Analysis of Endophyte Toxins: Fescue and Other Grasses Toxic to Livestock

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ABSTRACT: Research on livestock toxicoses caused by Acremonium (endophyte)-infected grasses strongly implicate the ergopeptine alkaloids with A. coenophialum-infected fescue and paxilline and the lolitrem alkaloids with A. lolii-infected perennial ryegrass as the causative agents. Isolation, identification, and detection of these toxins involves extraction with appropriate solvents, clean-up procedures, and chromatographic methods with known standards. Thin-layer, high-performance liquid and gas chromatography along with ultraviolet and mass spectrometric (i.e., electron impact, chemical ionization, tandem mass) characterizations have been reported. These methods have varying degrees of success depending on the matrix from which the alkaloids have been extracted. Ergovaline is the primary ergopeptine alkaloid isolated from cultures of A. coenophialum and also from infected fescue grass and seeds toxic to livestock. Other compounds isolated from the endophyte-infected fescue include: lysergic acid amide (ergine), the clavine class of ergot alkaloids (chanoclavine I, agroclavine, elymoclavine, pennisclavine), the pyrrolizidine alkaloids (N-formylloline, N-acetylloline, N-methylloline, N-acetylnorloline), and the unique pyrrolopyrazine alkaloid peramine. The loline alkaloids and peramine have been more associated with the insect-deterrent properties of the endophyte-infected fescue than with livestock toxicoses. Also, both peramine and the ergopeptine alkaloids (ergovaline, ergotamine) have been isolated from A. lolii-infected perennial ryegrass. More recently, paxilline and lolitrem B have been detected in laboratory cultures of A. coenophialum isolated from tall fescue. The ergot alkaloids in endophyte-infected perennial ryegrass may be more related to decreased animal productivity (weight gains, reproduction problems), whereas the lolitrems cause the staggers syndrome. The detection, isolation, identification, and analyses of these compounds from Acremonium-infected grasses is presented.

Key Words: Acremonium, Endophytes, Alkaloids, Analytical Methods, Toxins, Grasses

Introduction

Toxicoses in livestock grazed on Acremonium spp.-infected grasses have a pronounced negative economic effect on animal production (Hoveland, 1993). The Acremonium grass endophytes are taxonomically aligned with the family Clavicipitaceae and live or spend their entire life cycle within the aerial portion of their grass host (Bacon and DeBatista, 1991). Several reviews have been published 1) on the economic perspectives with regard to livestock toxicoses (Stuedemann and Hoveland, 1988; Hoveland, 1993), 2) on mechanisms and mode of toxicity (Thompson and Porter, 1991; Porter and Thompson, 1992; Thompson and Garner, 1994), 3) on the chemistry and biochemical origins of the compounds associated with the grass endophytes (Bush et al., 1993; Garner et al., 1993; Rowan, 1993; Porter, 1994), and 4) on the biological and agronomic aspects of endophyte-host grass associations (Bacon, 1988; Bacon and Siegel, 1988; Siegel et al., 1990; Bacon and DeBattista, 1991; Bacon and White, 1994). This review will focus on the analyses of compounds that have been directly or indirectly related to A. coenophialum Morgan-Jones & Gams (Morgan-Jones and Gams, 1982), the endophyte of tall fescue (Festuca arundinacea Schreb.), and A. lolii Latch, Christensen, & Samuels (Latch et al., 1984), the endophyte of perennial ryegrass (Lolium perenne L.).

Compounds Related to Acremonium-Infected Fescue and Perennial Ryegrass

Ergopeptine alkaloids, primarily ergovaline (Figure 1, Tables 1 and 2), and the indole isoprenoid lolitrem...
alkaloids, primarily lolitrem B (Figure 2, Table 2), have been associated with toxic A. coenophialum-infected tall fescue and A. lolii-infected perennial ryegrass, respectively. Both ergovaline and lolitrem B have been isolated from laboratory cultures and grasses infected by these endophytes.

Other alkaloids occurring with the endophyte-host grass associations are the loline alkaloids, primarily N-acetylloline and N-formylloline (Figure 3, Table 2) in A. coenophialum-infected tall fescue (Bush et al., 1982, 1993; Yates et al., 1990) and the pyrrolopyrazine alkaloid peramine (Figure 4, Table 2) in both infected fescue and perennial ryegrass (Rowan et al., 1986; Tapper et al., 1989; Siegel et al., 1990; Rowan, 1993). Although these compounds have been related to the insect-deterrent properties of the endophyte-infected grasses rather than animal toxicoses, they may augment the toxicity of the ergot and lolitrem alkaloids, respectively. Therefore, a secondary emphasis of this review will be placed on the isolation and identification of the lolines and peramine, along with the ergot and lolitrem alkaloids.

**A. coenophialum-Infected Fescue**

**Ergot Alkaloids.** The chemistry, biochemistry, and pharmacology of the ergot alkaloids from Claviceps spp.-infected grass seeds, cereal grains, and laboratory cultures have been the focus of research from time immemorial (Bove, 1970; Berde and Schild, 1978). The known “ergot alkaloids” produced by Claviceps spp. may be divided into five major classes: the clavine, lysergic acid, simple lysergic acid amides, ergopeptine, and ergopeptam alkaloids (Berde and Schild, 1978; Perellino et al., 1992, 1993). The ergopeptines, simple lysergic acid amides, and clavines (Figures 1, 5, and 6) have been isolated from A. coenophialum-infected tall fescue (Yates et al., 1985; Lyons et al., 1986; Petroski and Powell, 1991). Although ergovaline is the major ergopeptine alkaloid in A. coenophialum-infected tall fescue (Yates et al., 1985; Lyons et al., 1986; Yates and Powell, 1988; Rottinghaus et al., 1993), lysergic acid amide (or ergine; Figure 5) can exist in concentrations approximately equal to that of ergovaline (Richard A. Shelby,
Table 1. Major ions and atomic mass units (amu) for the ergopeptine (or ergot cyclol) alkaloids; AH, BH, and CH are the major ions (amu) resulting from isobutane chemical ionization mass spectrometry (CIMS) (see Figure 1 for corresponding ions)

<table>
<thead>
<tr>
<th>Group</th>
<th>R₂</th>
<th>MWᵃ</th>
<th>AH</th>
<th>BH</th>
<th>CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ergotamine group (R₁ = -CH₃)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ergotamineᵇ</td>
<td>-CH₂Ph</td>
<td>581</td>
<td>268</td>
<td>315</td>
<td>245</td>
</tr>
<tr>
<td>Ergosineᵇ</td>
<td>-i-Bu</td>
<td>547</td>
<td>268</td>
<td>281</td>
<td>211</td>
</tr>
<tr>
<td>beta-Ergosine</td>
<td>-sec-Bu</td>
<td>547</td>
<td>268</td>
<td>281</td>
<td>211</td>
</tr>
<tr>
<td>Ergovalineᵇ</td>
<td>-i-Pr</td>
<td>533</td>
<td>268</td>
<td>267</td>
<td>197</td>
</tr>
<tr>
<td>Ergobine</td>
<td>-Et</td>
<td>519</td>
<td>268</td>
<td>252</td>
<td>183</td>
</tr>
<tr>
<td>Ergoxine group (R₁ = -C₂H₅)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ergostine</td>
<td>-CH₂Ph</td>
<td>595</td>
<td>268</td>
<td>329</td>
<td>245</td>
</tr>
<tr>
<td>Ergoptineᵇ</td>
<td>-i-Bu</td>
<td>561</td>
<td>268</td>
<td>295</td>
<td>211</td>
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<tr>
<td>beta-Ergoptine</td>
<td>-sec-Bu</td>
<td>561</td>
<td>268</td>
<td>295</td>
<td>211</td>
</tr>
<tr>
<td>Erginine</td>
<td>-i-Pr</td>
<td>547</td>
<td>268</td>
<td>281</td>
<td>197</td>
</tr>
<tr>
<td>Ergobutine</td>
<td>-Et</td>
<td>533</td>
<td>268</td>
<td>267</td>
<td>183</td>
</tr>
<tr>
<td>Ergotoxin group (R₁ = -i-Pr)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Ergocristine</td>
<td>-CH₂Ph</td>
<td>609</td>
<td>268</td>
<td>343</td>
<td>245</td>
</tr>
<tr>
<td>alpha-Ergocryptine</td>
<td>-i-Bu</td>
<td>575</td>
<td>268</td>
<td>309</td>
<td>211</td>
</tr>
<tr>
<td>beta-Ergocryptine</td>
<td>-sec-Bu</td>
<td>575</td>
<td>268</td>
<td>309</td>
<td>211</td>
</tr>
<tr>
<td>Ergocornineᵇ</td>
<td>-i-Pr</td>
<td>561</td>
<td>268</td>
<td>295</td>
<td>197</td>
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<tr>
<td>Ergobutyrine</td>
<td>-Et</td>
<td>547</td>
<td>268</td>
<td>281</td>
<td>183</td>
</tr>
</tbody>
</table>

ᵃMW = molecular weight.
ᵇFound in endophyte-infected grass and cultures.

dpt. of Plant Pathology, Auburn Univ., Auburn, AL, personal communication). Lysergic acid amide probably results from the solvolytic cleavage of lysergylmethylcarbinolamide (Figure 5) and is not considered a true natural product (Groger et al., 1968; Floss, 1976). Both lysergic acid amide and lysergylmethylcarbinolamide have biological activity (Berde and Schild, 1978; Oliver et al., 1993) and should be considered (along with the minor ergot alkaloids) where fescue toxicity is concerned. Ergovinone (Figure 5), another simple lysergic acid amide, also has been isolated from endophyte-infected fescue seeds (Petroski and Powell, 1991). However, this compound may be from Claviceps contamination of the seeds (Yates and Powell, 1988) and additional studies are needed to verify whether ergonovine is indeed a constituent of A. coenophialum-infected tall fescue. Because ergonovine is found in conjunction with the ergopeptine alkaloids in Claviceps-infected wheat and ryegrass (Scott et al., 1992), it is possible this alkaloid may also be present in endophyte-infected grasses. The clavine alkaloids chanoclavine(s), penniclavine, elymoclavine, and agroclavine (Figure 6) have been isolated from endophyte-infected tall fescue (Lyons et al., 1986) and are precursors in the biosynthesis of the simple lysergic acid amides and the ergopeptines (Floss, 1976; Garner et al., 1993; Porter, 1994.

Isolation and Identification. There are a variety of procedures for the extraction, isolation, and identification of the ergot alkaloids from A. coenophialum-infected tall fescue. Current methods of choice involve extraction with either an aqueous tartaric or lactic acid solution (lactic acid seems to work best for the extraction of ergovaline from the infected fescue seed and forage). Table 2. Major alkaloidsᵇ associated with Acremoniumᵇ-infected tall fescue (TF) and perennial ryegrass (PRG) seed and forageᶜ

<table>
<thead>
<tr>
<th>Endophyte-grass</th>
<th>Alkaloids</th>
<th>Lolines</th>
<th>Ergovaline</th>
<th>Peramine</th>
<th>Lolitrems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-TFᵇ</td>
<td>1,800-5,000</td>
<td>2-6</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Al-PRGᵇ</td>
<td>—</td>
<td>5</td>
<td>5-40</td>
<td>5-10</td>
<td>—</td>
</tr>
</tbody>
</table>

ᵇConcentrations are micrograms of alkaloid/gram of material.
ᶜAc = A. coenophialum; Al = A. lolii.

dGallagher et al., 1985; Fannin et al., 1990; Siegel et al., 1990; Yates et al., 1990; Bush et al., 1982, 1993.

Figure 4. Peramine and its major ions (amu) resulting from electron impact mass spectrometry.
LYSEROGYLC METHYL-
CARBINOLAMIDE

LYSEROGYLC AMIDE
(ERGINE)

ERGONOVINE

Figure 5. Examples of some of the lysergic acid amide group of ergot alkaloids.

[Testereci et al., 1990], partition chromatography with chloroform or methylene chloride at an appropriate pH, column clean-up procedures using either silica, alumina, or an ion exchange resin, and identification and analysis using co-chromatography (TLC and/or HPLC) with ultraviolet or fluorescence detection. Mass spectrometry (MS) also has been employed for the identification, analysis, and quantification of ergot alkaloids.

The ergot alkaloids are extremely susceptible to photolytic and air oxidation, hydration, and epimerization at the C-8 position of the ergolene ring (Berde and Schild, 1978; Garner et al., 1993; Porter, 1994). Epimerization of the C-8 position occurs in either acidic or basic conditions and, therefore, the isolation of the C-8 epimers (designated with the suffixal -inine [i.e., ergovaline vs ergovalinine]) occurs in most extraction procedures. Decomposition and epimerization may be minimized by working under subdued or yellow light, concentrating extracts in vacuo at room temperature or less (i.e., ≤ 25°C), and by concentrating small volumes of extracts under a stream of nitrogen. Storing dried concentrates in amber vials under nitrogen at or below 0°C will help prevent further decomposition. Moubarak et al. (1993) have successfully stored ergovaline at -4°C for up to 12 mo. Furthermore, ergovaline decomposes rapidly when extracted from non-freeze-dried plant tissues (Garner et al., 1993). Thus, observing the correct precautions during sample collection, handling, and preparation for analysis are crucial for the meaningful isolation and quantitative analysis of the ergot alkaloids.

High-Performance Liquid Chromatography. Use of HPLC with either ultraviolet or fluorescence detection is rapidly becoming the preferred method for routine screening and analysis of the ergopeptine alkaloids in endophyte-infected grasses (Yates and Powell, 1988; Testereci et al., 1990; Hill et al., 1991, 1993; Rottinghaus et al., 1991; Moubarak et al., 1993; Zhang et al., 1994). For example, a rapid, simplified HPLC method for the analysis of the ergot alkaloids associated with A. coenophialum-infected tall fescue has been developed (Richard A. Shelby, Dept. of Plant Pathology, Auburn Univ., Auburn, AL, unpublished data). Extraction of infected seed or grass with alkaline methanol, followed by filtration, and direct HPLC analysis (fluorescence detection) with a mobile phase of either 60 or 70% alkaline methanol results in separation of ergovaline, ergovalinine, lysergic acid amide, and its isomer isolysergic acid amide (ergine), along with other minor ergopeptine alkaloids. Moubarak et al. (1993) have reported a unique preparative method for the isolation and purification of large quantities of ergovaline. This procedure involves a modification of the methods of Scott and Lawrence (1980), Testereci et al. (1990), and Rottinghaus et al. (1991): infected seeds are extracted with

Figure 6. Examples of some of the clavine group of ergot alkaloids isolated from A. coenophialum-infected tall fescue.
a 5% aqueous lactic acid solution and the ergot alkaloids are adsorbed onto SM-2 Biobeads (BioRad, Hercules, CA). Extraction of the Biobeads with methanol, followed by an HPLC clean-up procedure using a C-18 RP column (Vydac, Separations Group, Hesperia, CA), and HPLC analysis using gradient elution with acetonitrile:ammonium carbonate:methanol as the mobile phase results in pure ergovaline (≥ 95%). Zhang et al. (1994) have employed an amberlite XAD-2 exchange resin for a rapid clean-up step after extracting infected fescue seed with a 5% lactic acid:methanol (4:1, vol/vol) solution. Scott et al. (1992) and Rottinghaus et al. (1993) have reported additional extraction, clean-up, and HPLC procedures for the analysis of the ergopeptine alkaloids in cereal grains, flour, and feeds.

Thin-Layer Chromatography. After the ergot alkaloids have been extracted into a suitable organic solvent, TLC on silica gel remains one of the most powerful tools for the analysis and identification of these compounds. The major advantages of TLC are that several samples may be analyzed at the same time, TLC does not involve expensive instrumentation, and it may be used as a complement to MS in the confirmatory identification and analysis of mixtures of epimeric alkaloids (see below). Perellino et al. (1993) have reported a TLC procedure on silica gel for the separation of most all of the known ergot alkaloids. By developing the TLC plates in methylene chloride: isopropyl alcohol (92:8, vol/vol) three times (drying the plates between runs), these alkaloids separate into the isoisergic acid group (i.e., -ine epimers), the ergotoxine group, the ergoline group, and a mixture of the ergotamine and clavine groups. Removal of the silica from the area of the plates consistent with the known standards, extraction of the alkaloids from the silica using methanol:chloroform (1:4 or 1:1, vol/vol) (Porter et al., 1974; Perellino et al., 1993), and rechromatography in chloroform: methanol (9:1 or 4:1, vol/vol) separates ergovaline from the clavine alkaloids (agroclavine and chanoclavine) (Porter et al., 1979). Other solvent systems used for the TLC analysis of these compounds are listed in Table 3.

Visualization of the ergot alkaloids on a TLC plate may be accomplished with a hand-held UV light at 254 and 366 nm. The 9,10-double bond in the D-ring of the ergoline portion of the molecule (Figure 1) is conjugated with the indole nucleus and gives the ergopeptine alkaloids their characteristic bright, pale blue fluorescence (UV lambda max in methanol at approximately 315 and 242 nm). Those ergot alkaloids devoid of the 9,10-double bond give a characteristic dark blue absorption under 254 nm (UV lambda max in methanol at approximately 292, 280, 275, and 222 nm) indicative of the indole nucleus. Spraying the TLC plates with a solution of p-N,N-dimethylaminobenzaldehyde (van Urk's reagent; Stahl, 1969) followed by spraying with a 1% sodium nitrite solution (water:ethanol, 1:1 vol/vol) (Sprince, 1960) produces intense blue spots that are also characteristic of the ergot alkaloids. Colorimetric analysis at 590 nm of a crude alkaloid fraction relative to a known standard (i.e., ergonovine maleate; ergotamine tartrate) provides a method for quantifying total ergot alkaloids in crude extracts (Michelson and Kelleher, 1963). Individual ergot alkaloids may then be identified and quantified by a combination of TLC, HPLC, and/or MS.

Mass Spectrometry. Identification and quantitative analysis of the ergot alkaloids isolated from infected grass and from cultures using MS include electron impact (EI) (Porter et al., 1979), chemical ionization (CI) (Porter and Betowski, 1981; Porter et al., 1981), and tandem mass (MSMS) spectrometry (Plattner et al., 1983; Yates et al., 1985; Lyons et al., 1986; Porter et al., 1987). Under low resolution electron impact (70 eV), the ergopeptine alkaloids pyrolytically decompose into the lysergic acid amide, the cyclic peptide, and diketopiperazine fragments A, B, and C, respectively (Figure 1). These fragments then undergo EI and produce spectra characteristic of the ergoline ring and the peptide portion of the parent molecule. Fragments useful in the interpretation of these spectra occur at 70, 125, and 154 atomic mass units (amu), which are characteristic of the proline moiety (Porter et al., 1979; Bianchi et al., 1982). These ions, in combination with the lysergic acid amide ion at 267 amu (ion A, Figure 1), are indicative of an ergopeptine alkaloid. Using ergovaline for example, in addition to 267 amu, the other two major fragments associated with ions B and C (i.e., fragments indicative of the methyl substituent at R1 and the isopropyl substituent at R2, Figure 1, Table 1) occur at 266 and 196 amu, respectively. The major disadvantage with low resolution electron impact mass spectrometry (EI MS) (70 eV) in the analysis of the ergopeptine alkaloids is the low abundance (i.e., ≤ 1%) of the molecular ion and the diagnostic fragment ion B when R2 is an alkyl substituent (i.e., ethyl-, n-butyl-, isobutyl-, sec-propyl-, isopropyl; Figure 1, Table 1). Although fragments associated with the ergoline nucleus are isobaric with those fragments related to the cyclic peptide (B) and diketopiperazine

<table>
<thead>
<tr>
<th>Table 3. Solvent systems a effectively used for thin-layer chromatography on silica gel for separation and identification of ergot alkaloids b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃C₂H₅:PrOH (92:8; 90:10; 75:25)</td>
</tr>
<tr>
<td>CHCl₃:MeOH (90:10; 80:20)</td>
</tr>
<tr>
<td>CHCl₃:MeOH (90:10) in a saturated NH₃ atmosphere</td>
</tr>
<tr>
<td>CHCl₃:MeOH:NH₃ (94:5:1)</td>
</tr>
<tr>
<td>CHCl₃:Et₃NH (90:10)</td>
</tr>
<tr>
<td>Benzene:Dimethylformamide (86.5:13.5)</td>
</tr>
</tbody>
</table>

a All systems (vol/vol) are in saturated atoms, in tanks lined with Whatman No. 1 filter paper.

b Perellino et al., 1993; Porter et al., 1974, 1979, 1981.
Porter et al., 1979). Spectral interpretation of complex mixtures of the ergopeptines, however, becomes somewhat more difficult.

Isobutane chemical ionization mass spectrometry (CIMS) has been employed in the identification of the ergopeptine alkaloids (Porter and Betowski, 1981; Porter et al., 1981). This method involves an ion-molecule reaction of the alkaloids in the presence of a reagent gas (isobutane). The ion-molecule reaction results in reduced fragmentations, as seen with low resolution EIMS, produces simplified spectra, and thus circumvents interpretation difficulties of the more complex spectra and those resulting from mixtures of these alkaloids. Under isobutane CIMS, the lysergic acid amide (A), cyclic peptide (B), and diketopiperazine (C) molecules (Figure 1) abstract a proton from a t-butyl cation (which is generated in the mass spectrometer) and the resulting ion-molecule reaction produces spectra containing three major ions represented by AH, BH, and CH in Figure 1 and Table 1. These ions are 1 + amu greater than the parent fragments A, B, and C (Porter and Betowski, 1981; Porter et al., 1981; Plattner et al., 1983; Yates et al., 1985). The major fragments used to identify the known ergopeptine alkaloids by this method are outlined in Table 1. Although ergovaline and its C-8 epimer ergovalinine produce the same three major fragments at 268 (AH), 267 (BH), and 197 (CH) amu and cannot be distinguished by this method, both compounds (as with the other ergopeptines and their C-8 epimers) separate nicely using TLC and/or HPLC analysis (Porter et al., 1974, 1979; Yates and Powell, 1988; Rottinghaus et al., 1991, 1993; Perellino et al., 1993). Tandem mass spectrometry (Plattner et al., 1983) also has been employed in the separation and analysis of ergopeptine alkaloids from both endophyte-infected tall fescue and perennial ryegrass (Yates et al., 1985; Lyons et al., 1986; Rowan and Shaw, 1987). In an overly simplified description, MS/MS (which also is conducted in the presence of a reagent and/or a target gas) uses one stage of mass separation to isolate the individual alkaloids of interest in a crude extract; depending on the ionization mode, this usually involves the molecular ion, a protonated molecular ion, or a molecular anion; a second stage of mass separation is then employed to analyze the product and/or daughter ions (also dependent on the reagent and/or target gas). The fragmentation mechanisms for the ergopeptine alkaloids utilizing MS/MS with isobutane as the reagent gas and argon as the target gas are analogous to that described for isobutane CIMS and are described in detail (Plattner et al., 1983). Major advantages of this system are that complex mixtures of these compounds can be analyzed without prior cleanup, only small samples of extracted material are needed, and isomeric species (i.e., ergosine vs beta-ergosine; Figure 1, Table 1) can be distinguished. However, MS/MS analysis requires expensive instrumentation not readily available to most laboratories, and like CIMS, this system cannot distinguish between epimeric ergopeptine alkaloids. Subsequently, MS/MS is not practical for routine screening of toxic or infected grasses. Field desorption MS also has been used in the identification of the ergopeptine alkaloids (Bianchi et al., 1982) and provides extremely simplified spectra along with intense molecular ions.

Quantification and Toxicity

Cornell et al. (1990) have reported ergovaline concentrations at 50 ng/g of infected grass is sufficient to produce some signs of fescue toxicosis in cattle stressed by heat. Spiers (1993) demonstrated similar thermoregulatory imbalances in rats dosed with ergovaline. Dyer (1993) and Oliver et al. (1993) have reported analogous in vitro vaso-activities of ergovaline and lysergic acid amide, respectively. Thus, although ergovaline is an exceptional marker associated with A. coenophialum-infected fescue, ergovaline toxicosis in livestock most probably is augmented by the total concentration of the ergot, and possibly other alkaloids in the infected grass.

Ergovaline concentrations in infected tall fescue seeds and forages have ranged from 2 to 6 µg/g (Table 2). Total concentrations of ergot alkaloids in A. coenophialum-infected tall fescue vary with the season and amount of nitrogen fertilization (Lyons et al., 1986; Belesky et al., 1988; Lyons et al., 1990; Rottinghaus et al., 1991; Arachevaleta et al., 1992), and these are factors that should be considered prior to sample collection and analysis for alkaloid content. Whether the relative concentrations of individual ergot alkaloids vary with season or nitrogen fertilization is currently unknown.

Loline Alkaloids. The loline alkaloids in A. coenophialum-infected tall fescue are produced either by the plant in response to the fungus and/or as a defense mechanism in response to insect herbivory that may involve interactions between both the endophyte and its host grass. Moreover, Petroski et al. (1990) have suggested the lolines may be allelopathic, thus improving the infected-grass's ability to compete with other grasses. The chemistry, occurrence, and biological effects of the loline alkaloids and associated endophyte-grass interactions have been reviewed (Pewell and Petroski, 1992; Bush et al., 1993). The lolines are directly related to A. coenophialum-infected grasses and have not been identified in A. loli-infected grasses (Siegel et al., 1990).

Capillary gas chromatography (GC) using either flame ionization (FID) and/or MS detection (MSD) (Yates et al., 1990; Pewell and Petroski, 1992; Tepaske et al., 1993) is the current method of choice
for the analysis of the loline alkaloids. Extraction of ground seed (approximately 5 g) with methylene chloride:methanol:ammonia (75:25:0.5, vol/vol/vol), followed by filtration, provides extracts ready for GC/FID or GC/MSD analysis. Forage samples may be extracted similarly, but prior to analysis, a sulfonic acid solid phase clean-up step of the forage extracts is necessary to remove substances that interfere with the assay. The detection limit for N-acetylloline and N-formylloline is 10 ng. Tepaske et al. (1993) have described a similar GC/MSD procedure for the analysis of the loline alkaloids in bovine urine and plasma. Although the loline alkaloids do not provide a well-defined molecular ion in the mass spectrum (70 eV), their separation under GC conditions and characteristic mass fragmentation patterns (Powell and Petroski, 1992) allows for their unequivocal identification and quantification.

Petroski et al. (1990) and Powell and Petroski (1992) reported a method for the separation of the loline and the ergot alkaloids from endophyte-infected tall fescue whereby an alkaloidal extract is subjected to column chromatography using Sephadex LH-20. The loline alkaloids pass through the column and the ergot alkaloids are recovered by exhaustive elution of the LH-20 with methanol. Also, Petroski and Powell (1991) employed counter current chromatography for the separation of milligram quantities of N-methylloleine, N-acetylloline, and N-formylloline from an endophyte-infected tall fescue seed.

Total lolines (defined as N-formyl- and N-acetylloleine) occurring in endophyte-infected tall fescue seed (3,263 µg/g) and forage (1,723 µg/g) were quantified using GC/FID (Yates et al., 1990). Concentrations of these alkaloids (as with the ergot alkaloids) in forages vary with season, the amount of nitrogen fertilization, grazing pressure, and the amount of insect herbivory (Bush et al., 1982, 1993; Belesky et al., 1987; Eichenseer et al., 1991). Further studies are needed to determine whether the loline alkaloids significantly contribute to fescue toxicosis in livestock.

A. lolii-Infected Perennial Ryegrass

Paxilline and the Lolitrem B Alkaloids. Current evidence suggests that the indole-isoprenoid lolitrems are almost as diverse as the ergot alkaloids. Paxilline and lolitrem B (Figure 2) are the two major alkaloids associated with perennial ryegrass staggers (Gallagher et al., 1985; Weedon and Mantle, 1987; Miles et al., 1992; Fletcher et al., 1993; Penn et al., 1993). Studies involved with determining the biosynthesis of paxilline and lolitrem B resulted in the identification of alpha-paxitriol, lolitriol, and the lolitrems A, C, D, and E (Miles et al., 1993). These additional minor lolitrems may contribute to the overall toxicity of paxilline and lolitrem B.

Gallagher et al. (1985) reported an isolation and screening method for lolitrem B in A. lolii-infected perennial ryegrass. One gram of oven-dried, milled grass was extracted with chloroform:methanol (50 mL; 2:1, vol/vol) for 1 h. One milliliter of the extract was dried under nitrogen, reconstituted in methylene chloride (2 mL) and subjected to a cleanup step on silica. The eluent (100 µL) from the silica was then analyzed by HPLC on a Zorbax Silica column (DuPont, Wilmington, DE), using methylene chloride: acetonitrile (80:20, vol/vol) as the mobile phase. Recovery of lolitrem B was 93 to 97% with detection limits at .5 ng (fluorescence detection). This HPLC method has been used to screen up to 80 samples/d. The amount of lolitrem B in the infected grass necessary to elicit the staggers syndrome is only 5 ppm (Gallagher et al., 1985).

Peramine. Although the correlation of peramine (Figure 4) as the major insect deterrent in A. coenophialum-infected tall fescue and A. lolii-infected perennial ryegrass has been reported (Rowan and Tapper, 1989; Tapper et al., 1989; Siegel et al., 1990), mammalian toxicity to permine has not been determined. The isolation and analysis of peramine from infected grasses presents some unique problems because of the guanidino moiety attached to the aliphatic side chain on the pyrrolopyrazine ring. Tapper et al. (1989) have reported a method for the analysis of both peramine and lolitrem B using a two-phase extraction system in which 100 mg of freeze-dried, ground grass is first extracted with methanol: chloroform (3 mL) and followed by concurrent extractions with hexane and water (3 mL each). The aqueous and organic phases are separated by centrifugation. Lolitrem B was analyzed in the organic phase as previously reported (Gallagher et al., 1985), whereas peramine, after minor cleanup using ion-exchange chromatography, was analyzed in the aqueous phase by HPLC with a mobile phase of acetonitrile:guanidinium formate (pH = 3.7). Recovery of peramine is 93 to 100%, with detection limits at 1 µg/g of infected grass. Alternatively, peramine may be analyzed in the aqueous phase, after an ion-exchange cleanup step with two minicolumns connected in series. The first column (BioRad AG 2 x 8, 200–400 mesh) is in the hydroxide form and the second column (Analytichem Bond Elut CBA) is in the carboxylic acid form. After the aqueous phase is aspirated onto the columns, they are washed with 80% aqueous methanol (3 mL), the columns separated, and peramine eluted from the acid column with aqueous methanol and formic acid. Peramine is then analyzed by TLC using chloroform:methanol:acetic acid:water (20:10:1:1, vol/vol/vol/vol) as the developing solvent (Tapper et al., 1989). Fannin et al. (1990) have reported a rapid, sensitive, reverse-phase TLC method for the analysis and quantification of peramine in crude extracts of several endophyte-infected grasses, and Rowan et al. (1986) have defined the characteristic low-resolution mass fragmentation (70 eV) of peramine (Figure 4).
As with the loline alkaloids, further studies are needed to establish whether peramine augments the toxicities of the ergot and(or) lolitrem alkaloids or whether peramine causes a unique mammalian toxicosis of its own.

Summary

The major toxins associated with *Acremonium*-infected grasses and with animal toxicoses are the ergot and lolitrem alkaloids. Ergovaline and lysergic acid amide (and/or) lysergylmethylcarbinolamide are the major ergot alkaloids in *A. coenophialum*-infected tall fescue. Paxilline and lolitrem B are the major lolitrem in *A. lolii*-infected perennial ryegrass. Peramine is the major insect deterrent in both infected grasses, whereas the loline alkaloids seem to be primarily associated with *A. coenophialum*-infected tall fescue.

Identification and analysis of the ergot alkaloids in endophyte-infected grasses are effectively accomplished by a combination of TLC and HPLC and the use of EIMS and CIMS. High performance liquid chromatography with either ultraviolet or fluorescence detection is the preferred method for routine screening of endophyte-infected plants for the ergopeptide alkaloids, the lolitremes, and peramine. The loline alkaloids in infected tall fescue are more effectively analyzed by GC with FID or MSD. The advantages of GC/MSD analyses for the lolines are that retention times are consistent with known standards and that their characteristic mass spectra allow for unequivocal identification and quantification.

Animal toxicosis caused by consuming *A. coenophialum*-infected tall fescue has been reported to occur at levels of 50 ng of ergovaline/g of grass, whereas *A. lolii*-infected perennial ryegrass produces the staggers syndrome at 5 μg of lolitrem B/g of grass. The diverse number of biologically active alkaloids that appear in the endophyte-infected grasses no doubt will reduce the overall quantity necessary to produce either tall fescue and/or perennial ryegrass toxicosis in livestock. Therefore, the total concentrations of the ergot and(or) lolitrem alkaloids should be considered where livestock are grazed on infected grass pastures.

Implications

Chemical analysis of endophyte toxins in *Acremonium*-infected fescue and other grasses toxic to livestock has consistently added to our knowledge for combating these problems. Improvements in extraction efficiency, chromatography, and instrumentation have provided information on the number and classes of alkaloids associated with fescue toxicosis and ryegrass staggers in animals on these *Acremonium*-infected grasses. Continued improvements with isolation and chromatographic techniques should provide sufficient quantities of toxins necessary for further animal testing. These studies should then provide answers to both the individual and combined activities of the toxins isolated from the infected grasses. Moreover, chemical analyses and animal studies will eventually define whether fescue toxicosis results from the total concentration of the ergot alkaloids and whether toxicosis is augmented by either or both the loline alkaloids and peramine in infected tall fescue. Animal studies also will define whether the lolitrem-induced ryegrass staggers in livestock is augmented by the ergot alkaloids and peramine in infected perennial ryegrass.

Literature Cited


