Nematotoxic effects of endophyte-infected tall fescue toxins and extracts in an *in vitro* bioassay using the nematode *Pratylenchus scribneri*

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Abstract

Biotypes of the *Neotyphodium coenophialum*-tall fescue grass symbiota are provided with enhanced protection from grazing vertebrate herbivores due to the production of toxic secondary metabolites. However, considerable controversy exists concerning this symbiotum and its toxicity to nematode species. A sterile *in vitro* system was developed to determine the interactive nature of known toxins specific to this mutualistic association and compounds within grass extracts known to be nematotoxic. The *in vitro* assay used *Pratylenchus scribneri,* the lesion nematode, as the target organism to determine the interactive nature of ergot alkaloids, the pyrrolizidine alkaloid (the lolines), total phenolic fractions, and specific phenolic compounds. The *in vitro* assay is described along with methods for testing toxicity. The results indicate that only two of three ergot alkaloids were toxic to *P. scribneri*, and there were possible potentiating or synergistic effects with other alkaloids and water soluble polyphenolics. HPLC analysis and UV mass spectrometry of root extracts revealed the presence of two major polyphenolics, chlorogenic and di-caffeoylquinic acids, both of which are natural constituents of this and other plants and have known toxicity to several species of nematodes. Further, it was determined that there were quantitative differences between the total phenolic and specific phenolic contents in roots of endophyte infected and noninfected tall fescue, cultivar Jesup. This *in vitro* assay offers a rapid and routine screen for acute testing chemical components of the tall fescue-endophyte symbiotum for toxicity to this nematode species.

Keywords: Chlorogenic acid, di-caffeoylquinic acids, ergot alkaloid, lolines, nematode, polyphenolics, *Pratylenchus scribneri,* pyrrolizidine alkaloid

Introduction

Biotypes of *Neotyphodium*-tall fescue grass symbiota are provided with enhanced protection from grazing vertebrate herbivores and insects due to the production of toxic secondary metabolites. However, considerable controversy exists concerning the involvement of the *Neotyphodium coenophialum* and resulting toxicity to nematode species. Early observations indicated possible resistance of Kentucky-31 to several species of nematodes (McGowan *et al.* 1961; Hoveland *et al.* 1975; Pedersen *et al.* 1988). These early observations have been substantiated for some nematode species (Pederson & Rodriguez-Kabana 1984; Townsend *et al.* 1984; West *et al.* 1988; Halisky & Myers 1989; West *et al.* 1990), although the chemical identity of repelling toxins remain unknown or not clearly established. Identifying the chemical agent or agents responsible for any and all nematocidal responses, particularly as it relates to the fungus, is highly desirable. Ultimately the chemical identities should indicate any fungal contribution from the symbiotum components, or any interactive relationship with the host grass for increased toxicity. This approach may also determine if the livestock toxins are distinct or synergistic with specific nematotoxic compounds. Additionally, such a system should also have the potential for rapidly identifying native and novel strains of *N. coenophialum* that are more aggressive to species of nematodes, hopefully extending control across the many trophic species of these obnoxious plant pathogens. The outcome will provide a focus for technological exploitations of forage grasses for nematode resistance. However, a rapid and routine technique is required to identify the substance or substances responsible for nematotoxic biological activity in symbiotic tall fescue and other symbiota.

We describe here a sterile *in vitro* system developed to determine the interactive nature of known toxins specific to the tall fescue mutualistic association and compounds known to be nematotoxic in plants. This bioassay was used successfully to determine the toxicity of several classes of mycotoxins to several species of nematodes (Ciancio 1995; Nitao *et al.* 1999; Nitao *et al.* 2001). The *in vitro* assay system developed used the lesion nematode *Pratylenchus scribneri* as the target organism to determine the interactive nature of the test compounds, which include the ergot alkaloids, the pyrrolizidine alkaloid (the lolines), and extracts and fractions from roots of tall fescue.

Materials and Methods

Pratylenchus scribneri nematode bioassay

Bioassays were conducted under sterile *in vitro* conditions following the procedure of Meyer *et al.* (1990). Briefly, it consisted of testing purified compounds as well as total root extracts and fractions of endophyte-infected and non-infected tall fescue cv. Jessup on the mobility of second-stage juveniles (J2) of *P. scribneri* (Fig. 1). Harvested eggs of P. *scribneri* were

Figure 1 Procedure of the *In vitro Bioassay* used for detecting antagonism of extracts and compounds from endophyte/tall fescue to the lesion nematode *Pratylenchus scribneri* (see text for specific details)

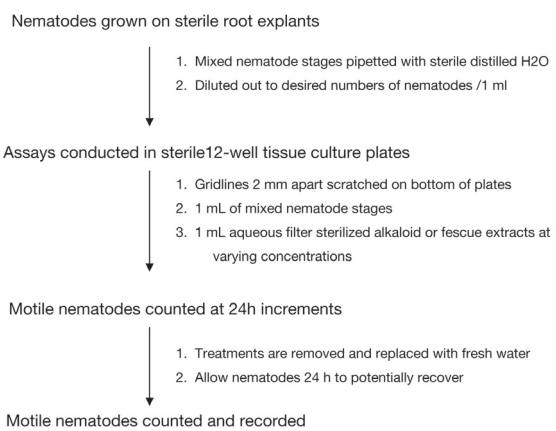
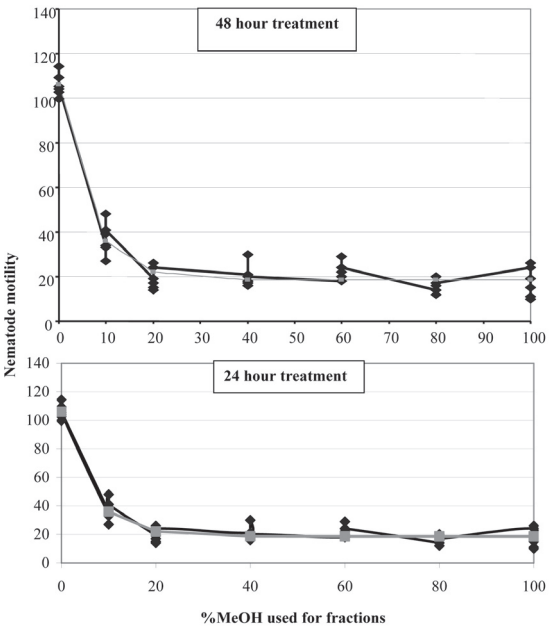


Figure 2 Regression analysis of results of the bioassay of methanolic fractions of endophyte-infected tall fescue cv. Jessup total root extracts against *Pratylenchus scribneri* over 24 and 48 hour exposure periods. Fractions were significant at P<0.05.

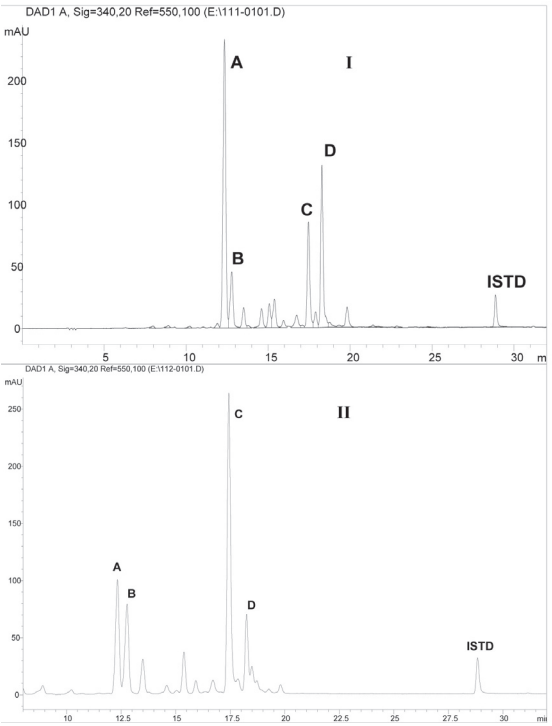


surface disinfected and the resulting J2 were maintained as sterile cultures on sterilised maize roots explants grown on Gamborg's BS medium (Nuettel & Rebois 1985). All assays were conducted in sterile 12-well polystyrene tissue culture plates incubated at 25°C. One ml of J2 was added to each well (approximately 25 to 140 J2/well, depending on the experiment), followed by adding 1 ml of sterile distilled water or buffer solution for control groups. Experimental groups contained appropriate dilutions of extracts in water. All experiments were tested with control treatments of water and solvents. The plates were incubated for 48 h, after which all liquid was removed from the wells, and replaced immediately with fresh sterile water. The plates were incubated for an additional 24 h, and motile nematodes were counted under low power of a dissecting microscope. It was observed that nematodes recovered their motility after 24 h in the fresh water rinse. Acute toxicity as defined here was a measure of the effects of compounds or fractions on the motility of second-stage juveniles of *P. scribneri*. *Pratylenchus scribneri* cultures were maintained in the greenhouse on roots of maize plants. All experiments were repeated four times and differences between endophyte-infected and non-infected extracts were significant with t-test (0.05).

Preparation of plant extracts

Endophyte infected and noninfected tall fescue, cv. Jessup, was grown in pot culture in the greenhouse. After 22 weeks, they were harvested, and the roots rinsed to remove soil. Plants were stored under refrigeration until analysis. Roots, 45 g, were ground in 500 ml of 100% MeOH using a Polytron tissue grinder, filtered (Whatman #2), and the MeOH evaporated under vacuum. The resulting residues were weighed and a stock solution made up in distilled water adjusted to produce extract solutions between 115 and 2,400 ppm. These solutions were filter sterilised (0.2 µm) before use. The assays were conducted as indicated above.

Figure 3 HPLC chromatogram of a methanolic root extracts of endophyte I, and noninfected, II, tall fescue cv. Jessup. Key to peak identities: A, 3-caffeolyquinic acid, B and C, unidentified caffeoylquinic isomers; D, 3,5-dicaffeoylquinic acid; ISTD (internal standard), chrysin.



Identification of compounds.

Ergonovine, ergocornine, and α–ergocryptine were purchased from Sigma, St Louis, MO, USA. The loline alkaloids were obtained as a mixture (n-acetyl loline and N-formyl loline alkaloids), which was extracted from tall fescue seed. Identification of fractions in compounds were generated with an HP 1050 diode array, an HPLC linear gradient from 10% MeOH in 35 min, using a Beckman Ultrtasphere ODS (C_{18}) column 2.5 x 250 mm with a UV detector at 350 nm. The total root extract, 1 150 ppm, was poured onto a C_{18} column and 0, 10, 20, 40, 60, 80, and 100% MeOH fractions were collected (Fig. 2). The MeOH was evaporated and water added to adjust each MeOH fraction back to approximately 1 150 ppm. Experiments were repeated four times and differences between endophyte-infected and non-infected extracts were significant with t-test (0.05).

Results and Discussion

HPLC and UV mass spectrometry analysis of root extracts revealed the presence of many polyphenolic substances of which the major compounds identified were chlorogenic and di-caffeoylquinic acids (Fig. 3). A concentration of 1 150 ppm was chosen as an optimal concentration for further testing of this methanolic fraction against the bioassay. The *in vitro* assay system revealed that methanolic root extracts and sub-fractions of *N. coenophialum*-infected tall fescue roots exhibited adverse effects on *P. scribneri* activity while non-infected root extracts did not show any signs of adverse activity on this nematode.

Table 1 Bioassay of total root extracts of endophyte-infected and noninfected tall fescue cv. Jessup against motility of *Pratylenchus scribneri* scored at 24 and 48 hours. A total of 45 g of roots were used from 22 wk old pot grown plants. All experiments were replicated four times and results presented as an average. Differences between infected and noninfected fractions were significant at P=0.05. See text for extraction protocol.

Extract concentration (ppm)	Nematode Motility			
	---- 24 hours ----		---- 48 hours ----	
	Noninfected	Infected	Noninfected	Infected
115	117	99	93	40
287.5	105	95	85	21
862.5	103	97	92	25
1 150	98	80	80	26
1 725	92	68	77	35
2 400	90	63	68	20

Table 2 Bioassay of ergot and loline alkaloids on motility of *Pratylenchus scribneri* bioassayed 24 and 72 hours following exposure, measured in fresh water. All experiments were replicated four times and results presented as an average. Differences between infected and noninfected fractions were significant at P=0.05. See text for extraction protocol.

		Motility							
		---- 5* ----		---- 50 ----		---- 100 ----		---- 250 ----	
Treatments	Control	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Loline	29	8.25	2.5	3.5	0.25	0	0	0	0
Ergonovine	29	5	4.75	4	2	2.5	1.25	1	0.25
Ergocornine	29	13.25	9	12.5	8.25	12.5	6.25	12.25	4
α-Ergocryptine	29	9.25	2.5	4	1.75	0	0	0	0

*Concentration in µg/ml

Within corresponding fractions from infected and noninfected root extracts, the differences were significant at P=0.05. Motility of J2 was not recovered in any of the treatments after the extended observation period.

Healthy intact roots release into soils exudates containing hydrophobic and hydrophilic substances, including various classes of compounds such as organic acids, peptides, amino acids, sugars, phenols, and other miscellaneous compounds. The roots of grasses, tall fescue in particular, are noted producers of exudates that are speculated to be stimulatory, inhibitory, or inactive relative to competing organisms. Phenolic compounds are one such class that serves as deterrents of several soil pathogens and invertebrate pests (McKeehen *et al.* 1999; Wu *et al.* 2001). The water soluble phenolic acid fractions were established as biologically active in the nematode bioassay. Therefore, the phenolic acid fractions are candidate compounds potentially involved in resistance or tolerance to nematodes capable of parasitizing tall fescue.

The fungal endophyte *N. coenophialum*, associated with tall fescue, produces various secondary metabolites that are potentially toxic to nematodes. Increasing the exposure time of nematodes to total extracts from endophyte-infected roots resulted in decreased nematode motility. However nematode motility was not affected by exposure to extracts from the non-infected roots.

The results from this bioassay indicate that it is suitable for testing whole extracts of tall fescue, which we have shown contain natural metabolites that are toxic to *P. scribneri* (Table 1). The caffeoylquinic acid isomers are phenolic acids with known biological activity to many invertebrate pests, including nematodes (Wu *et al.* 2001). Interestingly, the production of phenolics has been reported in *Epichloe typhina* (Koshino *et al.* 1988). While the occurrence of the polyphenolics in tall fescue shows no qualitative difference relative to endophyte status, we show here that there is a quantitative difference. Endophyte-infected and non-infected roots had the same caffeoylquinic acid derived polyphenolics, but at varying concentrations. Based on an analysis of the HPLC peaks, chlorogenic acid in roots of endophyte-infected and noninfected was present at 558 and 339 ppm respectively. The caffeoylquinic acid isomer in endophyte-infected and noninfected tall fescue roots was found at 118 and 281 ppm respectively, while the other unidentified caffeoylquinic acid isomer was present at 191 and 813 ppm respectively. The other polyphenolic acid, 3, 5-Dicaffeoylquinic acid, was present at 285 and 200 ppm respectively, but the biological significance to these two differences relative to nematode activity has not been determined.

Three commercially available alkaloids, ergonovine, ergocornine, and α–ergocryptine, were tested for toxicity to *P. scribneri* (Table 2). After 72 h, nematode toxicity was only observed in those treatment groups exposed to ergocornine at concentrations higher than 50 µg/ml. After the observation periods were completed, all liquid was removed and replaced with fresh sterile water. Motile nematodes were counted after 24 and 96 h following the water replacement. It was observed that nematode motility did not recover after 96 h rinse with ergonovine, and α–

Table 3 Bioassay of combinations of ergot and loline alkaloids on the motility of *Pratylenchus scribneri* bioassayed at 24 and 72 hours following exposure, measured in fresh water. All experiments were replicated four times and results presented as an average. Differences between infected and noninfected fractions were significant at P=0.05. See text for extraction protocol

		Motility							
		---- 10* ----		---- 50 ----		---- 100 ----		---- 250 ----	
Treatments	Control	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Loline + ergocornine	21	3.5	0.25	0.25	0	0.25	0	0	0
Loline + α-ergocryptine	21	5.5	1.25	0	0.75	0	0	0	0
Ergocornine + α-ergocryptine	21	6.5	0.25	4	0	0.5	0	0	0
Loline + α-ergocryptine + ergocornine	21	2.25	0	0	0	0	0	0	0

*Concentration in µg/ml

ergocryptine. However, the nematodes exposed to ergocornine either remained motile or regained motility in numbers similar to control groups.

Experiments designed to determine potentiating and or synergistic effects of the previously tested alkaloids involved mixing positive ergot alkaloid with the loline alkaloids at various concentrations (Table 3). Assays were conducted as described in sterile 12-well polystyrene tissue culture by adding 1 ml (approximately 25 J2/well) and 1 ml of an alkaloid mixture. After 72 h, J2 motility was greatly decreased over those observed when each compound was used alone. At 10 µg/ml, the loline + α-ergocryptine mix had an average of 1.25 motile J2 and at 50 µg/ml there was an average of 0.75 motile J2 was observed. At 10 µg/ml the loline + ergocornine and ergocornine + α-ergocryptine mixtures had an average of 25% motile J2. All experimental liquids were removed and replaced with fresh sterile water, where motility observations were conducted over a 24 to 96 h post exposure period.

The results of tests on purified alkaloids indicate that there is toxicity in specific ergot alkaloids along with the loline alkaloids. Based on this *in vitro* bioassay two of the ergot and loline alkaloids are acutely toxic to this species of lesion nematode. The toxicities recorded were nematocidal and not static responses since water rinses did not restore nematode motility after a 96 hours extended observation period. This is particularly interesting since these fungal secondary metabolites occur in symbiotic tall fescue as a mixture, suggesting that high concentrations in tall fescue are not necessary to kill this lesion nematode. However, the *in planta* distribution suggests that the ergot alkaloids are present in root tissue at low concentrations, if at all. Thus, the potentiating effects with the lolines alkaloids are strongly suggestive of a probable mechanism for *P. scribneri* inhibition. The results of this study indicate that further testing of these compounds, alone and in combinations with additional alkaloids and the phenolics are warranted. These compounds will be further investigated, along with ergovaline, the principle ergot alkaloid found in symbiotic tall fescue, for their potential individual and synergistic activities against *P. scribneri* and ultimately other nematode species.

Conclusion

The *in vitro* bioassay described can be used to detect acute toxicities of compounds and fractions from tall fescue to *P. scribneri,* a nematode that is parasitic on tall fescue. Although its action on nematode reproduction is not assessed by the assay, the results can be used as an indirect measure of reproductive toxicity by recording the effects on nematode egg hatch (Meyer *et al.* 1990). The assay is rapid, allowing the testing of specific purified compounds for nematode toxicity, but also identifying grass or fungal crude extracts for more detailed chemical examinations. This assay can also be used for several other nematode species associated with tall fescue, including species of *Meloidogyne, Heterodera,* and *Xiphinema* (Nitao *et al.* 1999). The results indicate that while constituents within the ergot alkaloid fraction are toxic to this species of nematode, there are possible potentiating effects observed when other alkaloids are mixed, suggestive of prior observations of toxicity on specific insects (Siegel *et al.* 1991). Further, total phenolic and specific phenolic contents were determined in roots of endophyte infected and noninfected tall fescue cv. Jesup. HPLC analysis and UV mass spectrometry of root extracts revealed the presence of two major polyphenolics that were identified as chlorogenic and di-caffeoylquinic acids, both of which have known toxicity to several species of nematodes. Correlations of *in planta* nematode toxicity with this bioassay are warranted, as well as detailed studies on specific and relevant ergot alkaloids and other phytotoxins known to occur in roots of tall fescue.

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