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ISOLATION OF FEEDING DETERRENTS AGAINST ARGENTINE STEM WEEVIL FROM RYEGRASS INFECTED WITH THE ENDOPHYTE *Acremonium loliae*

D.D. ROWAN[1] and D.L. GAYNOR[2]

[1]*Applied Biochemistry Division*
[2]*Entomology Division*
DSIR, Palmerston North, New Zealand

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Abstract—Infection of ryegrass (*Lolium perenne*) by an endophytic fungus (*Acremonium loliae*) confers resistance against the Argentine stem weevil (*Listronotus bonariensis*). Extracts from ryegrass clones, infected and uninfected with *A. loliae*, were compared in a feeding choice bioassay, and several fractions were identified which affected stem weevil feeding behavior. One stem weevil feeding deterrent, peramine $C_{12}H_{17}N_5O$, has been isolated from infected ryegrass and partially characterized as a basic indole derivative. Extracts from cultured *A. loliae* had no effect on stem weevil feeding behavior nor was peramine detected in the fungal cultures examined. Peramine and the other active substances are hydrophilic in contrast to the lipophilic properties reported for the neurotoxic lolitrems also isolated from ryegrass infected with *A. loliae* and associated with causing ryegrass staggers disorder in livestock. It is suggested that ryegrass staggers and stem weevil feeding deterrency may arise by different biochemical mechanisms.

Key Words—Perennial ryegrass, *Lolium perenne*, Gramineae, Argentine stem weevil, *Listronotus bonariensis*, Coleoptera, Curculionidae, *Acremonium loliae*, *Acremonium coenophialum*, endophyte, bioassay, induced resistance, feeding deterrent, peramine.

INTRODUCTION

Perennial ryegrass (*Lolium perenne L.*), the dominant New Zealand pasture grass, can be severely damaged by Argentine stem weevil [*Listronotus bonariensis* (Kuschel), Coleoptera: Curculionidae]. In the spring, diapausing adults respond to increasing daylength and begin laying eggs in the leaf sheath at the

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base of ryegrass tillers. The larvae penetrate the stem and develop through four instars during which time they mine and kill up to four tillers. There are two generations per year, with populations peaking in the spring in the South Island and in the summer in the North Island. The adult weevils feed on the tips of leaves but do little damage to mature plants.

Perennial ryegrasses resistant to stem weevil have been identified (Kain et al., 1982a,b), and this resistance has been linked to the presence of an endophytic fungal infection in the resistant plants (Prestidge et al., 1982). The resistance of endophyte-infected ryegrass derives in part from selective feeding by the adult weevils. Gaynor and Hunt (1983) showed that there was less feeding by adult weevils on plants infected with endophyte, and this led to fewer eggs and larvae and to less damage. The endophytic fungus associated with stem weevil resistance of ryegrass, *Acremonium loliae* Latch, Christensen and Samuels, is seed transmitted and closely related to *Acremonium coenophialum* Morgan-Jones and Gams, found in tall fescue, *Festuca arundinaceae* (Schreber) (Morgan-Jones and Gams, 1982; Latch et al., 1984). A second, less common, *Gliocladium*-like endophyte has also been isolated from ryegrass (Latch et al., 1984) but, when ryegrass was artificially infected with this fungus, no resistance to stem weevil was detected (Gaynor et al., 1983).

Endophytic infection of ryegrass has been reported to be associated with resistance to eight species of sod webworms (*Crambus* spp.) (Funk et al., 1983a), to billbugs (*Sphenophorus* sp.) (Funk et al., 1983b) in the United States, and to pasture mealy bug, *Pseudantonina poae* (Mask.), in New Zealand (W.D. Pearson, DSIR, Lincoln, personal communication). Tall fescue infected with *A. coenophialum* has been shown to be resistant to stem weevil (Barker et al., 1983) and to oat aphid, *Rhopalosiphum padi* (L.) (Latch et al., 1985). Endophyte-infected ryegrass, however, is not resistant to *R. padi*. Endophytic fungi have also been reported from Chewings fescue (*F. rubra* L. *sensu lato*), Bermuda grass [*Cynodon dactylon* (L.) Pers.] and a number of other species (Latch et al., 1984). However, only for Chewings fescue are there indications of endophyte-associated insect resistance (Funk et al., 1983b).

The presence of *A. loliae* in ryegrass is also associated with a specific livestock disorder known as ryegrass staggers (Fletcher and Harvey, 1981). This disorder occurs sporadically in stock grazing ryegrass pasture infected with endophyte and is characterized by tetanic muscle spasms and a hypersensitivity to external stimuli. Ryegrass staggers is of considerable importance to agriculture in Australia and New Zealand (Cunningham and Hartley, 1959; Gallagher et al., 1984). The presumed causative agents of ryegrass staggers, a series of tremorgenic neurotoxins named lolitrems, have recently been isolated from extracts of "toxic" pasture and seed (Gallagher et al., 1981, 1982). The lolitrems are structurally similar to the other known tremorgenic mycotoxins such as the penitrems and the janthitrems (Gallagher et al., 1984). Infection of tall fescue

pasture with *A. coenophialum* in the United States is also associated with a complex of stock disorders referred to as fescue foot, fat necrosis, and summer slump or fescue toxicity (Johnson, 1983).

Recent research suggests that endophyte-infected ryegrass and tall fescue is less palatable and may also adversely affect growth and reproduction of stock (Johnson, 1983; Fletcher, 1983; Fletcher and Barrell, 1984). We decided to identify the biochemical mechanisms by which endophytic infection confers stem weevil resistance to the infected plant. This should indicate the feasibility of producing insect-resistant ryegrasses which have no associated animal toxicity and also suggest possible approaches to this goal, e.g., via plant selection or reinfection with mutant endophytes.

METHODS AND MATERIALS

The preference of adult weevils for endophyte-free plants, coupled with the consequent increase in larval damage, led us to develop a bioassay sufficiently sensitive to detect adult weevil feeding preferences between extracts from endophyte-infected and uninfected ryegrasses. "Grasslands Nui" perennial ryegrass was used throughout, and clones free of *A. loliae* were obtained by treating infected ryegrass with benomyl fungicide using the method of Latch and Christensen (1982). The plants were repotted several months prior to testing and checked for endophyte infection microscopically and by an ELISA technique (Musgrave, 1984). For large-scale extraction, the plants were subdivided and grown in the field for several months as spaced plants.

Feeding Choice Bioassay. Ryegrass from either infected or uninfected clones was harvested using an electric sheep-shearing handpiece to collect all material above ground level and stored at −20°C until required. Frozen grass was chopped into 2-cm lengths, blended twice in 95% ethanol, and the resulting extract filtered through Celite and evaporated to dryness under reduced pressure. This extract was redissolved in chloroform–ethanol–water (5:2:1), and a known volume was added to cellulose powder (Whatman CC31) to give a concentration of 2.5 g extract/4 g of cellulose powder. The cellulose powder was dried on a rotary evaporator at 40°C and then on a vacuum pump at 40°C for 1 hr.

Four grams of this cellulose powder was stirred rapidly into a hot mix made from agar (4 g), sucrose (5 g), and water (90 ml). After cooling, 35–40 agar disks were cut with a cork borer (1 cm diameter × 3 mm thickness) and used in a feeding choice bioassay to determine the feeding preference of adult weevils. Subsequent fractions isolated after solvent partitioning or chromatography were assayed at concentrations proportional to their concentration in the 95% ethanol extract.

For the feeding choice bioassay, one adult weevil in an 8-cm-diameter Petri dish was presented with two agar disks, one containing extract from an endophyte-infected ryegrass and the other extract from an uninfected clonal replicate. Dishes were replicated a minimum of 35 times and maintained at 16°C with 16 hr daylength for 72 hr. The amount of feeding on each disk was assessed by scoring blind on a 0–3 scale the amount of frass produced by the weevil (0, having no frass; 1, having 1–2 small clumps of frass; 2, having 4–10 clumps of frass; 3, having large areas of the disk covered in frass). The difference in feeding scores between the two agar disks of each Petri dish was calculated and the significance of the deviation of these differences from zero (i.e., no difference in feeding) was tested using the Wilcoxon signed rank test (Siegel, 1956). The mean difference in feeding scores (MFS) was determined for all the Petri dishes in the test. A positive MFS value indicates more feeding on the disks containing extract from uninfected ryegrass, while a negative MFS value indicates more feeding on the disks containing extract from infected plants.

Feeding Deterrent Bioassay. Activity in the feeding choice bioassay could be the result of either the presence of a feeding deterrent in the extract from the infected ryegrass or higher levels of some feeding stimulant in the extract from uninfected ryegrass. To distinguish between these possibilities, a change was made in the preparation of the cellulose powder used in the feeding choice bioassay. The 95% ethanol extract from uninfected ryegrass was partitioned between 80% ethanol and petroleum ether. The 80% ethanol phase was evaporated to dryness, weighed, redissolved in chloroform–ethanol–water (1 : 1 : 1), and adsorbed onto cellulose powder at 2 g of extract/4 g of cellulose powder. To this cellulose powder was added either sufficient purified extract of an active fraction from an infected plant to give a normal plant concentration (plus solvent, 15 ml total volume), or just solvent (15 ml). The resulting two suspensions were evaporated to dryness and the two portions of cellulose powder used to prepare the two agar disks for the feeding choice bioassay. This was then used to determine whether the previously active fraction still deterred feeding when incorporated back into the ethanolic extract, thus suggesting the presence of a feeding deterrent. To check whether an imbalance of feeding stimulants was involved, the above procedure was repeated but using instead the corresponding extract from a clonal plant uninfected with *A. loliae*.

Phase Partitioning. The 95% ethanol extract, prepared as previously described, was partitioned between chloroform–methanol–water (1 : 1 : 1), then further partitioned between *n*-butanol–water (1 : 1), or petroleum ether–80% ethanol (1 : 1) following the sequences outlined in Figures 1 and 2. All phases were backwashed with the appropriate solvents. The backwashes were partitioned against each other and recombined with their parent phases.

Chromatography. The fractions obtained from the above partitioning were further separated by chromatography following the sequences outlined in Fig-

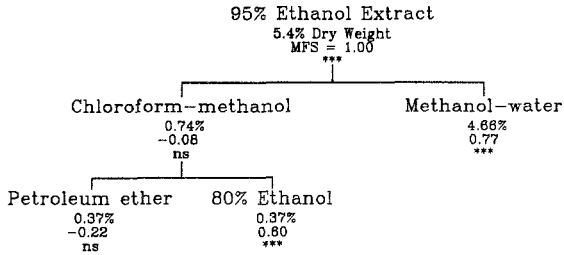

FIG. 1. Phase separation of ryegrass extract showing partitioning of mass (% dry weight), mean difference of stem weevil feeding scores when offered a choice of extracts from infected or uninfected ryegrass (MFS), and the significance of the difference (ns = not significant, *** = $P < 0.001$). A positive MFS value indicates more feeding on the agar disks containing extract from uninfected ryegrass.

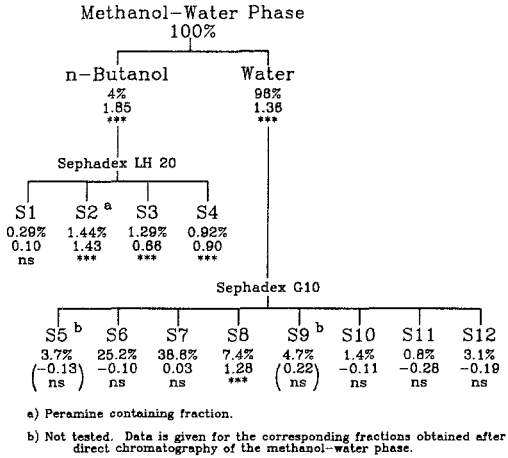

FIG. 2. Phase separation and chromatography of the methanol–water extract showing for each fraction: partitioning of mass (as a % of methanol–water extract), mean difference of stem weevil feeding scores when offered a choice of extracts from infected or uninfected ryegrass (MFS), and the significance of the differences (ns = not significant, *** = $P < 0.001$).

ures 2 and 3. Chromatographic fractions were routinely examined by silica gel thin-layer chromatography (TLC) using a vanillin–sulfuric acid spray reagent, and fractions containing similar components were bulked together for the bioassay. Extracts from both infected and uninfected plants were chromatographed separately, under, as far as possible, identical conditions.

For Sephadex G-10 chromatography (Figure 2), the sample (2.8 g) was eluted from a 25 × 920-mm column of Sephadex G-10 (V_0 180 ml) using 1%

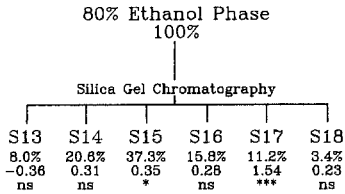

FIG. 3. Fractionation of 80% ethanol extract showing for each fraction: partitioning of mass (as a % of 80% ethanol extract), mean difference of stem weevil feeding scores when offered a choice of extracts from infected and uninfected ryegrass (MFS), and the significance of the differences (ns = not significant, * = $P < 0.05$, *** = $P < 0.001$).

n-butanol–water. The flow rate was 60 ml/hr, and 10-ml fractions were collected. Fractions eluting between 160 and 200, 200 and 330, 330 and 450, 450 and 580, and 580 and 1000 ml were bulked on the basis of their UV adsorption at 280 nm.

For Sephadex LH-20 chromatography (Figure 2), a ratio of 1 g sample to 55 g Sephadex LH-20 was used. The column was eluted with 80% ethanol.

For silica gel chromatography (Figure 3), a sample of the 80% ethanol phase (12.2 g) was applied to a column of silica gel (400 g) packed in 50% petroleum ether–chloroform. The column was eluted successively with chloroform (1.3 liters), chloroform–ethanol (9:1) (6.3 liters, two fractions collected); chloroform–ethanol (8:2) (3.5 liters); chloroform–ethanol–95% ethanol (7:3:3) (3 liters); then (1:2:2) (2.3 liters); then 95% ethanol (1.5 liters).

Isolation of Peramine. The stem weevil feeding deterrent, which we have named peramine, was detected in fraction S2 (Figure 2) as a blue staining spot ($R_f \sim 0.3$) after TLC and spraying with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde–hydrochloric acid). TLC was performed on aluminum-backed silica gel-60 using the lower phase of a chloroform–methanol–water (7:13:8) partition.

Fraction S2 was rechromatographed on Sephadex LH-20 with 80% ethanol until no significant further purification could be achieved. Further chromatography on Sephadex LH-20 (100:1 w/w) using 95% ethanol–dichloromethane (1:9) was used to remove pigments. Fractions containing peramine were eluted using a gradient of 95% ethanol–dichloromethane (2:8 to 1:1). This material was dissolved in 0.01 M ammonium bicarbonate buffer, pH 7.8, containing 2% *n*-butanol and applied to a column of CM-Sephadex in the same buffer. The column was washed with ammonium bicarbonate buffer until the last yellow band had eluted. Peramine was then eluted with 1% acetic acid, pH 4.6, containing 2% *n*-butanol.

Purification of peramine by high-performance liquid chromatography (HPLC) was achieved on a 5-μm C-18 Rad-Pak 8 × 100-mm column fitted with a RCSS C-18 Guard-Pak insert. A linear solvent gradient from 60% to

90% methanol–water over 20 min was used. Both solvents were 0.005 molar in sodium *n*-heptanesulfonate. The flow rate was 1 ml/min with UV detection at 230 and 280 nm. Peramine eluted as the single major peak after 27 min. To remove the bulk of the sodium *n*-heptanesulfonate prior to mass spectrometry, the material was partially desalted using G-10 Sephadex, eluting with water. Subsequent elution with methanol gave peramine.

Flatbed paper electrophoresis was run at pH 10.8 using a 0.1 M sodium carbonate buffer and 5-hydroxy- and 5-methoxytryptamine as standards.

Acetylation of Peramine. Peramine (~0.5 mg) was dissolved in acetic anhydride (0.5 ml) and pyridine (0.5 ml) and kept at room temperature for 6 hr until TLC indicated no peramine remained. Excess reagents were removed with toluene under reduced pressure, and the residue was taken up in dichloromethane and filtered. Preparative silica gel TLC using acetone–dichloromethane (1:3) gave diacetyl-peramine as the major product: electron ionization mass spectrometry (EIMS) *m/z*: 331(M^+), 272, 247, 230, 213, 205, 188, 175 (100%).

Bioassay with Cultured Endophyte. Mycelium from a liquid potato–dextrose culture of *A. loliae* endophyte was blended in 95% ethanol, filtered, evaporated, and made up as a standard solution as for the plant extracts. The hyphal extract was added to cellulose powder onto which had been adsorbed extract from endophyte-free ryegrass as described for the feeding deterrent bioassay above. Agar disks containing hyphal extract plus extract from endophyte-free ryegrass were compared with disks containing only extract from endophyte-free ryegrass. Bioassay concentrations corresponded to 0.7, 4, 7, and 28 mg fungal mycelium/g agar.

RESULTS

Crude Extracts. When adult stem weevils were presented with a choice of agar disks containing 95% ethanol extract either from ryegrass infected with *A. loliae* or from an uninfected clonal replicate, there was significantly less feeding (MFS = 1.00) on the disks containing the extract from infected ryegrass (Figure 1). When this extract was solvent partitioned with chloroform–methanol–water, activity was found in the more polar methanol–water phase (MFS = 0.77). When the inactive chloroform–methanol phase (MFS = −0.08) was further partitioned between 80% ethanol and petroleum ether (Figure 1), activity was found in the 80% ethanol phase (MFS = 0.60), suggesting a masking effect by inactive petroleum-soluble substances.

Isolation of Peramine. The methanol–water phase was partitioned between *n*-butanol and water (Figure 2), and activity was found in both phases. The *n*-butanol phase was chromatographed on Sephadex LH-20 and yielded three active fractions S2, S3, and S4. Fractions from both infected and uninfected plants

were compared by TLC using a variety of spray reagents. The only observed difference between the extracts from infected and uninfected plants was a blue-staining compound detected with Ehrlich's reagent only in fraction S2 from infected ryegrass. This blue-staining material, which we have named peramine, was purified by repeated Sephadex LH-20 chromatography and further purified on Sephadex LH-20 using a gradient of 95% ethanol in dichloromethane. This purified material was active in the feeding choice bioassay of 10 ppm (MFS = 1.13; $P < 0.001$) and 1 ppm (MFS = 0.50; $P < 0.001$). It was also tested in the feeding deterrent bioassay and was found to deter weevil feeding at 10 ppm (MFS = 0.49; $P < 0.001$). The concentration of this fraction in fresh plant tissue from infected plants was estimated to be approximately 8 ppm.

Peramine, homogeneous by TLC and essentially pure by HPLC, was obtained from the partially purified material by ion-exchange chromatography using CM-Sephadex. This material was tested in the feeding choice bioassay by incorporation into agar–cellulose powder disks containing sucrose as the only stimulant (sucrose agar) and was active at 1.3 ppm (MFS = 1.45, $P < 0.001$, concentration based on UV adsorption at 285 nm; ϵ taken as 10,000 based on indole). Ammonium acetate, a coeluant with peramine from the CM-Sephadex column, was also tested at a nominal 18 ppm against sucrose agar in the feeding choice bioassay and was inactive (MFS = 0.18).

A final purification of peramine was achieved by reversed-phase HPLC which removed a number of minor UV adsorbing impurities. A phase modifier, sodium *n*-heptanesulfonate (0.005 M), was added to the mobile phase in order to obtain retention of peramine on the C-18 stationary phase. The phase modifier proved difficult to separate from the small quantities of peramine present, reducing the preparative value of this separation step. Peramine, obtained after HPLC, was desalted on Sephadex G-10 and showed identical UV, low-resolution mass spectra, and chromatographic behavior on TLC to material obtained after CM-Sephadex chromatography.

Peramine showed UV maxima at 232 and 285 nm, suggestive of a 2,3-disubstituted indole (de Jesus et al., 1983). Paper electrophoresis indicated peramine was a strong base, retaining a positive charge at pH 10.8. Peramine gave a positive reaction with Dragendorff's reagent (1-μg sample on TLC) but no reaction with ninhydrin (8-μg sample size). High-resolution electron ionization mass spectrometry (EIMS) established $C_{12}H_{17}N_5O$ as the formula for the highest mass ion (*m/z* 247.1404, $C_{12}H_{17}N_5O$ requires 247.1432). A second high mass ion, measured as $C_{11}H_{15}N_3O$ (*m/z* 205.1214, $C_{11}H_{15}N_3O$ requires 205.1214) corresponded to the product of a cyanamide loss. Attempts made to obtain the FAB-mass spectrum of peramine gave only ions arising from the matrix used (glycerol, glycerol HCl, thioglycerol, PEG). The molecular formula of peramine was confirmed as $C_{12}H_{17}N_5O$ by acetylation which gave a diacetyl derivative *m/z* 331 (M^+, $C_{16}H_{21}N_5O_3$). Characteristic acetamide (*m/z* 272) and ketene (*m/z* 247, double ketene) losses were also observed.

Activity of Water Phase. The water phases from both infected and uninfected plants were chromatographed on Sephadex G-10, and the fractions obtained compared in the choice bioassay (Figure 2). Fraction S8, from within the included volume of the Sephadex column, was the only active fraction. Fractions from both infected and uninfected plants were examined by TLC, but no differences were observed under UV irradiation or with ninhydrin, vanillin, Ehrlich's, fast blue, or sulfuric acid as spray reagents. No peramine was observed. When methanol was added to aqueous solutions of S8, inorganic salts were precipitated. The remaining methanol-soluble components were predominantly glucose and fructose as determined by ^{13}C nuclear magnetic resonance spectroscopy ([^{13}C]NMR). No major differences in the [^{13}C]NMR spectra were observed for samples obtained from either infected or uninfected plants. Samples of S8 were consistently inactive in the feeding deterrent bioassay. The activity in the choice bioassay may result from a higher concentration of feeding stimulants in the extract from uninfected ryegrass. In light of these bioassay results and since no chemical differences were detected between extracts from infected and uninfected plants, this fraction was not further examined.

Activity of 80% Ethanol Phase. Silica gel chromatography of the 80% ethanol phase yielded two active fractions (S15 and S17) (Figure 3). Fraction S15 appeared to have marginal activity and was not further investigated. Fraction S17, obtained from endophyte-infected ryegrass, was an active feeding deterrent (MFS = 0.83, $P < 0.001$), while the corresponding fraction from an uninfected plant was inactive (MFS = −0.07). Fraction S17 from the infected plants was further purified by silica gel, Sephadex LH-20 (80% ethanol) and CM-Sephadex ion-exchange chromatography. TLC revealed the presence of peramine, suggesting that the activity of the 80% ethanol phase may be due to the same components as occur in the *n*-butanol phase, indicating an incomplete initial chloroform–methanol–water partition.

Bioassay of Cultured Acremonium. Extracts of mycelium of *A. loliae* grown in culture did not affect weevil feeding in the feeding deterrent bioassay at concentrations corresponding to 0.7, 4.0, 7.0, and 28.0 mg hyphae per gram of agar. The MFS values were, respectively, 0.03, −0.10, 0.19, and −0.10.

DISCUSSION

The results obtained with the feeding choice bioassays show that ryegrass infected with *A. loliae* contains extractable substances which affect the feeding behavior of adult Argentine stem weevil. Activity is concentrated in the more polar phases and can be further resolved by chromatography. A comparison of chromatographic fractions from plants infected and uninfected with *A. loliae* revealed an active feeding deterrent in the *n*-butanol phase. This feeding deterrent, which we have named peramine, was only found in extracts from ryegrass

infected with *A. loliae*. It was not detected in extracts from uninfected plants or in liquid cultures of *A. loliae* (D.D. Rowan, unpublished results). Peramine, $C_{12}H_{17}N_5O$, has been isolated and shows UV adsorptions and chromogenic reactions typical of a substituted indole derivative. Peramine deterred feeding by adult Argentine stem weevil at 1.3 ppm.

Peramine is chemically different from the tremorgenic lolitrems also isolated from ryegrass infected with *A. loliae* and which are suspected of causing sporadic outbreaks of ryegrass staggers in livestock. The molecular formula of lolitrem A ($C_{42}H_{55}NO_8$) and the structures of lolitrems B ($C_{42}H_{55}NO_7$) and C ($C_{42}H_{57}NO_7$) have been published (Gallagher et al., 1981, 1984). Lolitrems are nonpolar, neutral compounds containing a highly substituted indole ring system and are structurally related to the known aflatrem, penitrem, and janthitrem mycotoxins (Gallagher et al., 1984). Their biological activity against insects has not yet been reported. Based on the solvent partitioning studies of Gallagher et al., (1977) and on the HPLC retention data for lolitrem B (Gallagher et al., 1985), we would expect lolitrems to elute in fractions S13 or S14 (Figure 3). Fractions S13 and S14 were not active in the stem weevil choice bioassay, nor could the characteristic mass fragments for lolitrems (*m/z* 348) (Gallagher et al., 1981) be detected in either of the active fractions, S15 and S17. We therefore suggest that the neurotoxic lolitrems, if present in our extracts, are not important as feeding deterrents.

The amount of endophyte in ryegrass varies between 5 and 200 mg/g, with typical concentrations ranging from 30 to 50 mg/g (Musgrave, 1984). Extracts from cultured *A. loliae* were not active against Argentine stem weevil at concentrations corresponding to 28 mg hyphae/g agar. However, as bioassays on ryegrass extracts show activity even at one tenth the natural concentration of plant material, any feeding deterrent activity in the cultured endophyte should be detectable at the levels used in these tests. Neither peramine nor the lolitrem neurotoxins (R.T. Gallagher, personal communication) have yet been detected in liquid cultures of *A. loliae*. This suggests that peramine, the lolitrems, and other stem weevil active substances are not constitutive in the fungal mycelium. However, they might still be produced by *A. loliae* when growing endophytically or under as yet undefined culture conditions. Alternatively, they may be plant metabolites produced in response to the endophyte.

As peramine and the other stem weevil active fractions appear to differ chemically from the lolitrems, it may be possible to produce an endophyte-infected ryegrass resistant to stem weevil but which does not produce ryegrass staggers in livestock. An understanding of the mechanisms whereby endophytic infection of ryegrass confers resistance to infected plants may also be relevant to research on the insect resistance of other grasses infected with endophyte. Finally, the stem weevil resistance of ryegrass infected with endophyte can be seen as another example of a plant resistance to a pest arising from the effects of a previous fungal challenge.

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REFERENCES

BARKER, G.M., POTTINGER, R.P., and ADDISON, P.J. 1983. Effect of tall fescue and ryegrass endophytes on argentine stem weevil. *Proc. 36th N.Z. Weed Pest Control Conf.* 36:216–219.

CUNNINGHAM, I.J., and HARTLEY, W.J. 1959. Ryegrass staggers. *N.Z. Vet. J.* 7:1–7.

DE JESUS, A.E., STEYN, P.S., VAN HEERDEN, F.R., VLEGGAAR, R., WESSELS, P.L., and HULL, W.E. 1983. Tremorgenic mycotoxins from *Penicillium crustosum:* Isolation of penitrems A–F and the structure elucidation and absolute configuration of penitrem A. *J. Chem. Soc. Perkin Trans.* 1:1847–1856.

FLETCHER, L.R. 1983. Effects of presence of *Lolium* endophyte on growth rates of weaned lambs, growing on to hoggets, on various ryegrasses. *Proc. N.Z. Grass. Assoc.* 44:237–239.

FLETCHER, L.R., and BARRELL, G.K. 1984. Reduced liveweight gains and serum prolactin levels in hoggets grazing ryegrasses containing *Lolium* endophyte. *N.Z. Vet. J.* 32:139–140.

FLETCHER, L.R., and HARVEY, I.C. 1981. An association of a Lolium endophyte with ryegrass staggers. *N.Z. Vet. J.* 29:185–186.

FUNK, C.R., HALISKY, P.M., JOHNSON, M.C., SIEGEL, M.R., STEWART, A.V., AHMAD, S., HURLEY, R.H., and HARVEY, I.C. 1983a. An endophytic fungus and resistance to sod webworms: association in *Lolium perenne* L. *Bio/technology* 1:189–191.

FUNK, C.R., TIMPO, E.E., GOVINDARAJAN, S., and HALISKY, P.M. 1983b. Implications of endophyte-modified performance in grass breeding. 47th Annual Report of Forage Research in Northeastern United States, Agricultural Research Service, USDA, 47:3.

GALLAGHER, R.T., KEOGH, R.G., LATCH, G.C.M., and REID, C.W.W. 1977. The role of fungal tremorgens in ryegrass staggers. *N.Z. J. Agric. Res.* 20:431–440.

GALLAGHER, R.T., WHITE, E.P., and MORTIMER, P.H. 1981. Ryegrass staggers: Isolation of potent neurotoxins lolitrem A and lolitrem B from staggers producing pastures. *N.Z. Vet. J.* 29:189–190.

GALLAGHER, R.T., CAMPBELL, A.G., HAWKES, A.D., HOLLAND, P.T., MCGAVESTON, D.A., PANSIER, E.A., and HARVEY, I.C. 1982. Ryegrass staggers: The presence of lolitrem neurotoxins in ryegrass seed. *N.Z. Vet. J.* 30:183–184.

GALLAGHER, R.T., HAWKES, A.D., STEYN, P.S., and VLEGGAAR, R. 1984. Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: Structure elucidation of lolitrem B. *J. Chem. Soc. Chem. Commun.* 1984:614–616.

GALLAGHER, R.T., HAWKES, A.D., and STEWART, J.M. 1985. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* 321:217–226.

GAYNOR, D.L., and HUNT, W.F. 1983. The relationship between nitrogen supply, endophytic fungus, and Argentine stem weevil resistance in ryegrasses. *Proc. N.Z. Grass. Assoc.* 44:257–263.

GAYNOR, D.L., ROWAN, D.D., LATCH, G.C.M., and PILKINGTON, S. 1983. Preliminary results on the biochemical relationship between adult Argentine stem weevil and two endophytes in ryegrasses. *Proc. 36th N.Z. Weed Pest Control Conf.* 36:220–224.

JOHNSON, T. (ed.) 1983. Proceedings of Tall Fescue Toxicosis Workshop. Cooperative Extension Service, University of Georgia College of Agriculture, Athens, Georgia. pp. 1–26.

KAIN, W.M., SLAY, M.W., and WYETH, T.K. 1982a. Identification, occurrence and development of Argentine stem weevil (*Hyperodes bonariensis* Kuschel) resistance in perennial ryegrass (*Lolium perenne* L.), pp. 265–271, *in* K.E. Lee (ed.). Proceedings of the 3rd Australasian Conference on Grassland Invertebrate Ecology. S.A. Government Printer, Adelaide.

KAIN, W.M., WYETH, T.K., GAYNOR, D.L., and SLAY, M.W. 1982b. Argentine stem weevil (*Hyperodes bonariensis* Kuschel) resistance in perennial and hybrid ryegrasses. 1. Field resistance in perennial ryegrass (*Lolium perenne* L.). *N.Z. J. Agric. Res.* 25:255–259.

LATCH, G.C.M., and CHRISTENSEN, M.J. 1982. Ryegrass endophyte, incidence, and control. *N.Z. J. Agric. Res.* 25:443–448.

LATCH, G.C.M., CHRISTENSEN, M.J., and SAMUELS, G.J. 1984. Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon* 20:535–550.

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.

MORGAN-JONES, G., and GAMS, W. 1982. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe typhina*, new taxa in one of the two new sections of *Acremonium*. *Mycotaxon* 15:311–318.

MUSGRAVE, D.R. 1984. Detection of an endophytic fungus of *Lolium perenne* using enzyme-linked immunosorbent assay (ELISA). *N.Z. J. Agric. Res.* 27:283–288.

PRESTIDGE, R.A., POTTINGER, R.P., and BARKER, G.M. 1982. An association of *Lolium* endophyte with ryegrass resistance to Argentine stem weevil. *Proc. 35th N.Z. Weed Pest Control Conf.* 35:119–122.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.