J. Agric. Food Chem. 1993, 41, 2299–2303

Analyses of Selected Endophyte-Infected Grasses for the Presence of Loline-Type and Ergot-Type Alkaloids

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Selected endophyte-free and endophyte-infected grasses from the genera *Festuca*, *Lolium*, *Hordeum*, *Stipa*, and *Poa* were analyzed for the presence of loline- and ergot-type alkaloids. Loline alkaloids were analyzed by capillary GC, and ergot-type alkaloids were analyzed by reversed-phase HPLC. None of the endophyte-free samples contained detectable levels of either of these alkaloid types. Endophyte-infected grass samples gave widely variable alkaloid concentrations. *N*-Formylloline was the predominant loline alkaloid, and ergovaline was usually the predominant ergot-type alkaloid in these samples.

Clavicipitaceous grass endophytes of the genus *Acremonium* have a worldwide distribution (White, 1987). This mutualistic symbiotic relationship often benefits the infected grasses through inducement of resistance to environmental hazards including drought and herbivory by insects and mammals (Hill *et al.*, 1991). Unfortunately, the presence of the *Acremonium* endophyte in grasses reduces animal performance. As examples, sheep grazing on perennial ryegrass (*Lolium perenne* L.) infected with *Aremonium lolii* Latch, Christensen & Samuels, in New Zealand sometime suffer from ryegrass staggers (Mortimer and di Menna, 1985), and cattle and sheep grazing tall fescue (*Festuca arundinacea* Schreb.) infected with *Acremonium coenophialum* Morgan-Jones & Gams often exhibit fescue toxicosis (Stuedemann and Hoveland, 1988). Production losses attributed to endophyte-infected tall fescue are estimated to be between $200 and $800 million annually to cattle producers in the United States (Hoveland, 1990).

A large number of different grasses have been shown to be endophyte-infected, and such relationships have been known for nearly a century (White and Cole, 1985). The most well-known and thoroughly studied grass–endophyte associations involve ryegrasses (e.g., *Lolium temulentum* L., *Lolium remotum* Schranck, and *L. perenne*) and fescues (e.g., *F. arundinacea* and red fescue, *Festuca rubra*). Recently, fungal endophytes have also been identified in other grasses, namely *Stipa* (White and Morgan-Jones, 1987; Petroski *et al.*, 1993), *Hordeum* (Wilson *et al.*, 1992), and *Poa* species (Siegel *et al.*, 1990).

Acremonium-infected grasses produce a large number of different metabolites. Lolitrem B (considered to be the primary causative of ryegrass staggers), peramine, loline (Figure 1), and ergovaline are representative and have been most often studied [for references see Buckner and Burris (1983), Siegel *et al.* (1990), and Powell and Petroski (1992)], although other compounds probably also contribute to the problems observed in grazing animals. Comparative studies using endophyte-free (EF) and endophyte-infected (EI) tall fescue have implicated both loline- and ergot-type alkaloids (Figure 2) as chemical causatives of fescue toxicosis (Sanchez, 1987). Reliable methods for quantitative analyses of plant materials for

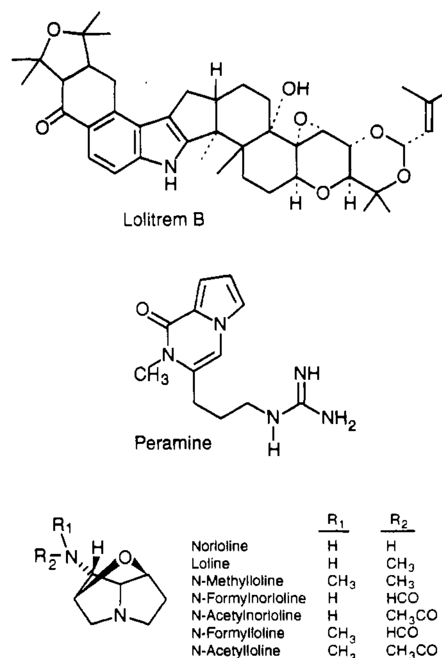

Figure 1. Lolitrem B, peramine, and loline-type alkaloids.

the loline alkaloids by capillary GC (Yates *et al.*, 1990) and the ergot-type alkaloids by RP HPLC (Yates and Powell, 1988) and by tandem MS (Yates *et al.*, 1985; Lyons *et al.*, 1986) were reported previously. Presented here are the results of quantitative analyses of EI and EF grass materials from selected *Festuca*, *Lolium*, *Stipa*, *Hordeum*, and *Poa* species for both the loline- and ergot-type alkaloids.

The loline alkaloids do not cause classical pyrrolizidine toxicity, but neither their direct or indirect mammalian toxicity nor their role in fescue toxicosis is currently known (Bush *et al.*, 1993; Powell and Petroski, 1992b). *N*-Formylloline (NFL) and *N*-acetylloline (NAL) are the major (Kennedy and Bush, 1983) and loline (L), norloline (NL), and *N*-acetylnorloline (NANL) (Yates *et al.*, 1990) are the minor naturally occurring loline alkaloids in tall fescue.

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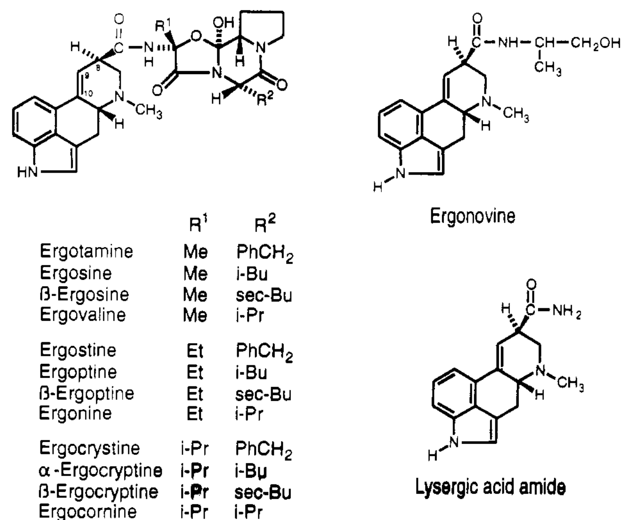

Figure 2. Ergot-type alkaloids.

Previously known sources and biological activities of the ergot-type alkaloids have been reviewed elsewhere and will not be discussed here (Rehacek and Sajdl, 1990). There is general agreement that these compounds adversely affect animal performance since symptoms often mimic those of classical ergotism (Bacon *et al.*, 1986). While ergovaline (EV) is usually the major ergot-type alkaloid found in EI tall fescue (Yates and Powell, 1988), ergosine (ES), ergotamine (EA), ergocryptine (EC), ergonovine (EN), and lysergic acid amide (LAA) are often present, as well. The incidence, identity, concentration, and relative ratios of the loline- and ergot-type alkaloids have been extensively studied in tall fescue, but this is not true of most other grasses.

EXPERIMENTAL PROCEDURES

Instrumentation, chromatographic conditions, sample preparations, experimental details, response factors, and recoveries for the loline-type (Yates *et al.*, 1990; TePaske *et al.*, 1993) and the ergot-type alkaloids (Yates and Powell, 1988; Yates *et al.*, 1985) are described elsewhere.

Instruments. Gas chromatography was performed with a Hewlett-Packard 5980A instrument equipped with flame ionization detectors. Programmed-gradient reversed-phase HPLC was performed with a Spectra Physics SP8800 ternary pump with a Varian Fluorochrom detector. Detector responses were integrated using a Spectra Physics SP4290 integrator. For peak identification, mass spectra were recorded in the EI mode at 70 eV in a Hewlett-Packard MSD 5970 mass spectrometer with sample introduction through a gas chromatograph.

Standard Compounds. Saturated pyrrolizidine alkaloids native to EI tall fescue were prepared from loline (Petroski *et al.*, 1989). 2-Phenylmorpholine (PM) used as a reference standard was purchased from Aldrich Chemical Co. Inc., Milwaukee, WI. Ergotamine, ergocryptine, and ergonovine were purchased from Sigma Chemical Co., St. Louis, MO. Ergovaline was a gift from Dr. George Rottinghaus, University of Missouri, Columbia, MO, and LAA was a gift from Richard Petroski (USDA/ARS, NCAUR).

Samples. Endophyte presence in grass materials was determined microscopically as described by Wilson *et al.* (1991). EI *Stipa robusta* samples were supplied by Dr. James F. White, Jr., Auburn University at Montgomery, Montgomery, AL, and by Dr. Keith Clay, Indiana University, Bloomington IN. The EI *Festuca versuta* seed was also supplied by James F. White, Jr., collected in June 1986 at Zilker Park in Austin, TX; a voucher specimen (Albers #43PHOOOLL) was deposited in the Lundell Herbarium, University of Texas, and a specimen of the *Acremonium* endophyte was also deposited (ATCC 58558). One of the *L. perenne* (cv. Repell) seed samples and the *Poa alsodes* materials were also supplied by Keith Clay. No corresponding EF *F. versuta*, *S. robusta*, or *P. alsodes* samples were available for analysis. Dr. Daryl D. Rowan, DSIR, Palmerston North, New Zealand, supplied the *L. perenne* G. nui seed. KY-31(a) and KY-31(b) *F. arundinacea* seed (standard 1991 and 1990, respectively) were supplied by Dr. Lowell T. Frobish, Alabama Agricultural Experiment Station, Auburn University, AL; KY-31(c) was supplied by Dr. John A. Paterson, University of Missouri, Columbia, MO; and KY-31(d) was supplied by Dr. David F. Nutting, University of Tennessee at Memphis, Memphis, TN. Freeze-dried *F. arundinacea* forage samples were supplied by Dr. Nicholas S. Hill, University of Georgia, Athens, GA. EI and EF seed samples of plant introductions (identified by PI numbers in Tables I and II) were selected from five *Lolium* and two *Hordeum* species. The samples had been stored at the seed storage facility (4 °C, 30% relative humidity, dew point 10 °C) of the USDA, ARS Western Regional Plant Introduction Station for 1–19 years. Unless indicated otherwise in Tables I and II, forage samples of *Hordeum* plant introductions were obtained from field-grown plants. All plants providing forage samples were at least 2 years old. Voucher specimens are on deposit and endophytes from these collections are in culture (Dr. Walter Kaiser, ARS, USDA Plant Introduction Station) at Pullman, WA.

Seed of *Lolium* came from *Acremonium*-infected and uninfected plant introduction lines, as previously identified by Wilson et al. (1991). The *Acremonium* endophyte in the *L. perenne* accessions was typical of *Acremonium lolii*, whereas the identity of the *Acremonium* endophytes in the other *Lolium* species was not established by Wilson et al. (1991). However, the *Acremonium* endophytes in these ryegrasses and *L. perenne* may be different species [see Latch et al. (1987)]. *Hordeum* seed and forage samples were acquired from *Acremonium*-infected and uninfected plants. The identity of isolates of *Acremonium*-like endophytes from *Hordeum bogdanii* and *Hordeum brevisubulatum* ssp. *violaceum*, which exhibit wide variation in cultural and morphological characteristics, has not been established (Clement *et al.*, 1992).

Sample Preparations. Forage materials were air-dried at ambient temperatures (21–25 °C for 7–10 days) unless otherwise denoted in the sample description. Samples of 1 g each were powdered to pass through a No. 20 mesh prior to extraction.

Loline Alkaloid Analyses. Extraction solvent consisted of CH_2Cl_2/MeOH/NH_4OH (75:25:0.5). PM standard (50.0 μg/mL) and extraction solvent were added to the powdered materials (10 mL/g) in a screw-cap glass centrifuge tube, the foil-lined cap was tightly sealed, and the tube was placed on an orbital shaker (170 rpm) overnight (16 h) at room temperature. The mixture was centrifuged at 2500 rpm for 25 min, and 1 mL was removed and placed in a screw-cap vial. Samples were then sealed and stored at −15 °C until analyzed by GC (2.0-μL injections).

Ergot-Type Alkaloid Analyses. Extraction solvent consisted of 99.5:0.5 MeOH/NH_4OH. Ergocryptine standard (5.0 μg) and extraction solvent were added to powdered materials (10 mL/g) in 500-mL round-bottom flasks, and the flasks were then stoppered and placed on an orbital shaker (170 rpm) overnight (16 h) at room temperature. Samples were routinely filtered through Whatman No. 54 paper; extraction solvent was removed under reduced pressure, dissolved in 1.00 mL of MeOH, vortexed for 30 s, and then stored at −15 °C until analyzed. Samples were warmed to room temperature prior to analysis. All operations were conducted under UV-shielded fluorescent light to avoid degradation of ergot alkaloids and other UV-sensitive compounds. A second sample, without standard, was also prepared and analyzed for samples thought to contain endogenous ergocryptine.

RESULTS AND DISCUSSION

None of the EF samples analyzed in this study contained measurable concentrations of loline- or ergot-type alkaloids; all samples discussed in the remainder of this paper are EI. The results of the loline- and ergot-type alkaloid analyses of the EI grass samples are presented in Tables I and II, respectively, with concentrations in parts per million (micrograms of alkaloid per gram of sample). Seed sample concentrations were not corrected for endogenous

Table I. Loline Alkaloid Concentrations in Endophyte-Infected Grasses

grass species	alkaloid concentration (ppm)				
	L	NML	NANL	NFL	NAL
F. arundinacea (s)[a]					
(a) KY-31, 1991	–	–	–	750	100
(b) KY-31, 1990	65	–	–	544	95
(c) KY-31, Missouri	126	196	107	1590	330
(d) KY-31, Tennessee	142	187	123	1812	389
F. arundinacea (f)[a]					
(a) Hill, Georgia	–	27	54	323	101
(b) Hill, Georgia	–	18	30	222	68
(c) Hill, Georgia	–	60	41	470	179
(d) Hill, Georgia	–	38	46	360	71
F. versuta (s)					
White, 1986, Texas	+[b]	+	421	1622	+
H. bogdanii (s)					
PI 440413	–	–	–	–	–
PI 314696	–	–	–	–	–
PI 269406	–	–	–	–	–
H. bogdanii (f)					
PI 314696[c]	–	–	–	+	–
PI 314696[d]	–	–	–	–	–
PI 440413	–	–	–	–	–
PI 269406	–	–	–	–	–
H. brevisubulatum spp. *violaceum* (s)					
PI 401386	–	–	–	–	–
H. brevisubulatum ssp. *violaceum* (f)					
PI 401386	–	–	–	–	–
H. brevisubulatum ssp. *violaceum* (f)					
PI 440420	–	–	–	+	–
L. rigidum (s)					
PI 250805	–	–	–	204	41
L. temulentum (s)					
PI 249725	29	59	–	503	90
L. persicum (s)					
PI 222807	–	–	–	518	132
L. multiflorum (s)					
PI 410154	–	–	10	30	12
L. perenne (s)					
PI 205278	–	–	–	–	–
PI 462339	–	–	–	–	–
cv. Repell	–	–	–	–	–
cv. Repell	–	–	–	–	–
G. nui, New Zealand	–	–	–	–	–
P. alsodes (s)					
Clay, 1992, N. Carolina	–	–	–	+	+
P. alsodes (f)					
Clay, 1992, N. Carolina	–	–	–	–	–
S. robusta (f)					
Clay, 1989, New Mexico	–	–	–	+	–
White	–	–	–	+	–

[a] (s), seed sample; (f), forage sample. [b] –, metabolite not detected; +, metabolite present. [c] Greenhouse grown. [d] Field grown.

Table II. Ergot Alkaloid Concentrations in Endophyte-Infected Grasses

grass species	alkaloid concentration (ppm)					
	EN	LAA	EV	ES	EA	EC
F. arundinacea (s)[a]						
(a) KY-31, 1991	?[b]	?	4.4	0.4	1.1	–[c]
(b) KY-31, 1990	?	?	3.1	?	?	–
(c) KY-31, Missouri	?	?	1.1	0.9	0.7	–
(d) KY-31, Tennessee	?	?	4.8	0.8	1.6	1.8
F. arundinacea (f)[a]						
(a) Hill, Georgia	–	–	1.4	0.2	0.1	–
(b) Hill, Georgia	?	?	1.4	–	–	–
(c) Hill, Georgia	?	?	1.3	–	–	–
(d) Hill, Georgia	–	–	1.5	–	–	–
H. bogdanii (s)						
PI 440413	–	–	–	–	–	–
PI 314696	–	–	–	–	–	–
PI 269406	–	–	–	–	–	–
H. bogdanii (f)						
PI 314696[d]	–	–	0.8	–	–	–
PI 314696[e]	–	–	–	–	–	–
PI 440413	–	–	–	–	–	–
PI 269406	–	–	–	–	–	–
H. brevisubulatum ssp. *violaceum* (s)						
PI 410386	–	–	–	–	–	–
H. brevisubulatum ssp. *violaceum* (f)						
PI 401386	–	–	–	–	–	–
H. brevisubulatum ssp *violaceum* (f)						
PI 440420	–	–	0.3	0.1	0.1	–
L. rigidum (s)						
PI 250805	–	–	–	–	–	–
L. temulentum (s)						
PI 249725	–	–	–	–	–	–
L. persicum (s)						
PI 222807	–	–	–	–	–	–
L. multiflorum (s)						
PI 410154	–	–	–	–	–	–
L. perenne (s)						
PI 205278	–	–	tr[b]	–	–	–
PI 462339	?	?	14.2	–	–	–
cv. Repell	?	?	0.8	–	–	–
cv. Repell	–	–	2.7	–	–	–
P. alsodes (s)						
Clay, 1992, N. Carolina	–	–	–	+[c]	–	+
P. alsodes (f)						
Clay, 1992, N. Carolina	–	–	–	+	–	+
S. robusta (f)						
Clay, 1989, New Mexico	–	32.1	–	–	–	–
White	–	8.0	–	–	–	–

[a] (s), seed sample; (f), forage sample. [b] ?, sample-related interference makes metabolite presence uncertain. [c] –, metabolite not detected; +, metabolite present. [d] Greenhouse grown. [e] Field grown. [f] tr, metabolite present in trace quantities.

moisture. A minus sign (–) in Tables I and II indicates that the indicated compound was not detected in the sample. A plus sign (+) denotes that the compound was detected by GC–MS but that the concentration was below measurable limits. Identities of all compounds presented in Table I were confirmed by GC–MS. Forage and seed materials are differentiated by (f) and (s), respectively.

As expected, some epimerization of the ergot-type alkaloids occurred (e.g., some ergovaline was converted to ergovalinine) during analysis. Data listed in Table II represent the combined concentration of the two epimers. Peak identities were confirmed by cochromatography (HPLC) with standard compounds. *F. versuta* could not be analyzed for ergot-type alkaloids by HPLC due to sample-related interferences. Insufficient quantities of *L. perenne* G. nui seed were available for ergot-type alkaloid analysis.

Neither the *P. alsodes* nor *H. bogdanii* forage or *Hordeum* seed materials (Table I) contained loline alkaloids, and only trace quantities were found in *H. brevisubulatum* ssp. *violaceum* and *S. robusta* forage and *P. alsodes* seed samples. By contrast, all of the fescue and ryegrass samples, except *L. perenne*, contained measurable levels of loline alkaloids. Loline alkaloids have been previously isolated from *Lolium*, *Festuca*, *Poa*, *Stipa*, and *Adenocarpus* genera (Siegel *et al.*, 1990; Petroski *et al.*, 1989, 1992). To our knowledge, this represents the first report of loline alkaloids from the genus *Hordeum* and from *Lolium rigidum*, *Lolium persicum*, and *Lolium multiflorum*. Loline (L) was previously isolated from *Lolium temulentum* (Dannhardt and Steindl, 1985), but it may have been formed by deacylation of NFL and NAL during extraction. NFL was the primary loline alkaloid in these samples.

The primary ergot-type alkaloids found in the samples analyzed were as follows (Table II): EV in *L. perenne* seed, *F. arundinacea* seed and forage and forage of two *Hordeum* samples, and LAA in *S. robusta*. No ergot-type alkaloids were detected in the other four *Lolium* species

or in any of the *Hordeum* seed materials. To our knowledge, this represents the first report of ergot-type alkaloids from the genera *Hordeum* and *Poa* other than those contaminated by *Claviceps* species (ergot).

In agreement with previous results, EI tall fescue samples all contained both loline- and ergot-type alkaloids (Kennedy and Bush, 1983; Yates *et al.*, 1990; Siegel *et al.*, 1990; Yates and Powell, 1988), and the EV, ES, and EA concentrations in these samples are comparable to literature values. In contrast, the loline alkaloid concentrations are somewhat lower than those found in previous studies. Tremendous differences have been observed in loline alkaloid levels among fescue samples, and endogenous levels have been shown to be affected by seasonal variations, management practices, storage practices, and sample age (Kennedy and Bush, 1983; Seigel *et al.*, 1990). Any or all of these variables may have influenced our results.

Endogenous EV concentrations in the perennial ryegrass samples varied from a trace to 14.2 ppm. Lolitrem is considered the primary cause of ryegrass staggers; however, EV may also affect animal performance on perennial ryegrass. *L. perenne* (PI 462339) might serve as an excellent source of EV for toxicological testing. None of the five *L. perenne* samples examined herein and only one of the eight samples analyzed by Siegel produced loline alkaloids (Siegel *et al.*, 1990). The latter was from a plant artificially infected with *A. coenophialum*. To our knowledge, loline alkaloids have not been reported in naturally infected *L. perenne*. This observation suggests that the loline alkaloids do not cause ryegrass staggers and that further chemical investigations of perennial ryegrass may provide insights into the biological origin of the loline alkaloids (plant, fungus, or both).

The *P. alsodes* samples were unusual in that ES and EC were present and EV and EA were absent. High concentrations of ES, EC, or EA often indicate the presence of ergot contamination (Porter *et al.*, 1987); however, no ergot was found during thorough optical examination of these grass materials. LAA (*S. robusta*) and NANL (*F. versuta*) were major constituents of the samples analyzed but are only minor constituents of tall fescue. However, animals that graze these grasses may also display toxic effects. It is important to analyze *Acremonium*-infected grasses for all of the ergot-type and loline alkaloids and not just for the major constituents of tall fescue. Some of the loline alkaloids in association with endophyte-infected tall fescue have been reported to adversely affect aphid survival (Eichenseer *et al.*, 1991).

In summary, the concentrations and identities of ergot-type and loline alkaloids among these selected endophyte-infected grass samples were extremely variable. Knowledge of the different alkaloid profiles in EI grasses may assist in the identification of *Acremonium*-infected grasses, provide information concerning insect resistance, and suggest solutions to animal toxicity problems.

ACKNOWLEDGMENT

We acknowledge Ronald Plattner (USDA/ARS, NCAUR) for GC–MS analyses and Tomya Wilson (USDA/ARS, NCAUR) for technical assistance. Ergovaline was a gift from Dr. George Rottinghaus, and LAA was a gift from Dr. Richard Petroski. Seed and forage samples were supplied by Drs. James White, Daryl Rowan, Keith Clay, David Nutting, Lowell Frobish, John Paterson, and Nicholas Hill.

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Received for review January 21, 1993. Revised manuscript received September 17, 1993. Accepted September 28, 1993.[⊗]

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[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1993.