Rapid Tentative Identification of *Rhizoctonia* spp. Associated with Diseased Turfgrasses

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ABSTRACT

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Fluorescence microscopy using the DNA-binding probe 4′,6′-diamidino-2-phenylindole (DAPI) was used successfully to determine number of nuclei per hyphal cell of *Rhizoctonia* spp. that cause patch diseases of turfgrasses. The procedure included growth of the hyphae from diseased tissue onto agar-coated microscope slides followed by rapid fixation with 3% formaldehyde. Samples were rinsed with distilled water and flooded with a 1-ppm solution of DAPI, then destained by flooding the sample with distilled water. Most of the water was then removed, a drop of glycerin was applied followed by a coverslip, and the sample was viewed using ultraviolet excitation with epiillumination and Neofluar objectives. Nuclei within isolates of *R. solani*, *R. cerealis*, *R. zeae*, and *R. oryzae* were clearly differentiated by the method. This method combines speed and reliability as a diagnostic tool.

Several *Rhizoctonia* spp. have been demonstrated to induce "patch" diseases of turfgrasses, including *R. solani* Kühn, *R. cerealis* van der Hoeven, *R. zeae* Voorhees, and *R. oryzae* Ryker (1,8). In transition zone climates, these diseases can occur on both cool- and warm-season turfgrass species (9).

There is variation in environmental conditions under which these species induce disease. *R. solani* generally is active under warm (24–28 C), humid conditions on cool-season grasses. However, *R. solani* AG-4 was active in cool (about 20 C), humid weather in the spring and fall on Tifton 419 bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burtt-Davy) in North Carolina (9). *R. cerealis* induces yellow patch disease of both cool- and warm-season turfgrasses under cool (10–20 C), humid conditions (1). *R. zeae* and *R. oryzae* have thus far only been identified as turf pathogens under warm to hot (>32 C) weather conditions (8). However, these are generalizations and disease occurrence associated with these different pathogens may overlap, hence the specific identification of the pathogen(s) is important.

At present, diagnosis of Rhizoctonia patch diseases is based on field symptoms, lesion characteristics, and identification of the *Rhizoctonia* species. Isolate identification is based on number of nuclei in cells, hyphal cytological characteristics, color of mycelium, and sclerotial characteristics (9). The primary characteristic that must be determined to distinguish *R. solani* from *R. cerealis* is number of nuclei per cell, because *R. solani* is multinucleate and *R. cerealis* is binucleate. *R. zeae* and *R. oryzae* also have been reported to be multinucleate but are distinguished from *R. solani* by the color of the mycelium in culture, the unique sclerotial characteristics, and a granular appearance of cytoplasm when viewed microscopically (9,10,12).

Numbers of nuclei within hyphal cells have been determined by rapid-staining procedures using trypan blue in lactophenol (2), aniline blue in glycerin (11), and safranin O in KOH (13); other methods have included HCL-Giemsa staining (5,6) and nuclear fluorescence with acridine orange (13). The rapid procedures have been criticized for lack of reliability for different isolates (3), and Giemsa staining, though reliable, is very time consuming (6). Acridine orange was reported to work well for distinguishing *R. solani* from binucleate *Rhizoctonia* but was not evaluated for *R. zeae* or *R. oryzae*, in which nuclei are very difficult to observe (13). Because turfgrasses may be infected by these four *Rhizoctonia* spp. (4,9), this study was initiated to evaluate a single rapid method to determine nuclear number, which could be used to aid in differentiating these pathogens.

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MATERIALS AND METHODS

Isolates of *Rhizoctonia* used in developing the technique were as follows: *R. solani* (AG-1) isolated from bluegrass in Connecticut, *R. cerealis* from bluegrass and bentgrass in Connecticut and North Carolina, *R. zeae* from tall fescue in North Carolina, and *R. oryzae* from rice (provided by F. Lee, University of Arkansas). One isolate of each species was grown on sterile moist fescue seed for 2 wk before inoculation of turfgrasses (9).

Kentucky bluegrass (*Poa pratensis* L.), and tall fescue (*Festuca arundinacea* Schreb.) were used as hosts and were grown in dense swards in pots in the greenhouse. Inoculations were made by placing 1 g (fresh wt) of infested fescue seed in the center of each pot of grass and covering with a plastic bag. Inoculated grasses were incubated in a 20–28 C environment in the greenhouse. Lesions appeared on the grasses about 4–7 days after inoculation. Small pieces of grass bearing lesions were excised, washed in sterile distilled water three times, blotted dry on sterile paper, and placed on glass slides that had been dipped in molten water agar (2%) to coat the slides. These slides were supported on glass rods placed in petri dishes. Five milliliters of sterile distilled water was placed in each petri dish to supply humidity and encourage fungal growth from the lesions. Other specimens were prepared similarly on slides but without the agar coating. In addition, grass blades with lesions were placed on water-agar petri dishes, and plugs of mycelium and agar were taken directly from the margins of active growth.

Fungal hyphae with characteristic acute branching of distally developing hyphae typical of *Rhizoctonia* were present in all cases about 18 hr after placement of lesions onto slides or media. The following protocol, adapted from Hoch and Staples (7), was used to stain *Rhizoctonia* nuclei: hyphae were fixed by flooding the hyphae with 3% formaldehyde for 2 min, then rinsed with sterile distilled water by flooding for 1 min. Hyphae were flooded for varying times (1–10 min, in 1-min increments) with 1 ppm of the fluorescent DNA-binding fluorochrome 4′, 6′-diamidino-2-phenylindole (DAPI) in distilled water, then rinsed for varying times (1–10 min, in 1-min increments) to remove excess DAPI from the agar and hyphae. Excess water was drained from the hyphae and a drop of glycerin was placed on the hyphae, then a cover glass was placed over the glycerin. The specimens were immediately viewed with ultraviolet light, using a Zeiss epiillumination system with Zeiss filter set no. 02

containing exciter filter G 365, dichromatic beam splitter FT 395, and barrier filter LP 420. Fungi were viewed and photographed with a Zeiss Neofluar 63× oil immersion objective and Ilford XP1 film at 800 ASA and developed at 800 ASA. *Rhizoctonia* spp. also were stained with acridine orange and viewed using appropriate ultraviolet excitation and filters after the method of Yamamoto and Uchida (13).

RESULTS AND DISCUSSION

Nuclei were easily differentiated using DAPI for all species of *Rhizoctonia* (Fig. 1). Nuclei of *R. solani* (Fig. 1A) and *R. cerealis* (Fig. 1B) were easily viewed in the cytoplasm, which appeared homogeneous. Nuclei of *R. zeae* (Fig. 1C) and *R. oryzae* (Fig. 1D) were also easily differentiated, but the cytoplasm was granular in appearance, with occasional distinct oil globules that did not fluoresce. Nuclei were distinguished within hyphae of *R. zeae* and *R. oryzae* rapidly and without doubt. Nuclei within these two species have been stained with difficulty using other methods (9,11). Septa could be observed by supplementing transmitted light with ultraviolet light. Varying the amount of transmitted light helped to distinguish nuclei as well as dolipore septa without moving the specimen or the objectives.

Intensity of fluorescing nuclei was optimal at 5 min of DAPI staining (in the absence of agar on slides) and 3 min of rinsing to destain or at 10 min of staining (in the presence of agar) and 4–5 min of rinsing to destain. The agar-coated slides were more effective than slides without agar, because the agar resulted in more abundant hyphal growth and provided support for the hyphae as they were manipulated through the staining procedure. However, the agar tended to absorb the DAPI, hence additional rinsing time was necessary to remove the excess stain. Staining nuclei using mycelial plugs from agar cultures also was successful, with a protocol of 10 min of exposure to DAPI and a water rinse of 5 min. Acridine orange gave satisfactory results, but the nuclei were less clearly distinguishable than similar preparations treated with DAPI.

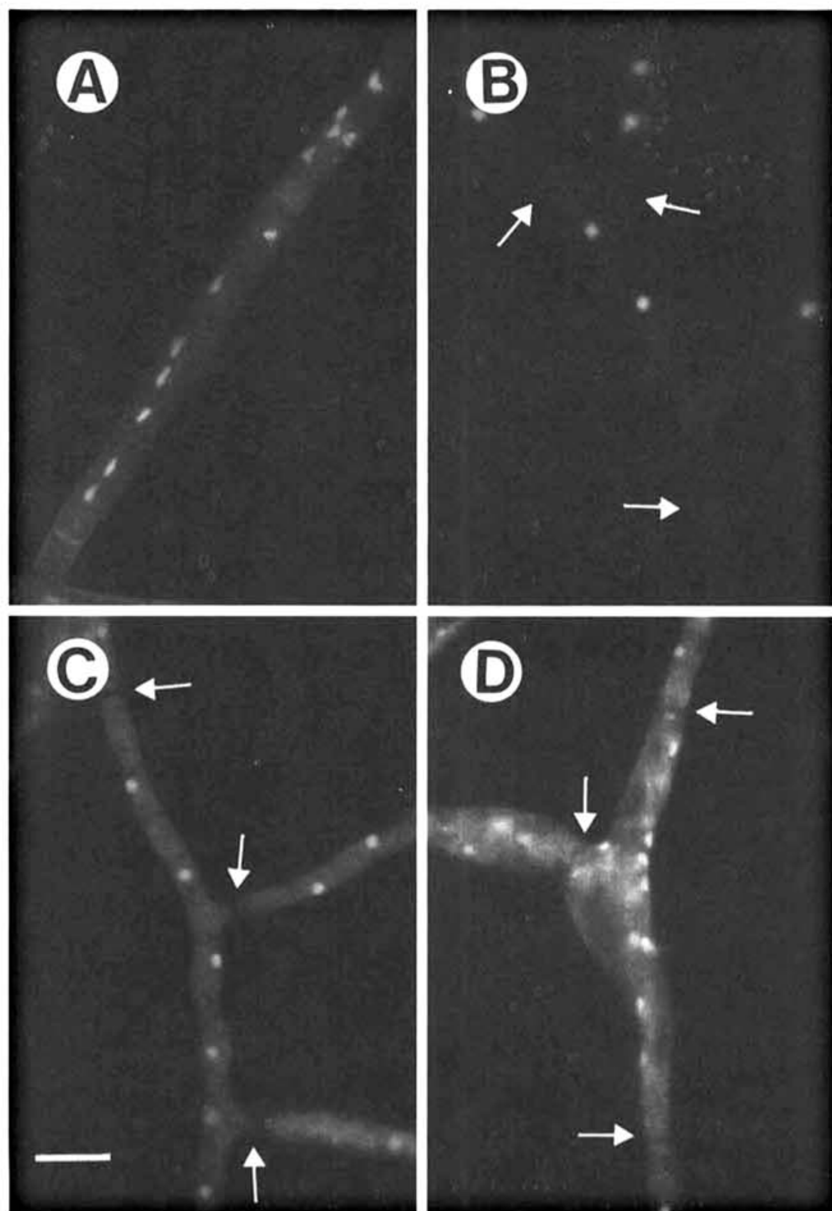

Fig. 1. Nuclear staining of *Rhizoctonia solani, R. cerealis, R. zeae,* and R. oryzae with DAPI: **(A)** *R. solani* showing seven nuclei in a single cell, **(B)** *R. cerealis* with binucleate hyphal cells, **(C)** *R. zeae* with multinucleate hyphal cells, and **(D)** *R. oryzae* with multinucleate hyphal cells. Arrows indicate location of septa. Scale bar = 10 μm.

This technique has been used on diseased turfgrass specimens submitted to the disease clinic at the Connecticut Agricultural Experiment Station. It was successful in rapidly distinguishing *R. solani* isolates from binucleate *Rhizoctonia* associated with diseased turf. By using the slide incubation technique or agar cultures coupled with DAPI staining, *Rhizoctonia* can be tentatively identified within 24 hr of receipt of the specimen. The limiting factor is the time required to induce sufficient mycelial growth for staining.

It should be noted that this method allows only tentative identification and will not differentiate *R. cerealis* from other binucleate *Rhizoctonia* that may be associated with turfgrasses. To date, anastomosis grouping with CAG 1 tester isolates is the only method to absolutely distinguish *R. cerealis* from other binucleate *Rhizoctonia* (1). Likewise, isolation and notation of cultural characteristics of suspected *R. zeae* and *R. oryzae* is required to aid in identification of these species. However, these fungi (but not *R. solani* or binucleate *Rhizoctonia*) react with phenolic substances such as lactophenol, in which the mycelium turns dark brown or black within 2–3 days after placement of the phenol onto the mycelium in pure cultures (9). This phenomenon has been associated with many (>50) isolates, appears stable, and may be useful in diagnosis.

The DAPI technique can be used as an aid in differentiating *Rhizoctonia* spp. in pathosystems other than turf diseases. At present, I am using the technique to rapidly separate *R. solani* from binucleate *Rhizoctonia* spp. isolated from strawberry roots. The DAPI-stained nuclei do not fade rapidly, thus a relatively large number of unknown isolates can be prepared at the same time. The method should prove useful for other studies in which exact counts of nuclei are needed.

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