Detecting Viable *Acremonium* Endophytes in Leaf Sheaths and Meristems of Tall Fescue and Perennial Ryegrass

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ABSTRACT

Welty, R. E., Azevedo, M. D., and Cook, K. L. 1986. Detecting viable *Acremonium* endophytes in leaf sheaths and meristems of tall fescue and perennial ryegrass. Plant Disease 70:431-435.

A method was developed to detect viable hyphae of the endophytic fungi *Acremonium coenophialum* or *A. loliae* in seedlings of tall fescue or perennial ryegrass, respectively. Captafol 4F (479 g a.i./L) at 7,489 μg a.i./ml of deionized water was used to treat seeds of two strains of tall fescue (KY-82-3G1-316 and KY-37G1-307) and perennial ryegrass (cultivar Repell). The fungicide retarded or eliminated the growth of seedborne species of *Alternaria*, *Cladosporium*, and *Epicoccum* but did not influence the recovery of *A. coenophialum* or *A. loliae* from seedlings. Captafol at 14,978 μg a.i./ml stunted the roots of 3-wk-old tall fescue seedlings; shoots were not affected. No phytotoxicity was observed at 7,489 and 3,745 μg a.i./ml. When used with seedling staining and microscopic examination, the method detects viable endophyte in leaf sheaths and meristems of 3-wk-old seedlings, reduces incubation time required for seedling examination, and eliminates greenhouse culture. Seed technologists were trained to detect endophyte hyphae by this technique. Several media, including potato-dextrose, cornmeal, and bran-malt agars, were suitable for growth of *A. coenophialum* or *A. loliae* in culture.

A relationship has been established between poor animal performance and the *Acremonium* endophytes of tall fescue (*Festuca arundinacea* Schreb. (1,2,9,18) and perennial ryegrass (*Lolium perenne* L.) (20). Cattle in the southeastern United States, where tall fescue is grown on 14 million hectares, often develop symptoms of toxicosis during summer months from grazing plants infected with *A. coenophialum* Morgan-Jones & Gams. In New Zealand, where perennial ryegrass is grown on 7 million hectares of pasture, sheep often develop a condition called ryegrass-staggers from grazing plants infected by *A. loliae* Latch, Christensen, & Samuels.

In Kentucky, 97% of 200 fields of tall fescue were infested with endophyte-infected plants (19); in Alabama, 95% of the tall fescue pastures were infested (12). Ninety-one percent of 193 seed samples from separate tall fescue seed-production fields in Missouri contained 50% or more endophyte-infected seeds (17). In Oregon in 1983–1984, 90% of 297 and 57% of 81 seed samples from individual seed fields of tall fescue and ryegrass (annual and perennial), respectively, contained less than 5% endophyte-infected seeds (Gene M. Milbrath, *personal communication*). Fescue toxicosis alone has been estimated to cost cattle producers in the Southeast $50–200 million annually (3,8). The only mode of natural fungus transmission known is by planting infected seed (16,19), and control of seedborne infections in tall fescue can be achieved by seed-treatment fungicides, heat treatments, or storage for 7 days at 49 C or 11 mo at 20 C (21,24).

Replanting pastures with endophyte-free seed, or seed with low levels of endophyte, offers an inexpensive method of control. As the need for endophyte-free seed increases, knowing the level of endophyte in seed lots is important.

Mycelium of *A. coenophialum* in seeds of tall fescue is coarse, convoluted, usually unbranched, and often located between the aleurone layer and the nucellus (7,16,20,24). The identity of the hyphae can be confirmed by growing infected seeds in the greenhouse for 6–10 wk, then culturing leaf sheaths in cornmeal agar (CMA) or potato-dextrose agar (PDA) (19). *A. coenophialum* can also be isolated from surface-sterilized tall fescue seed cultured on CMA or PDA (5,20). At present, the level of endophyte in seeds is detected by staining and microscopic examination (4,7,16,23,24) or enzyme-linked immunosorbent assay (ELISA) (11). Neither method distinguishes viable from nonviable hyphae. Because the endophyte dies in seeds during storage (21,24) or can be reduced by seed-treatment fungicides (21,24), a reliable test for the presence of viable endophyte is needed. The current detection method is a modification of an earlier procedure (14) and requires growing seedlings in the greenhouse for 6–10 wk, collecting first- and second-nodal tissue, staining hyphae and tissue, and examining specimens with a microscope (1,4,16,19,20,24) or detecting the endophyte by ELISA (11,19).

Endophyte identity is confirmed by culturing infected tissue on PDA or CMA and microscopic examination after 3–5 wk of incubation at room temperature (20–25 C). The disadvantages of the grow-out procedure are the length of time involved and the need for greenhouse culture.

The objectives of this study were to compare the growth of isolates of *A. coenophialum* and *A. loliae* on several common laboratory agar media, evaluate several methods of direct isolation of *A. coenophialum* from tall fescue seed in agar, and modify existing techniques for determining viable endophyte hyphae in seedlings of tall fescue and perennial ryegrass to reduce the time needed for the assay. A secondary objective was to provide a test that could be performed by seed analysts in conjunction with traditional routine tests and would not require expensive equipment (6).

MATERIALS AND METHODS

Growth in culture. Three strains of *A. coenophialum* from tall fescue and one strain of *A. loliae* from ryegrass were grown on 10 agar media, and their growth rates (mm/14 days) were compared. Tall fescue strain 1-24 was isolated from an infected culm from a field planting in Linn County, OR; strain G1-307 was isolated from an infected seedling of tall fescue strain KY-37G1-307 growing from seed in the greenhouse; and strain KY-1 was obtained from M. C. Johnson, Lexington, KY. Ryegrass strain RG-1 of *A. loliae* was isolated from an infected seedling of cultivar Repell of perennial ryegrass growing from seed in the greenhouse. The cultures were maintained on PDA at 4 C.

Agar media were prepared and sterilized, then 25 ml of each was poured aseptically into a sterile petri dish. All ingredients were from Difco except the

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Technical Paper 7542 of the Oregon Agricultural Experiment Station.

Accepted for publication 14 October 1985.

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All-Bran (Kellogg) in the 2% bran-2% malt-extract agar (BMA). Other media tested were routinely used in the laboratory for isolating and growing fungi, including CMA; Czapek-Dox agar (CzDA); 2% malt-extract agar (MA); 2% malt extract, 3% sucrose, 0.1% peptone, 0.25% yeast extract, and 2% agar (M-43); nutrient agar (NA); PDA; tomato juice agar (TJA); 2% agar (WA); and yeast-morphology agar (YMA). Plugs of mycelium (7 mm in diameter) were cut from the margins of 4-wk-old cultures on CMA and placed in the center of each petri dish. Dishes were sealed with Parafilm M stripes and incubated at 25 C in the dark. Temperature was recorded on a 7-day thermograph. The study was arranged in a completely randomized design with four replicates. Mycelial growth was measured every 2 wk for 10 wk from the margin of the plug to the margin of the colony in four directions, and the averaged values were used for the replicate.

Direct isolation from the seeds. Infected seeds were evaluated with other surface-sterilization methods and several common laboratory media supplemented with nutrients and antibiotics. In most tests, 25 seeds were placed in each of four dishes to evaluate the disinfection technique or the medium. All seeds were incubated at 25 C in dark. Cultures were examined at 3- or 4-day intervals throughout the incubation period. Materials and methods varied with the experiments and are presented with the results.

Seedling assays. A fungicide and bactericides were tested as seed treatments to retard or eliminate the growth of unwanted seedborne fungi and bacteria that interfere with endophyte detection. Endophyte-infected seeds were planted and incubated at 12–28 C to determine how incubation temperature influenced movement of the endophyte hyphae from the seed to the seedling. Materials and methods varied with the experiments and are presented with the results. Finally, successful procedures were applied to a seedling assay for detecting endophytes in tall fescue and perennial ryegrass seedlings.

RESULTS AND DISCUSSION

Growth in culture. Mycelium (Table 1) of the three strains of *A. coenophialum* from tall fescue grew fastest on BMA or PDA and slowest on WA or CzDA. Mycelial growth of the isolate of *A. loliae* from ryegrass was fastest on WA or CzDA and the slowest on NA or TJA. The density of mycelium on these media was variable. Generally, mycelium was dense on CzDA, PDA, TJA, M-43, YMA, or BMA and thin or sparse on CMA, MA, NA, or WA. *A. loliae* grew as fast or faster than *A. coenophialum* on all media except TJA, NA, and YMA. CMA was selected as the basal medium for testing procedures for direct isolation of endophytes from tall fescue and ryegrass seeds. Both species of *Acremonium* grew well on this medium, and although it did not provide the fastest growth rate of the media tested, it was a suitable compromise for selecting a single medium. Other media suitable for growth of both *A. coenophialum* and *A. loliae* included PDA or BMA. Linear growth in culture of both species of *Acremonium* is extremely slow compared with that of other fungi found in seeds (species of *Alternaria, Cladosporium, Epicoccum*, and *Fusarium*).

Direct isolation from seeds. CMA was supplemented with 0.1% glucose, 0.1% yeast extract, 0.05% casitone, and 0.05% casein hydrolizate. Seeds of tall fescue strain KY-82-3G1-316 were surface-sterilized in 2% NaOCl for 4 min, rinsed with sterile distilled water, blotted dry with sterile paper towels, and aseptically cultured on the various media.

Bacteria grew rapidly over the surfaces of the seeds and onto the surfaces of all media. No fungi grew from the seeds. After 6 days of incubation, the dishes were discarded.

Next, seeds of strain KY-82-3G1-316 were surface-sterilized in 1% NaOCl for 10 min, treated as before, and cultured on CMA or CMA containing 2% malt extract and 0.2% yeast extract plus 50 μg/ml of tetracycline or 50 μg/ml of cycloheximide.

Bacteria grew from seeds cultured on CMA and CMA plus cycloheximide but not from seeds cultured on CMA plus tetracycline. Fungi grew from seeds on all three media and filled the petri dishes after 10 days of incubation. Sporulating fungi included species of *Alternaria, Cladosporium*, and *Epicoccum*. These fungi are commonly associated with grass seeds (15). Clark et al (4) and Conger and McDaniel (5) also reported fungal contamination when attempting to isolate *A. coenophialum* from tall fescue seed. None of the fungi that grew from the seeds was *A. coenophialum*. The bacteria were not identified.

To determine the effects of these antibiotics on growth of *A. coenophialum*, agar plugs cut from the margins of 4-wk-old cultures were transferred to CMA, CMA plus 50 μg/ml of tetracycline, or CMA plus 50 μg/ml of cycloheximide and incubated for 4 wk in dark at 25 C. Growth of *A. coenophialum* was similar on CMA or CMA plus tetracycline, but growth was prevented on CMA plus cycloheximide. When the agar plug of mycelium from CMA plus cycloheximide was transferred aseptically to CMA, the mycelium did not resume growth. The study was repeated with similar results. We concluded that 50 μg/ml of cycloheximide in vitro was fungicidal to *A. coenophialum*, 50 μg/ml of tetracycline in vitro did not retard the growth of *A. coenophialum*, and a solution containing 50 μg/ml of tetracycline could be used to treat tall fescue seed to prevent the growth of some seedborne bacteria but allow the growth of *A. coenophialum*. Care should be used when adding tetracycline because Clark et al (4) found some inhibition of growth of *A. coenophialum* caused by 150 mg/L of this antibiotic.

Because species of *Alternaria, Cladosporium*, and *Epicoccum* were associated with seeds of both grass species, and because growth of these fungi was retarded in culture and in the field by captafol (Difolatan 4F) (22), an experiment was done to evaluate the effectiveness of captafol in controlling the growth of seedborne fungal contaminants that interfered with endophyte detection in seeds cultured on an agar medium.

When seeds of tall fescue strain KY-82-3G1-316 were cultured on CMA with 2% malt extract, 0.2% yeast extract, 50 μg/ml of tetracycline, and 1,000 μg/ml of captafol, seedborne contaminants (mainly species of *Alternaria*) grew from the seed but not onto the culture medium. After 4 wk of incubation in dark at 25 C, *A.*

Table 1. Growth rate of *Acremonium coenophialum* on 10 agar media at 25 C in the dark

	Increase in colony (mm/14 days) of isolate[x]				
	Tall fescue				Ryegrass
Agar	1-24	G1-307	KY	Mean[y]	(RG-1)
Bran-malt	4.6	5.1	4.6	4.8	5.1
Cornmeal	4.0	3.7	3.7	3.8	5.7
Czapek-Dox	1.1	0.7	1.3	1.0	6.0
Malt extract	3.1	3.5	3.0	3.2	5.1
M-43	1.1	3.4	2.6	2.4	5.0
Nutrient	2.0	2.0	1.3	1.8	1.3
Potato-dextrose	4.3	4.2	4.4	4.3	4.7
Tomato juice	3.5	3.4	3.8	3.6	2.1
Water	1.0	1.1	0.9	1.0	6.1
Yeast morphology	3.9	3.7	3.9	3.8	3.0
Mean[z]	2.9	3.1	3.0		4.4
C.V (%) = 14.9					

[x] Each value is the average of four measurements from the edge of the plug of mycelium to the margin of the colony taken at 2-wk intervals for 10 wk for each of four replicates.
[y] Media means for the tall fescue isolates that exceed LSD 0.76 are different ($P = 0.05$).
[z] Means for tall fescue isolates are not significantly different ($P = 0.05$).

coenophialum did not grow out of the seeds.

None of these techniques were suitable for direct isolation of *A. coenophialum* from tall fescue seed. Direct isolation of endophytes from seeds requires more severe seed-sterilization procedures to eliminate seedborne contaminants but not the endophyte, and further testing was done.

Seeds of KY-37G1-307 were surface-sterilized by modifying a procedure previously published (5). Seeds were put in 50% H_2SO_4 for 30–35 min (vigorous stirring), rinsed in sterile distilled water, rinsed again in 70% ethanol, rinsed again in sterile distilled water, washed (vigorous stirring) in 5.25% NaOCl (containing one drop Tween 20 per liter), drained, and air-dried in a sterile transfer hood for about 1 hr. Seeds were aseptically transferred to test tubes containing PDA and placed for germination in an incubator on a 24-hr cycle of 15 C for 16 hr in the dark followed by 25 C for 8 hr in the light. Tubes were examined at 2- to 3-day intervals for 4–5 wk.

Seedborne contaminants (including species of *Alternaria, Cladosporium, Epicoccum*, and *Fusarium*) often grew from the seeds. These tubes were usually discarded after 10–14 days of incubation. In test tubes free of these fungi, *A. coenophialum* began growing from infected seedlings after about 3 wk of incubation. In a test to determine if a quantitative measure could be made of viable endophyte with this method, 116 seeds were cultured; after 14 days, 14 cultures had been discarded. In a second test using 101 seeds, 44 were contaminated and discarded. In the first and second test, respectively, the endophyte grew from 95 of the remaining 102 seeds and from 46 of the remaining 57 seeds. The procedure is suitable for obtaining pure cultures of endophyte, but seedborne contaminants that survive surface-sterilization limit its use for determining the level of viable endophyte infection in a seed lot. Because this strain of tall fescue was previously found to have about 98% of its seeds containing viable hyphae of the endophyte, it seems apparent that the endophyte was also present in seeds previously discarded with the seed contaminants. The fact that the endophyte grew from 93% (test 1) and 81% (test 2) of the seeds that were not contaminated indicates that surface-sterilization also removed the endophyte. Futher testing with serial treatment with acid, alcohol, and hypochlorite solutions to eliminate contaminants and not eliminate the endophyte is needed before a method using direct isolation from seed is successful for determining viable endophytes in tall fescue seeds.

Seedling assays. Infected seeds of tall fescue strain KY-37G1-307 were planted in a flat containing soil:peat:perlite (1:1:1, v/v) and incubated at 18–25 C in the greenhouse for 2 wk. The flats were then moved to incubators at 12, 16, 20, 24, and 28 C with a 12-hr light/dark cycle. Plants were watered and fertilized to maintain vigorous growth.

When the flats were moved to the incubators, 50 seedlings were pulled gently from the soil mix and washed in running tap water to remove debris. Most of the roots were removed and discarded with the unused endosperm and remnants of the lemma and palea. The root-shoot interface, 1–2 cm of shoot and 2–5 mm of root, was digested and stained by slightly modifying a previously published procedure (14). Sections were soaked in 5% NaOH and 0.1% trypan blue overnight (about 16–18 hr). Next, sections were washed in distilled water and boiled gently in the lactophenol with 0.1% trypan blue for 20 min. Sections were rinsed in distilled water, squash-mounted in two or three drops of glycerine:water (1:3, v/v), and examined at ×100–1,000. At weekly intervals for 6 wk, 25 seedlings from each temperature were prepared and examined as described. Some seedlings were lost during preparation for staining, and results are expressed as infected seedlings per number of seedlings examined.

When seedlings from the greenhouse were stained and examined, endophyte hyphae were found in the meristems (Fig.

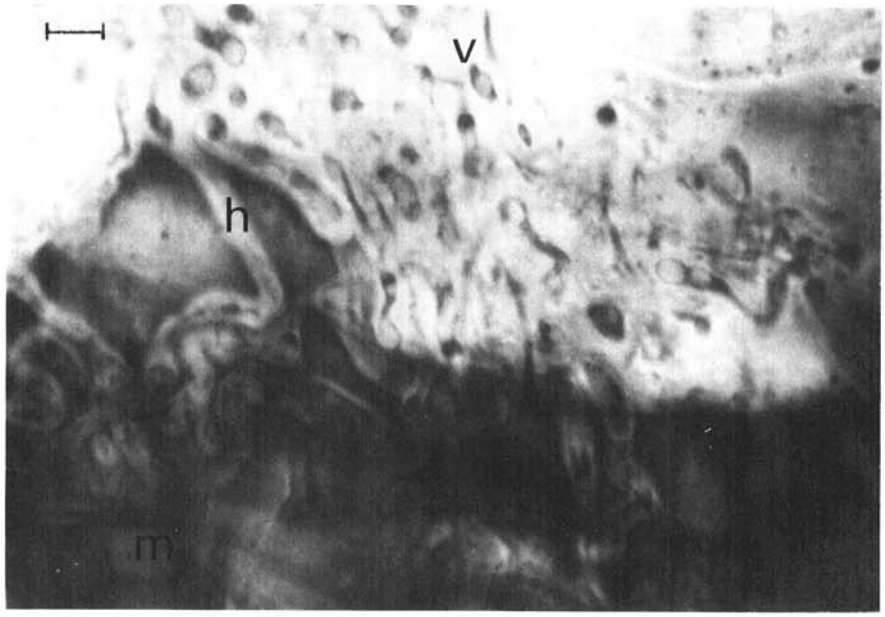

Fig. 1. Intercellular hyphae (h) and vesicles (v) of *Acremonium coenophialum* growing in the meristem (m) of tall fescue. Scale bar = 5 μm.

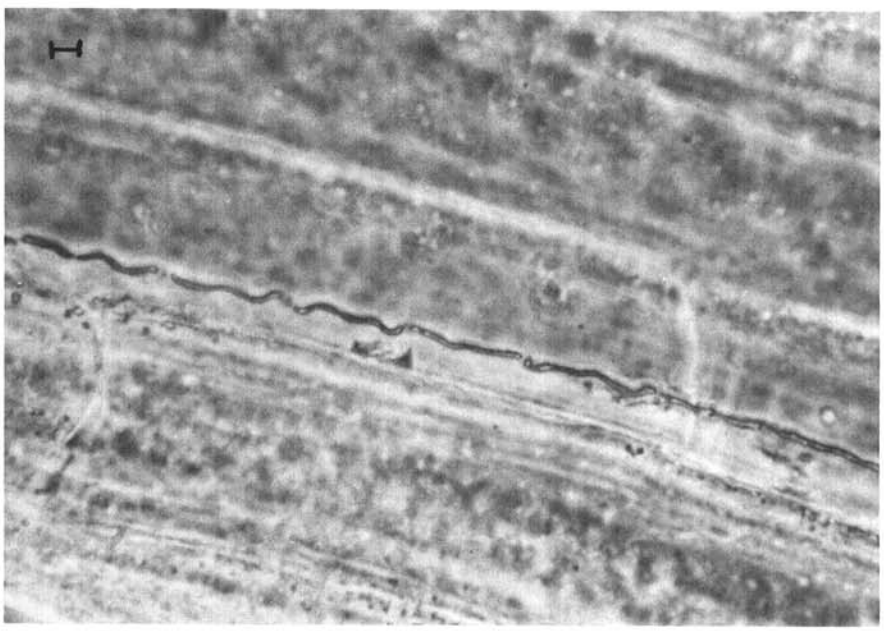

Fig. 2. Intercellular hyphae of *Acremonium coenophialum* growing in the leaf sheath of tall fescue. Scale bar = 10 μm.

1) and leaf sheaths (Fig. 2) of 25 of 38 seedlings (66%). After 7 days at 12–28 C (Table 2), 97–100% of the seedlings contained endophyte hyphae. Incubation continued for five additional weeks, and the percentage of endophyte-infected seedlings did not change. The effect of controlled temperatures on initial movement of the endophyte from seed to seedling was not determined because 66% of the shoots already contained the endophyte. By 3 wk (2 wk in the greenhouse and 1 wk in the incubators), movement of the endophyte from seed to seedling was nearly complete. The study also shows that the endophyte hyphae remain in the shoot meristem throughout the 8 wk of incubation at the temperatures tested. Vesicles were frequently observed distal to the meristem (Fig. 1). Their function is unknown, but they resemble "pinched-off" protoplasm that lacks a secondary thickened cell wall. Vesicles may function to disseminate the fungus within the plant.

During seedling preparation, mats or strands of mycelium and conidia of other fungi adhering to seedlings and seed remnants were also fixed and stained. Conidia of species of *Alternaria*, *Fusarium*, and *Epicoccum* could be identified. Darkly pigmented hyphae were also observed. (Hyphae of *A. coenophialum* is hyaline.) Captafol was evaluated again, this time as a seed-treatment fungicide to retard the growth of unwanted seedborne fungi.

Seeds of tall fescue strains KY-37G1-307 and KY-82-3G1-316 were soaked 1 hr in captafol at 14,978, 7,489, and 3,745 μg a.i./ml in distilled water (i.e., captafol 4F diluted 1:32, 1:64, and 1:128, respectively). As a control, seeds were soaked in distilled water. Seeds were dried overnight on paper towels and placed (6) on blotter paper moistened with a 0.1% solution of KNO_3. Seeds were chilled at 5 C in the dark for 7 days to break dormancy and moved to a growth chamber for 16 hr at 15 C in the dark and 8 hr at 25 C in the light (50 $\mu E/m^2/sec$). After 3 wk total of incubation, 50 seedlings were stained and examined at ×100–1,000 as previously described. The study was repeated.

Seedlings from seeds treated with captafol at 14,978 μg a.i./ml had severely stunted roots and normal shoots; no fungi grew from the unused endosperm, lemma, or palea. At 7,489 μg a.i./ml, roots and shoots showed no evidence of phytotoxicity and no fungal growth on seed remnants. At 3,745 μg a.i./ml, roots and shoots appeared normal, but strands and mats of pigmented hyphae were found on seed remnants. The one-way analysis of variance among captafol concentrations resulted in no significant differences ($P = 0.05$) in seed germination or endophyte detection in both strains of tall fescue seedlings (Table 3).

In a second test of seed treatment with captafol, seeds of tall fescue strains KY-37G1-307 and KY-82-3G1-316 and ryegrass cultivar Repell were treated with 7,489 μg a.i./ml of captafol, dried, and germinated as previously described. Seedlings were digested, stained, and examined as previously described.

Endophyte hyphae (averaged for two tests) were detected in 99, 100, and 91% of the seeds of tall fescue strains KY-37G1-307 and KY-82-3G1-316 and Repell ryegrass, respectively (Table 4). Endophyte hyphae (averaged for two tests) were detected in 90, 10, and 97% of the seedlings of tall fescue strains KY-37G1-307 and KY-82-3G1-316 and Repell ryegrass, respectively. Seeds of tall fescue strain KY-82-3G1-316 were frozen and accidentally thawed, then refrozen and rethawed before being used in this test (M. R. Siegel, *personal communication*). This may account for the loss in endophyte viability.

We now use captafol (7,489 μg a.i./ml) diluted with water as standard seed soak for a grow-out test to determine viable endophyte content of tall fescue and ryegrass seed. The procedure successfully detects changes in endophyte survival and seed germination.

When seedling sections (1–2 cm) of root-shoot from seeds of tall fescue or ryegrass previously treated with captafol (7,489 μg a.i./ml) were surface-sterilized with 1% NaOCl, rinsed with sterile distilled water, and cultured on cornmeal-malt extract-yeast extract agar containing 1,000 μg/ml of captafol, some sections yielded bacteria that soon overgrew the plant tissue. Tall fescue or ryegrass sections free of bacteria yielded *A. coenophialum* or *A. loliae*, respectively.

Table 2. Number of seedlings containing hyphae of *Acremonium coenophialum* per number of seedlings examined[a] after incubation at 12–28 C for 1–6 wk

Weeks incubated[b]	Temperature (C)				
	12	16	20	24	28
1	31/32	25/25	24/24	26/26	31/32
2	23/24	19/19	24/24	22/24	20/20
3	16/18	25/26	26/26	24/24	18/19
4	25/25	22/22	24/24	24/25	23/23
5	25/27	22/22	24/24	25/25	21/22
6	24/24	23/23	25/25	25/25	24/24

[a] Stem sections (1–2 cm) soaked in 5% NaOH + 0.1% trypan blue for 16–18 hr, washed with distilled water, boiled 20 min in lactophenol containing 0.1% trypan blue, rinsed, squash-mounted in one or two drops of glycerine:water (1:3, v/v) and examined at ×100–1,000.
[b] Two weeks after planting in the greenhouse, 25 of 38 seedlings contained hyphae of *A. coenophialum* in the meristems and leaf sheaths.

Table 3. Germination and detection of *Acremonium coenophialum* in seedlings of two strains of tall fescue treated with three rates of captafol

Concentration[a] (μg a.i./ml)	KY-37G1-307		KY-82-3G1-316	
	Germination (%)	Infected (%)	Germination (%)	Infected (%)
0	94[b]	95	75	10
3,745	82	89	72	10
7,489	90	97	80	13
14,978	87	96	84	8

[a] Captafol (478 g a.i./L) diluted with distilled water.
[b] Average based on two runs of 50 seeds each; means within columns for captafol concentrations are not significantly different from each other ($P = 0.05$, one-way ANOVA).

Table 4. Detection of *Acremonium coenophialum* in seeds or seedlings of tall fescue and ryegrass previously treated with captafol (7,390 μg a.i./ml)

Species	Strain or cultivar	Stained seed[a]		Stained seedling	
		Infected/ examined[b]	%	Infected/ examined	%
Festuca arundinacea	KY-37G1-307	99/100	99	87/97	90
	KY-82-3G1-316	100/100	100	7/67	10
Lolium perenne	Repell	91/100	91	87/90	97

[a] Seeds or seedlings placed in 5% NaOH and 1% trypan blue for 16–18 hr, rinsed with distilled water, boiled gently in lactophenol containing 0.1% trypan blue for 20 min, rinsed with distilled water, squash-mounted in one or two drops of glycerine:water (1:3, v/v), and examined at ×100–1,000.
[b] Seeds or seedlings containing hyphae typical of *A. coenophialum* examined at ×100–1,000. Results combined from two tests of 50 seeds each.

This procedure is used to obtain cultures of these fungi and to confirm the identity of the fungi by comparing characteristics with descriptions of *A. coenophialum* and *A. loliae* (13). However, this procedure is not reliable for detecting percent infection of viable endophyte, because some discarded sections with bacteria may or may not contain endophyte hyphae.

To determine if cycloheximide seed treatment could eliminate or reduce viable endophyte in seeds of tall fescue (KY-37G1-307) and ryegrass (Repell), seeds were soaked in 100 μg/ml of cycloheximide for 4 min, treated with 7,489 μg a.i./ml of captafol, and germinated and examined as previously described. As a control, seeds of both species were soaked 4 min in distilled water before captafol treatment and subsequent germination and examination. Fifty seedlings of tall fescue and ryegrass yielded 40 and 42 endophyte-infected seedlings, respectively. Cycloheximide treatment reduced viable endophyte content about 12–15%. Further testing at higher concentrations may reduce further viable endophyte content in grass seeds.

Conclusions. A method was devised to reduce the competition of seedborne *Alternaria, Cladosporium*, and *Epicoccum*, which facilitated the observation of *A. coenophialum* or *A. loliae* in 3-wk-old seedlings of tall fescue or ryegrass, respectively. The seedling staining method reported here is similar to the method used to detect endophyte hyphae in seeds. When endophyte detection methods in seeds or seedlings are coupled with acceptable sampling procedures (10,23), seed lots can be accepted or rejected depending on the needs of domestic or foreign markets. The advantage of this grow-out procedure over the existing one is that the incubation time is reduced from 6–10 to 3 wk, growth in greenhouse is eliminated, and a standard Association of Official Seed Analysts germination procedure is used (6). The test can be implemented with minimum expense for equipment and supplies, and a seed technologist already trained to detect endophyte hyphae in seeds can detect endophytes in seedlings. It is suggested, however, that for both seed and seedling detection methods, a plant pathologist or a mycologist should train and supervise personnel doing the test to help avoid the mistakes and confusion associated with observing hyphae of other seedborne and seedlingborne fungi.

Laboratories equipped to detect endophyte in seeds and seedlings by ELISA may also find this method useful because it reduces time and eliminates greenhouse culture.

LITERATURE CITED

1. Bacon, C. W., Porter, J. K., Robbins, J. D., and Luttrell, E. S. 1977. *Epichloë typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34:576-581.
2. Bush, L. P., Cornelius, P. C., Buckner, R. C., Varney, D. R., Chapman, R. A., Burrus, P. B., II, Kennedy, C. W., Jones, T. A., and Saunders, M. J. 1982. Association of *N*-acetyl loline and *N*-formyl loline with *Epichloë typhina* in tall fescue. Crop Sci. 22:941-943.
3. Carlson, G. E. 1983. Tall fescue problem—past and present. Pages 3-5 in: Proceedings of the Forage and Turfgrass Endophyte Workshop. Ore. State Univ. Ext. Serv., Corvallis. 100 pp.
4. Clark, E. M., White, J. F., and Patterson, R. M. 1983. Improved histochemical techniques for the detection of *Acremonium coenophialum* in tall fescue and methods of in vitro culture of the fungus. J. Microbiol. Methods 1:149-155.
5. Conger, B. V., and McDaniel, J. K. 1983. Use of callus cultures to screen tall fescue samples for *Acremonium coenophialum*. Crop Sci. 23:172-174.
6. Copland, L. O., ed. 1981. Rules for testing seeds. J. Seed Technol. 6:1-125.
7. Funk, C. R., Halisky, P. M., Johnson, M. C., Siegel, M. R., Stewart, A. V., Ahmad, S., Hurley, R. H., and Harvey, I. C. 1983. An endophytic fungus and resistance to sod webworms: Association in *Lolium perenne* L. Bio/Technology 1:189-191.
8. Hemken, R. W. 1983. Animal response and livestock production when feeding tall fescue. Pages 13-17 in: Proceedings of the Forage and Turfgrass Endophyte Workshop. Ore. State Univ. Ext. Serv., Corvallis. 100 pp.
9. Hoveland, C. S., Schmidt, S. P., King, C. C., Jr., Odom, J. W., Clark, E. M., McGuire, J. A., Smith, L. A., Grimes, H. W., and Holliman, J. L. 1983. Steer performance and association of *Acremonium coenophialum* fungal endophyte on tall fescue pasture. Agron. J. 75:821-824.
10. Johnson, M. C., Anderson, R. L., Kryscio, R. J., and Siegel, M. R. 1983. Sampling procedures for determining endophyte content in tall fescue seed lots by ELISA. Phytopathology 73:1406-1409.
11. Johnson, M. C., Pirone, T. P., Siegel, M. R., and Varney, D. R. 1982. Detection of *Epichloë typhina* in tall fescue by means of enzyme-linked immunosorbent assay. Phytopathology 72:647-650.
12. Lacefield, G. D. 1983. The endophyte of tall fescue. Seed World 121:18-20.
13. Latch, G. C. M., Christenson, M. J., and Samuels, G. J. 1985. Five endophytes of *Lolium* and *Festuca* in New Zealand. Mycotaxon 20:535-550.
14. Morton, D. J. 1961. Trypan blue and boiling lactophenol for staining and clearing barley tissues infected with *Ustilago nuda*. Phytopathology 51:27-29.
15. Neergaard, P. 1977. Seed Pathology. Vols. 1 and 2. Macmillan, London. 1,187 pp.
16. Neill, J. C. 1941. The endophytes of *Lolium* and *Festuca*. N.Z. J. Sci. Technol. A23:185-195.
17. Rycyk, F., Jr., and Sharpe, D. 1984. Infection of tall fescue seed in Missouri by the endophyte *Epichloë typhina*. Plant Dis. 68:1099.
18. Schmidt, S. P., Hoveland, C. S., Clark, E. M., Davis, N. D., Smith, L. A., Grimes, H. W., and Holliman, J. L. 1982. Association of an endophytic fungus with fescue toxicity in steers fed Kentucky 31 tall fescue seed or hay. J. Anim. Sci. 55:1259-1263.
19. Siegel, M. R., Johnson, M. C., Varney, D. R., Nesmith, W. C., Buckner, R. C., Bush, L. P., Burrus, P. B., II, Jones, T. A., and Boling, J. A. 1984. A fungal endophyte in tall fescue: Incidence and dissemination. Phytopathology 74:932-937.
20. Siegel, M. R., Latch, G. C. M., and Johnson, M. C. 1985. *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: Significance and control. Plant Dis. 69:179-183.
21. Siegel, M. R., Varney, D. R., Johnson, M. C., Nesmith, W. C., Buckner, R. C., Bush, L. P., Burris, P. B., II, and Hardison, J. R. 1984. A fungal endophyte of tall fescue: Evaluation of control methods. Phytopathology 74:937-941.
22. Spurr, H. W., Jr., and Welty, R. E. 1972. Incident of tobacco leaf microflora in relation to brown spot disease and fungicidal treatment. Phytopathology 62:916-920.
23. Welty, R. E., Milbrath, G. M., Faulkenberry, D., Azevedo, M. D., Meek, L., and Hall, K. 1986. Endophyte detection in tall fescue by seed staining and ELISA. Seed Sci. Technol. In press.
24. Williams, M. J., Backman, P. A., Clark, E. M., and White, J. F. 1984. Seed treatments for control of the tall fescue endophyte *Acremonium coenophialum*. Plant Dis. 68:49-52.