

Procedure for Isolating the Endophyte from Tall Fescue and Screening Isolates for Ergot Alkaloids

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A procedure was developed to isolate and determine ergot alkaloid production by *Acremonium coenophialum*, the endophytic fungus of tall fescue. The procedure established that macerated leaf sheath or pith from inflorescence stem placed either in a liquid medium or on a corn meal-malt extract agar medium produced isolated mycelium and characteristic conidia within a 3- to 3.5-week period. Once isolated, each fungus was placed in another liquid medium, M104T, where competent strains produced total ergot alkaloids ranging from 38 to 797 mg/liter. Several isolates were negative for ergot alkaloid synthesis. The production of ergot alkaloids by individual isolates was unstable; isolates rapidly degenerated in their ability to produce ergot alkaloids during subculture. However, the procedure as presented allows the assessment of an isolate for ergot alkaloid synthesis during its initial isolation.

An association consisting of the endophytic fungus *Acremonium coenophialum* Morgan-Jones et Gams and the tall fescue grass, *Festuca arundinacea* Schreber, was identified (2) and later established as being toxic to ruminant herbivores (7, 18). More recently, this association has been shown to be a deterrent of insect pests (5, 8, 10, 16). The association is widespread (19), and there are additional benefits derived from this fungus-grass relationship (17, 20) which, along with those stated, suggest that this association is mutualistic (3). The poor performance problems and toxicities in animals which ingest infected tall fescue were prevented when animals were grazed on endophyte-free grass. However, the ecological success and survival of endophyte-free fescue have been questioned (3); therefore, efforts are being directed toward developing forage grasses, as well as turf and conservation grasses, that have agronomic benefits associated with endophytes.

To accomplish this, it is necessary not only to establish the nature of the benefits within the association but also to identify those useful agronomic benefits derived from the fungus. To define the nature of the benefits derived exclusively from the fungus, a procedure must exist that will allow for isolation of endophytes from hosts and determine physiological characteristics. Two studies (6, 9) have defined the growth requirements of an identical strain of the fescue endophyte which had been maintained and subcultured in laboratories, but neither study addressed the requirement for isolating the endophyte from forage grasses for subsequent physiological characterization. Therefore, a procedure for isolating the fungus directly from tall fescue into liquid or agar medium is presented. Since ergot alkaloids have been associated with an agronomically important aspect of this association (12), this paper also describes a medium which measures the competence of an isolated fungus to produce these alkaloids.

MATERIALS AND METHODS

Endophyte isolation procedure. Infection of field- and greenhouse-grown plant materials used in this study was determined by a staining procedure (2). Infected material was freshly collected by cutting leaves 0.5 cm from the crown of the grass. Leaf blades were removed, and the

resulting sheaths were washed free of all debris. Sheaths were again washed and cut into approximately 3-cm sections. Sheaths were placed in sterile disposable cups, 30 ml of full-strength bleach (5.25% hypochlorite) was added, and the sheaths were agitated for 5 min. The bleach was poured off, and sheaths were rinsed once in sterile distilled water. Grass tissues (sheath or inflorescence stem) were aseptically placed in sterile petri dishes, and 0.5-cm segments were cut from the ends of each sheath and discarded. Sheaths were halved, and one half of each sample was teased (macerated) throughout its length with a fine needle to expose internal plant tissue. The remaining half of each sheath was not teased but was used directly for comparison of tissue preparation. Inflorescence stems were treated similarly, except that they were cut longitudinally into halves and that the central pith tissue was removed for inoculations.

One to several pieces of either teased or unteased segments were placed in 125-ml triple-baffled shake flasks, each containing 50 ml of a liquid medium (M102) consisting of: sucrose, 30 g; malt extract, 20 g; peptone (Difco Laboratories, Detroit, Mich.), 2 g; yeast extract, 1 g; MgSO₄, 0.5 g; KCl, 0.5 g; KH₂PO₄, 1.0 g; chloramphenicol, 0.05 g; and distilled water, 1,000 ml. The medium was adjusted to a pH of 6.0 with 10 N NaOH. The remaining segments of each tissue type were partially inserted into corn meal-malt extract (CMM) agar. Inoculated shake flasks were sealed with styrofoam plugs and placed on a gyratory shaker (200 rpm, 1-cm circular orbit). Fungus-infected grass tissues in flasks or petri dishes were incubated at 24°C in constant darkness for 4 weeks or until mycelium was evident. In flasks where grass tissue took longer than 4 weeks to produce fungi, evaporation was prevented by covering the styrofoam plugs with Saran Wrap.

Culture identification. The identities of fungi isolated from grasses were established by culture on CMM agar (1). Briefly, an inoculum obtained from the liquid medium used in the isolation procedure described above was placed on CMM agar, incubated in 12-h light-dark cycles at 24°C, and observed by microscopy (×100 or ×250 magnification) at 7-day intervals. Endophytes isolated from grasses that were placed directly onto CMM agar were observed in the same way. The presence of the conidia on conidiophores in a

typical T configuration is suggestive of the endophyte. The identity of the endophyte was confirmed by using published descriptions (11, 14). All fungi used for ergot alkaloid synthesis were transferred to and maintained in M102 at room temperature either in flasks of M102 or in tubes of CMM agar.

Ergot alkaloid production and analysis. Isolates of fungi used to determine ergot alkaloid production competence were obtained from 14- to 21-day-old cultures grown in M102, as described above. Ergot alkaloid production was accomplished on a medium (2) modified specifically for the tall fescue endophyte: sorbitol, 100 g; glucose, 40 g; glutamic acid, 10 g; KH_2PO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; yeast extract, 3.0 g; DL-tryptophan, 0.8 g; and distilled water, 1,000 ml. This medium (M104T) was adjusted to pH 5.6 with 10 N NaOH, and 100 ml was added to 500-ml triple-baffled cotton-stoppered flasks. Flasks were inoculated with 1 ml of inoculum from M102 that had been washed by centrifugation and suspended in sterile distilled water. Inoculated flasks were incubated at 24°C in darkness for 14 days on a gyratory shaker and were finally incubated as stationary cultures until ergot alkaloids were detected, usually within an 8-week period.

Ergot alkaloids were determined by adjusting the pH of the culture medium to one in a range of 9.5 to 10.0 with 10 N NaOH. The culture (mycelium and medium) was homogenized for 1 min, and ergot alkaloids were extracted with two 100-ml portions of CHCl_3 . The CHCl_3 extracts were combined, and alkaloids were partitioned into two 100-ml portions of 2% tartaric acid. The tartaric acid solution was made basic (to a pH of 9.5 to 10.0) with NaOH, and ergot alkaloids were removed from the basic tartaric acid by extraction with two 50-ml portions of CHCl_3 . The CHCl_3 fractions were combined and evaporated to dryness on a rotary evaporator, and the residue was taken up in 5 ml of tartaric acid. Total ergot alkaloids were determined colorimetrically on a dilution of the tartaric acid extract (13) by using a standard solution of ergotamine tartrate as a reference standard. The identities of the alkaloids were established by cochromatography with reference standards and other analytical procedures (15).

RESULTS

On CMM agar, the endophyte grew from one or both ends of plant tissue, although occasionally the fungus also grew from the side of tissue. On agar, growth was slow, and the fungus only reached a diameter of 1 mm in 6 weeks. In liquid medium, the initial inoculum that formed on one end of the plant tissue completely filled the flask during shake culture. Conidia were produced in abundance under both culture conditions, especially by fresh subcultured isolates.

The endophyte was isolated from all tissue preparations used in this study (Table 1). The tissue preparation that yielded the most endophyte was pith removed from the inflorescence stem, and the best results were obtained when this tissue type was placed in the liquid medium M102. Inflorescence stem tissue placed in M102 required the least average isolation time. The tissue preparation that yielded the least endophyte was nonmacerated sheath tissue placed in M102; this type of tissue required the longest isolation period, regardless of the isolation medium.

Endophytes isolated either on CMM agar or in M102 produced ergot alkaloids when transferred to M104T. However, the total amount of alkaloid produced on each medium varied. The mean totals of ergot alkaloids produced by 83

TABLE 1. Effect of tissue type and preparation on time of endophyte isolation on agar and liquid medium

Tissue and treatment	% yielding endophyte ^a	Average isolation time (wk) ^b
Unteased sheath on CMM agar	20	4.5
Teased sheath on CMM agar	35	3.5
Unteased sheath in M102	15	5.5
Teased sheath in M102	29	3.5
Inflorescence pith on CMM	62	3.5
Inflorescence stem on CMM	48	3.5
Inflorescence pith in M102	81	3.0
Inflorescence stem in M102	46	3.5

^a Represents mean percent of a total of 50 samples for each treatment; samples represent different geographical regions, including different fescue varieties.

^b Recorded as either the time necessary for fungus to reach a colony diameter of 0.5 mm on the agar medium or the time until the first visible appearance in the liquid medium.

strains initially isolated either on CMM agar or in M102 were 215 and 476 mg/liter, respectively. Endophytes isolated in M102 always produced significantly ($P = 0.05$) higher yields of total alkaloids than those isolated on CMM agar. Endophytes isolated from tissue on CMM agar would not grow when transferred directly to M104T. Such fungi had to be transferred to M102 before they could be assessed for ergot alkaloid synthesis in M104T medium. The average decrease in total ergot alkaloid production by isolates from CMM agar may be related to the decrease in production by most isolates during subculturing (Table 2), since CMM agar-isolated fungi were subcultured twice as frequently as those initially isolated into M102.

The time required for maximum accumulation of ergot alkaloids (Table 3) varied among isolates obtained from either procedure. Not all isolates produced ergot alkaloids, even though several were isolated from the same pasture. The amount of fungal dry weight produced in M104T by each isolate varied significantly and was not related to total ergot alkaloid synthesis but increased as each isolate was subcultured (Table 2).

DISCUSSION

The procedure reported here allows for the isolation of the tall fescue endophyte directly from the host and determines

TABLE 2. Endophyte stability for in vitro ergot alkaloid production during subculture

Isolate	Total ergot alkaloid produced (mg/liter) in subculture ^a :				
	0	1	5	10	20
RRC 347	772	578 (0.482)	515	321	356 (0.874)
RRC 358	38	ND (0.573)	ND	ND	ND (1.322)
RRC 321	292	215 (0.382)	ND	ND	ND (1.021)
RRC 241	221	ND (0.521)	ND	ND	ND (1.321)
RRC 360	391	ND (0.422)	ND	ND	ND (1.723)
RRC 320b	362	210 (0.722)	39	ND	ND (2.045)
RRC 347wc	521	409 (0.631)	ND	ND	ND (1.221)

^a Each subculture was made after 14 days of growth in M102 and was transferred either to fresh M102 for growth (dry weights) or to M104T for ergot alkaloid determination. ND, None detected. Numbers in parentheses refer to the means of three replications of dry mycelial weight (grams per 100 ml) produced after 21 days of growth of subcultures 1 and 20.

TABLE 3. Variations of time required in selected isolates of endophytes for total ergot alkaloid production

Isolate	Location ^a	Total ergot alkaloids (mg/liter)	Optimum time (wk)
RRC 321	3	199	12
RRC 238bR	1	586	6
RRC 347	2	797	6
RRC 238w	1	ND	
RRC 358	1	ND	
RRC 346F	4	387	12
RRC 346	2	38	10
RRC 238	1	458	15
RRC 241	2	ND	
RRC 336	5	ND	
RRC 239	1	ND	
RRC 335	5	ND	
RRC 254	2	568	15
RRC 254b	7	291	15
RRC 323	3	321	8
RRC 320b	6	151	8
RRC 320	8	ND	
RRC 320a	6	456	7
RRC 347W	5	ND	
RRC 347wc	5	312	7
RRC 254c	7	ND	
RRC 260	9 ^c	ND	

^a Pasture locations: 1, Mansfield; Ga.; 2, Jonesboro, Ga.; 3, Buford, Ga.; 4, Franklin, Ga.; 5, West Virginia; 6, southern Illinois; 7, Arkansas; 8, Virginia; and 9, Missouri.

^b All isolates were strains obtained from leaf sheaths in liquid M102 and represent subculture 2. ND, None detected within an 18-week period.

^c Isolated from Kenhy variety fescue; all others from Kentucky 31 tall fescue.

its potential to produce ergot alkaloids. The data indicated that placement of macerated inflorescence stem tissue into liquid M102 is the method of choice. Only 19% of macerated tissue, regardless of type, did not yield endophytes on culture. However, since inflorescence stems are available only during the spring, macerated tissue from sheaths placed in liquid M102 is acceptable. Nonmacerated sheath placed into M102 was the tissue preparation that yielded the least growth of fungi. In general, nonmacerated tissue, regardless of type and isolation medium, yielded fewer endophytes than did teased tissue and required longer incubation periods. The isolation medium did not influence isolation time; however, tissue type was important in this respect.

In both liquid M102 and CMM agar, the endophytes readily produced conidia typical for *A. coenophialum* (11, 14). Therefore, these media may be used to identify this species or to distinguish it from other species of endophytes which may exist on tall fescue or other forage grasses (19). Most isolates initially produced conidia abundantly, but this ability declined and was lost upon subculture.

The data in this study indicate that competence in ergot alkaloid synthesis by the strains of endophyte degenerates rapidly. This indicates that endophytes must be tested soon after isolation and that other physiological functions might also degenerate. This phenomenon is similar to the decline of mycotoxin synthesis by other fungi during subculture (4), which is primarily genetically determined, but related to culture conditions. In other endophytic fungi, e.g., *Balansia* species, most isolates retain this ability when subcultured onto similar media (1). In many isolates of fescue endophytes, the initial mycelium obtained is multinucleate (unpublished data); therefore, subsequent subculturing might produce through selection a mixed culture of nutri-

tional variants, many of which characteristically have faster growth rates. Degeneration of ergot alkaloid synthesis in a strain might be prevented by obtaining a single conidial isolate on CMM agar and carrying it through the screening procedure as described here.

More mycelial dry weight was produced by subcultured strains than by initial isolates, which possibly reflects a faster growth rate completely atypical of initial isolates. Apparently, these subcultured isolates were adapting to in vitro conditions of culture, which suggests that laboratory nutritional studies of such isolates may reflect neither the in vivo nor in vitro requirements. This is supported by data which indicate that the fungus would not grow from the grass initially in a less complex medium, M104T, but would do so when subcultured as an inoculum cultured first in M102, a very complex medium. The ergot alkaloid production medium, M104T, which is similar to that mentioned earlier (2, 3), supported growth of all isolates and produced mycelial dry weights comparable with those reported on other less complex media (6, 9) designed to determine the nutritional requirement of a strain of this fungus. However, it is not known whether these media (6, 9) are suitable for the synthesis of ergot alkaloids.

This study indicates that there are physiologically distinct strains of endophytes in tall fescue and that one physiological characteristic, ergot alkaloid synthesis, may be rapidly lost during culture, while yet another, growth rate, may be selected for during laboratory culture. The procedure as presented allows for the rapid isolation and assessment of isolates which will prevent this problem. The behavior of these modified fungi when reintroduced into tall fescue is unknown. If the loss in ergot alkaloid synthesis remains and the pest resistance mechanism is maintained, such isolates when reintroduced into fescue might be important starting materials for studies on the improvement of tall fescue for agronomic qualities associated with endophytes (3).

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