter. Hyphae of *P. tritici-repentis* were visible under the FITC-selective filter and sometimes also visible under the TRITC-selective filter (Figs. 1–6). Dual-stained sections from straw tissue that had been inoculated with *L. roseipellis* alone showed no FITC-stained hyphae. As an additional check on the method, the occurrence of clamp connections on some hyphae of *L. roseipellis* allowed us to verify that this basidiomycete did not stain with the antibody-linked FITC.

Affinity of the hyphae of *P. tritici-repentis* for the WGA-TRITC stain was generally greater when it was in intimate contact with *L. roseipellis*, perhaps because the latter fungus (which we had



Figs. 1–6. Dual-stained sections of wheat culm colonized by *Pyrenophora tritici-repentis* and *Limonomyces roseipellis*. L = hyphae of *L. roseipellis*, P = hyphae of *P. tritici-repentis*. Each pair of photographs (1–2, 3–4, 5–6) was taken from a single field of view but observed and photographed with either FITC fluorescence filters (1, 3, and 5) or TRITC filters (2, 4, and 6) in place. Hyphae that appear bright under FITC fluorescence were green, and hyphae that appear bright under TRITC fluorescence were yellow-red. Magnification for all photographs = ×660. **1–2,** Two hyphae of *L. roseipellis* growing in close association with *P. tritici-repentis*; **3–4,** *L. roseipellis* producing appressorium-like structures (A) against the hyphal wall of *P. tritici-repentis*; **5–6,** penetration of *P. tritici-repentis* by *L. roseipellis*. Invasion structures (I) and clamp connection (C) are visible on hyphae of *L. roseipellis*.

previously determined to be chitinolytic) (11) degrades hyphal walls of *P. tritici-repentis* and exposes the chitin residues.

Although the TRITC-stained hyphae were visually distinguishable from the counterstained plant cell walls, the sensitivity spectrum of the color photographic films we used made it difficult to record the distinction between the deep red (plant walls) and yellow-red (fungal walls). However, the distinction between FITC-stained *P. tritici-repentis* and TRITC-stained *L. roseipellis* is quite clear with color film; thus, double exposures with the FITC-selective filter and TRITC-selective filter enabled production of single photographs in which both colors appeared, showing the differentially stained fungi.

Interaction of *L. roseipellis* with *P. tritici-repentis*. As these two fungi grew toward one another in inoculated straw, they became closely associated spatially. *L. roseipellis* sometimes grew directly alongside and in contact with *P. tritici-repentis* (Figs. 1 and 2). We occasionally found points of contact between the two fungi at which *L. roseipellis* appeared to be forming appressorium-like structures against the wall of *P. tritici-repentis* (Figs. 3 and 4). We also found clear evidence that *L. roseipellis* had penetrated hyphae of *P. tritici-repentis* (Figs. 5 and 6). These latter interactions were commonly found in sections of straw where the two fungi had been in contact for no more than 24 hr, as determined from sequential plating of straw segments. Where the two fungi had been associated for a longer period of time, many hyphae of *P. tritici-repentis* appeared to be degraded, and some were internally colonized by *L. roseipellis*.

Utility of the dual-stain fluorescence method. With this technique, we have been able to observe the spatial relationships of these two fungi as they colonize sterilized plant tissue and the nature of their interaction (viz., penetration of *P. tritici-repentis* by *L. roseipellis*). Further studies to quantify growth patterns should thus be possible.

This same staining method, if applied with an antibody having greater specificity, could be used to study the organisms in naturally colonized material. In place of the combined indirect fluorescence staining and the general stain, one could use two antisera, one for *P. tritici-repentis* and another for *L. roseipellis*, each directly conjugated to one of the two fluorochromes. This would obviate the need for the lectin-based general stain, and allow color differentiation of two fungi even in the presence of other fungi.

In order to better observe the spatial relationships between the fungi, we cut fairly thick (16 μm) microtome sections. The use of water-soluble wax, which permitted thorough dewaxing of these thick sections and, thus, penetration of the aqueous stains, was important to the success of the method. Also, the use of toluidine blue-O as a counterstain was essential to use of the TRITC-conjugated stain in plant tissue. Other fluorochromes, such as the ultraviolet-light stimulated AMCA (7) may also be useful when they become available as conjugates of compounds with affinity for fungal hyphae.

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