APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1977, p. 576-581

Vol. 34, No. 5
Printed in U.S.A.

Epichloë typhina from Toxic Tall Fescue Grasses

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Received for publication 21 April 1977

Epichloë typhina, a clavicipitaceous systemic phytopathogen, was isolated from two varieties and three hybrids of tall fescue (*Festuca arundinaceae*). The morphology of the fescue isolates was compared with *E. typhina* isolated from bent grass (*Agrostis perennans*). In all isolates, conidia were identical and were typical of *E. typhina*. In fescue grasses the endophyte failed to produce stromata, but on bent grass the fungus seasonally produced stromata, typical of the genus. Cattle grazing the fescue grasses showed signs of the fescue toxicity syndrome, the *E. typhina* was found in frequencies of 100%; in grasses from pastures in which cattle showed no signs of the syndrome, frequencies were 0 to 50%. Nutritional factors in vitro were more complex for the isolates from fescue than for the isolate from bent grass. These studies suggested that *E. typhina* includes biotypes that might be involved in the toxicity syndrome. The fescue biotypes grew poorly on media, and yields were inadequate for toxicity studies. However, the bent grass isolate grew well on three media, and extracts from two of these were toxic to chicken embryos. All isolates produced in vitro the nontoxic fungal steroid tetraenone [ergosta-4,6,8(14),22-tetraen-3-one], which has been isolated from toxic fescue grasses.

The fescue toxicity syndrome of cattle (6) is a sporadic but serious problem in all sections of the United States where varieties of tall fescue grass (*Festuca arundinaceae* Schreber) are grown as forage. Although the fescue toxicity syndrome has been experimentally induced in cattle by intraruminal or intraperitoneal injection of grass extracts (4, 17), the chemical identity of the toxic compound is unknown. On the basis of the clinical signs and the sporadic and seasonal nature of the syndrome, a vasoconstricting mycotoxin has been implicated (18). Indeed, the severe clinical signs of the syndrome, gangrene of the tail tip, hooves, and ears, are similar to those of the gangrenous-type alkaloid poisoning caused by the alkaloids from species of *Claviceps*. Species of *Claviceps* have been ruled out as the cause of the fescue toxicity syndrome (18). The involvement of fescue alkaloids in the syndrome has been examined. Perloline, one of several alkaloids of tall fescue, has been implicated from in vitro studies as a possible cause of poor performance of cattle that graze fescue during the summer (3). The effect of perloline on sheep was examined, but the effect of feeding this compound to cattle has not been determined. However, cattle grazing fescue hybrids selected for low perloline content showed signs of the summer syndrome of toxic fescue (J. Bond, J. B. Powell, D. J. Undersander, L. I. Colbert, and R. R. Oltjen, Abstr. Annu. Meet. Am. Soc. Anim. Sci., Southern Division, Abstr. 61, 1977).

Many fungi have been studied as causes of the fescue toxicity syndrome (19). Recently, the isolation of *Balansia epichloë* from grasses of a toxic fescue pasture and the in vitro toxicity of this clavicipitaceous systemic phytopathogen were reported (2, 10). Moreover, studies on the identity of toxic alkaloids produced by *B. epichloë* (11) and the possible involvement of other species of *Balansia* in the poor performance of animals grazing infected grasses prompted a search for other endophytic fungi in toxic grasses. We now report the isolation and cultivation of another clavicipitaceous endophyte, *Epichloë typhina* (Fries) Tulasne, from hybrids and varieties of toxic tall fescue and the demonstration of in vitro toxicity of one isolate from *Agrostis perennans* (Walter) Tuckerman, bent grass.

MATERIALS AND METHODS

Examination of samples. Toxic and nontoxic fescue pastures in the United States were randomly sampled (see Table 1 for sources) and grass tillers were kept moist and as fresh as possible until analyzed in the laboratory. Grasses were examined from pith scrapings removed from longitudinally slit culms of fertile tillers. For uniformity, the internode of the

culm between the grass crown and first node was used for examination. The pith was placed on a microscope slide, stained with the dye solution (50 ml of lactic acid and 100 ml of 0.1% aqueous aniline blue [8]), warmed in a flame, and microcopically examined. Grasses were scored positive when intercellular hyphae were seen. The prevalence of *E. typhina* in samples was expressed as frequency (percentage). The identity of the endophyte in fescue culms was confirmed as *E. typhina* after isolating and culturing the fungus as described below and comparing with *E. typhina* from bent grass similarly isolated and cultured.

Isolation and cultural methods. Short segments of grass culms used for isolation were surface sterilized by shaking them in 6% sodium hypochlorite for 3 min, rinsed in sterile distilled water, and incubated on Difco potato-dextrose agar (PDA) at ambient temperatures until fungal growth was visible, usually in 3 to 4 weeks. Strains 238, 239, 245, 246, 249, and 250 were isolated by this procedure (see Table 2). *E. typhina* 9091 was obtained from ascospores germinated on PDA. This isolate was found parasitizing bent grass, *A. perennans*, on which it seasonally produced stromata typical of the disease. Strains of *E. typhina* were maintained at 4°C on corn meal-malt extract agar (CMM). This medium was prepared from the following: corn meal agar (Difco), 17.0 g; malt extract (Difco), 20 g; yeast extract, 2 g; and distilled water, 1,000 ml. Both CMM and PDA media contained (per liter): streptomycin sulfate, 50 mg; and chloramphenicol, 50 mg.

Three liquid media were used for growth studies of seven strains of *E. typhina* (Table 2). One medium, M96, consisted of: mannitol, 10 g; sucrose, 30 g; soluble starch, 15 g; malt extract, 10 g; yeast extract, 2 g; ammonium succinate, 6 g; $MgSO_4 \cdot 7H_2O$, 2 g; K_2SO_4, 0.68 g; and distilled water, 1,000 ml. The second medium, M43, consisted of: sucrose, 30 g; malt extract, 20 g; peptone (Difco), 1 g; yeast extract, 2.5 g; and distilled water, 1,000 ml. The third medium, M102, contained: sucrose, 30 g; malt extract, 20 g; peptone (Difco), 2 g; yeast extract, 1 g: $MgSO_4$, 0.5 g; KCl, 0.5 g; KH_2PO_4, 1.0 g; and distilled water, 1,000 ml. The pH of each medium was adjusted to 6.0 with 1 N HCl or 10 N NaOH. Cultures were incubated in 100 ml of medium for 2 to 3 weeks on a gyratory shaker (150 rpm, 1-cm circular orbit) at 24 and 26°C in 500-ml triple-baffled shake flasks.

Fungal extracts. The entire contents of the flasks of 3-week-old cultures were blended in an equal volume of methanol in a Waring blender for 10 min and filtered. The residue was extracted in 100 ml of methanol and filtered, and the two filtrates were combined. The filtrate was evaporated to dryness, and the residue was taken up in 25 ml of sterile distilled water. The distilled water extract was adjusted to pH 6.0 to 6.5 with 1 N NaOH and then filter sterilized with a 0.22-μm membrane (Millipore Corp.). Antibiotics were added to the culture solution before it was injected into eggs (1).

Toxicity bioassays. The chicken embryo bioassay was used to determine toxicity (16). Twenty fertile White Leghorn chicken eggs were injected with culture extract into the yolk (0.05 ml) prior to incubation and onto the air sac (0.1 ml) after 5 days of incubation. Eggs were incubated in a Humidaire model 50 incubator at 40°C and 89% relative humidity and turned every hour. Filtrates from uninoculated culture medium were extracted as described above, and similar dosages were injected onto air sacs for controls.

Tetraenone analysis. Strains of *E. typhina* were cultured for 3 weeks on M43 as described above, and the mycelium was analyzed for the fungal steroid tetraenone [ergosta-4,6,8(14),22-tetraen-3-one] (10).

RESULTS

In infected fescue grasses the hyphae of *E. typhina* were intercellular and ran vertically between the host pith cells (Fig. 1A). The hyphae were coarse, unbranched, and contorted (Fig. 1B). Slide preparations in which the pith cells were separated demonstrated the intercellular position of the hyphae (Fig. 1C). Hyphae were narrow and straight in the hybrid fescue G1-307, developed for low perloline alkaloid content (Fig. 1D). Hyphae also were straight in pith scrapings of bent grass. Occasionally two hyphae were coiled in the intercellular spaces (Fig. 1C). Intracellular hyphae were not observed.

The frequency of *E. typhina* in toxic fescue samples was 100% regardless of the type of grass, but in nontoxic samples it varied from 0 to 50% (Table 1). Frequency and toxicity apparently were related; i.e., samples with 100% frequency were all toxic. Frequency represents only qualitative differences among samples and does not distinguish between the amounts of hyphae in samples. For example, the fungus was found in the Kentucky fescue hybrid G1-307 and two of the Georgia samples of Kentucky-31 (Ky-31) with an equal frequency of 100% (Table 1), but hyphal mass in these two samples differed. Pith tissue from the fescue hybrid G1-307 had a much greater number of intercellular hyphae than did that of Ky-31. Similarly, the hybrid grass from Maryland G1-306 contained more intercellular hyphae than did one sample from the Virginia variety, Kenhy; however, the fungus was found in equal frequencies (50%) in both (Table 1).

The endophyte grew out after 2 to 3 weeks of incubation on agar medium from surface-sterilized stems or pith scrapings of the fescue grasses; the fungus grew out from bent grass in 2 to 3 days. On CMM medium, all isolates produced elliptical conidia typical of *E. typhina* on short tapering phialides, and the conidia (7 to 10 by 1.5 to 3.5 μm) are capable of immediate germination. The growth rate was lower for strains isolated from fescue grasses than for the strain isolated from bent grass. Colonies of *E. typhina* from fescue on CMM and PDA were white, restricted, and somewhat wrinkled and attained diameters of 1 to 1.5 cm in 4 weeks at room temperature. Colonies of *E. typhina* from

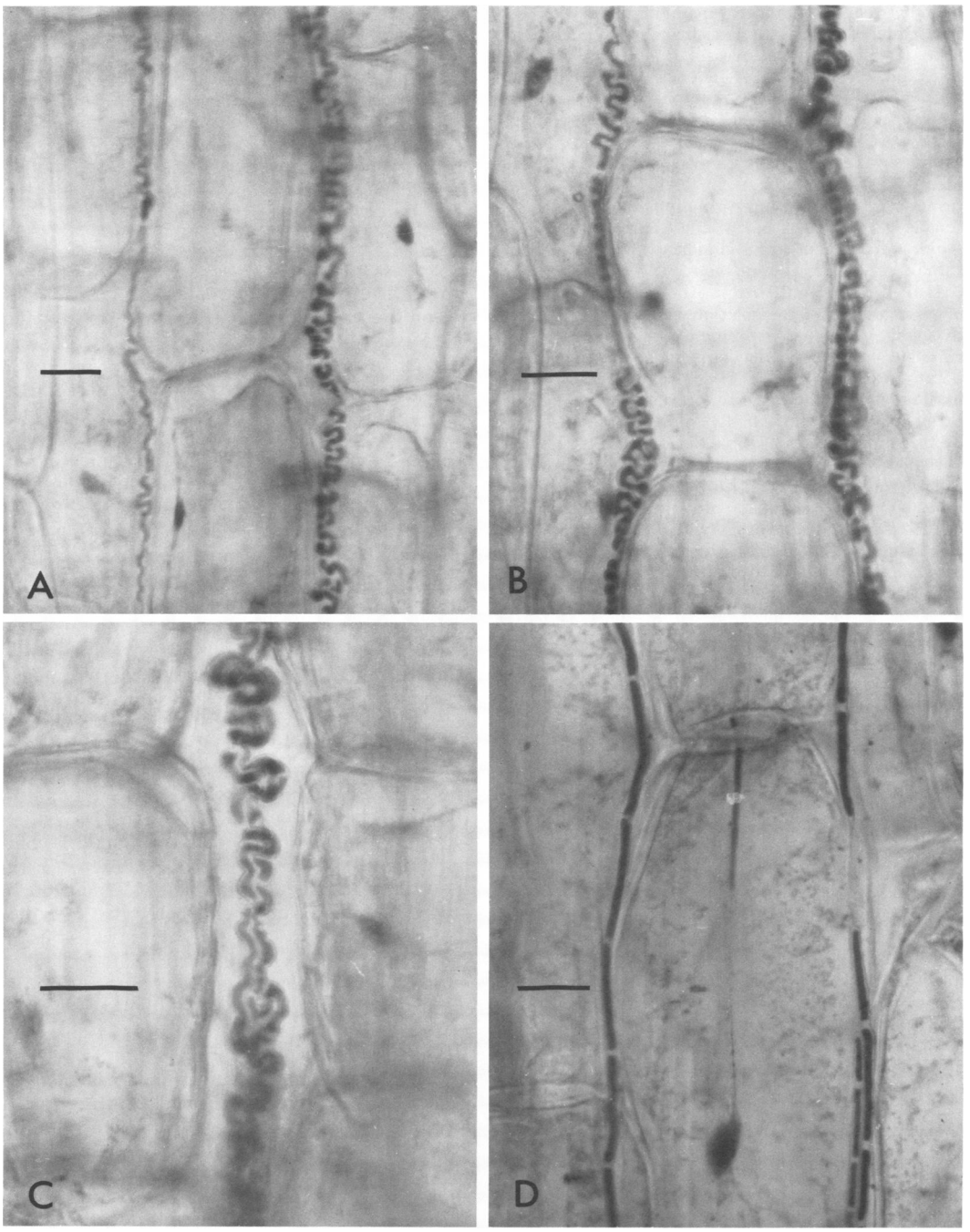

FIG. 1. *Light micrographs of stained mycelium of E. typhina in culm pith tissue from fescue grasses. (A and B) Two magnifications of contorted hyphae running parallel to pith cells of a toxic Georgia Ky-31 fescue sample. (C) Two coiled contorted hyphae in intercellular spaces of separated pith cells of a toxic Georgia Ky-31 fescue. (D) Narrow smooth hyphae in pith cells of a low perloline hybrid fescue, G1-307, from Maryland. Bars = 20 μm. (A) ×420; (B and D) ×537; and (C) ×672.*

bent grass were white and with growth at marginal areas first submerged and then with somewhat flocculent aerial mycelium, attaining a diameter of 8 cm in 4 weeks at room temperature. After repeated subculturing, the number of conidia produced by all strains decreased.

In submerged culture on media M43, M96, and M102, the growth (dry weight) of strains isolated from fescue was less than that of the isolate 9091 from bent grass (Table 2). Growth differed among the fescue isolates. Strains isolated from Kenhy and Ky-31 varieties of fescue produced more mycelium (dry weight) after 2 weeks on medium than did strains that were isolated from low (G1-307) and high (G1-306) perloline fescue varieties. On medium M43 all strains produced the steroid tetraenone (amounts not quantitated). Additionally, strain 246 differed from strains 238 and 9091 in its pH optimum in submerged culture on medium M43. The maximum dry weight of mycelium produced by strain 246 was 185 mg at pH 5.0, while the dry weights for strains 238 and 9091 were 205 and 637 mg of mycelia, respectively, but at pH 7.0.

Due to the sparse growth on all media for the fescue isolates of *E. typhina,* toxicity studies were not possible. Data from toxicity studies of strain 9091 from bent grass are shown in Table 3. Extracts of cultures on media M102 and M96 were toxic, but those of cultures on medium M43 were not. The culture extract of medium

TABLE 1. *Frequency of E. typhina in varieties and hybrids of tall fescue from pastures with and without signs of the fescue toxicity syndrome*

Sample location	Source[a]	Fescue type	Frequency (%)	Type of fescue toxicity syndrome
Georgia	CWB	Ky-31	100 (50/50)[b]	Summer syndrome[c] and gangrene of extremities
Georgia	CWB	Ky-31	10 (5/50)	None
Georgia	DB	Ky-31	100 (50/50)	Gangrene of extremities
Georgia	CWB	Ky-31	0 (0/50)	None
Kentucky	RCB	G1-306[d]	30 (3/10)	None
Kentucky	RCB	G1-307[e]	100 (10/10)	Summer syndrome
Maryland	JB	G1-307	100 (25/25)	Summer syndrome
Maryland	JB	G1-306	50 (15/30)	None
Missouri	GG	Ky-31	100 (15/15)	Gangrene of extremities
Missouri	GG	Ky-31	25 (5/20)	None
Virginia	HB	Kenhy	100 (20/20)	Gangrene of extremities
Virginia	HB	Kenhy	50 (10/20)	None

[a] CWB, C. W. Bacon, Field Crops Laboratory, R. Russell Agriculture Research Center, USDA, ARS, Athens, Ga; DB, David Bedell, Cooperative Extension Service Rural Development Center, University of Georgia, Tifton, Ga.; RCB, R. C. Buckner, Agronomy Department, University of Kentucky, Lexington, Ky.; JB, James Bond, Ruminant Nutrition Laboratory, Nutrition Institute, USDA, Beltsville Research Center, Beltsville, Md.; GG, G. Garner, Department of Agricultural Chemistry, University of Missouri, Columbia, Mo.; HB, Harry Bryant, Virginia Forage Research Station, Virginia Polytechnic Institute, Middleburg, Va.

[b] Numbers in parentheses indicate a ratio of the tillers found infected with the endophyte to the total number of tillers obtained from a source.

[c] Signs of the summer syndrome include rough hair coat, loss of weight, and rapid breathing (12).

[d] Rye grass-tall fescue hybrid selected for high perloline content.

[e] Rye grass-tall fescue hybrid selected for low perloline content.

TABLE 2. *Tetraenone production and mycelial dry weight of strains of E. typhina on culture media*[a]

Strain	Sample type and source[b]	Dry wt (mg)			Tetraenone
		M43	M96	M102	
238	T Ky-31 (CWB)	161	83	52	+
239	NT Ky-31 (CWB)	163	81	50	+
245	T Kenhy (HB)	164	86	52	+
246	T G1-307 (JB)	81	46	24	+
249	NT G1-306 (JB)	102	55	31	+
250	NT Kenhy (HB)	155	76	50	+
9091	Bent grass	634	527	875	+

[a] Dry weight determined after 2 weeks of incubation of medium; tetraenone determined by thin-layer chromatography (10) after 3 weeks of incubation on M43.

[b] See Table 1 for references to sample source and location; T, a toxic grass sample; NT, a nontoxic grass sample.

TABLE 3. *Toxicity of E. typhina 9091 to chicken embryos*

Medium	Chicken embryo death/total eggs[a]	
	Before incubation	After incubation
M43	0/20	0/20
M96	20/20	9/20
M102	20/20	20/20

[a] Each dosage is an average of two replications; each replication consists of 20 eggs. The background mortality of these media was 0%.

M96 was more toxic when injected into the yolk before incubation of eggs than when injected on the air cells after 5 days of incubation.

DISCUSSION

An intercellular systemic mycelium was seen microscopically in culm pith tissue of fescue grasses, and after isolation on agar it resembled *E. typhina* in colony appearance and in production of conidia typical of the genus and its conidial state described as *Sphacelia typhina* (Persoon) Saccardo (13). *E. typhina* has been reported on a number of grasses in North America (7, 15) and in five species of *Festuca* (7), but the reports were based on the presence of the conspicuous external stromata of the fungus on the infected grasses. Yellow to orange stromata surround the sheath of the flag leaf at the apex and produce in succession conidia borne on phialides on the surface of the stroma and ascospores borne in perithecia immersed in the stroma. The inflorescence is partially or completely suppressed. These are the typical symptoms, commonly called the "choke disease," as exhibited by infected bent grass in Georgia. The wide-spread occurrence of *E. typhina* as an endophyte in tall fescue has been unnoticed previously because the infections are symptomless. Consequently, *E. typhina* has not been investigated as a possible cause of the fescue toxicity syndrome. Similar symptomless infections of rye grass and fescue with *E. typhina,* however, were reported in Great Britain (14) and New Zealand (8, 9). This symptomless infection is not the rule in the genus *Festuca* as mature stromata have been found on five species of fescue grasses (7).

E. typhina undoubtedly is comprised of a number of biotypes. Sampson (14) found differences in growth rates between isolates from infected *Festuca rubra* and *Dactylis glomerata.* These differences in growth rates reflect differences in nutritional requirements and suggest that the biotypes isolated from fescue are more nutritionally dependent, in their parasitic habit, than is the biotype isolated from bent grass. Even with the limited growth, all fescue isolates produced the nontoxic fungal steroid tetraenone, which previously was reported as present in toxic fescue samples and absent in nontoxic samples and which was used as a field indicator of toxicity (10). In that study the fungus that produced tetraenone on the fescue grasses was not determined, but an endophyte was suspected. The apparent relation between frequency of *E. typhina* as an endophyte in fescue and toxicity of the grass suggests the possibility of involvement of *E. typhina* in the fescue toxicity syndrome. A quantitative assessment of relative density of mycelium in the samples of various fescues might strengthen this relationship. Our study indicates that one biotype of this endophyte is toxic to chicken embryos. Proof of toxicity in *E. typhina* may be complicated by inherent differences in biotypes of the fungus and by host and seasonal influences on fungal metabolism as in the related genus *Claviceps* (5). The nature of the nutrients provided by different hosts might affect the metabolism of the fungus in the same way as variations in artificial culture media influenced the production of toxic metabolites by *E. typhina* 9091 (Table 2). Differences in hyphal morphology (Fig. 1D) indicate either a difference in biotype or a host-induced effect on growth pattern.

The fact that infections of fescue are symptomless means that no fungal reproductive structures are involved in the transmission of the endophyte. Sampson, however, demonstrated that *E. typhina* growing as an endophyte in symptomless infections of *F. rubra* was transmitted in the seed of the host (14), and seed transmission seems to be the most probable source of the infection observed in fescue in the United States. If the seed is the primary source of infection in fescue, endophyte-free plants might be produced from seed that has been stored for a year or more (9). Alternatively, the endophyte might be eliminated by treatment with a systemic fungicide.

ACKNOWLEDGMENTS

We thank D. Bedell, J. Bond, H. Bryant, R. C. Buckner, and G. Garner for samples for fescue grasses; R. Bennett for technical assistance; and F. W. Gould, Department of Range Science, Texas A&M University, College Station, Tex., for identification of grasses.

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