A Rapid Staining Method for Detection of Endophytic Fungi in Turf and Forage Grasses

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ABSTRACT

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Because of the increased interest in endophyte-infected grasses, an efficient detection method was deemed necessary. To facilitate screening a large number of individual seeds and plants, a rapid, safe staining procedure was developed using rose bengal. Previous methods were time-consuming and often required careful boiling of the plant tissues. The rose bengal staining procedure is simple and effective and gave excellent visual results. Minimal staining times of 30–60 sec gave good fungal differentiation even in thick tissues, and no additional fixing was necessary. Seeds and dried tissue also were stained effectively.

Endophytic fungi of turf and pasture grasses have been known to exist for many years (10,11). Some of these fungi, particularly *Epichloë typhina* (Pers.) Tul., produce external fruiting structures and infected plants can be readily identified (11). Other endophytic fungi, such as the *Acremonium* spp., produce no obvious fruiting structures or external signs of disease (7,8). Detecting the presence of these non-choke-inducing endophytic fungi in turf and pasture grasses is essential as significant effects occur in grasses containing such fungi. In particular, enhanced insect resistance and adverse livestock effects have been reported (12). Although it is necessary to be able to detect the presence of endophytes in pastures, it is also important to identify individual infected plants or seeds for plant breeding work and in determining the effects of infection on the plant.

Several different stains have been used to detect endophytic fungi in grasses. Epidermal peels from leaf sheaths, pith scrapings, and seeds have been examined, with seeds requiring additional sectioning or prolonged soaking in strong acids or alkalis and then squashing. Sampson (11) used cotton blue or gentian violet followed by Gram's iodine solution. Others used lactophenol-cotton blue (3,5), lactophenol-trypan blue (4), and aniline blue (1,10) for staining the mycelium. Clark et al (2) soaked seeds in lactic acid to soften them and to remove the lemma and palea, afterward staining the seeds with aniline blue-lactic acid. The main objections to these staining methods include poor visualization of the fungus, extended staining times or boiling, and the use of hazardous materials, in particular, trypan blue and phenol. A nonmicroscopic procedure uses an enzyme-linked immunosorbent assay (ELISA). This test detects the presence of fungal antigens in seed or plant tissues (6,9) and is somewhat limited because of its sophisticated nature. To determine percentage of infection, each seed or tiller must be individually analyzed; this is very time consuming and often impractical.

The objective of this study was to overcome the shortcomings of other methods by developing a simple, safe, rapid method of detecting small amounts of fungus within individual infected plants and seeds.

MATERIALS AND METHODS

A staining solution of rose bengal was developed and tested in pith, leaf sheath, seed, and dried vegetative tissue of perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Schreb.), and several species and subspecies of fine fescue (*Festuca* spp.).

Three staining solutions were made: 1) standard solution, 0.5% rose bengal (Fisher Cert. C. I. 45440) dissolved in 5% aqueous ethyl alcohol (w/v); 2) alkaline solution, 2.5% sodium hydroxide dissolved in standard solution (w/v); and 3) aqueous solution, 0.25% rose bengal in water (w/v). Shelf life of the standard and aqueous solutions was about 5–6 mo. Alkaline solution was prepared immediately before using because of its shorter shelf life (1 to 2 days). Pith and fresh leaf sheath tissues were stained using standard solution. Alkaline solution was used for softening and staining seeds and air-dried tillers. Aqueous solution was used for completing the staining of softened seeds and air-dried tillers.

Inner epidermis of a leaf sheath was peeled off and placed on a glass slide, preferably peeled side up. Pith tissue was scraped from within the lower portion of flowering stem. One to two drops of standard solution were then applied to the sample. Staining time was usually 30–60 sec, but could be as short as 15 sec in fresh, well-peeled samples. The sample was covered with a cover slip and excess stain was then drawn off.

Seeds were soaked in alkaline staining solution (approximately 20 ml/g sample) for approximately 8–24 hr, depending on the size and condition of the seed. For example, fine fescue required 8–12 hr, whereas tall fescue needed 16–24 hr. Intermediate-sized ryegrass seeds required 12–16 hr. Soaking in alkaline staining solution was complete when one or two representative seeds were soft enough to crush under a cover slip. When the seeds were soft enough, they were washed gently in distilled water and then placed in the aqueous staining solution for 3–6 hr to complete the staining. Seeds were then ready for examination or were stored for later examination in the aqueous solution at 10–15 C for up to 3–5 mo.

Frequently, many more plant samples need to be taken than can be processed at one time. To alleviate this problem, samples were air dried and stained later. Whole, green tillers were removed, air dried, and stored at room temperature. The dried tillers were cut into lengths of approximately 10–15 mm, and then soaked, stained, and stored as for seeds. However, the initial soaking time was 16–24 hr, depending on the age and condition of the sampled plants. Larger tillers and older plants required the maximum soaking times. Hay or straw tissue also could be stained successfully using this method.

The stained tissues were placed on glass slides and then covered with cover slips; seeds and dried tillers were crushed thinly under the cover slip. Slides were viewed at 100–400× using a green interference filter mounted over the light source. The endophytic

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fungus appeared red or pink and the background was green. The aleurone cells in seeds and some other cellular debris were red, but distinct from the characteristic endophytic hyphae.

In some cases, tissues were overstained, especially thick samples, larger seeds, or tall fescue samples. With overstained or nondistinct fresh tissue, 1 or 2 drops of distilled water were pulled under the cover slip and excess stain drawn off with blotting paper. Sometimes, 1 or 2 drops of sodium hydroxide solution (2% in distilled water) were used to reduce excess stain; this provided even clearer viewing. When seeds were overstained, excess stain was precipitated out with 1 or 2 drops of glacial acetic acid (70%) per milliliter of aqueous solution. It was swirled and then the solution was poured off immediately and washed twice with distilled water. Seeds were then either examined or stored in distilled water in the refrigerator at 10–15 C for up to 6 mo.

To make a comparison between rose bengal and another staining method, fresh tissue and seeds were stained with lactophenol-trypan blue. Peeled epidermal tissue was placed on a slide and 3 or 4 drops of lactophenol-0.1% trypan blue stain were applied to the sample. After 3–5 min the slides were heated gently over a flame. One or two drops of water were then added, excess stain was wiped away, and the sample was covered with a cover slip. Pith tissue was stained similarly. Seeds were soaked overnight in 5% NaOH-0.1% trypan blue solution, rinsed, and then stained by boiling gently for 12–15 min in lactophenol-0.1% trypan blue. The lactophenol contained lactic acid, glycerol, liquified phenol, and water (1:1:1:5). To our knowledge, lactophenol-trypan blue or any other stains have never been used to stain dried tillers.

The accuracy of the rose bengal staining procedure was checked by examining several lots of perennial ryegrass seed, and a number of Chewings fescue (*F. rubra* L. subsp. *commutata* Gaud.) and hard fescue (*F. longifolia* Thuill.) plants for the endophyte. Replicate samples were tested with both the trypan blue and rose bengal stain and percent infection was computed on the basis of number of samples with endophytic mycelium per number of samples examined. Chi-square tests were performed to find the differences in percent infection between the staining methods.

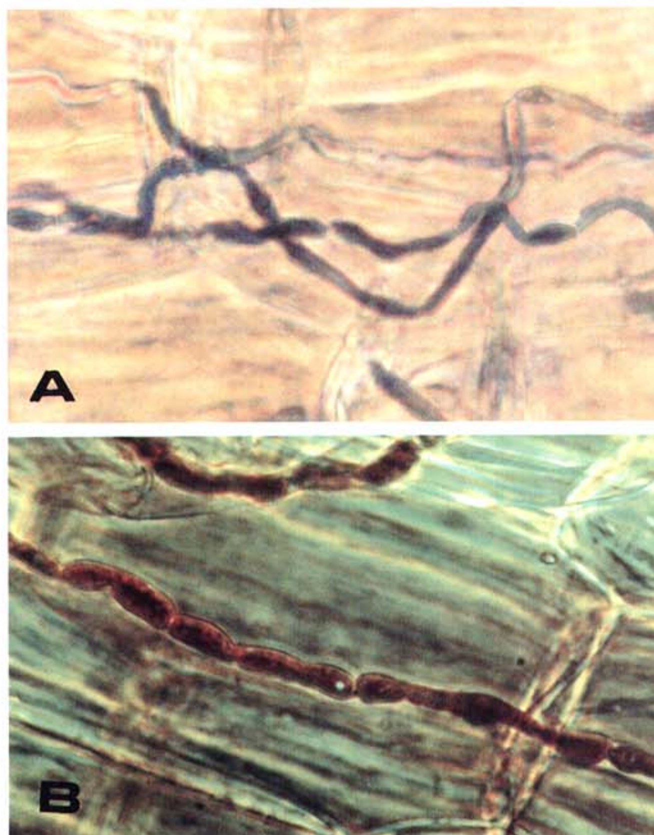

Fig. 1. Comparison of endophyte in tall fescue leaf sheath. **A,** Stained with trypan blue (400×), and **B,** stained with rose bengal (400×).

RESULTS AND DISCUSSION

Our results show that rose bengal stain can be used for staining endophytic fungi in plant tissue and is as accurate as the trypan blue staining method. Figure 1 compares endophyte stained with rose bengal stain and trypan blue stain. Tall fescue leaf sheaths (Fig. 1B) stained with rose bengal allowed better visualization of the mycelium as compared with trypan blue (Fig. 1A). Rose bengal was an improvement over trypan blue because it was extremely quick (30–60 sec compared with 3–5 min for trypan blue), avoided boiling and additional fixing of specimen, and was safer to use. The green filter enhanced the contrast. The same advantages were found with pith scrapings from senescent (Fig. 2A) and fresh stems. The technique for staining endophyte in dried tillers was, to the best of our knowledge, first developed in our laboratory. Figure 2B shows dried tillers stained as well as fresh tillers (leaf sheath or pith) when rose bengal stain was used. Because samples can be stored and processed later, rose bengal stain offers an additional advantage.

For staining seeds, the initial soaking time for both trypan blue and rose bengal was the same. The final soaking time with rose bengal stain, however, was longer. This was much preferred over boiling with hazardous chemicals in the trypan blue staining method. Furthermore, the contrast and visualization of hyphae was better with rose bengal (Fig. 3A and B).

Table 1 presents a comparison of the percent infection of seed lots and tillers with both trypan blue and rose bengal staining methods. Two selections of hard fescue gave identical results in both methods. The other three selections of hard fescue and the perennial ryegrass seed lots showed a little variation, but none of them were statistically significant ($P = 0.05$). All of the Chewings fescue selections showed a higher percent infection with the rose bengal staining method, but the differences between the two methods were not statistically significant ($P = 0.05$).

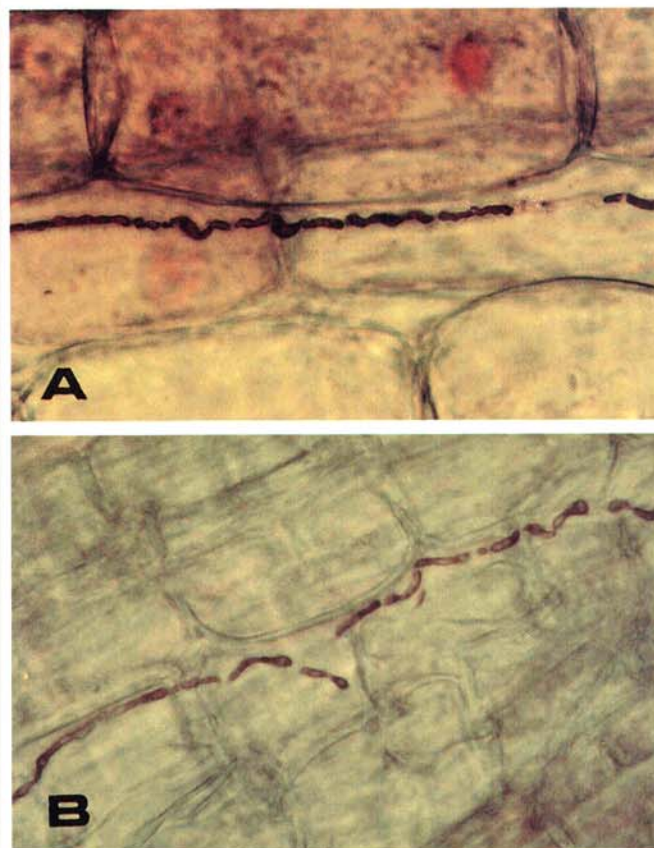

Fig. 2. Endophytic hyphae in stem tissue of tall fescue stained with rose bengal. **A,** Pith from senescent stem (200×), and **B,** air-dried tiller (200×).

TABLE 1. Comparison of trypan blue and rose bengal stains for detecting endophyte in tillers of hard fescue and Chewings fescue and in seed lots of perennial ryegrass

	Trypan blue			Rose bengal			
Entry	Samples examined (no.)	Samples with endophyte (no.)	Infection (%)	Samples examined (no.)	Samples with endophyte (no.)	Infection (%)	Adjusted χ^{2a}
Hard fescue							
A79-365	6	6	100	9	8	89	0.045
A79-390	6	5	83	9	9	100	0.045
A79-315	7	7	100	7	7	100	··· b
A79-199	5	5	100	14	14	100	··· b
A78-41	6	6	100	9	8	89	0.045
Chewings fescue							
A78-40	12	9	75	29	23	79	0.012
A78-141	15	11	74	30	28	93	1.947
A78-20	12	9	75	15	13	87	0.073
Perennial ryegrass							
M9-3-PR-3-A1	50	30	60	50	35	70	0.703
M9-3-PR-12	52	38	73	53	37	70	0.024
M9-3-PR-13	50	39	78	50	36	72	0.213
M2-3-14	50	38	76	55	43	78	0.001
M9-3-PR-14	50	42	84	50	37	74	0.964
M9-3-PR-15	50	33	66	55	39	71	0.111
M9-3-PR-4-1	50	37	74	55	41	74	0.026
J9-2-250 PT	30	26	87	75	59	79	0.443

[a] Chi-square tests were used to evaluate differences between staining methods: $\chi^2(0.05) = 3.841$. No significant differences were found between staining methods for any entry.
[b] Statistical analysis was not performed because observed and expected results were identical.

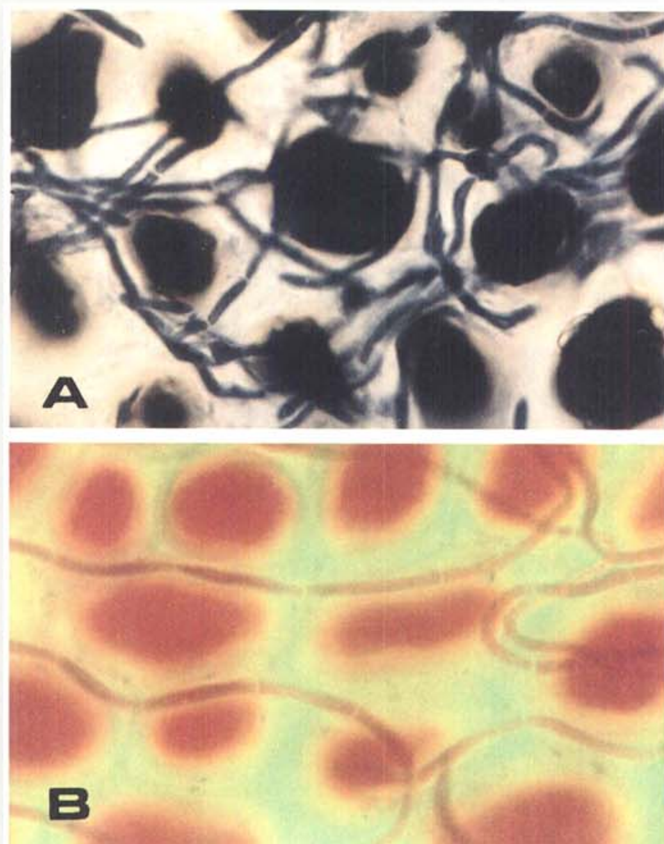

Fig. 3. Endophyte-infected seed stained to show hyphae. **A,** perennial ryegrass with trypan blue (400×), and **B,** hard fescue with rose bengal (400×).

Microscopic examination of rose bengal stained tissue was an effective and accurate method for detecting the presence of endophytic fungi in perennial ryegrass, tall fescue, and fine fescues. The technique was simple and quick and required no additional fixing of stained tissue. The stain components and procedures were relatively safe.

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