Variability of Ergovaline in Seeds and Straw and Endophyte Infection in Seeds Among Endophyte-Infected Genotypes of Tall Fescue

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ABSTRACT

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Individual plant genotypes of tall fescue (*Festuca arundinacea*) cultivar Kentucky 31 infected (E+) or not infected (E−) by the endophyte *Acremonium coenophialum* growing for 2 yr in field plots were evaluated for ergovaline in seeds and straw and for percent endophyte infection in seeds. Ergovaline (analyzed by HPLC) in straw from 25 E+ genotypes ranged from 8 to 1,268 ng/g in 1991 and from 0 to 478 ng/g in 1992; mean ergovaline concentration in straw from E+ genotypes in 1991 (292 ng/g) differed (F value = 7.42, P = 0.008) from that in 1992 (130 ng/g). Ergovaline concentration in seed ranged from 2 to 6,064 ng/g in 1991 and from 3 to 8,659 ng/g in 1992; mean ergovaline concentration in seeds from E+ genotypes was similar (F value = 0.21) in 1991 (2,509 ng/g) and 1992 (2,295 ng/g). Percent endophyte-infected seeds (stained and examined microscopically) among E+ genotypes was variable and ranged from 2 to 100% in 1991 and from 0 to 100% in 1992, but means were statistically similar (F value = 0.2) between years (84% in 1991 and 80% in 1992). This study provides evidence that among E+ genotypes of tall fescue growing in the field, variation exists in production of ergovaline and percent endophyte-infected seed. Furthermore, ergovaline in straw from E+ genotypes varies between years.

Additional keyword: ergopeptides

Considerable research has been done on the relationship between tall fescue (*Festuca arundinacea* Schreb.) and *Acremonium coenophialum* Morgan-Jones & W. Gams (20), a fungal endophyte that grows intercellularly in foliage. Previous studies include detecting the fungus in host tissue; evaluating effects of endophytes on plant growth, morphology, and persistence; feeding avoidance by insects; and determining toxicity to livestock (3,4,6,17,33,36). *A. coenophialum* has been associated with a tall fescue toxicity syndrome in cattle since 1977 (5). Ergopeptide alkaloids, principally ergovaline, are found in foliage of endophyte-infected (E+) tall fescue but not in endophyte-free (E−) tall fescue (4,7,17,25). *A. coenophialum* produces ergovaline in culture (24), and isolates are known to vary in total ergot alkaloid production (2). Ergovaline has been found in blood serum of steers grazing E+ tall fescue but not in steers grazing E− tall fescue (14). The interaction of tall fescue with *A. coenophialum* has been identified as symbiotic mutualism because both plant and endophyte benefit from the relationship (33).

Seasonal variation in ergopeptide alkaloids (predominantly ergovaline) in tall fescue cv. Kentucky 31 has been reported in Missouri (28) and Georgia (7). Lyons et al (19) sampled the same pasture of E+ tall fescue twice and found more ergovaline in leaf blades and sheaths in December than in June. Hill et al (16,17) reported variability for alkaloid concentration among E+ tall fescue genotypes grown in a greenhouse. Nitrogen fertilization is known to increase ergopeptine alkaloid concentration in tall fescue (7,19). Research on ergovaline concentration in field-grown tall fescue has been done with mixtures of E+ and E− plants used for hay or pasture (15,19,28).

When forage and turf grasses are grown for seed in Oregon, about 1 million tons of residue (leaves and panicles, minus seeds) are produced annually (22). Previously, these residues (hereafter referred to as straw) were destroyed by field burning (11). However, smoke is a nuisance and raises concerns about environmental pollution. A 1991 Oregon legislative decision created a schedule to decrease field burning from 101,215 ha to 26,315 ha by 1997. As an alternate use, grass straw is becoming a valuable source of winter feed for beef cattle, and a new industry is developing for exporting grass seed straw to Pacific rim counties for use as feed supplements (11,22).

During the past 10 yr, commercial plant breeders have developed fescue cultivars with increasing numbers of E+ plants. It is generally accepted that endophyte-infected tall fescue plants have improved plant persistence and performance (17,36). However, the presence of ergovaline produced by the endophyte in these cultivars may limit use of straw for feed. Nothing is known about the concentration of ergovaline in straw from seed production, and no information is available about the variability of ergovaline concentration in straw from individual E+ genotypes growing in the field.

Although it has been reported that individual plants of E+ tall fescue produce more seed than E− plants (9,27), we know of no reports on the variability in percent endophyte-infected seeds harvested from individual plants of E+ tall fescue.

The purpose of this study was to determine the amount of ergovaline in seed and straw and percent endophyte infection in seeds from individual E+ genotypes of tall fescue growing in the field.

MATERIALS AND METHODS

Field plants. Foundation seed of tall fescue cv. Kentucky 31 was stored at 4 C in sealed containers until used. Seed was placed on blotter paper moistened with 0.1% KNO_3 and incubated in a germination chamber with alternating cycles of 25 C with 8 hr of light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 15 C with 16 hr of dark.

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After 2 wk, individual seedlings were transplanted to single cone-shaped plastic containers (3.8 × 21 cm) containing fine-grade vermiculite. Cones were placed in racks, and racks were placed in a mist chamber in a greenhouse at 20 ± 5 C for 7 days until plants were well rooted. Racks were moved to a growth chamber at 25 C with 16 hr of light (489–502 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 20 C with 8 hr of dark. Plants were watered daily and treated weekly with 0.81 g/L of 20-20-20 (N-P-K, 473 ppm N) liquid general-purpose fertilizer supplemented with soluble trace element mix at the rate of 5.3 ml/kg of N-P-K 20-20-20 to maintain vigorous growth.

After 7 wk in the growth chamber, foliage was cut 10 cm above the vermiculite surface and removed; 5 wk later, foliage was cut and removed again. Plants were moved to a greenhouse at 20 ± 5 C for 4 wk and "hardened off" outside on the north side of a headhouse for 2 wk. Foliage was cut 3 cm above the vermiculite surface, and plants were transplanted on 9 April 1990 on 1-m centers in 14-m rows spaced 1 m apart in a field of Chehalis-Willamette sandy loam with gravel (pH 6.7–6.9) at the Botany Farm near Corvallis, Oregon. Plants were treated with 18-18-18 (N-P-K, 100 kg/ha of N) fertilizer at transplanting and irrigated to establish the plants. The fertilizer rate was similar to what seed producers apply and was 27 kg/ha of N higher than what is recommended (23). Glyphosate isopropylamine salt (1.12 kg a.i./ha) was applied 3 wk before transplanting to control weeds.

In late July 1990, foliage was cut with a rotary mower and allowed to remain on the field. Plants were fertilized in fall 1990 and spring 1991 and again in fall 1991 and spring 1992 with 18-18-18 (N-P-K, 100 kg/ha of N). In both years, weeds were controlled by mechanical cultivation or an application of 4 kg a.i./ha of diuron between 1 October and 15 November to control grass seedlings and a mixture of 227 g a.i./L of 2,4-dichlorophenoxyacetic acid and 215 g a.i./L of 2-(2-methyl-4-chlorophenoxy) propionic acid to control broadleaf weeds. During the study, plants were not treated with insecticides. Stem rust developed in the plants during May to July in each year but was not controlled by fungicides.

Seed heads and straw were collected from individual plants in June 1991 and 1992 when seeds were ripe, i.e., when seed moisture (wet-weight basis) reached about 30%. Collections were put into burlap bags and allowed to dry at ambient air temperatures until seed and straw moisture reached 10%. Seeds were hand-threshed, cleaned, and stored at room temperature until examined for endophyte. Subsamples from each plant were analyzed for ergovaline in seed and straw and percent endophyte infection in seeds. After subsamples were taken for analyses, remaining foliage was cut with a rotary mower and allowed to remain on the field.

Selection of plants for the study. On 7 June 1991, five tillers were removed at the crown from each of 40 plants, eight plants in each of five rows. The basal end of each tiller was cut to 5-cm lengths, sectioned longitudinally, and cut into five 1-cm cross sections to provide 10 1-cm sections for each tiller. The mesophyll of the leaf sheath from each 1-cm section was scraped onto a glass slide, stained with aniline blue or rose bengal (5,29,37), and examined at 160–400× for hyphae typical of *A. coenophialum*, a common procedure used to detect endophyte hyphae in tillers of E+ tall fescue.

Of the 40 plants tested, 25 E+ plants were selected for study on the basis of presence of endophyte hyphae in one or more of the five tillers examined. These plants were numbered 1 though 25. A tiller was determined to be E+ when endophyte hyphae were found in one 1-cm section. Endophyte infection of a tiller was confirmed by examining three or more 1-cm sections for endophyte hyphae. Sixteen plants (numbers 1 through 16) had five E+ tillers; five plants (numbers 17 through 21) had four E+ tillers; two plants (numbers 22 and 23) had three E+ tillers; one plant (number 24) had two E+ tillers; and one plant (number 25) had one E+ tiller.

If no endophyte hyphae were found in any of the 10 sections from each of five tillers (i.e., 50 1-cm sections), the plant was considered E−. Five E− plants were selected for the study and were numbered 26 through 30. Location of the 30 plants in the field was recorded, and subsamples of seed and straw were collected and analyzed in 1991 and 1992.

Ergovaline analysis. The basal ends of tillers from individual E+ or E− plants were cut into 20- to 25-cm lengths, chopped in a mechanical shredder/chipper, and stirred to provide a uniform mixture of straw. Then, 10 g of the straw mixture from each plant was ground to a fine powder in a Cyclotec Sample Mill (Tector Inc., Hoganas, Sweden) and stored at −20 or −70 C until analyzed; 10 g of seed from each plant were similarly ground to a fine powder and stored. Tall fescue is a host for *Claviceps purpurea* (Fr.:Fr.) Tul., but seed collected from each genotype in both years were free of ergot sclerotia.

Duplicate 1-g subsamples of ground tissue were mixed with ergotamine tartrate as an internal standard, extracted with chloroform, and partially purified using silica solid phase extraction tubes. Each subsample was analyzed by reverse phase HPLC using fluorescence detection (λ_{ex} = 250 nm, λ_{ex} = 420 nm) (12). For the standard curve to be valid over three orders of magnitude, data were fit to a second order polynomial by a computer program using the Marquardt-Levenberg algorithm (Sigma-Plot, Jandel Scientific, Corte Madera, CA). The average within-run variation was 2.44% CV (n = 102), and the average run-to-run variation for controls was 9.7% CV (n = 46). Ergovaline concentrations were expressed as nanograms per gram. When ergovaline concentration exceeded scale on the first run, analyses were repeated with 0.5 or 0.25 g of ground plant material. All samples had two or more replicate runs, and means of runs are presented.

Analysis of seed for endophyte. Fifty to 100 seeds from each plant were stained with trypan blue and examined at 160–400× for endophyte hyphae (29,35,37).

Nitrogen analysis. Quantification of plant reduced N was performed on subsamples (0.5 g each) of ground straw using the micro-Kjeldahl procedure as described by Nelson and Sommer (21). Source of ground straw was the same as that used for the ergovaline analysis. Results were expressed as percent total nitrogen per sample.

Statistical analysis. Data for experimental variables (ergovaline in seed, ergovaline in straw, or percent endophyte-infected seed) from each E+ plant (numbers 1 through 25) and from each E− plant (numbers 26 through 30) were compared. Means for 25 E+ plants were analyzed as paired comparisons between years in a two-way ANOVA, with 25 plants serving as observations and year serving as replication. Data for each experimental variable were analyzed separately. In both years, percent nitrogen in straw from each plant was correlated with concentration of ergovaline and percent E+ seed. Correlation coefficients (r values) were tested for significant differences from 0 using Student's t (P = 0.05, 28 df = 2.048).

RESULTS

Ergovaline analysis. Ergovaline concentration in straw from each of 25 E+ plants was variable among plants and between years (Fig. 1A). Data were arranged to present results for plants with five of five tillers E+ (numbers 1 through 16), beginning with the plant with the highest concentration of ergovaline. Plants with four of five tillers E+ were presented next, followed by plants with three of five tillers E+, etc., until data for E− plants were presented (numbers 25 through 30). This was done to facilitate individual plant comparisons between years. (The same order of plant ranking was retained for Figures 1B and C.) Straw from plants 1 through 22 contained higher concentrations of ergovaline than straw from plants 23, 24, and 25. Ergovaline concentration in plants 23, 24, and 25 ranged from 8 to 33 ng/g in 1991 and from 0 to 2 ng/g in 1992. Only straw from plant number 22 con-

tained more ergovaline in 1992 than in 1991. Mean ergovaline concentration in straw from plants 1 through 25 in 1991 (292 ng/g) was significantly higher (F value = 14.97, $P = 0.001$) than in 1992 (130 ng/g). Although ergovaline was recovered in 1991 from straw of five plants labeled as E− (ergovaline concentrations in plants 26 through 30 were 3, 3, 1, 3, and 1 n/mg, respectively), this concentration is considered below the critical amount (approximately 10 ng/g) for accurate detection. In 1992, plants 28 and 29 contained 1 ng/g of ergovaline, also below the critical amount for accurate detection. Trace amounts of ergovaline may be false-positives because of the sensitivity of the protocol used for the analysis.

Ergovaline concentration in seed from each of 25 E+ plants also was found to be variable among plants and between years (Fig. 1B). Ergovaline concentration in seeds from plants 1 through 22 ranged from 1,485 to 6,064 ng/g in 1991 and from 1,118 to 8,659 ng/g in 1992. Ergovaline concentration in seeds from plant 23 was 3 ng/g in 1991 and 30 ng/g in 1992, and that in seeds from plants 24 and 25 was 2 ng/g in 1991 and 9 and 3 ng/g, respectively, in 1992. Plants 23, 24, and 25 also had the lowest concentrations of ergovaline in straw. Mean ergovaline concentration in seeds from 25 E+ plants in 1991 (2,509 ng/g) was not statistically different (F value = 0.49) from that in 1992 (2,295 ng/g). Seed from 15 of 25 plants (numbers 1, 4, 5, 6, 8, 9, 11, 12, 13, 15, 16, 17, 18, 20, and 21) contained more ergovaline in 1991 than in 1992. Ergovaline concentration in seed of plants 26, 27, and 28 was 1, 1, and 5 ng/g, respectively, in 1991 and 27, 33, and 19 ng/g, respectively, in 1992. Ergovaline concentration in seed of plants 29 and 30 was 0 ng/g in 1991 and 5 and 4 ng/g, respectively, in 1992.

Analysis of percent endophyte-infected seed. Percent endophyte infection in E+ plants 1 through 25 was variable and ranged from 1 to 100% in 1991 and from 0 to 100% in 1992 (Fig. 1C). In 1991, percent E+ seed from 20 of the plants numbered 1 through 25 was ≥90%; in 1992, percent E+ seed from 19 of the plants numbered 1 through 25 was ≥90%. In 1991, seed harvested from plants 23, 24, and 25 contained 1, 2, and 3% E+ seed, respectively, but seed from these plants was endophyte-free in 1992. Plants 23, 24, and 25 had the fewest number of E+ tillers (three, two, and one of five, respectively) of the plants selected for study and had the lowest concentration of ergovaline in seeds and straw when analyzed in 1991 and 1992.

Except for plants 6, 20, and 22, percent E+ seeds from plants 1 through 25 was generally similar in 1991 and 1992. Mean endophyte infection for plants 1 through 25 was 84% in 1991 and 80% in 1992, respectively, and was not statistically

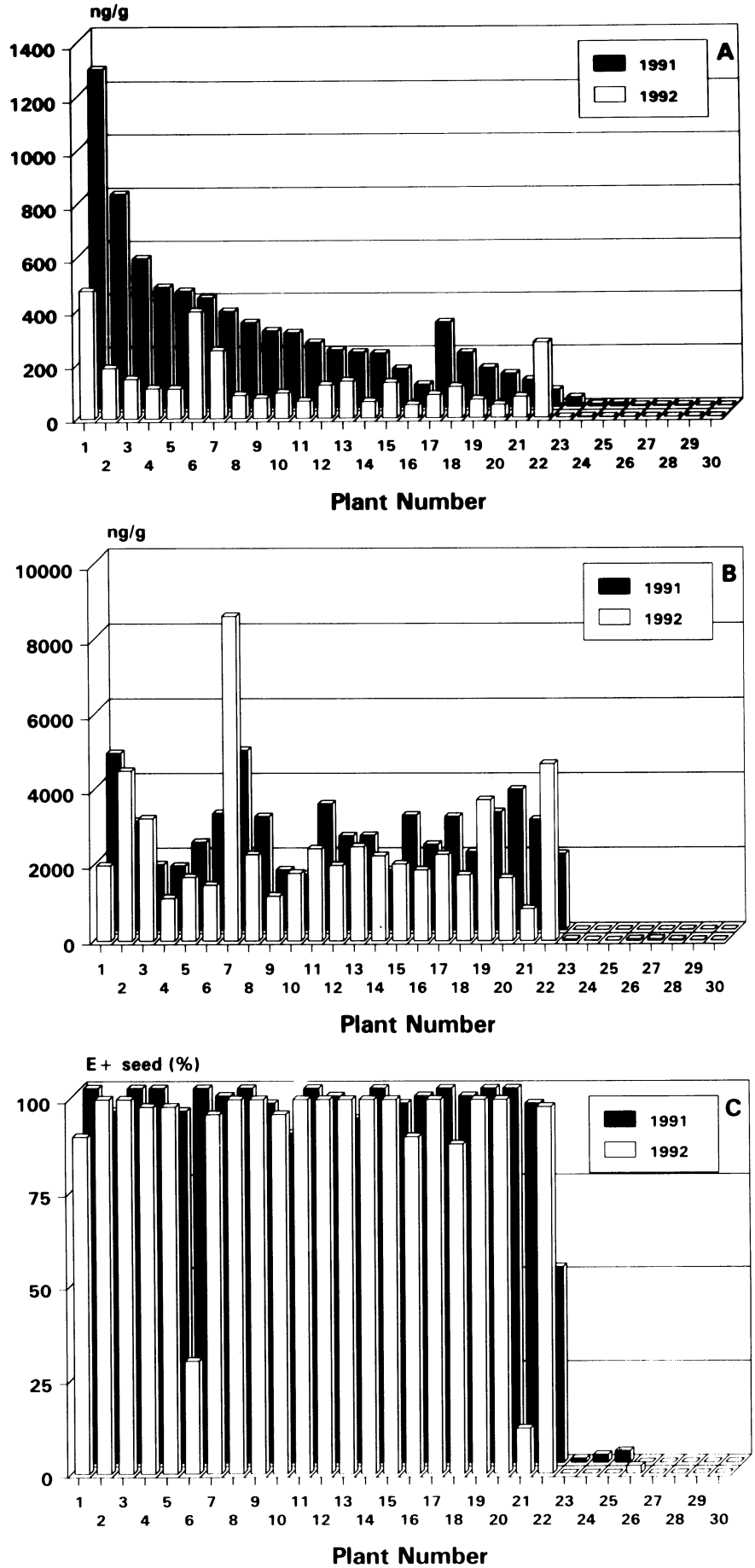

Fig. 1. Ergovaline concentration (ng/g) in **(A)** straw and **(B)** seed and **(C)** percent endophyte-infected seed from 30 plants of Kentucky 31 tall fescue growing in a field near Corvallis, Oregon, in 1991 and 1992.

different (F value = 0.82).

When the study began, endophyte hyphae were not observed in the five tillers of plants 26 through 30. Recovery of 2% E+ seed from plant 26 in 1992 indicates this plant was E+.

Nitrogen analysis. In 1991, nitrogen from ground straw of plants 1 through 25 ranged from 0.22 to 0.58%, with a mean of 0.33%. In 1992, nitrogen from these plants ranged from 0.23 to 0.49%, with a mean of 0.38%. Percent nitrogen means for 25 plants in 1991 were significantly smaller (F value = 5.85, $P = 0.023$) than in 1992. In 1991, nitrogen in plants 26 through 30 ranged from 0.28 to 0.41%, with a mean of 0.38%. In 1992, nitrogen in these same plants ranged from 0.33 to 0.55%, with a mean of 0.40%. These means were not statistically different (F value = 1.98, $P = 0.230$).

There was no significant correlation between percent nitrogen in straw and percent endophyte-infected seeds collected in 1991 ($r = -0.20525$) or 1992 ($r = -0.08817$) or between percent total nitrogen in straw and ergovaline concentration in straw collected in 1991 ($r = -0.03227$) or 1992 ($r = 0.09029$). However, there was a significant correlation between percent seed infection and ergovaline concentration in straw in 1991 ($r = 0.55793$) and 1992 ($r = 0.41818$).

DISCUSSION

Considerable variation was observed in ergovaline concentration in seed and straw and in production of E+ seed among E+ genotypes of tall fescue. While the cause for plant-to-plant variation was not established in this study, these data support findings of others that ergovaline concentrations vary among E+ genotypes (17). Furthermore, these data establish that ergovaline in seed and straw from E+ plants varies greatly from year to year. Ergovaline concentration in straw was at or below 200 ng/g in 10 of 25 plants in 1991 and 21 of 25 plants in 1992 (plants originally thought to be the only E+ plants in the study). Straw from six plants in 1991 and from one plant in 1992 had ergovaline concentrations ≥400 ng/g. These results indicate that analysis for ergovaline is needed if straw from E+ plants is used for feed; such analysis would allow livestock producers to develop strategies for avoiding potentially toxic feed.

Grass seed is not used generally for livestock feed. However, screenings (shriveled or small seed) from the seed-cleaning process are sometimes processed into pellets and used for livestock feed. Seed screenings from E+ plants are likely to contain ergovaline, and their use for feed should be discouraged because of this problem.

Although E− plants of tall fescue and other grasses can become infected by endophytic fungi by inoculation in controlled conditions (10), it is widely accepted that tall fescue plants do not become infected by the endophyte in nature. The endophyte is disseminated by planting E+ seed (4,6,32–34). Recovery of 2% E+ seed from plant 26 in 1992 indicates this plant, originally scored E−, was probably E+. When these results were obtained for plant 26, another 50 seed were stained and examined, and results were similar. It is unlikely that plant 26 became infected in the field between 1991 and 1992. Furthermore, ergovaline does not occur in E− plants of tall fescue (7,19,25). In 1992, recovery of ergovaline from plant 26 (19 ng/g), plant 27 (33 ng/g), and plant 29 (27 ng/g) indicates that these plants, originally scored E−, were probably E+. In both years, trace amounts (1–5 ng/g) of ergovaline were recovered from subsamples of seed or straw from plants 28 and 30, again below the limit of accurate detection (10 ng/g for this study).

Ergovaline and endophyte detection in three plants (numbers 26, 27, and 29) originally thought to be E− provides evidence that examining a small number of stained tiller pieces for endophyte hyphae may not be an acceptable method for determining if tall fescue plants are E−. Failure to detect endophyte hyphae in tillers or seeds of E+ tall fescue could also result from an inability of hyphae to grow from the crown meristem into the tiller or differentiating inflorescence (37). Erratic infection of tillers by other species of *Acremonium* has been observed among other E+ grass hosts examined as herbarium specimens (37). If hyphal staining is the only method used to identify E− plants, sample size is critical and should be large enough to establish a confident decision, perhaps 50–100 tillers per plant. Perhaps using HPLC or immunoassay (26) for detection of ergovaline in tall fescue tissue would be a better method than seed-staining or tiller-staining for determining if a plant is E+ or E−.

Nitrogen fertilization has been reported to influence the ergopeptide alkaloid concentration in the foliage and seeds of tall fescue in pastures in Georgia (6,17) and Missouri (28). Data from this field study provide evidence that when ample nitrogen is provided to realize an optimum fertilizer response for grass seed production, nitrogen fertilization is not related to concentration of ergovaline in straw from individual E+ tall fescue plants.

Further studies are needed to verify variable plant responses in concentration of ergovaline and percent endophyte infection observed here in field-grown plants. However, these results support those of Hill et al (17), that growth responses (e.g., ergovaline production, tiller production, dry matter production, crown weight, etc.) are not always the same for all E+ tall fescue plants. Direct (1) and indirect (8,13,18,30,31) evidence of host specificity among endophytes and grass hosts is supported by observations of others and was recently reviewed (10). A specific host genotype × specific endophyte strain interaction might regulate production and/or translocation of ergovaline within the plant. A host genotype × specific endophyte strain interaction may also influence the manner by which endophyte hyphae within crown meristems grow into developing tillers or spikelet primordia. Such a host/fungus interaction might also explain field-to-field variation in concentration of ergovaline and percent E+ seeds for a cultivar produced within the same growing season (R. E. Welty, A. M. Craig, and M. D. Azevedo, *unpublished*).

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