Techniques

Practical Nuclear Staining Procedures for Rhizoctonialike Fungi

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ABSTRACT

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An HCl-Giemsa staining procedure and two rapid direct staining methods were used to stain nuclei of multinucleate and binucleate Rhizoctonialike fungal isolates. Isolates were cultured on thin layers of Difco potato dextrose agar (DPDA) in plastic petri plates. Disks cut from the cultures were transported through the HCl-Giemsa staining process in tissue carrier mounts. Stained disks were mounted in dark corn syrup. The DPDA cultures also were stained directly in the plates with either 0.5% aniline blue or 0.05% trypan blue in lactophenol. In either case, the mycelium to be stained first was wetted with an acidified wetting agent to facilitate staining. Cover slips were added, and the stained hyphae were microscopically examined in the plates with a dry × 40 objective. Of the staining methods tried HCl-Giemsa consistently gave better results.

Determination of the numbers of nuclei in vegetative cells aids in distinguishing between *Rhizoctonia solani* Kühn and Rhizoctonia-like fungi in the absence of the perfect state (6–8). Parmeter et al (8) investigated Rhizoctonialike cultures and separated them into groups with: (i) multinucleate vegetative cells and (ii) binucleate cells. In agreement with Flentje et al (4) all *R. solani* isolates were multinucleate. Parmeter and Whitney (7) reviewed much of this literature.

Collectively, the procedures for staining nuclei of *R. solani* and Rhizoctonialike fungi with HCl-Giemsa (1,2,4,8,10,13) seemingly have little interrelationship. Some of the procedures involve permanent mounting of stained preparations for detailed cytologic studies; for simple enumeration purposes, schedules can be less complex.

Other nuclear stains, as well as phase contrast microscopy, also have been used with *R. solani*, but reference will be limited to two rapid staining procedures suitable for doliform pore detection and nuclear staining: (i) an aniline blue staining technique reported by Tu and Kimbrough (12) and (ii) the method of Sanders et al (11) based on trypan blue staining. Burpee et al (3) recently published an expanded account of the latter method.

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The objective of this study was to make these staining procedures, especially HCl-Giemsa, more practical and reliable for routine nuclear staining as an aid to identification of *R. solani*.

MATERIALS AND METHODS

General. Rhizoctonialike isolates were cultured on Difco (Difco Laboratories, Detroit, MI) potato dextrose agar (DPDA) in 9-cm diameter plastic petri plates containing 7 ml of the medium. Transfers were made to one side of plates and, when growth was sufficient, a disk was cut in the colony with an 11-mm diameter cork borer. The disk then was transferred with a curved section lifter to a Tims® (Lab-Line Inc., Melrose Park, IL) deep-bottom tissue carrier (2.54 × 2.54 × 0.64 cm). The holes in the carriers were enlarged to 0.4 mm diameter to speed drainage. Groups of four or five carriers (number fitting readily into a 9-cm petri dish bottom) could be taken conveniently through the staining schedule together. The culture plates from which disks were removed were retained for subsequent rapid, direct staining of isolates in the plates with aniline blue and trypan blue stains.

HCl-Giemsa schedule. Culture disks were fixed in a 3:1 mixture of 95% ethanol and glacial acetic acid in a wide-mouth jar (9 × 11 cm) with lid for 10 min (volume was sufficient to cover the carriers completely, ie, 4–16 culture disks), transferred to (i) 95%

ethanol for 15 min, (ii) acetone for 20 min, (iii) 95% ethanol. Usually after overnight storage in 95% ethanol or after at least 15 min, the disks were transferred to petri dishes containing: (i) 70% ethanol for 15 min, (ii) 50% ethanol for 15 min, (iii) 25% ethanol for 15 min, and (iv) tap water for 15 min. Then disks were hydrolyzed in 1N HCl for 8 min at 60 C (in a covered storage dish, 10 × 8 cm). After hydrolysis, the disks were washed in three changes of distilled water, 5 min per change, placed in 1:1 buffer-distilled water for 5 min and then in full strength pH 6.8 buffer for 10 min (buffer consisted of a mixture of equal parts of 2.8 g Na_2HPO_4 in 1,000 ml of distilled water and 2.4 g of KH_2PO_4 in 1,000 ml of distilled water). Disks were stained for at least 2 hr and, more commonly, 15–18 hr (overnight) in Giemsa stain. The stain was prepared with three drops of commercial Giemsa stain solution per milliliter of buffer. Limited use was made of a stock solution prepared from dry powered Giemsa, according to Furtado's method (5). Volumes required to prepare the final staining solution varied with the source of concentrated Giemsa solution. Tissure carriers were then transferred to water in a petri dish bottom and rinsed cautiously with a slow stream of cold tap water for 1–2 min to avoid disk damage or loss and remove stain scum completely. Tissue carriers were then placed in buffer for 5–60 min; the time was determined by periodically checking differentiation of nuclei by microscopic examination (× 100–200 magnification) of disks floated in buffer. Culture disks were transferred to slides with a section lifter, and free liquid buffer was absorbed with bibulous paper. Then the disks were mounted in Karo® dark corn syrup (blue label).

The final Giemsa staining solution must be freshly prepared, and other reagents must be renewed regularly. After acetone treatment and during hydration, disks tend to float and oscillate rapidly; care must therefore be taken to avoid loss of identification of disks. Carriers can be stacked so as to retain the disks within them; deep liquid levels can be avoided during hydration.

Rapid staining in plates. Six to eight drops of wetting solution (1,000 ml of distilled water, 1 ml of Tween 20®, wetting agent, acidified with 1 ml of 85% lactic acid) were distributed on each of two locations per culture plate, avoiding areas of abundant aerial mycelium if possible, and spread and rubbed lightly with a bent spatula until the hyphae were thoroughly wetted. Those areas were stained with 1 drop of 0.5% aniline blue in lactophenol or with 1 drop of 0.05% trypan blue in lactophenol. Cover slips were placed over the treated areas and a small amount of unstained mycelium. After 5–30 min, these areas were examined microscopically using a dry × 40 objective. The gradations in staining intensity from unstained to deeply stained hyphae facilitated detection of cells with distinctly stained nuclei.

RESULTS AND DISCUSSION

The purpose of the nuclear staining was to differentiate between multinucleate and binucleate Rhizoctonialike isolates. Multinucleate and binucleate isolates were stained with trypan blue (Figs. 1 and 2, aniline blue (Figs. 3 and 4), and HCl-Giemsa (Figs. 5–8). HCl-Giemsa was definitely superior to trypan blue and aniline blue in reliability and quality. For example, the nuclei in the runner hyphae (Fig. 7) were stained clearly by Giemsa, but runner hyphae nuclei were much less frequently stained by the other stains. However, because Giemsa was much more time consuming (1–2) days vs. 5–30 min), rapid methods have a definite advantage for initial screening. Even if refrigerated, preparations stained by rapid methods generally lose quality with time, ie, overnight, and thus should be photographed soon after preparation to preserve details.

Doliform pore septa are an identifying characteristic of *R. solani* and related basidiomycetes (7). Aniline blue (12) and trypan blue (11) staining help to distinguish these septa. Giemsa also can aid in visualization of doliform pore septa (Fig. 5).

Many variations in technique were tried before this HCl-Giemsa schedule was developed. Culturing methods that depend on growth of Rhizoctonialike isolates on agar-coated slides (4), moist cover slips (1,2,), cellophane (8), or hyphal mats (13) were unreliable because hyphae were frequently lost, particularly during hydrolysis, before completion of a schedule. Use of Haupt's adhesive did little to improve retention of hyphae. Furthermore, hyphae in these thin cultures dried rapidly and became distorted during brief exposures to air. Dried hyphae did not recover turgor upon further processing and stained very darkly, and nuclei could not be observed. For detailed cytologic studies, the single-plane, sparse hyphae obtained by these techniques are useful, but they are poorly suited for routine processing of large numbers of cultures when only nuclear numbers need be ascertained.

The adoption of thin DPDA plates evolved from Saksena's (10) technique. Use of plates with a thin agar layer instead of attempting to slice a thin layer from an agar block cut from a culture was simpler and gave good results. The tissue carriers permitted more than one disk to be processed concomitantly and prevented handling damage of disks. Hyphae tended to be less vacuolated on PDA than on corn meal agar. More consistent results were obtained with the lengthened hydration series than with Saksena's (10) shortened schedule. Inclusion of acetone improved staining (1). Storage of culture disks in 95% ethanol for longer than overnight cannot be recommended generally, but disks stored over a weekend may still stain satisfactorily. Final mounted preparations tend to be rather thick, but areas for good observation can be located and photographed (Figs. 5–8). The nuclei (Fig. 8) have good contrast and stand out clearly, although the hyphae are darkly stained. Overstained preparations can be differentiated in distilled water, acidified with several drops of glacial acetic acid (9), or left in the final buffer for longer periods. In either case, destaining should be monitored microscopically. A final buffer soak before mounting was needed to give permanence to Karo®-mounted slides. Nuclei can still be readily observed in most slides prepared 8–10 mo previously; the slides generally show little evidence of deterioration. Although the Giemsa schedule can be completed in a single day, using the minimum 2-hr staining interval, overnight staining generally is more convenient and results in better preparations.

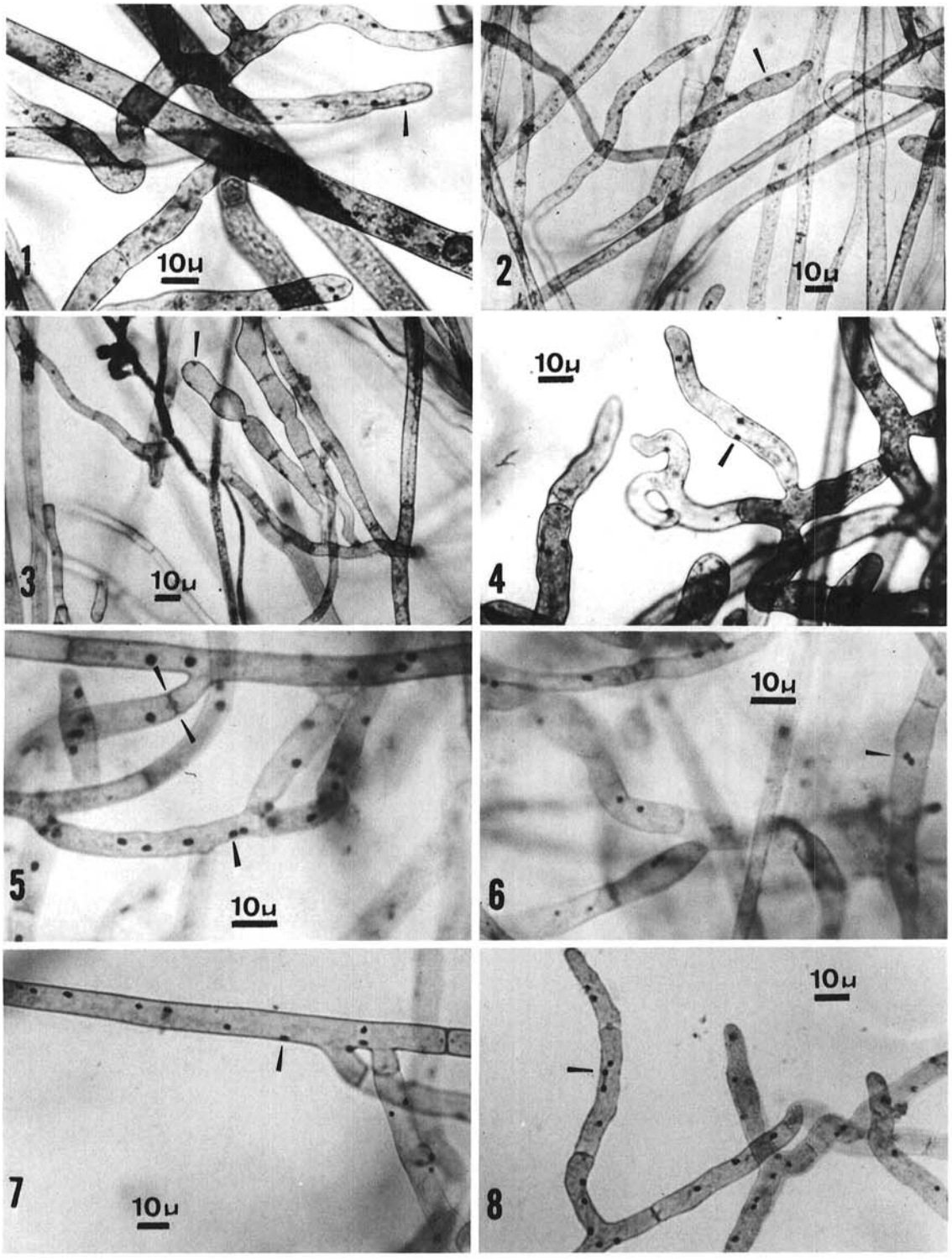
Sanders et al (11) used 2% water agar cultures in their technique for direct staining of culture plates. I found highly vacuolated hyphae of many thalli of Rhizoctonialike isolates on water agar, which interfered with observations of nuclei. Furthermore, in limited tests, substitution of other brands of agar for Difco Bacto-Agar did not consistently reduce vacuolation or improve staining, contrary to the results of Burpee et al (3). Even without vacuolation, the nuclei did not always stain well. Because *R. solani* hyphae are difficult to wet, a wetting agent was applied and improved staining of nuclei. Acidifying the wetting agent solution further improved stain penetration and gave more consistent staining of nuclei than did the wetting solution alone. Aniline blue (0.5%) in lactophenol (combined with the acidified wetting agent) gave better direct staining of nuclei than did the 0.5–1.0% aniline blue in 50% glycerine, slightly acidified with HCl, recommended by Tu and Kimbrough (12). Vacuolation problems were reduced on thin-layered DPDA plates and good staining of nuclei was achieved with either aniline blue (Figs. 3 and 4) or trypan blue (Figs. 1 and 2) in lactophenol. Occasionally, aniline blue would stain nuclei of isolates that trypan blue would not and vice versa. Neither stain was adequate for nuclei of a few isolates. Giemsa always stained nuclei, but some isolates stained better than others.

The question of whether the stained organelles were nuclei or nucleoli may be resolved by reviewing ancillary data on the three stains and procedures presented here. Considering HCl-Giemsa first, HCl hydrolyzed the chromatin of the nuclei, which then reacted with Giemsa stain as evidenced by a dark blue coloration of the stained organelles. According to Saksena (10), HCl-Giemsa stained chromatin deeply and nucleoli only faintly. In addition, Brushaber et al (2), Flentje et al (4), Parmeter et al (8), and Tu and Kimbrough (13) have reported on HCl-Giemsa staining of nuclei of *R. solani*. Tu and Kimbrough (12) reported that aniline blue reacts with the nuclei of *Thanatephorus cucumeris* (Frank) Donk and related fungi. Subsequently, nuclear phenomena in basidial structures, as well as nuclear numbers, were investigated using aniline blue staining (14). Fewer studies have been made with trypan blue, but nuclear staining with trypan blue was verified indirectly by comparing nuclear number determinations with those

obtained by a modified Giemsa staining method (11).

Additional evidence that nuclei rather than nucleoli were stained was based on comparisons of nuclear size with dimensions for *R. solani* nuclei. Flentje et al (4) reported that under phase contrast, nuclei were 2.0–3.1 μ and oval but changed shape with movement in the cell. Tu et al (15) reported that nuclei stained with aceto-orcein are approximately 1.9–2.4 μ. Giemsa-stained nuclei in my study were approximately 2.0–2.4 μ, except for those of the binucleate isolate (Fig. 6), which were 1.4–1.7 μ. Even these lower dimensions exceed those given by Flentje et al (4) for an object considered a nucleolus observed in *R. solani* under phase contrast. Approximate dimensions for trypan blue stained nuclei were 2.0–2.5 μ, which were similar to the dimensions of aniline blue stained nuclei.

Thus, the evidence indicates that organelles of multinucleate and binucleate Rhizoctonialike isolates that were stained (Figs. 1–8) were indeed nuclei.



Figs. 1–8. Photomicrographs of multinucleate and binucleate Rhizoctonialike isolates depicting nuclei (arrows point to respresentative nuclei) stained by three staining techniques. **Figs. 1 and 2,** Trypan blue stain: **1,** multinucleate isolate; **2,** binucleate isolate. **Figs. 3 and 4,** Aniline blue stain: **3,** binucleate isolate; and **4,** multinucleate isolate. **Figs. 5–8,** HCl-Giemsa stain: **5,** multinucleate isolate (nucleus indicated by one arrow, doliform pore septum by two arrows); **6,** binucleate isolate; **7,** multinucleate runner hypal cell (10 nuclei visible); and **8,** darkly stained multinucleate isolate exhibiting good contrasts.

Nearly 200 isolates were stained using the HCl-Giemsa schedule and aniline blue techniques (fewer were stained with trypan blue). This development of practical nuclear staining techniques for determination of nuclear numbers in vegetative cells of large numbers of isolates of Rhizoctonialike fungi meets the need of plant pathologists.

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