Infection of Tall Fescue with *Acremonium coenophialum* by Means of Callus Culture

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ABSTRACT

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Calli initiated from peduncle tissue of endophyte-free tall fescue plants and maintained on a culture medium were inoculated with the endophytic fungus *Acremonium coenophialum*. This was accomplished by making an incision into the middle of calli 1–2 cm in diameter and inserting a small amount (<1 mm^2) of mycelium followed by a 10-wk incubation. About 17% of the regenerated plants from these calli were infected as determined by microscopic examination of leaf sheaths and by enzyme-linked immunosorbent assay. Additional evidence for the successful establishment of *A. coenophialum* in these plants was the accumulation of pyrrolizidine alkaloids and conferment of resistance to the oat bird cherry aphid. *A. coenophialum* was reisolated from leaf sheaths of the artificially inoculated plants. Inoculations of perennial ryegrass calli with *Acremonium* spp. resulted in only endophyte-free plants upon regeneration of callus. We were unable to induce shoot regeneration in *Acremonium*-inoculated bluegrass and orchardgrass calli.

A seed-transmitted endophytic fungus (*Acremonium coenophialum* Morgan-Jones & Gams) present in tall fescue (*Festuca arundinacea* Schreb.) has been associated with fescue toxicity in cattle (1,9) and with resistance to some species of insects (11,17). A related endophyte (*Acremonium loliae* Latch, Christensen, & Samuels) found in perennial ryegrass (*Lolium perenne* L.) and implicated in a neurological disorder of sheep known as staggers (6) also confers insect resistance to the grass (2,7). Although the endophytes can be easily cultured on various media, it has proven considerably more difficult to artificially inoculate endophyte-free tall fescue and perennial ryegrass. Only recently have Koch's postulates been completed through inoculation of meristem tissue of 1-wk-old grass seedlings by Latch and Christensen (16). This paper describes the successful inoculation of endophyte-free tall fescue with *A. coenophialum* by means of callus culture, resulting in established endophyte-infected tall fescue plants. It also discusses attempts to infect calli of bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L.), and perennial ryegrass with *A. coenophialum* and *A. loliae*.

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MATERIALS AND METHODS

Plant materials. A Kentucky 31 (Ky 31) breeders' seed field on the University of Kentucky experiment station research farm at Lexington served as the source of endophyte-free tall fescue plants. Individual plants were brought into the greenhouse in January 1984 to initiate inflorescence production. Endophyte-free Grimalda perennial ryegrass and Boone orchardgrass plants as well as plants of three bluegrass cultivars, A-34, Adelphi, and Kenblue, from a turf trial were subsequently handled in the same manner to promote panicle development. The greenhouse was maintained at about 23 C with supplemental lighting to obtain a 12-hr photoperiod.

Callus culture. Calli were produced from peduncle tissue according to the procedure of Kasperbauer et al (14). Plants were selected when panicles were beginning to emerge above the flag leaf. Explants were of soft young tissue from the lower ends of rapidly elongating peduncles. This peduncle tissue was cut into segments 2–3 mm and placed on culture medium after surface-sterilization and removal of leaf sheaths (14). The basic culture medium was the same as that used by Kasperbauer and Collins (15), with modified levels of auxin. The concentration of 2,4-D (2,4-dichlorophenoxyacetic acid) used in the callus initiation and maintenance medium was 3 mg/L, whereas the levels of 2,4-D in the shoot-regeneration and rooting media were 0.25 and 0 mg/L, respectively. Kinetin and NAA (1-naphthaleneacetic acid) were not used. All other components of the media were the same (15), except the concentration of sucrose in the rooting medium was lowered from 20 to 10 g/L. The concentration of agar in all media was 8 g/L, and pH was adjusted to 5.9 before autoclaving.

Inoculation of callus. Isolates of *A. coenophialum* and *A. loliae* were obtained from leaf sheaths of Ky 31 tall fescue and Ellett perennial ryegrass, respectively (13). Cultures were maintained on a cornmeal agar medium as described previously (12).

Calli were subcultured at 4- to 6-wk intervals on the callus maintenance medium before inoculation. Tall fescue and perennial ryegrass calli were inoculated 10–12 wk after callus initiation, whereas bluegrass and orchardgrass calli were inoculated 20 wk after callus initiation.

Inoculation was accomplished by making an incision in the middle of calli 1–2 cm in diameter with a scalpel and inserting a small section (<1 mm^2) of mycelium obtained from the periphery of the fungal colony. Flame-sterilized instruments were used in a laminar-flow transfer hood. After incubation in the dark at 23 C for 10 wk, calli were transferred to shoot-regeneration medium and placed in culture chambers at 20 C with cool-white fluorescent lamps (60–70 μE m^{-2} s^{-1}) and a 12-hr photoperiod.

Plant establishment. Calli from which leafy shoots developed, usually within 4 wk on the shoot-regeneration medium, were transferred to rooting medium. After rooting, shoots were transplanted

Table 1. Summary of grass calli inoculations with *Acremonium coenophialum* and *A. loliae*

Grass	Endophyte	Calli inoculated (no.)	Calli producing shoots (no.)	Regenerated plants (no.)	Infected plants (no.)
Tall fescue	*A. coenophialum*	31	24	48	8
	A. loliae	18	8	9	0
Perennial ryegrass	*A. coenophialum*	17	4	5	0
	A. loliae	29	6	8	0
Bluegrass	*A. coenophialum*	9	0	0	0
	A. loliae	8	0	0	0
Orchardgrass	*A. coenophialum*	15	0	0	0
	A. loliae	15	0	0	0

into a potting mixture composed of 50% Pro-Mix BX (Premier Peat Moss Co., New Rochelle, NY), 30% loam, and 20% sand in 7.5-cm plastic containers. Transplants were placed under the greenhouse bench in transparent plastic bags for 4 days to prevent desiccation. The bags were partially opened for an additional 3 days to gradually condition the transplants to greenhouse humidity levels. After removal of the bags, transplants were set on top of the bench.

Determination of infection. The resultant plants obtained from the inoculated calli were tested for the presence of endophyte by some or all of the following methods: microscopic examination of leaf sheaths (4), enzyme-linked immunosorbent assay (ELISA) (10,12), accumulation of pyrrolizidine alkaloids (3), or an aphid feeding-preference assay (AFPA) using the oat bird cherry aphid (*Rhopalosiphum padi* L. (17). Plants were tested 4–5 mo after establishment. For the AFPA, plants were randomly arranged in trays with plants touching each other and *R. padi* aphids of all ages were scattered over plants. The trays were placed in a growth chamber at 20 C with a 12-hr photoperiod (90–100 μE m^{-2} s^{-1}), and aphids on each plant were counted after 18 days.

Completion of Koch's postulates. Leaf sheaths of plants found positive for endophyte presence according to the above tests were surface-sterilized in 20% Clorox for 3–5 min, rinsed in sterile water, and placed on potato-dextrose agar containing streptomycin sulfate and penicillin G at concentrations of 100 mg/L. The leaf sheath tissue was examined after 3 wk for growth of the endophytic fungus onto the agar surface.

RESULTS AND DISCUSSION

Calli were obtained from peduncle tissue of tall fescue, perennial ryegrass, orchardgrass, and bluegrass. There was no evidence of naturally occurring endophyte in any of these calli because all callus cultures were free of endophyte by visual observation. Callus cultures obtained from endophyte-infected plants show the presence of endophyte 3–4 wk after initiation (5,14; *personal observation*). Furthermore, representative uninoculated calli of each explant source of the original tall fescue and perennial ryegrass plants were tested by ELISA (10,12) and found negative for endophyte presence.

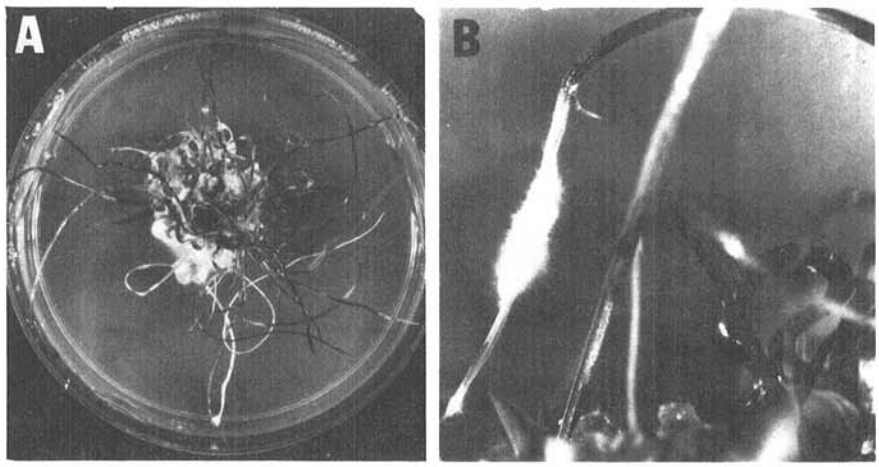

Fig. 1. Tall fescue callus 15 wk after inoculation with *Acremonium coenophialum*. **(A)** Entire callus with regenerated shoots. **(B)** Close-up of a regenerated shoot showing external mycelium of *A. coenophialum*.

The calli, regardless of grass species, appeared to serve as a suitable substrate for growth of both *A. coenophialum* and *A. loliae*, because mycelium was clearly visible on the surface of all calli by the end of the 10-wk incubation period after inoculation. Seventy-seven percent of the tall fescue calli inoculated with *A. coenophialum* produced shoots after transfer to the shoot-regeneration medium, and many calli produced multiple shoots and plants (Table 1, Fig. 1). In contrast, 44% of the tall fescue calli inoculated with *A. loliae* resulted in regenerated plants, and production of shoots from perennial ryegrass calli inoculated with *A. coenophialum* and *A. loliae* was only 24 and 21%, respectively (Table 1). We were unable to induce shoot production in the endophyte-inoculated bluegrass or orchardgrass calli (Table 1); however, abundant root regeneration occurred on many of the orchardgrass calli. Variations in the auxin component of the shoot-regeneration medium may be necessary to promote shoot formation in the bluegrass and orchardgrass calli.

A. coenophialum was present in eight (17%) of the plants regenerated from the *A. coenophialum*-inoculated tall fescue calli (Tables 1 and 2). These eight plants (C, D, H, J, K, N, O, and P) (Table 2) were traced to six separate calli, which in turn represented peduncle explants from three original tall fescue plants. Frequently, both endophyte-infected and endophyte-free plants were obtained from the same single inoculated callus (Table 2). All regenerated plants of tall fescue calli inoculated with *A. loliae* and of perennial ryegrass calli inoculated with *A. coenophialum* or *A. loliae* were free of endophyte, indicating that these callus inoculations were not successful (Table 1).

We obtained the same results with each of the methods used for determining the presence of *A. coenophialum* in the regenerated tall fescue plants (Table 2). Accumulation of pyrrolizidine alkaloids (*N*-acetyl and *N*-formyl loline) was only detected in plants that tested positive for endophyte presence by microscopic examination (Fig. 2A) and ELISA (Table 2). The mean number (± SD) of aphids observed on the endophyte-free tall fescue plants in the AFPA was 180 ± 66, about fivefold greater than the mean of 35 ± 18 observed on the endophyte-infected plants (Table 2). Recent studies suggest that the pyrrolizidine alkaloids in tall fescue are an insect-feeding deterrent (11). *A. coenophialum* was reisolated from leaf sheaths of regenerated plants C, D, H, and K (Table 2, Fig. 2B).

Table 2. Determination of infection by *Acremonium coenophialum* in plants obtained from inoculated tall fescue calli

Plant	Callus[a]	LSS[b]	ELISA[c]	*N*-acetyl and *N*-formyl loline (μg/g)	AFPA[d]
A	2	−	−	ND[e]	221
B	2	−	−	ND	269
C	2	+	+	838	26
D	2	+	+	1,029	38
E	2	−	−	ND	255
F	2	−	−	ND	180
G	4	−	−	ND	73
H	4	+	+	927	30
I	4	−	−	ND	151
J	17	+	+	286	52
K	20	+	+	540	8
L	35	−	−	ND	157
M	35	−	−	ND	132
N	35	+	+	307	66
O	40	+	+	275	32
P	40	+	+	207	24

[a] Callus from which plant was derived.
[b] Leaf sheath staining.
[c] Enzyme-linked immunosorbent assay.
[d] Aphid feeding-preference assay. Plants placed adjacent to each other in trays, and about 200 *Rhopalosiphum padi* aphids were scattered over the plants. Number of aphids on respective plants after 18 days.
[e] Not detected.

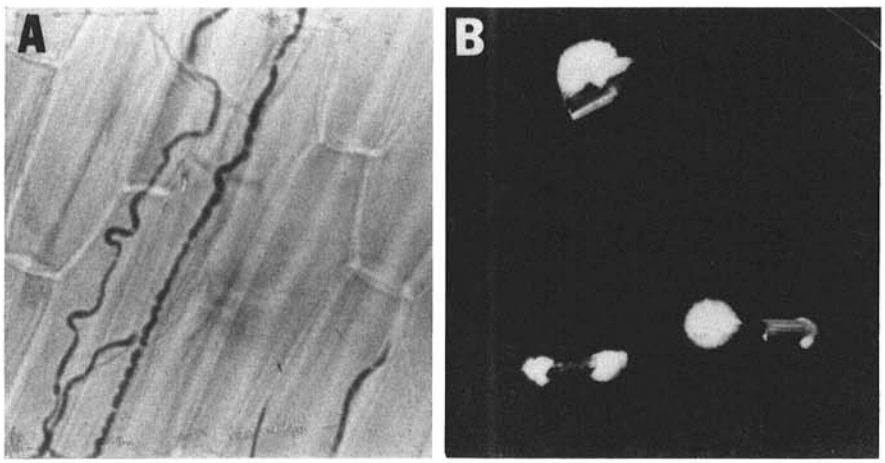

Fig. 2. Presence of *Acremonium coenophialum* in tall fescue plants regenerated from inoculated peduncle callus. **(A)** Hyphae of *A. coenophialum* running longitudinally in epidermal leaf sheath tissue. **(B)** *A. coenophialum* after 4 wk of growth from leaf sheaths on potato-dextrose agar.

Because *Acremonium* spp. appear to confer some benefits to the plant, such as insect resistance (2,11), introduction of these fungi into endophyte-free grasses may be desirable. This may be especially true for turfgrasses in which any associated harmful effects on livestock (1,6) would be of minor importance. We do not know, however, if the beneficial aspects of endophyte presence would necessarily be expressed in different endophyte-host grass species combinations. Inoculation of grass calli may be a useful alternative to the seedling-inoculation procedure of Latch and Christensen (16) for introducing these endophytic fungi into various grasses.

Tissue-culture technology has been widely used to investigate host-fungus interactions (8,18). The successful inoculation of endophyte-free tall fescue callus with its respective endophyte and the regeneration of this calli to obtain endophyte-infected plants offers new opportunities for studying the relationships between endophytic fungi and grasses.

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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. Barker, G. M., Pottinger, R. P., Addison, P. J., and Prestidge, R. A. 1984. Effect of *Lolium* endophyte fungus infections on behaviour of adult Argentine stem weevil. N.Z. J. Agric. Res. 27:271-277.
3. Bush, L. P., Cornelius, P. L., Buckner, R. C., Varney, D. R., Chapman, R. A., Burrus, P. B., 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.
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 seed samples for *Acremonium coenophialum*. Crop Sci. 23:172-174.
6. Fletcher, L. R. 1982. Observations of ryegrass staggers in weaned lambs grazing different ryegrass pastures. N.Z. J. Exp. Agric. 10:203-207.
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. Helgeson, J. P., and Haberlach, G. T. 1980. Disease resistance studies with tissue cultures. Pages 179-184 in: Tissue Culture Methods for Plant Pathologists. D. S. Ingram and J. P. Helgeson, eds. Blackwell Scientific Publications, Oxford. 272 pp.
9. Hemken, R. W., Jackson, J. A., Jr., and Boling, J. A. 1984. Toxic factors in tall fescue. J. Anim. Sci. 58:1011-1016.
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., Dahlman, D. L., Siegel, M. R., Bush, L. P., Latch, G. C. M., Potter, D. A., and Varney, D. R. 1985. Insect feeding deterrents in endophyte-infected tall fescue. Appl. Environ. Microbiol. 49:568-571.
12. 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.
13. Johnson, M. C., Siegel, M. R., and Schmidt, B. A. 1985. Serological reactivities of endophytic fungi from tall fescue and perennial ryegrass and of *Epichloë typhina*. Plant Dis. 69:200-202.
14. Kasperbauer, M. J., Buckner, R. C., and Bush, L. P. 1979. Tissue culture of annual ryegrass × tall fescue F hybrids: Callus establishment and plant regeneration. Crop Sci. 19:457-460.
15. Kasperbauer, M. J., and Collins, G. B. 1972. Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. Crop Sci. 12:98-101.
16. Latch, G. C. M., and Christensen, M. J. 1985. Artificial infection of grasses with endophytes. Ann. Appl. Biol. 107:17-24.
17. Latch, G. C. M., Christensen, M. J., and Gaynor, D. L. 1985. Aphid detection of endophyte infection in tall fescue. N.Z. J. Agric. Res. 28:129-132.
18. Trigiano, R. N., Van Dyke, C. G., Spurr, H. W., Jr., and Gray, D. J. 1984. Infection and colonization of tobacco callus by *Peronospora tabacina*. Phytopathology 74:280-285.