NEMATOTOXICITY OF *NEOTYPHODIUM*-INFECTED TALL FESCUE ALKALOIDS AND OTHER SECONDARY METABOLITES ON THE PLANT-PARASITIC NEMATODE *PRATYLENCHUS SCRIBNERI*

by

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ABSTRACT

Tall fescue (*Festuca arundinacea*) is a perennial, cool-season turf and forage grass species in the United States that covers over 20 million hectares of pastureland. *Neotyphodium coenophialum*, an endophytic fungus associated with cool-season grasses, enhances host fitness and imparts pest resistance to the grass. Biologically active alkaloids and other secondary metabolites are produced in this association that not only cause adverse effects on livestock, fescue toxicosis, but may also play a role in the reduction of plant-parasitic nematode populations. Currently there is little information available on the effects of these biologically active compounds on nematodes associated with tall fescue.

Therefore, this research examines the interaction of ergot and loline alkaloids, as well as polyphenolic compounds, from endophyte-infected tall fescue on toxicity to the lesion nematode, *Pratylenchus scribneri*. *In vitro* bioassays were performed to assess the effects of specifically identified compounds on *P. scribneri* motility, mortality, and

chemoreception. While separate greenhouse studies evaluated the effects of endophyte-infected tall fescue on *P. scribneri* viability. Root extracts served as nematistatic agents to the nematodes in the chemical submersion assays and affected nematode behavior by acting as repellents in chemoreception studies.

During individual tests, ergovaline and α-ergocryptine were nematicidal at 5µg/ml and 50µg/ml respectively. However, chemotaxis studies revealed α-ergocryptine as an attractant (1-20µg/ml) and repellent (50-200µg/ml). Ergovaline was an effective repellent (1-5µg/ml) and a nematicidal (10-200µg/ml). Nematistasis was observed in nematodes exposed to ergocornine and ergonovine, but was reversible. Ergonovine was a repellent for *P. scriberni*, while ergotamine was an attractant. N-formylloline (NFL) was nematicidal at 100-250µg/ml and reversibly nematistatic at 5-50µg/ml. However, NFL also affected nematode behavior similarly to α-ergocryptine, where it acted as an attractant (1-20µg/ml) and a repellent (50-200µg/ml). The loline mixture of NAL+NFL was also nematicidal (50-200µg/ml) and a repellent (1-20µg/ml). Alkaloid combinations were effectively nematicidal.

Greenhouse studies revealed that endophyte-infected tall fescue are essentially non-hosts to *P. scribneri* populations, with root populations averaging 3.3 to 17.3 nematodes per pot. While, soil populations ranged between 4,866.67 and 8,450 nematodes per pot. This work identified some of the biologically active compounds produced in endophyte-infected tall fescue as nematotoxic and should be further studied to enumerate their modes of action against other plant-parasitic nematodes.

INDEX WORDS: clavine, endophytic fungus, ergot alkaloids, ergopeptide, ergovaline, *Festuca arundinacea*, *in vitro* bioassay, lesion nematode, loline alkaoids, *Lolium*

arundinaceum, nematistatic, nematotoxic, *Neotyphodium coenophialum*; *Pratylenchus scribneri*, pyrrolizidine alkaloids, tall fescue

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B.S., South Carolina State University, 2001

M.S., Georgetown University, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR of PHILOSOPHY

ATHENS, GEORGIA

2008

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August 2008

DEDICATION

I dedicate this to mi abuela, Irma Rivera, and Larry "Shake" Robinson, Jr. two of the most extraordinary people in my life that have made an enormous impact and imprint on my heart, mind, and soul. They were apart of my life at different times, but they both pushed me to want more and to get more from life. These angels loved me and I thank the Lord everyday that he made it possible for me to know and love them. I am proud, honored, and blessed to have had them in my life.

My dedications would not be complete if I didn't acknowledge the heart warming presence of my love, PJ. Thank you honey!! For always being my rock. Your love balanced me and made me focus like never before. To the best friend I ever had, my love for you is eternal.

I dedicate my degree to these wonderful people. I love you now and I always will.

ACKNOWLEDGEMENTS

I would like to take this time to especially thank Dr. Charles Bacon for the opportunity to do better for myself and my family. He took a chance with me and encouraged me even when I doubted myself. Thank You Dr. B!! My committee: Dr. Anthony Glenn, Dr. James Noe, Dr. Patricia Timper, Dr. Nicholas Hill, and Dr. Albert Culbreath thank you for your guidance, support, and patience. I would also like to thank Dr. Susan Meyer, Paula Crowley, and Carol Masler for their advice and providing me with the nematodes used in my experiments. I can't forget about Dr. Maurice Snook and Trevor Mitchell for their assistance with the chemical analyses. And to Tony Edmund, thank you for the introduction to the USDA and to Dr. Bacon. That was the best student-mentor pairing you handled. The friendship we developed is a different kind that only you and I will ever comprehend. You're my boy Tony Edmund.

A special shout-out goes to Dorothy Hinton for her kind ear, sisterly advice and being a sounding board for research, as well. No one will ever understand what you mean to me. To my Guatemalan brother Edwin R. Palencia, I will miss you most of all. We have definitely grown together as scientists and as family. Edwin, I mean that you are my brother. And as unprofessional as this may sound, I will especially miss the TMRU travel crew of Dr. Bacon, Tony Glenn, Dorothy Hinton, and Edwin Palencia. Going to APS was that much better with the TMRU travel crew!!!

Big ups to the Grammy Family of Na'Im Moses, George Jackson, Willette Crawford, and Amanda Harvey. Look here in these pages and you will find each of you in a sentence or two. We did it. You supported me and my craziness. I love you!!!! Mandy aka the sarcastic bohemian psychiatrist, Na'Im aka Johnnie Cochran before the OJ trial, Dubs aka the food

scientist that started the salmonella outbreak on tomatoes and quite possibly the e-coli spread in lettuce, and George aka the Mad Veterinarian I just had to put your business out there. HAHA!!!

To my mother and my sister, I'm here because of you. I want better. Ma, your 5 and 6am wake-up calls got me here. You are the best alarm clock and cheerleader ever. It's your turn now. To my father, thank you for reintroducing yourself into my life. There is a Dr. Bacetty now. To my lovely nieces, Bianca and Christina, take this as an example to follow, life is what YOU make of it and don't let anyone or any situation make you think less of yourselves, your auntie Ada will always be here for you.

And last but definitely not least, my Green Leaf Pelion boys. The guys that welcomed me unconditionally into the fam and made me feel apart of something bigger. Thank you all. Thank you for your constant encouraging words, loving praise, and especially for your continuous presence in all aspects of my life both personally and professionally. Shake said it best "You don't need blood to be family." Family are people that will always make you feel at home and no matter where life has taken me or what life put me through Reggie, Manzell, Claude, Torrey, Calvin, Chris, Murray, Phillip, Claude Ray, Robert, Tommy, Sammy, and Shake were always there to welcome me home. When I felt like giving up you guys would call and ask how school was going and when you would see me again. You didn't even know it, but that small act alone often gave me the second wind I needed to keep going. Thank you!!! I love you. You realize it's been six years since we first met?!?! The best six years of my life. I can't say it enough I love you guys. I can't imagine any other years without you in them. Blood or no blood you guys are my family. I am your sister always and forever.

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CHAPTER 1

INTRODUCTION

A. Fungal Endophytes

Endophytes are symptomless fungi that colonize the living, internal tissues of their hosts, even though on other hosts the endophytic hyphae of related species may, after an incubation or latency period, cause disease (101). Endophytes have been detected in plant life growing in various climate zones around the globe. It is not uncommon to find endophytes in low-latitude tropical climates, mid-latitude temperate climates, and high-latitude climates like those of the boreal forests that extend from Alaska to New Foundland (121). Endophytic fungi have been isolated from mosses, ferns, palms, broad-leaf trees, herbaceous annuals, perennials (121), and in grasses worldwide (113).

There is a group of clavicipitaceous fungi (*Clavicipitaceae*) that form a mutualistic relationship with grasses (Pooidae). The endophytic fungus of interest is an asexual anamorphic *Neotyphodium* species that forms asymptomatic mutualistic associations with their hosts, and are vertically transmitted through the seed following colonization of the developing ovule (82,121). According to Schardl and Moon (89) the *Neotyphodium* endophytes are asexual versions of the sexual related *Epichloë* species. Several *Neotyphodium* endophytes are of hybrid origin and exist as combinations of two to three *Epichloë* or *Neotyphodium* ancestors. The *Neotyphodium* endophyte of tall fescue is one such hybrid and is the result of a complex interaction that resulted in the hybridization of three distinct species: *N. unicatum* tall fescue was infected by *Epichloë festucae*, and this process was repeated by the infection of this hybrid-tall fescue with yet another sexual species *E. baconii* that produced the current double hybrid *N. coenophialum* (89).

B. Tall Fescue – *Neotyphodium* Relationship

Turf and pasture grasses are monocotyledonous plants that naturally lack toxic secondary metabolites that would serve as an anti-herbivory function, thus protecting the plants from pests. This anti-herbivory characteristic, which is lacking in grasses, provides persistence in a stressful environment as a result of seasonal changes and in an ecosystem manipulated by human activity. To account for the lack of production and diversity of toxic metabolites, it is theorized that certain species of grasses developed alternative strategies to survive, which assisted in their widespread dominance in diverse habitats and persistence under grazing pressures.

One evolutionary strategy was to develop a symbiotic relationship with a systemic group of endophytes in the fungal family *Clavicipitaceae* (*Ascomycotina*) (24,113). This relationship exhibits features not usually found in other plant/microbial symbioses. Unique morphological adaptations developed including dense adventitious roots, a strategically placed and highly regenerative basal or intercalary meristem, resistance to certain fungal diseases, enhanced nutrient uptake, and growth stimulation resulting from stresses such as grazing or defoliation (90). Another adaptation resulting from the association with this group of fungi was the accumulation of unique classes of alkaloids, specifically the ergot alkaloids, which serve as toxins and deterrents to vertebrate and invertebrate herbivory.

The amount of land in the U.S. available as forage for grazing animals is nearly 2.4×10^8 ha and worldwide it is more than 10 times as large. Pasture and rangeland in the U.S. provides forage for nearly 10^8 cattle and more than 10^7 sheep (10). *Festuca arundinacea* (synonym *Lolium arundinaceum* (Schreber) Darbyshire; tall fescue) is one of the most important cool-season forage grasses in the USA and is cultivated on approximately 20 million hectares of pastureland, mainly in the eastern USA (4,12,22,55). Most tall fescue plants in natural plant

communities and older pastures are infected by the symptomless fungal endophyte (5) *Neotyphodium coenophialum* Glenn, Bacon, & Hanlin (30).

Benefits of Grass-Endophyte Relationship

The popularity of tall fescue is a result of its wide adaptation, ease of establishment, long productive season, and tolerance to abuse, drought, poor drainage, and adaptation to a wide range of soil pH (4,55). The infection of tall fescue with *N. coenophialum* and toxic effects on cattle was first described by Bacon et al. (6). Since *N. coenophialum* is vertically transmitted it is completely dependent on its host plant and their progeny for survival and dissemination (89). Hyphae are distributed in all plant parts, except leaf blades, and are present only in root tissues when seedlings are cultured on agar medium (5). This species grows intercellularly and completes its entire life cycle without any external signs of infection (6,7,91) (see **Fig. 1.1**).

Survival of a specific genotype of this fungus within a tall fescue population is dependent on the relative fitness requirements of the infected plant. This grass-endophyte association is a mutualistic relationship, and the fungus is non-pathogenic (4,6,7), which suggests that the fungus co-evolved with tall fescue (50). The fungus benefits by receiving nutrients, protection, reproduction, and dissemination. The grass benefits by increased growth and competitiveness, tolerance to drought and microbial infections, and it may be aided by *in planta* production of toxic secondary metabolites and alkaloids that confers grazing tolerance and persistence to mammalian and insect herbivory, and pathogens (4,6,22,38).

Animal Toxicoses

Neotyphodium-infected grasses are responsible for symptoms of toxicity in several ruminant and non-ruminant herbivores. Endophyte-infected grasses are nutritionally comparable to endophyte-free grasses, but exhibit a wide range of protective and fitness enhancements

relative to endophyte-free grasses (10,33). The positive effects of the endophyte on the tall fescue host, combined with its deleterious effects on grazing animals, present livestock producers with a biological dilemma. If they utilize endophyte-infected tall fescue for pastures, livestock suffer toxicosis conditions, but when they utilize endophyte-free tall fescue, they risk loss of the pasture due to environmental and/or pest stresses. Animals raised on endophyte-infected pastures typically have poor performance and exhibit fescue toxicosis, a disease syndrome that includes reductions in weight gain, reproductive capacity, and lactation. In the U.S., a 1988 study estimated that fescue toxicosis caused $600 million in cattle production losses annually (103). It is well established that all aspects of this toxicosis are due to the production of the ergot alkaloids by the fungus, although the specific alkaloid involved in the specific animal symptoms has not been established. Horse and sheep production have also been shown to be affected by the ergot alkaloids, a similar mechanism of action is suggested. However, there are some differences as the horse appears to be highly sensitive to toxins from this grass, indicating species differences and physiologies. Poultry is also sensitive to these alkaloids but to date this has not been demonstrated under laboratory conditions from fescue diets. Cattle production losses attributed to endophyte-infected tall fescue have been related to ergot-type alkaloids (mainly ergovaline or metabolites derived from this and other component ergot alkaloids via ruminant microbial transformation (37)) and possibly to the loline group of alkaloids (103). The main syndromes of cattle toxicosis are summer syndrome, fescue foot, and fat necrosis (77, 79, 103).

Summer syndrome is a condition occurring during the summer months of August to September when ambient temperatures are at their highest. It is characterized by poor animal weight gains, intolerance to heat, excessive salivation, rough hair coats, elevated body

temperatures, nervousness, lower milk production, and reduced conception rate (42, 79, 103). Fescue foot is the most dramatic visible symptom occurring on cattle grazing tall fescue (103). It is associated with colder environmental temperatures and is manifested first by a red line (hyperemia) at the coronary band of the hoof (107). Signs of fescue foot include rough hair coat, scouring, lameness, arched back, loss of weight, increased body temperature, increased respiration rate and a gangrenous condition arises at the feet, tail, and ears (118,103). The gangrene may result in necrosis and sloughing of the affected hoof (118). Fat necrosis is the formation of hard masses of necrotic mesenteric (107) fat surrounding the intestinal tract (103). Visible signs include loss of weight, scanty feces, and bloating (118). The necrotic lesions can disrupt normal digestion and also interfere with calving or renal function (103,107). The summer syndrome is economically severe due to its prevalence among all herds and its effects on cattle gain and calving.

Fescue toxicosis is also a problem to horses. There are approximately 688,000 horses in the U.S. that graze tall fescue (40). The toxins from the endophyte of tall fescue are the causative agents for reproductive abnormalities in gravid mares. The horse toxicity syndrome in mares includes increased gestation lengths, agalactia, foal and mare mortality, tough and thickened placentas, weak and dysmature foals, and reduced serum prolactin and progesterone levels (35). The impacts on horses are not as great as cattle since horses are not pastured on tall fescue to the same extent as cattle.

Toxicity to Insects

The secondary compounds produced by the tall fescue grass-endophyte association are known to affect many vertebrate and invertebrate pests. Studies have indicated that endophyte-infected tall fescue can deter feeding or lower densities of various insect species including:

Rhopalosiphum padi (L.), *Schizaphis graminum* (Rondani), *Diuraphis noxia* (Mordvilko), and in the Aphididae; *Agallia constricta* Van Duzee, *Draeculacephala antica* (Walk), and *Endria inimical* (Say) in the Cicadellidae; *Oncopeltus fasciatus* (Dallas) in the Lygaeidae; *Chaetocnema pulicaria* Melsheimer in the Chrysomelidae; *Sopdoptera frugiperda* (J.E. Smith) in the Noctuidae; *Crambus* spp. in the Pyralidae; *Listronotus bonariensis* (Kuschel) in the Curculionidae; *Tribolium castaneum* (Herbst) in the Tenebionidae; and *Popillia japonica* Newman in the Scarabaeidae (18,36,46,48,53,78,98).

C. Known Toxins

In planta production of defensive metabolites for protecting grasses from vertebrate and invertebrate herbivory and its association with toxicosis in grazing animals has lead to studies on the grass-endophyte mutualistic relationship. Endophyte-infected tall fescue results in an accumulation of alkaloids, many of which are toxic. The cultivars of tall fescue (primarily 'Kentucky-31') are estimated to be 90% and higher infected with *N. coenophialum* (10, 14). The endophyte is not uniformly distributed in all plant tissues and organs (94). Concentration and perhaps localization of alkaloids within the grass is dependent on the growth cycle of the grass (57). In this ubiquitous grass-endophyte relationship there are a number of biologically active secondary metabolites (70) known as alkaloids. The four major alkaloids produced by *Neotyphodium* endophytes include the ergot alkaloids, lolines (saturated 1-aminopyrrolizidines), peramine (pyrrolopyrazines), and the lolitrems (indole-diterpenes); followed by perloline (diazaphenantrene alkaloid) which is produced in endophyte-infected fescue (1,16,57,85,115,118). However, very little biological activity has been documented with the perloline alkaloid. The endophyte of tall fescue, *N. coenophialum* only produces the ergot, loline, and peramine alkaloids.

ERGOT ALKALOIDS

Chemistry

Ergot alkaloids comprise a group of indole alkaloids which are predominantly found in various species of the ascomycete *Clavicipitaceae* (31). Ergot producing fungi are the oldest known producers of mycotoxins that belong to the *Epichloë* and *Neotyphodium* genera (31), which can infect plants. Chemically the ergot alkaloids are 3,4-substituted indole derivates that have a tetracyclic ergoline ring structure (110) (see **Fig. 1.2**).

The ergots of *Neotyphodium*-infected tall fescue are classified into three groups: the simple amides of lysergic acid, clavines, and ergopeptide forms (31,57,110). The simple lysergic acid derivatives have a short peptide chain attached to the ergoline nucleus in amide linkage via a carboxy group at the carbon 8 position (110). The natural ergoline-derived alkaloids (110) have a double bond in the D-ring at either the 8,9- or 9,10- position of the ergoline ring (31,110) and the nitrogen in the D-ring is always methylated (110). Ergopeptides are alkaloids characterized by a modified tripeptide containing proline and an α-hydroxy- α –amino acid (31). Based on the amino acid composition the ergopeptide class is subdivided into the ergotamine, ergoxine, and ergotoxine groups (31,76,110) (see **Table 1.1**). The amino acid substitutions are located in the first two amino acid positions (I and II) of the cyclol-lactam chain, while the third amino acid position (III) is always proline (110) (see **Figure 1.3**).

The ergot alkaloid pathway arises from indole derivatives of tryptophan and mevalonic acid. There is an oxidative reaction sequence of chanoclavine to agroclavine followed by the irreversible conversion of agroclavine into elymoclavine (31). Elymoclavine (see **Fig. 1.4**) is then converted to lysergic acid by oxidation of carbon 17 hydroxyl to an acid functional group and shifts the double bond from the 8,9- position to the 9,10- position (76). Simple lysergic acid

derivatives and ergopeptides are synthesized from lysergic acid by amide formation at the carbon 17 position (76).

The ergot alkaloids found in *Neotyphodium*-infected tall fescue include ergonovine, ergotamine, ergovaline, and others (120) (see **Table 1.2**). The clavine alkaloids identified include chanoclavine and agroclavine (57,76, 119). Ergot alkaloids are produced by the fungus (1) and are distributed in the stems, leaves, and seeds (70) of tall fescue with lower concentrations occurring in the roots (96). The levels of ergot alkaloids in grasses and their seasonal distribution are dependent on the type of plant tissue, environment, and host-fungal genome interactions (2, 3, 9).

Biological Activity

The primary ergot alkaloids produced in the endophyte-tall fescue relationship are the ergopeptides, with ergovaline accounting for 60-80% of the ergot alkaloid fraction (57,119). Since ergovaline is an ergopeptide its activity in mammals is expected to include effects on the nervous, circulatory, reproductive, and immune systems, leading to high or low blood pressure, muscle contractions, hypothermia, altered secretion of pituitary hormones, reduced fertility, disturbances in sleep-wake cycles, lowered immune responses, and at high doses, hallucinations and gangrene of the extremities (67,77). Toxicity of the fescue ergot alkaloids has been determined on small laboratory animals, *in vitro* and *in vivo* (2, 25, 64, 102, 122), or evidence is suggested from feeding cattle and horses endophyte-infected seed and grass (11, 28, 43, 68, 73, 81).

The various toxic and pharmalogical effects of ergot alkaloids are caused by the structural homology of their tetracyclic ergoline ring system with the monoamine neurotransmitters epinephrine, norepinephrine, dopamine, and serotonin (31, 52, 67, 110).

Depending on its substituents attached to the carboxy group at C8 of the ergoline ring system, the D-lysergic acid has varying affinities towards the different neurotransmitter receptors (110) (see **Fig. 1.5**). The structural similarities between the ergolines and these biogenic amines may explain why some of the ergot alkaloids act as partial agonists and/or antagonists on receptor sites of neurotransmitters (31). Tudzynski et al. and Groger et al. have witnessed that the ergopeptide alkaloids exert vasoconstriction and sympatholytic-adrenolytic effects because of their high affinity for α-adrenergic receptors (31,110). The clavines and simple lysergic acid amides with a small side chain have less adrenergic activity but show strong anti-serotoninergic activity because of its high affinity for serotonin (5-HT) receptors (31,110).

Occurrence in Plants/Fungi

Ergot alkaloids are widely known secondary metabolites of fungi in the genus *Claviceps*, with host plants belonging to the families *Juncaceae*, *Cyperaceae*, and *Gramineae*. Ergot alkaloids are also produced in other unrelated fungi and higher plants. Fumigaclavine and festuclavine have been isolated from *Aspergillus fumigatus* Fres. Fumigaclavine was also found in *Rhizopus nigricans*. *Aspergillus* and *Penicillium* species produce various clavines, such as rugulovasines from a *Penicillium* species (31). Ergobalansine is synthesized by *Balansia obtecta* an endophyte of the annual grass species *Cenchrus echinatus* L. The Southwestern perennial grass, *Stipa robusta*, has also been found to be contaminated with *Neotyphodium*. *Balansia epichloë*, *B. claviceps*, *B. henningsiana*, *B. strangulus*, and *Epichloë typhina* are systemic fungi that produce clavine-type alkaloids. Cultures of *B. epichloë*, *B. strangulus*, and *E. typhina* have been reported producers of agroclavine, elymoclavine, penniclavine, and 6,7-secoagroclavine (31). Several ergot alkaloids are found in species of plants in the family Convovulaceae.

LOLINE ALKALOIDS

Chemistry

The pyrrolizidines (lolines) are a class of alkaloids that are of fungal origin, although they were considered to be products of the endophyte-host complex. Most pyrrolizidine alkaloids are esters of hydroxylated 1-methylpyrrolizidine and most of the hepatotoxic pyrrolizidine alkaloids are esters of unsaturated (double bond between Carbons 1 and 2) pyrrolizidine base that lacks the N-methyl group. The unsaturated forms act as hepatotoxins and carcinogens. The major pathways of metabolism via the liver are pyrrole formation, hydrolysis, and oxidation (15). The Graminae species that have an association with an endophytic fungus contain the most important saturated amino pyrrolizidines, the lolines and their derivatives, that contain an additional nitrogen at Carbon 1 and have a stable oxygen bridge between Carbons 2 and 7 of the base (15) (see **Fig. 1.6**). There are seven naturally-occurring lolines and derivatives that exist in *Neotyphodium* infected tall fescue (see **Table 1.3**), with N-formylloline (NFL) and N-acteylloline (NAL) (98) present at the greatest concentrations, regardless of the stage of plant growth.

Biological Activity

The biological activity of the individual lolines has not been tied to livestock toxicosis; however, alone or in combination, they exhibit strong anti-insect activity. The negative effects include feeding deterrents and toxic factors to the root feeding scarabaeid grub, *Popilla* sp., when fed alkaloid supplemented diets (15). Purified fractions of NFL and other loline derivatives also showed feeding deterrence and toxicity to the aphid (*Rhophalosiphum padi* L.), the greenbug (*Schizaphus graminum* Rondani), and to the milkweed bug (*Oncopeltus fasciatus* Dallas) (15). Pupal mass and survival of the parasitoid, *Euplectrus* sp., was reduced in fall armyworm larvae

fed synthetic diets containing loline alkaloids (13). Petroski et al. (74) found seed germination of *Lolium multiflorum* Lam. was significantly inhibited by NFL and not by the other NAL and N-metylloline derivatives. The biological activity of the two lolines has been suggested as having synergistic potentiating effects with the ergot alkaloids on animal toxicities (97). Feed intake, average daily gain, and serum prolactin are depressed in rats fed a partially purified fraction of loline alkaloids (15).

In *Neotyphodium*-infected tall fescue, loline alkaloids are randomly distributed among seeds, leaf blades, pseudostems, and roots (15,16,95). The concentrations of lolines found in root tissue were at lower levels than those detected in shoot tissue, but may be sufficient to help protect against some insects (15,96) especially if the activity is potentiated or synergistic. The amount of NFL detected in soils below tall fescue plants infected with *N. coenophialum* was four times the level required to inhibit germination of *Lolium multiflorum* (16). Translocation of loline alkaloids is indicated by the detection of NAL and NFL in roots and leaf blades (95). The proposed route of translocation is by way of the phloem, but may be upward in the xylem (15). Depending on the plant's growth cycle, the lolines accumulate to greater concentrations than the other alkaloids (105).

Occurrence in Plants/Fungi

Bush et al. estimated that loline alkaloids are found in approximately 3% of the flowering plants of the world and lolines are a source of human and animal exposure to plant toxins and carcinogens (15). The unsaturated pyrrolizidine forms can be found in plants from the *Crotalaria*, *Echium*, *Heliotropium*, *Senecio*, and *Symphytum* genera (15). While the saturated pyrrolizidine group have been found in several pasture and wild grasses, they are always associated with variants of *Neotyphodium occultans* (if they produce sexual fruiting bodies it is

an *Epichloë* sp.and if not it is otherwise a *Neotyphodium* sp. association) (88). The unsaturated lolines have been found in the following host-endophyte associations: *Lolium cuneatum*, *L. perenne*, *L. temulentum*, *Festuca arundinacea*, *F. gigantean*, *F. pratensis*, *F. argentina*, *Poa autumnalus*, and *Adenocarpus* species as well as numerous other grasses (15,88,104) (see **Table 1.4**).

PERAMINE ALKALOIDS

Chemistry

The novel pyrrolopyrazine alkaloid (peramine) occurs in many endophyte-grass relationships. This fungal metabolite consists of a lipophilic pyrrolopyrazine heterocyclic ring structure with a hydrophilic guanidinium side chain (see **Fig. 1.7**) (95). Peramine is less lipophilic than the other classes of alkaloids and may possibly explain its distribution pattern *in planta* (95). Peramine is derived from proline and arginine. It is related to other diketopiperazine-mycotoxins such as aspergillic acid and related pyrazine derivatives produced by *Aspergillus* and *Candida* species (83). Porter suggested that peramine biosynthesis occurs via cyclization and condensation reactions, with the final N-methylation via and N-methyltransferase and S-adenosylmethionine (76).

Biological Activity

Peramine is the only known pyrrolopyrazine alkaloid that is produced in endophyte-infected tall-fescue, perennial ryegrass, and other symbiotic grasses (16,84,98). Research has established peramine's primary activity as an insect feeding deterrent alone and synergistically with the ergot alkaloid (84,97). It has not been reported to have activity against mammalian herbivores or to be phytotoxic (95). When fed diets of peramine extracts from *N. lolii*-infected perennial ryegrass or pure peramine, feeding deterrence was observed with the Argentine Stem

Weevil, the cutworm *Graphaina mutans* Walker, and the beetle *Phaedon cochleariae* F. (83). Mammalian toxicity of peramine is low to nonexistent. Dosages for insect toxicity and/or deterrence ranges from 10μg g^{-1} to 100 μg g^{-1}, whereas dosages needed for an observable toxic response in mice was 1000mg kg^{-1} dose (83). This was definitely an artificial environment because concentrations of this magnitude do not naturally occur in nature: consequently, peramine is essentially nontoxic to mammals.

The distribution of peramine in endophyte-infected tall fescue and perennial ryegrass follows the distribution of the endophyte in the plant. Leaf sheaths contain greater concentrations of peramine and endophyte than leaf blades. The oldest green leaf sheath contained the highest concentrations when compared to the lower concentrations in youngest emerging leaf blade (95). Peramine is translocated from the endophyte into the plant intercellular spaces where it is either metabolized or mobilized (49). This phenomenon was exhibited in endophyte-infected tall fescue where about 1/3 of the peramine was present in the leaf blade where no endophyte was present (86).

Occurrence in Plants/Fungi

Peramine occurs widely in endophyte-infected grasses. This alkaloid is present in tall fescue infected with *N. coenophialum*, in ryegrasses and fescues infected with *E. typhina* and in seedlings of annual ryegrasses infected with *Neotyphodium*-like endophytes. In studies conducted by Siegel et al. (98), 35 grass-endophyte associations were screened for the alkaloids produced by the mutualistic relationship, only 23 of the plants produced peramine. Out of those 23 plants that produced peramine, 15 were naturally infected with their respective endophyte while 8 plants were artificially infected with their endophyte (see **Table 1.5**).

NEMATODES

Plant-parasitic nematodes have the potential to hamper the establishment and productivity of rangeland and pasture grasses, and may also affect plant forage quality (10). The association of symbiotic tall fescue with tolerance or resistance to some plant-parasitic nematodes is highly speculative and/or observational. Part of the problem is the fact that the endophyte status of tall fescue used in the early studies was unknown, which we now know is an important factor when addressing the nematode-grass association. Extensive field surveys and greenhouse experiments were performed on the effects of plant-parasitic nematodes on forage grass species without knowing the endophyte status of the grasses (41,61). McGlohon et al. identified ten genera of nematodes in soil samples from 217 forage crops where some of the grasses are now known to be infected with a fungal endophyte. This study also identified several pasture grasses that were highly susceptible to nematodes, e.g. Kentucky bluegrass (61). The nematodes identified included: *Hoplolaimus* (lance), *Trichodorus* (stubby root), *Tylenchorynchus* (stunt), *Helicotylenchus* (spiral), *Pratylenchus* (lesion), *Xiphinema* (dagger), *Criconemoides* (ring), and *Paratylenchus* (pin). Additional nematodes identified later include *Heterodera zeae* (cyst), *Meloidogyne marylandi* (root-knot), *Belonolaimus longicaudatus* (sting), and *Paratrichodorus minor* (stubby root) (71).

After the 1977 discovery of the endophyte status as a cause of animal toxicity (6) and the demonstration that the association of tall fescue infected with *N. coenophialum* resulted in a defensive mutualism (19), there have been several experiments to examine the influence of the endophyte on plant-parasitic nematodes. Research has suggested that endophyte presence in tall fescue is associated with reduced numbers of various plant-parasitic nematodes in the soil and on grass roots. Many nematodes are affected by the grass-endophyte relationship. The lesion

nematode, *Pratylenchus scribneri* Steiner, is commonly associated with forage grasses and has been shown to reproduce on tall fescue (10,47). *P. scribneri* is an obligate parasite which feeds and migrates in the cortical tissue of plant roots (10,47,112). They continuously travel within their hosts roots, feeding and moving from cell to cell (10,47). *P. scribneri* can also move in and out of the roots traveling freely in the rhizosphere (10). This movement leads to secondary invasion of roots by other soilborne pathogens, which can result in root necrosis (10).

West et al. (112) and Kimmons et al. (47) compared population densities of *P. scribneri* in the roots and soil surrounding tall fescue plants with and without the endophyte. They found fewer nematodes in the endophyte-infected grass roots than in endophyte-free roots, and fewer nematodes were in the surrounding soil of endophyte-infected grass than in endophyte-free grass. This population reduction supports previous findings that endophyte-infected grasses may be resistant or tolerant to nematodes (72). The sensitivity of nematodes to the presence of the endophytic fungus may be the result of one or more of the inhibitory alkaloid compounds produced by the fungus and/or the plant, and translocated to the root. Inhibitory compounds may be important in reducing the populations of some nematodes by affecting their feeding habits, survival, or reproductive rates (47). Despite these and other studies, doubt remains because there is still a question of whether the *Neotyphodium* endophyte causes the reduction in the densities of soil nematodes (34), probably due to the lack of an identifiable toxin from the fungus or substances due to the association. In a recent study, Panaccione et al. used gene knock-out technology to determine the effects of three ergot alkaloids, ergovaline, agroclavine, and setoclavine, on *P. scribneri* in a perennial ryegrass system infected with a hybrid *Neotyphodium* endophyte (Lp1) (69). The study used one dose, a low concentration of each of the alkaloids. The sources of the alkaloids were not indicated, although they prepared the setoclavine by

oxidizing the agroclavine with a crude extract of ryegrass leaf tissue (69). According to the authors, ergovaline caused a significant reduction in nematode numbers, and that nematode reduction followed the infection status of the grass. However, Panaccione et al. concluded that ergot alkaloids were not responsible for nematode toxicity (69). The work, in spite of its erroneous title and conclusion does show a response to ergovaline and that even a genetically modified fungal endophyte does have a significant effect on this nematode. In this case, the nature of the nematicide was not identified or acknowledged. The identity remains unknown and the work of Panaccione et al. (69) should be scrutinized, reevaluated, and substantiated due to the erroneous source and preparation of the alkaloids used, and a different host.

D. Statement of the Problem

Thus, the involvement of the endophyte in protecting tall fescue from nematode parasitism, while highly suggestive, is still in its beginning stages. The lack of chemical evidence and few plant-parasitic nematode species have been the subject of endophyte-infected tall fescue experimentation. This may be compounded from evidence that nematode resistance conferred by the endophyte may be dependent on the interaction between fescue and endophyte genotype (3), which has been documented in prior work on plant-parasitic nematodes (108). Finally, interactive roles of secondary plant or endophyte metabolites on nematode biology (i.e. reproduction and susceptible stage) have not been determined. This thesis examines the interaction of specifically identified compounds and fractions from endophyte-infected tall fescue on the toxicity to one species of nematode, cultured and tested *in vitro*, as well as the plant-chemical response observed in one genetically identical version of tall fescue challenged with nematode infection.

E. Project Objectives

The research proposed is designed to examine the *in vitro* toxicity of plant and endophyte secondary metabolites in the resistance of tall fescue to *P. scribneri*, which should provide some insight into the role these metabolites play *in planta*. This was undertaken by the establishment and use of an *in vitro* bioassay using *P. scribneri*, a major nematode species with an endoparasitic feeding style, and four broad research objectives. The basic hypothesis is that there are compounds in endophyte-infected tall fescue that are toxic or repellent to nematodes, and that these compounds can be identified using bioassays designed to detect acute toxicity or changes in nematode behavior.

Objective I: Identify chemical constituents of endophyte-infected and non-infected tall fescue and determine activity against plant-parasitic nematodes.

A. Determine the toxicity of plant extracts from endophyte-infected and endophyte-free tall fescue to lesion nematodes.

Objective II: Determine potentiating and/or synergistic effects of the alkaloids produced in the tall fescue-*Neotyphodium* relationship that may reduce nematode populations.

A. Ergot alkaloids – Ergocornine, α-ergocryptine, Ergonovine, & Ergovaline

B. Loline alkaloids – N-formylloline & Loline mixture

Objective III: Determine efficacy of natural plant products or fractions of the tall fescue-*Neotyphodium* relationship as effective chemoreception disruptors of lesion nematodes

A. Asses the attraction or repellency of total root exudates.

B. Assess the attraction or repellency of the fractions or purified alkaloids.

Objective IV: Determine if a relationship exists between endophyte-infection and the suppression of lesion nematode populations using a clonal population of tall fescue.

F. CHAPTER 1 DIAGRAMS

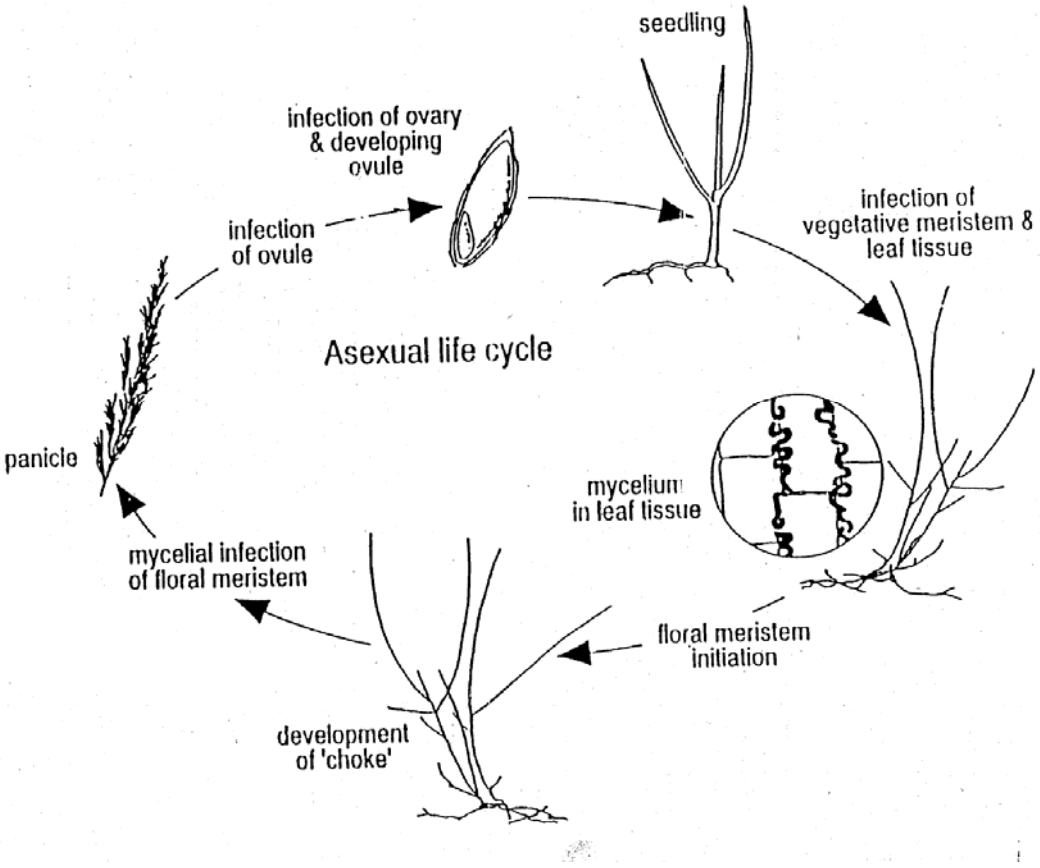

Figure 1.1. Asexual life cycle of *Neotyphodium sp.* infected grasses (Scott and Schardl, 1993)

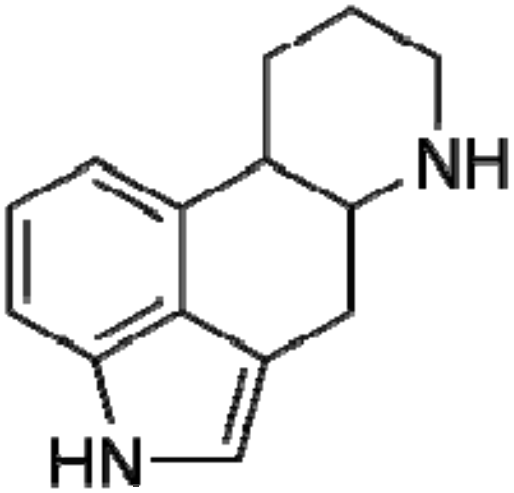

Figure 1.2. Ergoline ring structure

Table 1.1. Ergopeptide class alkaloids

	Ergotamine Group	*Ergoxine Group*	*Ergotoxine Group*
R_2	$R_1=CH_3$	$R_1=C_2H_5$	$R_1=CH(CH_3)_2$
CH_2-C_6H_5	Ergotamine	Ergotosine	Ergocristine
CH_2-$CH(CH_3)_2$	α-Ergosine	α-Ergoptine	α-Ergocryptine
$CHCH_3$-C_2H_5	β-Ergosine	β-Ergoptine	β-Ergocryptine
$CH(CH_3)_2$	Ergovaline	Ergonine	Ergocornine
CH_2CH_3	Ergobine	Ergobutine	Ergobutyrine

(Gröger and Floss, 1998)

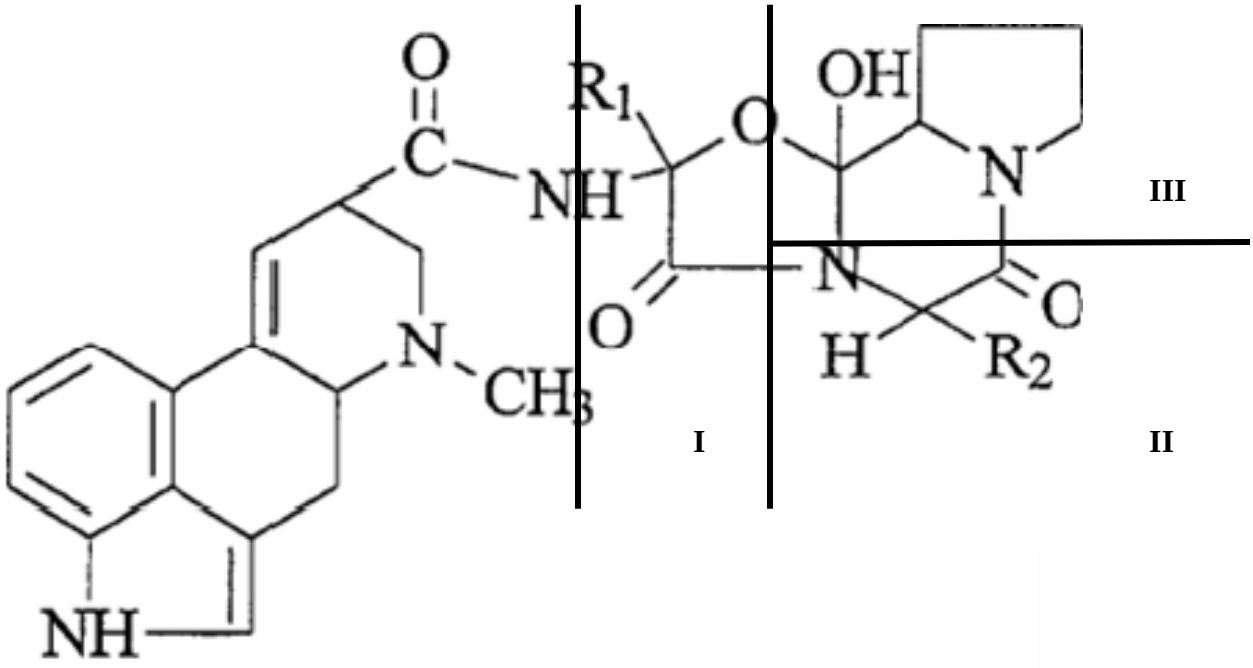

Figure 1.3. Amino acid substitutions of the cyclol-lactam chain attached to the ergoline ring structure. **I** and **II** are the first two amino acid positions for substitutions, and position **III** is always proline.

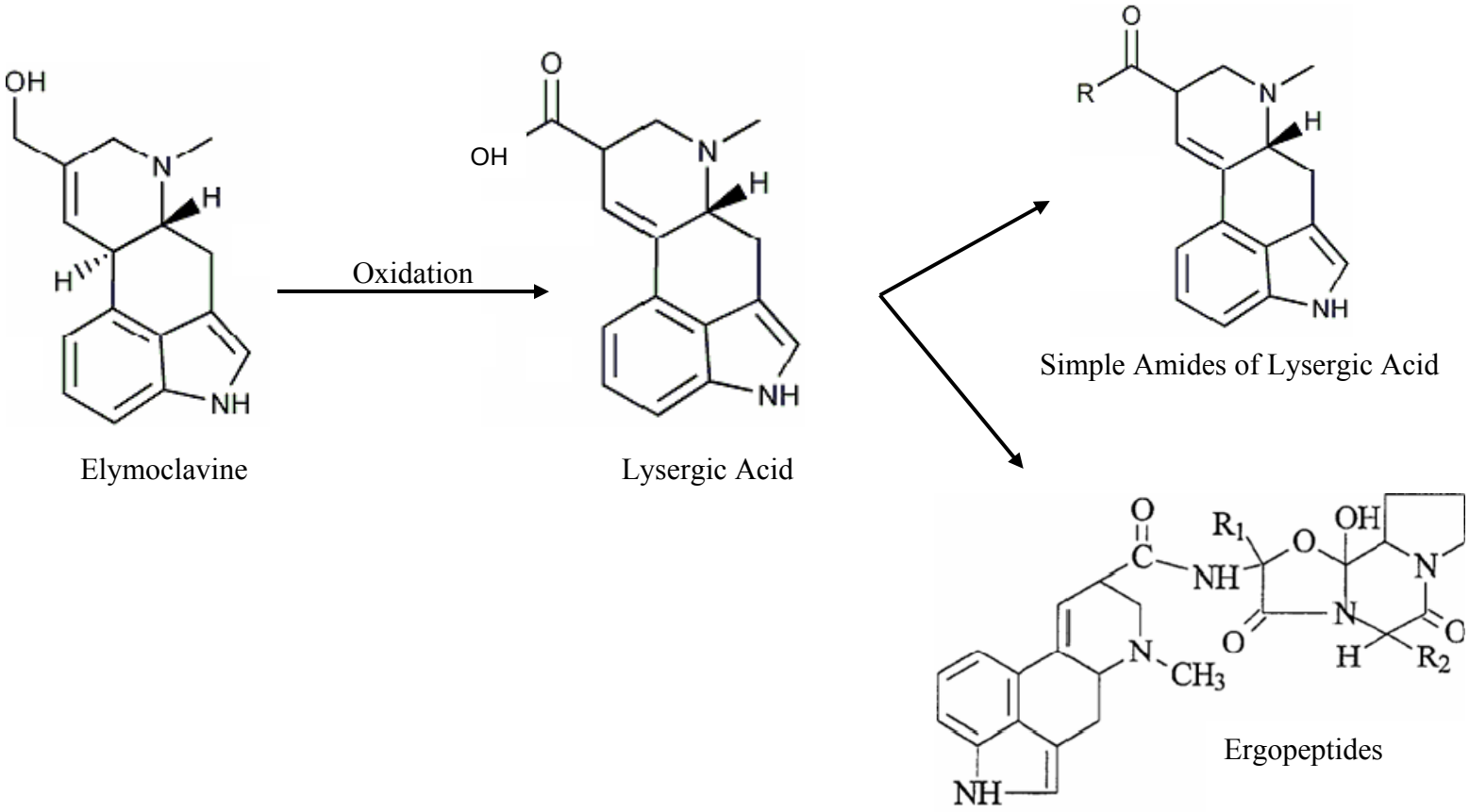

Figure 1.4. Intermediates of the ergot alkaloid pathway (Panaccione, 2005).

Table 1.2. Ergot alkaloids produced in *Neotyphodium*-infected tall fescue

Host Species & Cultivars	
Festuca arundinacea Schreb.	
Ergonovine	Ergovaline
Ergotamine	Ergocornine
Ergosine	α-ergocryptine
β-ergosine	β-ergocryptine
Ergoptine	Ergostine
β-ergoptine	

(Yates and Powell, 1988)

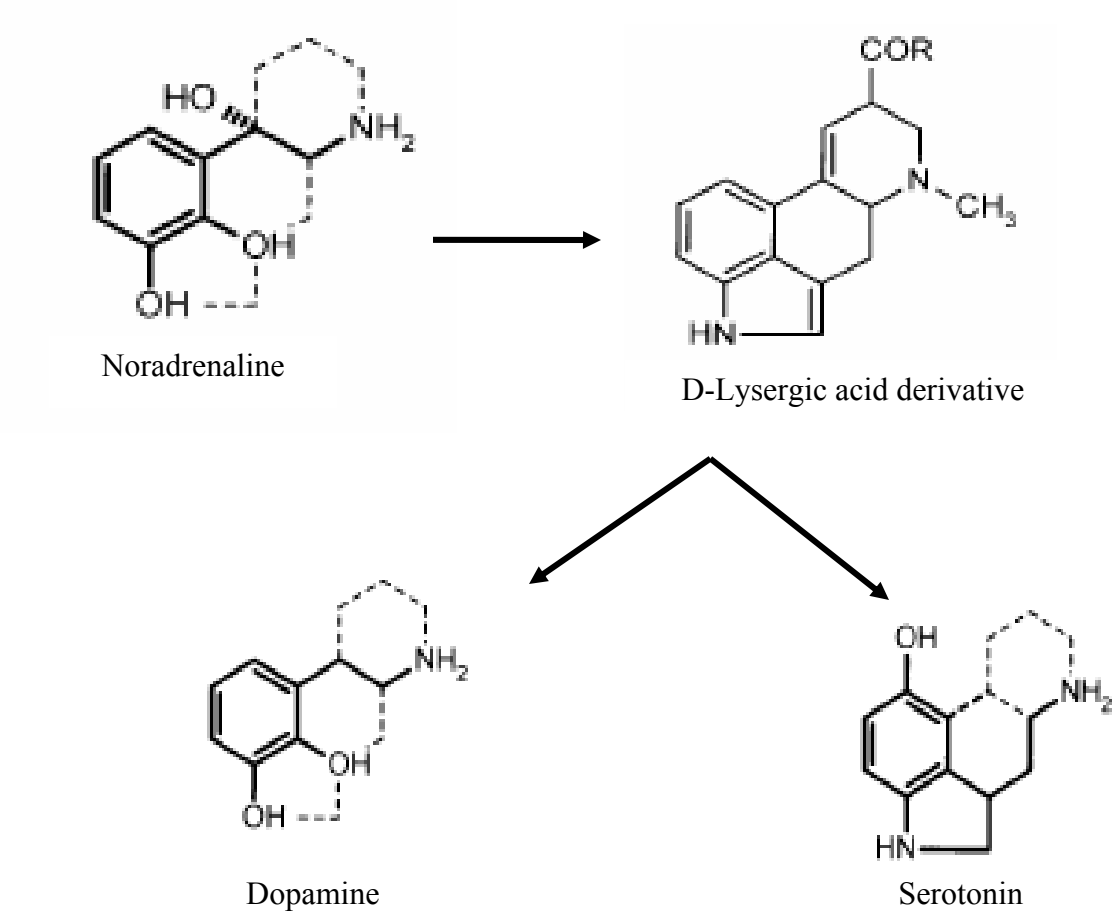

Figure 1.5. Structural similarities between ergoline ring structure and different neurotransmitters (dopamine, noradrenaline, serotonin). (Tudzynski et al., 2001).

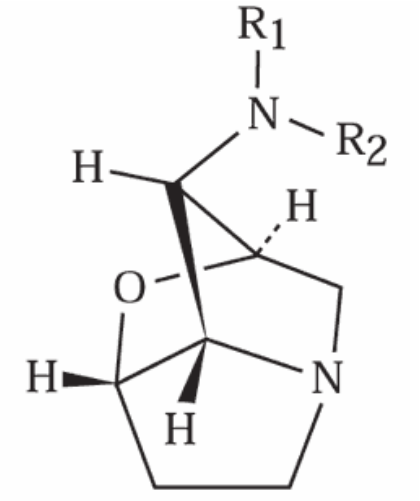

Figure 1.6. Saturated amino pyrrolizidine alkaloid structure

Table 1.3. Seven forms of the amino pyrrolizidine alkaloids

Forms	***R_1***	***R_2***
Loline	H	CH_3
N-formylloline (NFL)	CHO	CH_3
N-acetyloline (NAL)	CH_3CO	CH_3
N-methylloline	CH_3	CH_3
Norloline (Temuline)	H	H
N-acetylnorloline	CH_3CO	H
N-formylnorloline	CHO	H

Table 1.4. Endophyte-symbiotic grasses possessing lolines

Host Species	*Host Tribe*	*Endophyte Species*	*Origin*
Achnatherum robustum (Vasey) Barkworth	Stipeae	*Neotyphodium* sp.	USA
Agrostis hiemalis Britton et al.	Aveneae	*Epichloë amarillans* J.F. White	USA
Echinopogon ovatus P. Beauv.	Aveneae	*N. aotraroae* Moon et al.	New Zealand
Festuca versuta Beal	Poeae	*Neotyphodium* sp.	Texas
Hordeum bogdani Wilensky	Triticeae	*Neotyphodium* sp.	Central Asia
H. brevisubulatum (trin.) Link	Triticeae	*Neotyphodium* sp.	Central Asia
Lolium arundiaceum	Poeae	*N. coenophialum*	USA
L. arundinaceum	Poeae	*N. coenophialum*	Morocco
L. giganteum (L.) S.J. Darbyshire	Poeae	*E. festucae*	Europe
*L. multiflorum*Lam.	Poeae	*N. occultans*	South Africa
L. persicum Boiss. Et Hohen.	Poeae	*N. occultans*	Iran
L. pratense	Poeae	*N. unicatum*	Europe
L. pratense	Poeae	*N. siegelii* Craven et al.	Germany
Lolium sp.	Poeae	*Neotyphodium* sp.FaTG-3	Tunisia
L. rigidum Gaud.	Poeae	*N. occultans*	Egypt
L. temulentum	Poeae	*N. occultans*	Greece
Poa alsodes A. Gray	Poeae	*Neotyphodium* sp.	North Carolina
P. autumnalis Muhl.	Poeae	*Neotyphodium* sp.	Texas

(Schardl et al., 2007)

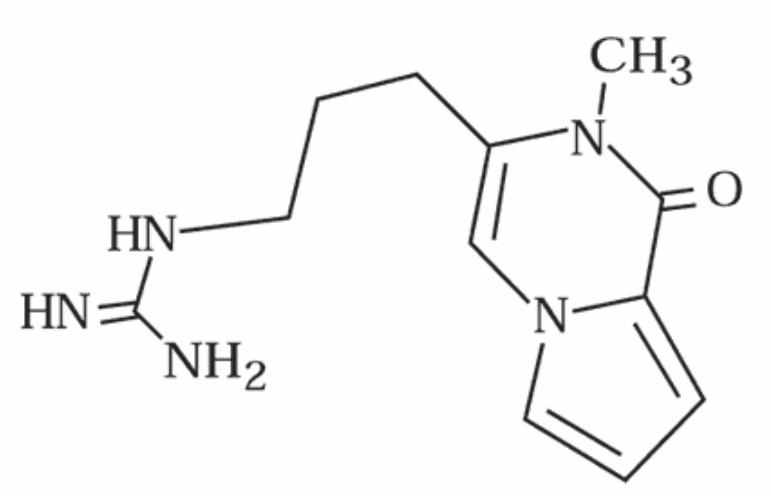

Figure 1.7. Peramine structure

Table 1.5. Endophyte-symbiotic grasses possessing Peramine

Host Species & Cultivars	*Endophyte Species*	*Origin of Endophyte*
Lolium perenne L.		
Nui	*N. lolii*	NI
Repel	*N. lolii*	NI
Ruanui	*N. coenophialum*	AI, *F. arundinacea* (KY 31)
Unknown cv.	*E. typhina*	NI
Gator	*E. typhina*	AI, *L. perenne*
Gator	*E. typhina*	AI, *F. longifolia*
Festuca arundinacea Schreb.		
G1-320 (Johnstone)	*N. coenophialum*	AI, *F. arundinacea* (KY 31)
G1-320 (Johnstone)	*N. coenophialum*	AI, *F. arundinacea* (Au-triumph)
G1-320 (Johnstone)	*N. lolii*	AI, *L. perenne* (Nui)
G1-320 (Johnstone)	*N. lolii*	AI, *L. perenne* (Repel)
G1-320 (Johnstone)	*E. typhina*	AI, *F. longifolia*
KY 31	*N. coenophialum*	NI
F. longifolia Thuill.		
SR-3000	*E. typhina*	NI
F. glauca Vill.		
Carolyn blue	*E. typhina*	NI
F. rubra commutata Guad.		
Longfellow	*E. typhina*	NI
Enjoy	*E. typhina*	NI
F. paradoxa (Desv.)	*Neotyphodium* sp.	NI
Poa ampla Merr.	*E. typhina*	NI
Poa autumnalus (Muhl.) Ell.	*N. coenophialum*	NI
Bromus anomalus Rupt.	*A. starrii*	NI
Elymus canadensis L.	*E. typhina*	NI
Sitanon longifolium Smith	*E. typhina*	NI
Agrostis hiemalis (Walt.) B.S.P.	*E. typhina*	NI

(Siegel et al., 1990)

CHAPTER 2

MATERIALS AND METHODS

A. Nematode Culture

The original culture of *P. scribneri* was generously provided by Dr. Susan Meyer (Beltsville, MD). The nematodes were cultured on corn root explants using the following procedure. Corn (Zea mays L.) cv. Pioneer 3223 seed were surface disinfected for 10 minutes in 100% bleach (5.25% sodium hypochlorite) followed by a 15 minute soak in 95% ethanol. Nine to ten surface sterilized seeds were placed onto 1.5% water agar (15g/L, Difco, Detroit, MI) plates and allowed to incubate in the dark at 25^{o}C for 3-5 days. Primary roots were cut and transferred to pH adjusted (5.7) Gamborg's B5 (Gibco, Carlsbad, CA) agar (15g/L) (80). Plugs from the original Meyer plate were transferred onto the fresh sterile roots. Prior to experiments *P. scribneri* were extracted from the culture plates by pippetting sterile, distilled water (ca. 25^{o}C) onto the surface of the B5 medium and transferring the nematode solution to sterile conical tubes for a stock solution. Through serial dilutions nematodes were diluted and counted to desired numbers for experimental use.

B. Plant Cultures

1. Root Extracts and Fractions Studies using Mixed Populations of Endophyte-infected and Non-infected Tall Fescue

Twenty 2 week-old tall fescue cv. Jesup seedlings (ten infected and ten non-infected with *N. coenophialum*) were obtained from Donald Wood (Dept. of Crop and Soil Sciences, University of Georgia, Athens, Ga) and transplanted to individual 8" plastic azalea pots filled with sterilized Fafard Professional Potting Mix. The pots were randomly arranged in the Russell Research Center greenhouse in Athens, Ga. Pots were watered 4 days per week to maintain soil

moisture and fertilized with Osmocote's Slow Release Plant Food (Marysville, OH) (19-6-12, NPK). Six weeks post transplanting, each pot was repotted into 7-gallon pots that would allow for extensive root growth for the entire length of the experiment (August 2003 – May 2004). Plants were randomly selected and screened for confirmation of endophyte infection by microscopic examination of the outermost leaf sheath (38).

2. *Nematode Suppression Study using an Individual Plant Genotype of Endophyte-infected and Non-infected Tall Fescue*

a. Tall fescue nematode inoculations. Sandy loam soil provided by Ted Holladay (Dept. of Plant Pathology, University of Georgia, Athens, GA) was pre-treated twice by steam heating for 3 hours. Subsamples of the soil were placed in sterile distilled water overnight and observed with a stereoscope for any signs of nematodes and insects. The soil was placed in ten 8" azalea pots and planted with 5 *Neotyphodium*-infected *F. arundinacea* in one set and 5 genetically identical endophyte-free *F. arundinacea* in another. These plants were initially cloned by Charles Bacon (USDA, Athens, GA) and subsequently maintained and supplied to us by Chris Schardl (Department of Plant Pathology, University of Kentucky). Each pot was sampled to microscopically screen for the presence or absence of endophyte hyphae in the outermost leaf sheath (38). Once the endophyte status was confirmed the pots were separated based upon whether or not they were to be inoculated with nematodes. Three plants of each endophyte status were inoculated with 1500 nematodes in 10ml water. In the soil two 1cm diameter x 2cm deep holes were bored onto opposite sides of the plants where 5ml of the nematode water was pipetted into each hole. The holes were covered with more steam sterilized sandy soil and the plants watered. The pots were randomly arranged in the Russell Research Center greenhouse in Athens, Ga. Pots were watered as needed to maintain soil moisture and

fertilized with Osmocote Slow Release Plant Food (Marysville, OH) (19-6-12, NPK). Experiments were repeated for statistical analysis.

b. Nematode assay. Sampling for nematode presence in soil was performed at 30, 45, and 60 days post nematode inoculation. The 30- and 45-day sample periods were performed as follows: a 600 cm^3 soil sample was taken from each of the three replicate pots using a core borer. Three soil cores from each pot were combined and holes were refilled with sterile sandy loam soil and watered. Total plant root damage was not assessed until the final 60-day sampling period so the entire plant could be sacrificed. The final 60-day time period was performed by removing approximately 900cm^3 of soil from each pot using a core borer.

c. Sucrose flotation centrifugation nematode extraction. A 100 cm^3 sub-sample of soil from each pot was suspended in 500ml of sterile distilled water, vigorously stirred for 20 seconds, and allowed to settle for 1 minute. The soil slurry was poured onto 40µm and 400µm nested sieves. Nematodes were then washed into a 150ml beaker and poured in 50ml centrifuge tubes. The tubes were centrifuged at 1400rpm (420G) for 5 minutes where the supernatant was carefully decanted. The supernatant was replaced with equal volume of the sucrose solution (454g sucrose/1L H_2O), tubes covered, and vortexed. The tubes were uncovered and centrifuged for 60 seconds at 1400 rpm (420G), where the sugar-nematode suspension was then decanted onto a 400µm mesh sieve. The nematodes were rinsed from the sieve into a beaker and 1ml was placed into a counting dish for approximate population counts (45).

d. Population density of *P. scribneri* inside tall fescue roots. The 500 cm^3 root-soil from the 30- and 45-day nematode soil sampling procedure was run through an elutriator and the separated roots were evenly distributed into small Kemwipe lined azalea pots. A misting chamber was setup with clean glass funnels with plastic tubing attached to the ends clamped at

the lower 2-3 inches of the tubing. Cold water was poured into the funnels to a level just below the small pots. The pots were placed on top of the funnels and the misting chamber door shut. The pots were water misted at a one minute interval for 30 seconds over a 48 hour period, which allows the lesion nematodes to escape the roots and flow with the water into the plastic tubing attached to the ends of the funnels and settle to the lower 2-3 inches of the tubing. After 48 hours the clamp is released to allow the lower 20ml to be captured into 50ml beakers. Counts of a 1ml sub-sample of each beaker were performed in counting dishes under a stereomicroscope. Nematode population densities in the roots for the final 60-day time period were extracted in a similar fashion, but on a larger scale. After the final soil sampling, the entire root system was submerged into water to release any remaining soil. The roots were then chopped with scissors and lightly rinsed to remove debris. Chopped roots from each pot were thoroughly mixed, weighed, and placed onto the mist chamber, as described above. However, since there was more root material placed into the mist chamber pots the mist chamber was allowed to run for 5 days (120 hours) instead of the standard 48 hours. Nematode sampling and counting were completed at 48, 72, and 120 hours as described above.

C. Sterile *in vitro* nematode bioassay protocol

An *in vitro* bioassay was developed to compare the effects of the various compounds produced in *N. coenophialum* infected *F. arundinacea* and non-infected *F. arundinacea* on nematode motility under sterile conditions. This bioassay was a modification of Meyer et al. (63) who demonstrated its effectiveness in bioassaying several mycotoxins and fungal extracts. Purified compounds as well as total root extracts and fractions of endophyte-infected and non-infected tall fescue cv. Jesup were tested against mixed stages of the lesion nematode, *P. scribneri* (see **Fig. 2.1**).

All assays were conducted in a Fisher Scientific Isotemp incubator at 26^{o}C in sterile 12-well flat bottomed polystyrene tissue culture plates (Corning Costar) previously scratched with 2mm gridlines on the bottom for ease of nematode counting. Nematodes were extracted from established culture plates as described above. One ml of the lesion nematodes was pipetted into each well of the tissue culture plates. Each well was randomly assigned a letter to match its corresponding alkaloid, total root extract, or fractions and control solutions, with each letter replicated 3-4 times. Following the letter assignments, 1ml of either the test compounds or water controls were pipetted into separate wells containing the nematodes. Plates were allowed to sit undisturbed at room temperature in the dark for the duration of an experiment.

Nematode motility was evaluated at 24, 48, and 72 hours after addition of the test solution. Nematodes were observed under a 10X objective of a dissecting scope and those that did not immediately appear to be motile were gently probed with a dissecting needle to initiate movement. If after probing the nematodes remained motionless they were deemed non-motile, following the procedure of Meyer et al (63). Following the final time period, all liquid was removed by pipette and replaced with fresh room temperature sterile distilled water. Nematodes were allowed to sit in the fresh water at room temperature for an additional 24-96 hours, where motility observations were repeated as previously described. Nematodes that did not immediately appear motile were gently probed with a dissecting needle to initiate movement. If after probing the nematodes remained motionless, they were considered dead.

D. Nematode bioassay of plant residues

1. *Total Root Extracts from Endophyte-Infected and Non-Infected Tall Fescue*

Twelve randomly selected 22-week-old plants of endophyte-infected and non-infected tall fescue cv. Jesup were harvested and gently rinsed to remove soil. Forty-five grams of roots

were ground in 500ml of methanol using a Polytron tissue grinder, filtered (Whatman #2), and the methanol was evaporated to dryness under a vacuum using a rotoevaporator at 45°C. The residues were weighed and a stock solution prepared by dissolving the residue in sterile distilled water and filter sterilized (Acrodisc Syringe Filter 0.2µm HT Tuffryn Membrane). The stock solutions were adjusted to yield final root extract concentrations of 115(*A*), 287.5(*B*), 862.5(*C*), 1,150(*D*), 1,725(*E*), and 2,400(*F*) µg/ml. The bioassays were conducted as previously described using approximately 130 lesion nematodes per well of the tissue culture plates with letter assignment of *A*-*F* for the methanol extracts and *G* for a control. Experiments were repeated four times using fresh plant residue batches and differences between endophyte-infected and non-infected extracts were determined with t-test (P = 0.05).

2. *Fractionation Studies of Endophyte-Infected Tall Fescue*

Fractions used for screening biological activity were prepared with an HP 1050 diode array, an HPLC linear gradient from 10% MeOH in 35 min., using a Beckman Ultrasphere ODS (C_{18}) column 2.5x250mm with a UV detector at 350nm. Material from the previous tall fescue cv. Jesup total root extract (1,150ppm) experiments were poured onto a C_{18} column and 0(*A*), 10(*B*), 20(*C*), 40(*D*), 60(*E*), 80(*F*), and 100(*G*)% MeOH fractions were collected. The MeOH was evaporated off and water added to adjust each fraction back to approximately 1,150ppm. The bioassays were conducted as previously described using approximately 130 lesion nematodes per well of the flat bottomed polystyrene tissue culture plates with letter assignment of *A*-*G* for the methanol fractions, *H* for a methanol control to test for activity against nematodes, and *I* as a water control. Experiments were repeated four times with fractions from the new root extracts and differences between fractions were determined with t-test (P = 0.05).

E. Bioassay of Specific Toxins for Nematocidal Activity

1. Bioassay of Individual Alkaloids Produced in the Neotyphodium-Tall Fescue Grass Association

Three independent experiments were conducted to determine the individual toxicity of ergot and loline alkaloids to *Pratylenchus scribneri*. Assays were again conducted in sterile tissue culture plates (Corning Costar) with 2mm gridlines scratched on the bottoms. *P. scribneri* populations were extracted from established culture plates as previously described. A nematode stock solution was prepared by diluting the population number down to approximately 30 nematodes per 1ml of sterile distilled water. A 1ml suspension of lesion nematodes was pipetted into each well and allowed to sit for 24 hours to allow for recovery from any manipulation shock. Stock solutions were prepared for each alkaloid (ergonovine-*A*, ergocornine-*B*, α-ergocryptine-*C*, loline-*D*) and diluted to test 5, 50, 100, and 250 µg/ml. Each concentration was repeated four times. On day 0, 1ml of the compounds was added to their assigned nematode wells where the plates were allowed to sit undisturbed at room temperature for the duration of the experiment. Nematode motility was conducted as previously described and evaluated at 24, 48, 72, and 96 hours after the solution was added.

2. Bioassay of Mixtures of Loline and Ergot Alkaloids

In this experiment the effects of adding a mixture of the ergot and loline alkaloids were tested. This assay was performed on *P. scribneri* using alkaloid mixtures at various concentrations. Ergocornine, α-ergocryptine, and loline were again used in this section as mixtures:

A. Loline + ergocornine + α-ergocryptine

B. Loline + ergocornine

C. Loline + α-ergocryptine

D. Ergocornine + α-ergocryptine.

Assays were repeated from earlier methods. A nematode stock solution was prepared by diluting the nematodes extracted from the B5 culture plates down to approximately 30 nematodes/ml of sterile distilled water. Lesion nematodes in 1ml of water were pipetted into wells and allowed to rest for 24 hours to allow for recovery from any manipulation shock. Stock solutions were prepared for each mixture (loline + ergocornine + α-ergocryptine -*A*, loline + ergocornine-*B*, loline + α-ergocryptine-*C*, ergocornine + α-ergocryptine -*D*) and diluted to test 5, 50, 100, and 250µg/ml. Each dilution of a mixture was replicated four times. Nematode motility assays were conducted as previously described and evaluated at 24, 48, 72, and 96 hours after the solution was added. Three independent experiments were performed for statistical analysis. All alkaloid compounds commercially purchased from Sigma.

F. Nematode Chemoreception Study

Since nematodes rely on chemoreception to locate potential hosts, it is important to know how the endophyte influences nematode behavior. To evaluate the effects on nematode behavior, a chemoreception *in vitro* assay was performed. This assay used ergot and loline alkaloids previously tested, as well as using extracts from roots of the clonal line of *Neotyphodium*- infected and non-infected tall fescue cv. Jesup. Observations were made using a Nikon SMZ1500 microscope with a Nikon DXM1200C digital camera attachment.

Compounds were determined to be an attractant or a repellent using a sixteen segment grid developed by Wuyts et al. (2006). The grid was placed on an agar plate to record the presence = 1 or absence = 2 of nematode tracks (117) (see **Figure 2.2**). The modified technique was employed to calculate the chemotaxis factor (Cf) by dividing the sum of scores of the

attractant zones by those of the repellent zones (see **Figure 2.3**). A Cf greater than 2 denoted an attraction, while values lower than 0.5 resulted in the compound being a nematode repellent.

1. *Attraction or Repellency Associated with Root Extracts of Endophyte-Infected and Non-Infected Tall Fescue*

Two independent pot culture experiments were conducted to identify the attractive or repellent nature to *Pratylenchus scribneri* of root compounds previously found to be leached into the rhizosphere from *Neotyphodium*-infected and non-infected plants. During the nematode suppression study root samples were taken on 0-, 30-, 45-, and 60-days for chemical analysis and the chemoreception study using the control non-nematode inoculated plants. Between 1.29 and 9.61 grams of roots were ground in 500ml of methanol using a Polytron tissue grinder, filtered (Whatman #2), and the methanol evaporated to dryness under a vacuum using a rotoevaporator at 45^{o}C. The residues were weighed and a stock solution prepared by dissolving the residue in sterile distilled water and filter sterilized (Acrodisc Syringe Filter 0.2µm HT Tuffryn Membrane). A comparative analysis of *N. coenophialum*-infected and non-infected roots was executed as described earlier using high-performance liquid chromatography (HPLC) and UV mass spectrometry. Stock solutions of the extracts were adjusted to yield final test concentrations of 400, 200, 100, 50, 20, 10, and 5µg/ml and their effects on nematode behavior compared by observing movement tracks on the agar. One hundred-µl of root extracts were pipetted into 1cm wells on opposite sides of a 5cm petri dish that contained 0.4% water agar. Approximately 6 nematodes were placed in the center of the agar and allowed to move freely for 2 hours while incubated in the dark at 25^{o}C and observations were made using the Nikon microscope-camera setup.

2. *Attraction or Repellency Associated with Alkaloid Compounds*

To determine at what concentration nematodes begin to become attracted and/or repelled to a potential nematicide, 100µl of ergot alkaloids including ergovaline, ergotamine, ergonovine, and α-ergocryptine, as well as a loline mix (NFL+NAL) and NFL. They were individually tested at concentrations of 200, 100, 50, 20, 10, 5, and 1µg/ml and their effects on nematode behavior compared by observing movement tracks in the agar, following the Wuyts procedure (117). Observations were conducted as previously described.

G. Statistical Analysis

Bioassay data were analyzed using regression analyses (SAS v. 9.1) to determine significant differences between root extracts and fractions of endophyte-infected or non-infected fescue and their effects on *P. scribneri* motility. Regression testing (SAS v. 9.1) was also used to determine significant differences of data generated from *in vitro* bioassays testing the effects of alkaloids, alone and in combinations, on *P. scribneri* motility. Duncan's test was performed when comparing nematode populations in the roots and soils of a genetic clone of endophyte-infected and non-infected fescue plants.

H. CHAPTER 2 DIAGRAMS

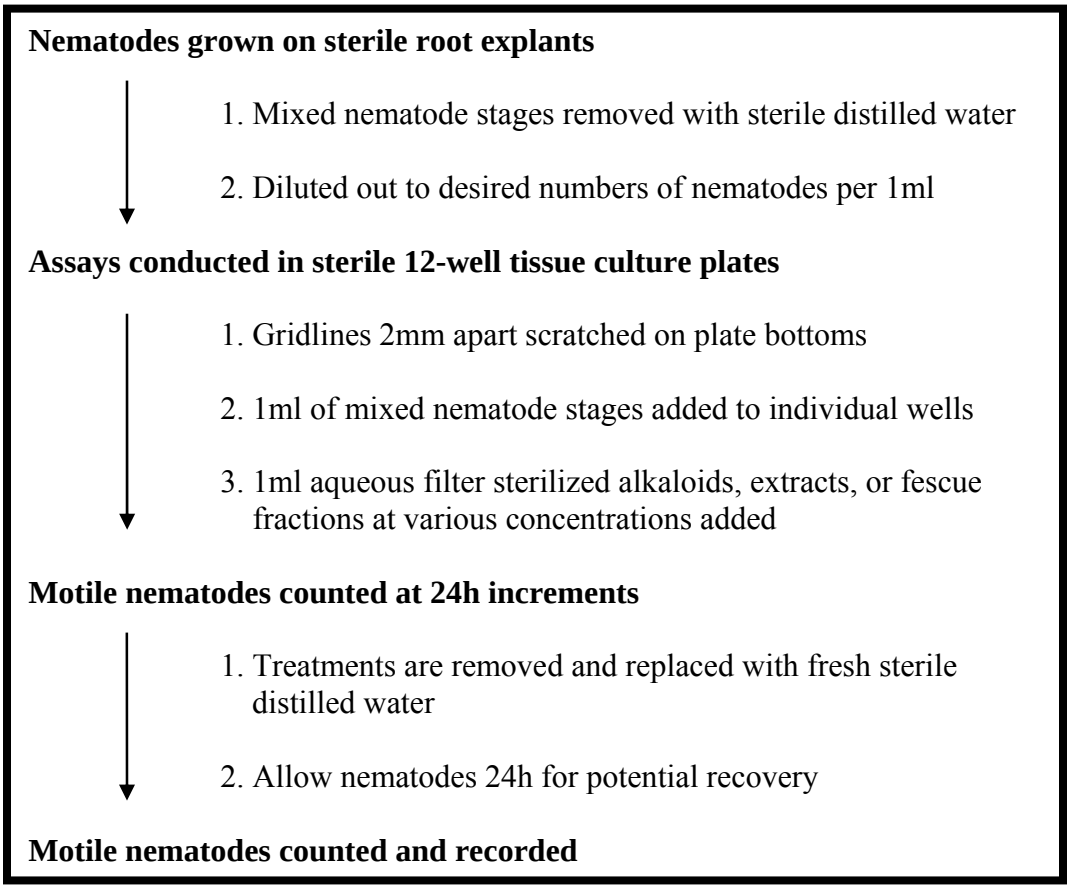

Figure 2.1. The sterile *in vitro* bioassay procedure

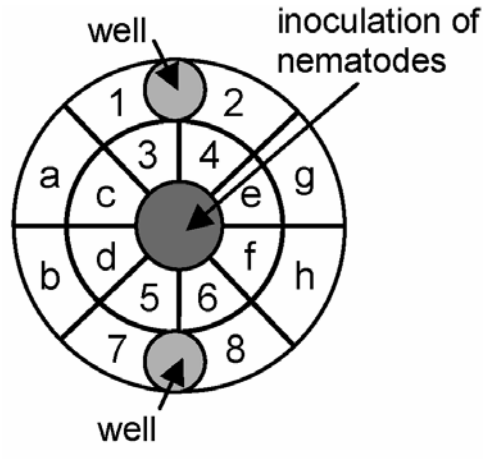

Figure 2.2 Diagram of chemoreception study grid

$$Cf = \frac{\text{attractant}}{\text{repellent}} = \frac{\text{scores for [1] + [2] + ... + [8]}}{\text{scores for [a] + [b] + ... + [h]}}$$

attractant	$Cf > 2.0$
repellent	$Cf < 0.5$
neutral	$0.5 \leq Cf \leq 2.0$

Figure 2.3. Chemotaxis factor (Cf) equation

CHAPTER 3

EXPERIMENTAL RESULTS

A. Chemical profile of compounds produced in *N. coenophialum*-infected and non-infected *F. arundinacea*

In order to identify candidate chemical compounds responsible for decreased *P. scribneri* populations associated with endophyte-infected tall fescue, a comparative analysis of *N. coenophialum*-infected and non-infected roots was executed using high-performance liquid chromatography (HPLC) and UV mass spectrometry. Screening root extracts revealed the presence of various polyphenolic substances of which the major compounds identified were chlorogenic and 3,5 - di-caffeoylquinic acids (see **Figure 3.1**). Endophyte-infected and non-infected fescue roots contained the same caffeoylqunic acid derived polyphenolics, but at varying concentrations. Analysis of the HPLC revealed peaks of chlorogenic acid in roots of endophyte-infected and non-infected fescue revealed concentration of 558 and 339 ppm respectively. The caffeoylquinic acid isomer (Peak B) in endophyte-i0nfected and non-infected fescue roots were found at concentrations of 118 and 281 ppm respectively, while the other unidentified caffeoylquinic acid isomer (Peak C) were present at 191 and 813 ppm respectively. The other polyphenolic acid, 3,5-dicaffeoylquinic acid, was present at 285 and 200 ppm in endophyte-infected and non-infected roots respectively. As depicted in figure 3.1, both the endophyte-infected and non-infected tall fescue extracts contain natural metabolites that are possible candidates for decreased nematode populations. Endophyte-infected and non-infected root extracts from the genetically identical fescue plants also revealed chlorogenic acid, 3,5 – dicaffeoylquinic acid, as well as unidentified Peak B (see **Table 3.1**). However, unknown Peak C identified in the diverse fescue populations was not present in the genetically identical plants.

B. Nematistatic effects caused by *N. coenophialum*-infected *F. arundinacea*

1. Bioassay of total root extracts

An *in vitro* bioassay was performed to determine if nematotoxicity is achieved with chemical compounds that are extracted from roots. The objective was to assess if a relationship exists between endophyte status, the chemical compounds produced in the roots, and decreased nematode populations. The results from this bioassay system indicate that increased concentrations of secondary metabolites produced in endophyte-infected tall fescue are indeed potentially toxic to *P. scribneri* (see **Figure 3.2**). Increasing exposure times of nematodes to endophyte-infected total root extracts resulted in decreased nematode motility. Exposing nematodes to non-infected root extracts did not produce the same or similar effects on motility. However, it is important to note that following the 48 hour submersion of nematodes in endophyte-infected extracts, a fresh water replacement step was included to detect reclamation of nematode motility. After a 24 hour fresh water bath nematode motility was regained to numbers exceeding those of the 24 hour treatment counts. This tells us that the chemical compounds present in the roots act as a paralyzing (nematistatic) agent and not as acute toxins. This is significant because in the natural root environment it is quite possible that plant-parasitic nematodes will be exposed to these compounds for longer than 48 hours and would eventually lead to nematode death.

2. Bioassay of methanolic fractions

An *in vitro* bioassay of total root extract sub-fractions against *P. scribneri* was performed. Results from the previous extract experiments revealed that at concentrations greater than 1,150 ppm there was relatively no change in the number of motile nematodes. Therefore, fractionation studies were conducted at an optimal concentration of 1,150 ppm. This *in vitro*

assay system revealed (see **Figure 3.3**) that methanolic root sub-fractions of *N. coenophialum*-infected tall fescue exhibited adverse effects on *P.scribneri* motility. However, there was no sign of an adverse effect on nematode motility when tested against sub-fractions of non-infected total root extracts. Motility was not recovered when the endophyte-infected fraction was replaced with fresh water.

C. Nematotoxic effects caused by alkaloids produced in *N. coenophialum*-infected *F. arundinacea*

1. In vitro bioassay of the individual alkaloids

Possible nematotoxicity of alkaloids produced in *N. coenophialum*-infected tall fescue was studied by employing the same *in vitro* bioassay system. The ergot and loline alkaloids were individually tested for toxicity to *P. scribneri* (see **Table 3.2**). At the lowest concentration, 5µg/ml, ergovaline treatments negatively affected nematode viability 100% after 72 hours. After 72 hours, 100% of nematode motility ceased in the NFL and α-ergocryptine treatments in excess of 50µg/ml. Motility in nematodes exposed to ergonovine was severly decreased when concentrations exceeded 250µg/ml. Ergocornine treatments caused a significant decrease in nematode motility, but complete ceasation of motility was not observed even at the highest concentration of 250µg/ml. Nematode motility did not recover following removal of ergovaline and 96 hour exposure to fresh water. However, some nematodes recovered in water following ergonovine treatments (5-100µg/ml). Nematodes exposed to ergocornine (all concentrations) and NFL (5-50µg/ml) recovered after incubation in water.

2. In vitro bioassay of alkaloid combinations

Experiments were designed to determine the potentiating and/or synergistic effects of the previously tested alkaloids found to be involved in nematotoxicity of *P. scribneri* (see

Table 3.3). At 10µg/ml, the loline+α-ergocryptine mix, had an average of 1.25 motile nematodes and at 50µg/ml there was an average of 0.75 motile nematodes observed. The 10µg/ml concentration of loline+ergocornine and ergocornine+ α-ergocryptine mixtures had an average of 0.25 motile nematodes. After 72 hours, the loline+ ergocornine+ α-ergocryptine mix had no motile nematodes across all concentrations. The alkaloid mixtures were removed and nematodes subjected to a fresh water bath for 24-96 hours, where motility was not recovered in any of the treatments.

D. Suppression of *P. scribneri* populations by *N. coenophialum*-infected *F. arundinacea* under greenhouse conditions

A pot study was used to determine if *N. coenophialum*-infected tall fescue can suppress *P. scribneri* populations. Sample root weights at 30 days post nematode inoculations were 7g/100cm^3 and 4 g/100cm^3 for the endophyte-infected and non-infected plant respectively. At 45 days, sample root weights for the endophyte-infected and non-infected plants totaled 9g and 5g, respectively. At 60 days, the average root weights for the endophyte-infected and non-infected plants were 97g/ pot and 58g/pot respectively.

When evaluating nematode populations inside roots (see **Table 3.4**) the 30 day counts for both the endophyte-infected and non-infected roots averaged to be nearly identical with 93 nematodes per pot sample. The major division in population numbers occurred at the 45 day sampling period. The endophyte-infected plants averaged between 40 and 46 nematodes, while the non-infected plants yielded an average between 227 and 326 nematodes. This trend was exhibited again at the final 60 day take down. The entire root system for each pot was used for these counts and revealed that nematode populations were severely depressed in the endophyte-infected plants while the non-infected plants served as fantastic hosts. Nematode

populations averaged from 3.3 to 17.3 nematodes per root system of the endophyte-infected plants. However, populations ranged between 1,073 and 2,222.67 nematodes per root system in the non-infected plants. That is nearly a 2-fold increase in *P. scribneri* root populations over the initial 1,500 nematode inoculation. Nematode populations in soil containing endophyte-infected and non-infected plants were also compared (see **Table 3.5**). At 30 days, nematodes numbers from soil containing endophyte-infected and non-infected plants averaged 66.67 and 433.34, respectively. There was a reversal in nematode populations between endophyte presence or absence at 45 days. Sample populations were between 1,133.33 and 1,316.67 nematodes in the endophyte-infected soils, whereas in the non-infected soils the average number of nematodes was 350. At day 60 populations in endophyte-infected soils ranged between 4,866.67 and 8,450 per pot, while the non-infected soils yielded nematode numbers between 466.67 and 750 per pot.

E. Nematode chemoreception assay observations

1. Chemosensory effects of total root extracts from N. coenophialum-infected and non-infected F. arundinacea

To reveal more about the biology of nematode suppression, an alternative chemical *in vitro* bioassay was performed. It was designed to evaluate the secondary metabolites within the roots of *N. coenophialum*-infected tall fescue and its effects on nematode behavior. From the results (see **Figure 3.4**) we've determined that nematode behavior is affected by the secondary metabolites produced in this fungal-grass association. After calculating the chemotaxis factors (Cf), extracts from days 0 and 30 exhibited a neutral effect on *P. scribneri* behavior in both the endophyte-infected and non-infected samples. Clear differences between endophyte status treatments occurred with extracts from the 45 and 60 day sampling periods. Day 45, the endophyte-infected extracts exhibited strong repellency at 100-400µg/ml

concentrations with Cf scores of 0, while the lower 5-50µg/ml concentrations were weaker repellents with Cf scores of 0.3. In stark contrast, non-infected root extracts were strong attractants across all concentrations tested (Cf = 3). Day 60, extracts exhibited similar strong nematode attraction across all concentrations (Cf = 3). However, in the endophyte-infected root extracts concentrations 50-400µg/ml were all strong repellents (Cf = 0), while 5-20µg/ml remained weak repellents (Cf = 0.3). Controls performed as expected, $CaCl_2$ was an attractant (Cf = 3); sterile distilled water had no effect on behavior because nematodes moved over the entire plate; and acetic acid (0.25%) was a strong repellent (Cf = 0). It is important to note that the concentration of acetic acid tested was decreased to 0.25% because preliminary tests revealed that 0.5 and 1% acetic acid acted as a nematicide to *P. scribneri*, where they never moved away from the inoculation site and died sometime during the 2 hour incubation period.

2. Chemosensory effects of alkaloids produced in N. coenophialum-infected F. arundinacea

Using the same chemoreception bioassay system, nematode behavior was examined when exposed to purified alkaloids that are produced in *N. coenophialum*-infected tall fescue (see **Figures 3.5a and b**). Ergovaline, the principal ergopeptide alkaloid of endophyte-infected fescue, is a strong repellent (Cf = 0) at the 100-200µg/ml concentrations and a weak (Cf = 0.3) repellent from 1-50µg/ml. Following a 2 hour incubation period *P. scribneri* in 10-200µg/ml ergovaline treatments were found clustered together dead inside the agar and alive at the 1-5µg/ml concentrations. Since ergotamine and α-ergocryptine are also ergopeptides it was expected that they would also act as nematode repellents. However, across all concentrations, ergotamine was an attractant (Cf = 2.5-4) and even more interesting is that this apparent attractant caused nematode mortality at concentrations of 50-200µg/ml. Alpha-ergocryptine was a weak (Cf = 0.2-0.4) nematode repellent between 50-200µg/ml and an attractant (Cf = 3)

between 1-20µg/ml. The lysergic acid amide, ergonovine, was an effective but weak (Cf = 0.25-0.3) repellent. Nematode mortality was not affected across concentrations. Pyrrolizidine alkaloids affected *P. scribneri* behavior in a similar fashion to the ergopeptides, ergovaline and α-ergocryptine. The loline mix (NFL + NAL) was a strong (Cf = 0) repellent between 10-200µg/ml and a weak (Cf = 0.3) repellent at 1-5µg/ml concentrations. As with ergovaline, nematode mortality was adversely affected. In the 20-200µg/ml treatments, *P. scribneri* were found clustered together and dead after apparently moving a short distance from its inoculation site. Mortality was not affected at the lower 1-10µg/ml concentrations. N-formylloline affected nematode behavior similarly to α-ergocryptine. The higher, 50-200µg/ml, concentrations were weak (Cf = 0.2-0.4) nematode repellents, while the lower, 1-20 µg/ml, treatments acted as attractants (Cf = 2-3). Nematode mortality was not adversely affected across concentrations. Controls (distilled water, $CaCl_2$, and 0.25% acetic acid) acted as previously tested on nematode behavior.

F. CHAPTER 3 DIAGRAMS

A. Chemical profile of compounds produced in genetically diverse populations of *N. coenophialum*-infected and non-infected *F. arundinacea*

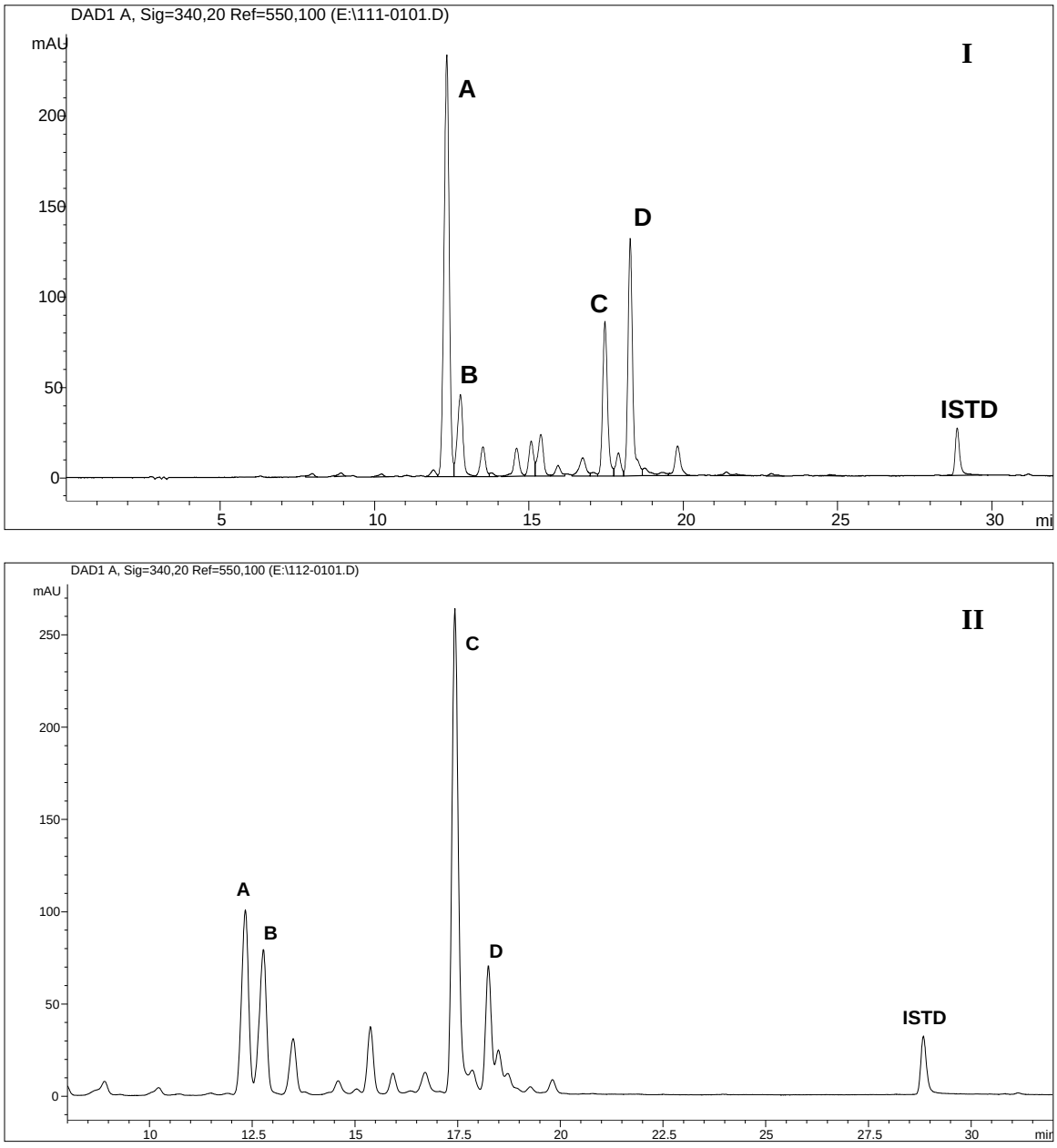

Figure 3.1. HPLC chromatograph of methanolic root extracts of endophyte-infected (I) and non-infected (II) tall fescue cv. Jesup. **A**, Chlorogenic acid; **B** and **C**, unidentified Caffeoylquinic isomers; **D**, 3,5-dicaffeoylquinic acid; **ISTD**, international standard chrysin.

Table 3.1. Polyphenol content in root tissue of a clonal line of *F. arundinacea* as determined by HPLC analysis.

Compound	*Endophyte Status*	*January*	*February*	*March*
Chlorogenic Acid	+	171.0	1092.5	517.7
	-	957.6	2205.0	342.8
3,5-Dicaffeoylquinic Acid	+	28.0	446.2	91.4
	-	509.7	503.6	54.8
Unidentified Compound B	+	61.0	405.3	90.5
	-	486.1	834.3	120.4
Caffeic Acid	+	2.9	12.0	5.0
	-	2.9	10.0	16.3

*Concentrations are in μg/g fresh root weight

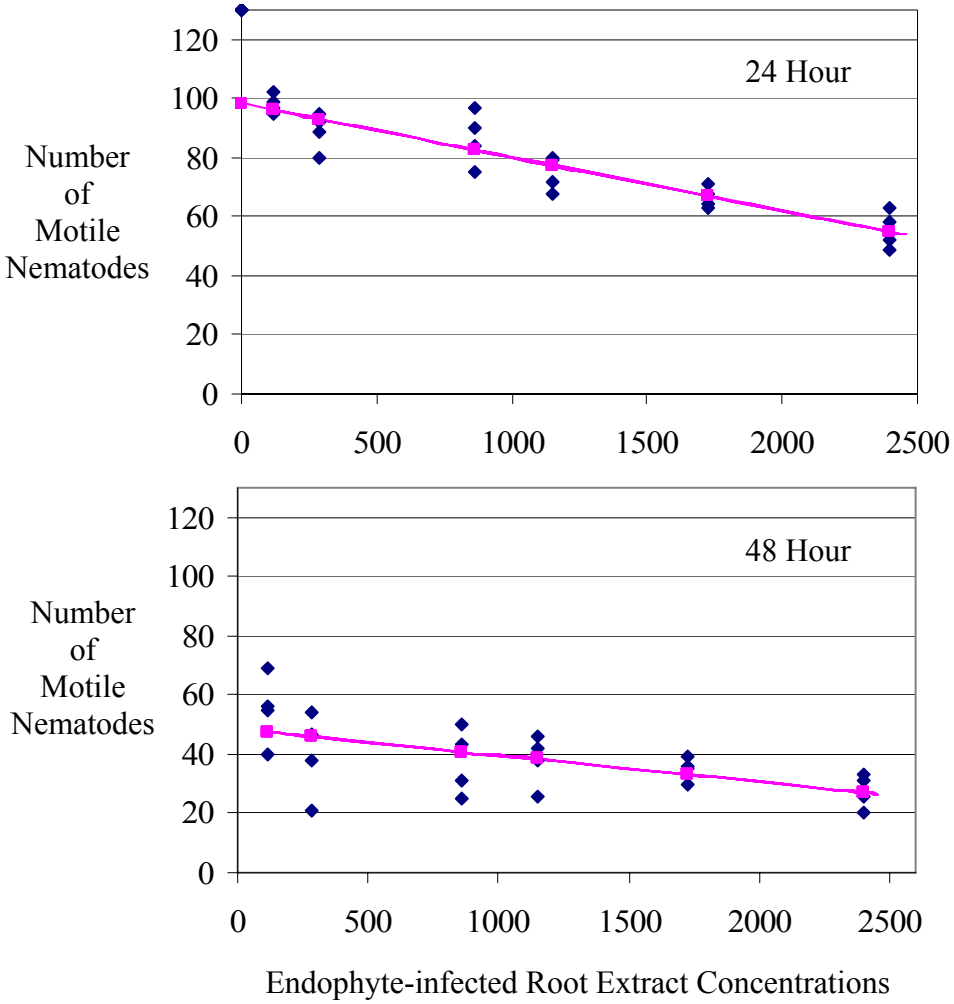

Figure 3.2. Bioassay of *N. coenophialum*-infected *F. arundinacea* cv. Jesup total root extract effects on *P. scribneri*. Nematode (approx. 140) motility observations recorded at 24 and 48 hour exposure periods, followed by fresh water replacements. Four independent experiments were performed and results presented as averages. When compared to water controls differences at each concentration are significant at $P < 0.05$ with a regression fit test.

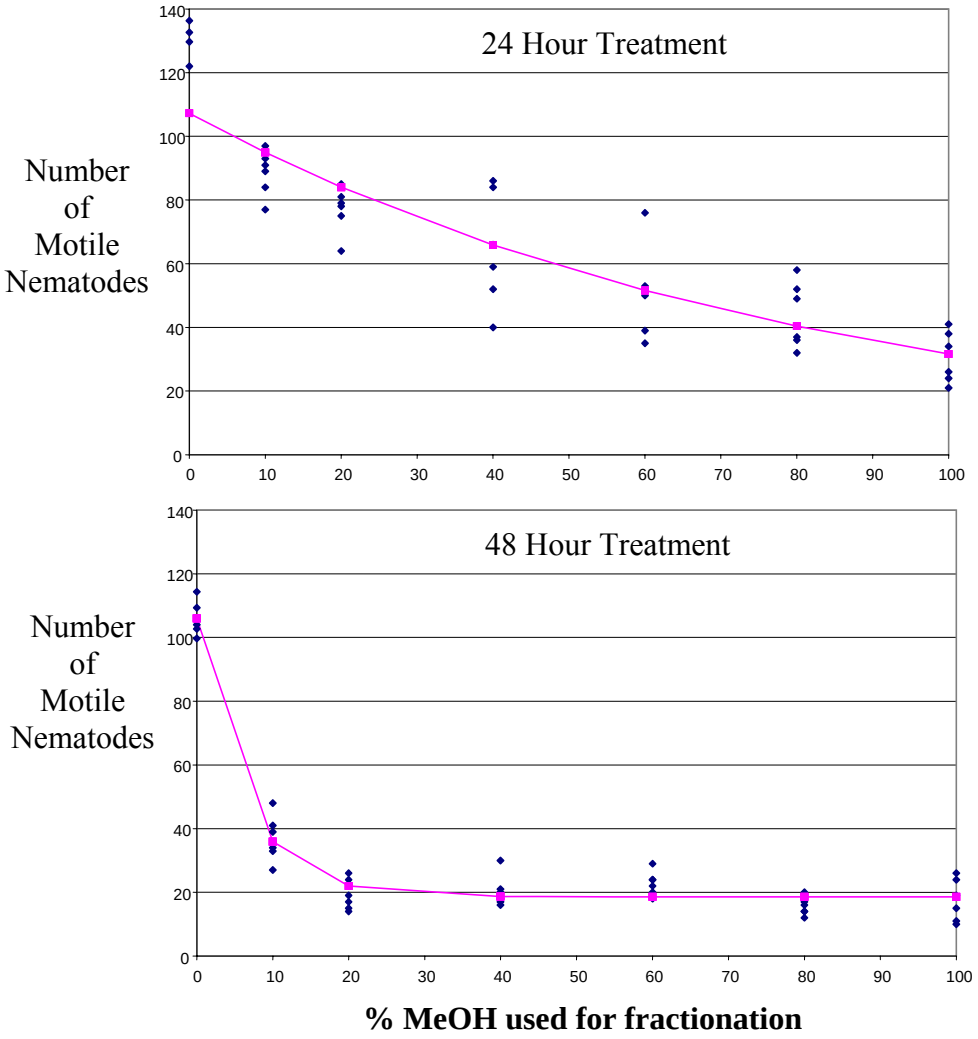

Figure 3.3. Bioassay of methanolic fractions of *N. coenophialum*-infected *F. arundinacea* cv. Jesup total root extracts effects on *P. scribneri*. The endophyte-infected total root extracts (1,150ppm) from the previous experiment were poured onto a C_{18} column and 0, 10, 20, 40, 60, 80, and 100% MeOH fractions were collected. MeOH was evaporated and sterile water added to adjust fractions to approx. 1,150 ppm. Nematode (approx. 140) motility observations recorded at 24 and 48 hour exposure periods. Four independent experiments were performed and differences between fractions are significant at $P < 0.05$ with a regression fit test.

Table 3.2. Motility bioassay of *Pratylenchus scribneri* following exposure to serial dilutions of ergot and loline alkaloids. Nematodes (approx. 30) were exposed to alkaloids for 24 to 72 hours, followed by fresh water replacement. Three independent experiments were performed with 4 replicates and results presented as averages. Using a regression fit test, when compared to controls differences between alkaloids were significant at $P < 0.05$.

	Nematode Motility							
Alkaloid	*-- 5* --*		*-- 50 --*		*-- 100 --*		*-- 250 --*	
Treatments	*24h*	*72h*	*24h*	*72h*	*24h*	*72h*	*24h*	*72h*
Control	29^{a}	29^{a}	29^{a}	29^{a}	29^{a}	29^{a}	29^{a}	29^{a}
Loline (NFL)	8.25^{c}	2.5^{d}	3.5^{d}	0.25^{d}	0^{d}	0^{d}	0^{d}	0^{c}
Ergonovine	5^{d}	4.75^{c}	4^{c}	2^{c}	2.5^{c}	1.25^{c}	1^{c}	0.25^{c}
Ergocornine	13.25^{b}	9^{b}	12.5^{b}	8.25^{b}	12.5^{b}	6.25^{b}	12.25^{b}	4^{b}
α-Ergocryptine	9.25^{c}	2.5^{d}	4^{c}	1.75^{c}	0^{d}	0^{d}	0^{d}	0^{c}
Ergovaline	1^{e}	0^{e}	0^{e}	0^{d}	0^{d}	0^{d}	0^{d}	0^{c}

*Concentration of alkaloid treatments are in μg/ml

Table 3.3. Motility bioassay of *Pratylenchus scribneri* following exposure to ergot and loline alkaloid combinations. Nematodes (approx. 30) were exposed to alkaloids for 24 to 72 hours, followed by fresh water replacement. Loline in this experiment is a mixture of N-acetylloline and N-formylloline. Three independent experiments were performed with four replicates and results presented as averages. Using a regression fit test, when compared to controls differences between alkaloid combinations were significant at $P < 0.05$.

	Nematode Motility							
Alkaloid	*-- 5* --*		*-- 50 --*		*-- 100 --*		*-- 250 --*	
Combinations	*24h*	*72h*	*24h*	*72h*	*24h*	*72h*	*24h*	*72h*
Control	21^{a}	21^{a}	21^{a}	21^{a}	21^{a}	21^{a}	21^{a}	21^{a}
Loline+Ergocornine	3.5^{c}	0.25^{c}	0.25^{c}	0^{c}	0.25^{b}	0^{b}	0^{b}	0^{b}
Loline + α-Ergocryptine	5.5^{b}	1.25^{b}	0^{c}	0.75^{b}	0^{c}	0^{b}	0^{b}	0^{b}
Ergocornine + α-Ergocryptine	6.5^{b}	0.25^{c}	4^{b}	0^{c}	0.5^{b}	0^{b}	0^{b}	0^{b}
Loline+Ergocornine + α-Ergocryptine	2.25^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{b}	0^{b}	0^{b}

*Concentration of alkaloid treatments are in μg/ml

Table 3.4. Populations of *P. scribneri* in *F. arundinacea* roots 8 weeks after inoculation. Nematodes (approx. 1,500) were inoculated into soil of a genetic clone of endophyte-infected and endophyte-free tall fescue cv. Jesup. Experiment was repeated with three replicates per experiment and results presented as averages. Differences between endophyte-status were significant at P = 0.05 with Duncan's multiple range test.

	Nematode Population Root Counts				
Endophyte Status	*Day 0*	*Day 30*	*Day 45*	*Day 60*	*Final R_f**
Endophyte-infected	1,500	93[a]	43[a]	1.2[a]	0
Endophyte-free	1,500	93[a]	276.67[b]	1734[b]	1.16

*R_r = final # of nematodes / # inoculated on Day 0

Table 3.5. Populations of *P. scribneri* in *F. arundinacea* soil 8 weeks after inoculation. Nematodes (approx. 1,500) were inoculated into soil of a genetic clone of endophyte-infected and endophyte-free tall fescue cv. Jesup. Experiment was repeated with three replicates per experiment and results presented as averages. Differences between endophyte-status were significant at P = 0.05 with Duncan's multiple range test.

	Nematode Population Soil Counts				
Endophyte Status	*Day 0*	*Day 30*	*Day 45*	*Day 60*	*Final R_f**
Endophyte-infected	1,500	66.67[a]	1,225[a]	6,791.56[a]	4.53
Endophyte-free	1,500	433.34[b]	350[b]	600[b]	0.4

*R_r = final # of nematodes / # inoculated on Day 0

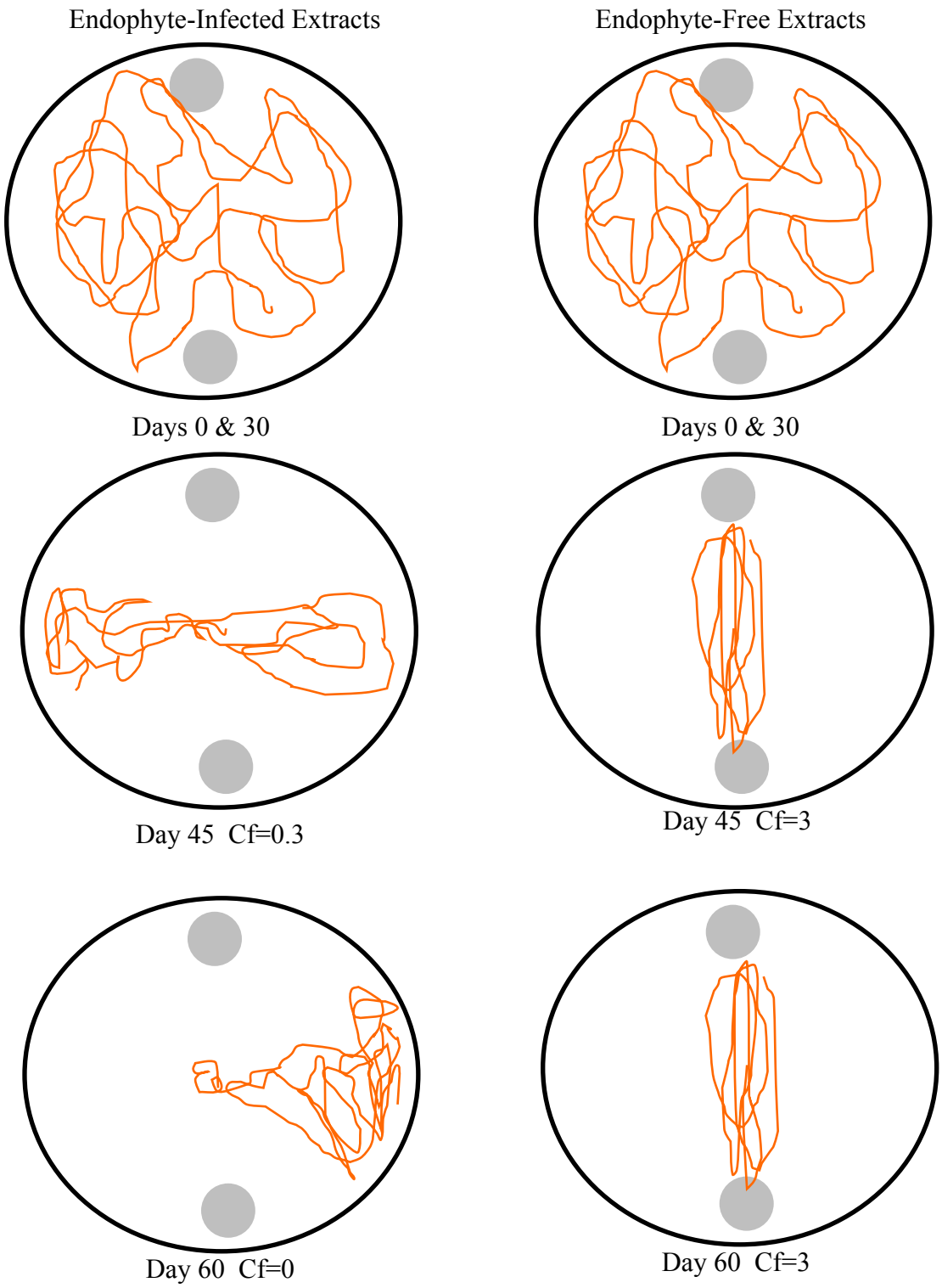

Figure 34. Diagram sketch of fescue extract effects on nematode behavior during chemotaxis study

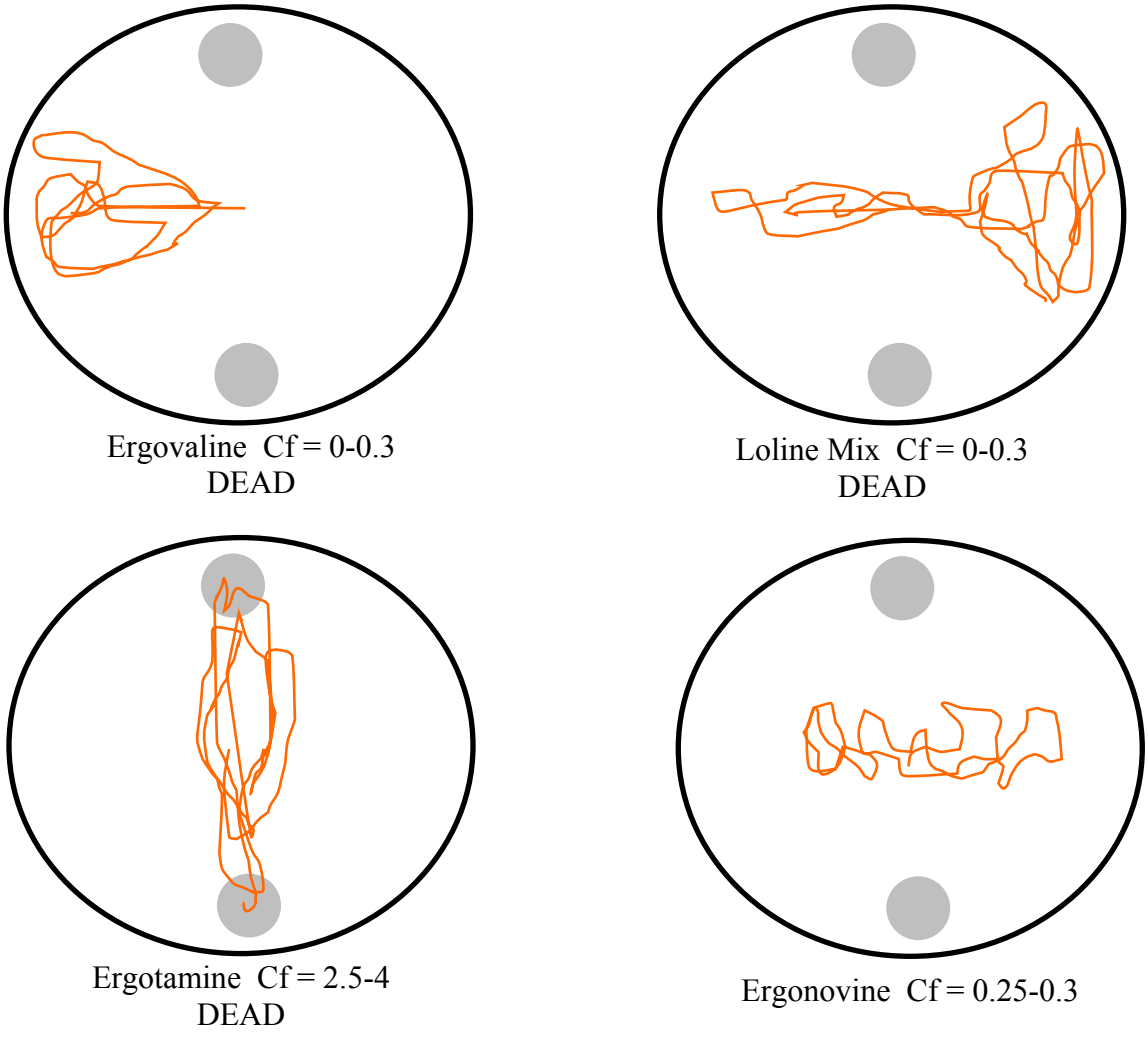

Figure 3.5a. Diagram sketch of alkaloid effects on nematode behavior during chemoreception study

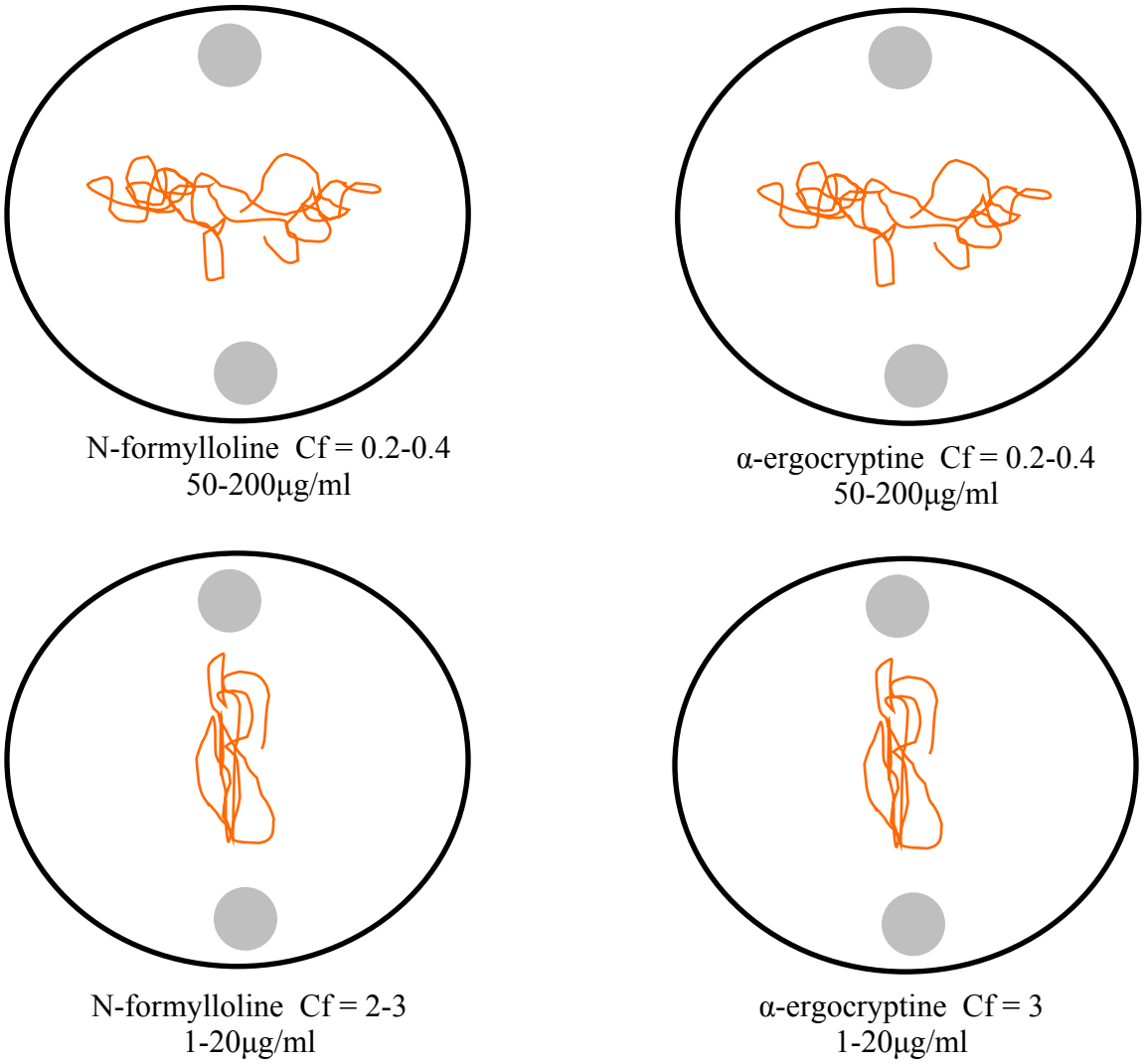

Figure 3.5b. Diagram sketch of alkaloid effects on nematode behavior during chemoreception study

CHAPTER 4

DISCUSSION AND CONCLUSION

Root-lesion nematodes (genus *Pratylenchus*) are economically important plant parasites worldwide with a wide host range of moncots and dicots. *Pratylenchus* spp. attack plant roots, tubers, and rhizomes (56) and are most destructive to plants in sandy soils. They have been reported as pathogens of corn, potato, alfalfa, strawberries, legumes, and turf grasses (23,26,47,109). Depending on species, host, and temperature, the life cycle can be completed in 5-8 weeks. Nematode eggs are laid inside roots and juveniles go through 4 molts in a single life cycle. All stages, except for the egg, are vermiform, motile, and infective to plant host roots. Lesion nematodes are migratory endoparasites that spend a greater percentage of their life within the roots, feeding intracellularly within the root cortex, however than can migrate in and out of the root and sometimes feed as ectoparasites.

Root damage is achieved when this nematode extends and retracts its stylet to break cells walls for entry and feeding, as well as its constant migration to new cells once the cytoplasmic contents of the previous cells are depleted. They enter by way of the regions of root hair development and the zone of elongation (125). Extensive endoparasitic feeding can result in changes in the root that include nuclear hypertrophy and eventual cell death. However, ectoparasitic feeding on root hairs has been reported in *Pratylenchus* spp. (124). Control of lesion nematodes is difficult to attain because they have a wide host range and they protected from nematicides while they are in the root. Therefore an effective means of control is the use of resistant plant varieties.

A major area of research interest is endophyte-infected tall fescue and its enhanced resistance to nematodes and insects. Pastures and meadows of *F. arundinacea* are commonly

associated with reduced populations of lesion nematodes (106). Other plant nematodes that are also negatively affected by the tall fescue symbiosis include *Helicotylenchus* spp. (spiral – semi-endoparasite), *Meloidogyne* spp. (root knot - endoparasite), *Belonolaimus* spp. (sting - ectoparasite), *Tylenchorynchus* spp. (stunt – semi -endoparasite), and *Paratrichodorus* spp. (stubby root - ectoparasite) (10,72,112). In these previous studies fewer nematodes were present in the soils of endophyte-infected tall fescue. The array of nematodes with varying feeding styles described previously lends to our hypothesis that reduced nematode populations in endophyte-infected tall fescue roots are possibly due to nematicidal compounds that may be encountered in the rhizosphere.

In this thesis, numerous experiments were conducted to elucidate the mechanism by which *N. coenophialum*-infected *Festuca arundinacea* reduces populations of *P. scribneri*. This was studied by testing various chemical compounds that are produced in the fungal-grass association in an *in vitro* bioassay system as well as pot studies. The *in vitro* assay system revealed that methanolic extracts and sub-fractions of *N. coenophialum*-infected tall fescue exhibited adverse effects on *P. scribneri* activity, while endophyte-free extracts showed negligible signs of affecting this nematode's activity. Here, a negative relationship exists between the polyphenols and nematode activity. As concentrations and exposure times to polyphenols contained in extracts increased nematode motility significantly decreased, even recovering motility to numbers near those of 24 hour treatments when subjected to a fresh water bath. Individual comparison studies of the polyphenols could not be performed because purified forms of each were not isolated from the extracts and only chlorogenic acid could be purchased from an outside retailer. Inhibition of motility was reversible in 20% of *P. scribneri* during *in vitro* studies involving chlorogenic acid, across all concentrations (10 – 1,000 ppm) (data not

shown). The induction of nematode paralysis is in agreement with previous studies of Wuyts et al., which documented several phenolics as nematistatic agents (117).

The roots of tall fescue grassesare noted producers of exudates that are speculated to be stimulatory, inhibitory, or inactive to organisms (20,59). Creek and Wade were the first to establish the excretion of phenolic compounds from the root of hydroponically grown tall fescue (20). These data showed that five of the phenolic compounds excreted were inhibitory to lettuce seeds including cinnamic, ferulic, gallic, gentistic and syringic acids. These analyses were performed without regards to the endophyte status of the plant and none of the identified compounds were tested against vertebrate and invertebrate pests. However, collectively these compounds are expected to have a wide range of activity to several organisms, nematodes included. Malinowski et al. provided the first evidence that the presence of the endophyte in grass provided for an enhanced amount of phenolic compounds in roots and suggested that may play a role in plants defense (59). Finally, root exudates form the basis for most allelopathic responses currently observed, which in the broad definition of allelopathy can be extended to account for inhibitory responses to nematodes, parasitic and free-living. Additionally, it was determined that under a deficiency of phosphorus, there was an even greater increase in total phenolic concentration (59).

We have shown that endophyte-infected total root extracts are comprised of allelochemicals that are potentially toxic to the lesion nematode, *P. scribneri*, since motility was disrupted. Hung and Rohde noted a relationship between phenols and nematode resistance in tomato (44). High phenolic content of some *Solanaceae* plants are positive indicators of resistance to *Heterodera rostochiensis* (29). Studies by Plowright et al. suggested that chlorogenic acid has a functional role in the resistance of rice to the stem nematode, *Ditylenchus*

angustus (75) and affects nematode coordination. Since our results indicate the extracts act as inhibitory (nematistatic) compounds, it is possible that the secondary metabolites involved act as chemical barriers to nematode penetration of endophyte-infected tall fescue roots. This is supported by statements from Wuyts et al. that polyphenolics were not toxic to *P. penetrans* juveniles, but did inhibit egg hatch presumably by its diffusion into the egg causing paralysis of the developing juvenile (117). Anti-nematodal activity has also been reported when studying extracts of members in the *Graminaea* family and its effect on *Bursaphelenchus xylophilus* (pine wood nematode) activity (58). *Graminaea* family members have been documented as producers of ergot and loline alkaloids (31).

Phenolic compounds have been associated with resistance in plants against nematode attack. Increases in the content of phenolic compounds in plants challenged by plant-parasitic nematodes have led researchers to draw conclusions of their effects on the soil dwelling pest. Possible mechanisms of anti-nematode effects exhibited by polyphenols include toxicity, antifeedant properties, egg hatch inhibition, motility inhibition, blocking the ability to recognize potential host plants, and repulsion in the presence of a susceptible host (123). In 1998, Baldridge et al. detected higher constitutive levels of polyphenols, caffeic and chlorogenic acid in particular, in alfalfa plants resistant to *P. penetrans* (8). Suggesting that caffeic and chlorogenic acids are possible compounds of interest in the control of plant-parasitic nematodes. The results from our study is in line with both studies in that *P. scribneri* motility was inhibited by chlorogenic acid and the constitutive levels of chlorogenic acids were higher in roots of plants that displayed resistance to lesion nematode populations.

Polyphenols are just a piece of the nematode resistance puzzle. Previous studies indicate that there is no single mechanism of chemical defense for nematode resistance. When one

begins to speak of endophyte-infected tall fescue grasses that are resistant or tolerant to vertebrate and invertebrate pests the topic of ergot and its alkaloids must be introduced. *Claviceps purpurea* is a seed replacement disease that produces a dried overwintering sclerotial structure known as an ergot. Rye, oats, cool-season grasses, and wheat are noted hosts of ergot producing fungi. Ergots have been documented in human lives since the Middle Ages. Numerous epidemics have been linked to ergot poisoning, ergotism, including St. Anthony's fire, which is descriptive of sufferers experiencing a burning sensation in their limbs. The black plague and ergotism have been linked because it was believed that continuous human consumption of ergot-infected wheat and rye weakened their immune systems. The gangrenous results are from the vasoconstrictive action that occurs, which is accompanied by reduced blood flow to the hands and feet (114). Currently ergots and their alkaloids have been exploited for their medicinal and biocontrol properties.

Based on their chemical diversity the toxins in sclerotia are being used as antagonists and/or agonists of numerous neurotransmitter receptors to aid in the relief of migraines, sleep aids, and in the treatment of diabetes and Parkinson's disease (114). The defensive properties the ergots deliver to plants infected with the ergot alkaloids is a target of many research proposals. In mammals, the ergot alkaloids mechanism of action is to provoke or inhibit biological responses by binding to a receptor to block/induce a natural response. It is this project's goal to utilize ergot alkaloids and its effects on neurotransmitters to aid in the control plant-parasitic nematodes.

The results from our motility bioassay designed to determine affects from individual as well as combinations of alkaloids are in agreement with Mackeen et al. (58) . At concentrations above 50μg/ml nematotoxicity is associated with individual ergopeptide, lysergic acid amide,

and pyrrolizidine (NFL) alkaloids. However, from the data presented here there appears to be a potentiating nematicidal effect on *P. scribneri* when they are exposed to a combination of ergopeptides (ergocornine and α-ergocryptine) and a loline mixture (NFL and NAL) at low concentrations of 10µg/ml. Since these alkaloids do occur in endophyte-infected tall fescue as mixtures and, albeit at concentrations that are lower in the roots than in the canopy (94), it is realistic to conclude that the phenomenon exhibited in this study is not a laboratory induced artifact, but is actually a common occurrence in the environment whereby plant-parasitic nematodes may be strongly affected in one or more stages of their life cycle. Where we were unsuccessful in identifying ergot and/or loline alkaloids in the roots of endophyte-infected tall fescue, other researchers have documented their findings. Bush et al. reported root concentrations of NFL in tall fescue from 274-290µg/g root (15), which are in range with the concentrations tested in this study. Panaccione et al. found ergot alkaloids at concentrations ranging from 0.04-0.06µg/g (69) and these plants did exhibit nematode resistance. Malinowski et al. noted that there is variability in ergot alkaloid production and the variability may be related to altered root morphology and changes in the rhizosphere of endophyte-infected tall fescue (59). Through individual screenings, we identified that above 50µg/ml NFL and α-ergocryptine are the major alkaloids that act as a *P. scribneri* nematicide. However, when tested as a combination they are effective nematicides at 50µg/ml. Singly, ergocornine did not affect nematode mortality, as compared to the other alkaloids. Interestingly, when tested as a potential nematicide in combination with loline and α-ergocryptine, our bioassay demonstrated that all three collectively, at 5µg/ml, have negatively affected nematode mortality by 100%.

Next, we examined the effect of endophyte infection of tall fescue on nematode infestation in order to examine if the adverse effects revealed in the *in vitro* bioassays were

reproducible *in planta*. Endophyte-infected roots and soil were assayed for nematode populations at 30, 45, and 60 days post inoculation and compared to samples taken from nematode inoculated endophyte-free fescue plants. Tall fescue infected with the endophyte was essentially free of infection by *P. scribneri*. This outcome is in agreement with the findings of Timper et al. (108), Gwinn and Bernard (32), and Kimmons et al. (47), where there were lower populations of *P. scribneri* in endophyte-infected fescue roots (32,47,108). However, Kimmons et al. (47) also stated that nematode populations on endophyte-free plants were at or below inoculum levels, which is opposite of the findings of this study where we found nematode populations on endophyte-free plants actually increased 2-fold.

Soil populations of *P. scribneri* are apparently affected by fescue's endophyte status (47). At the conclusion of our study there was a nearly 60% reduction of nematode populations found in soil of endophyte-free plants, while there was a 250-400% increase of nematode populations in soils of endophyte-infected plants. This differs from evidence presented by Timper et al. (108) that found few to no *P. scribneri* in soils from both endophyte-infected and non-infected plants. A plausible explanation for the differences between these two studies could be: 1) Timper et al. (108) used mixed populations of tall fescue grown up from seed, while we used a clone from a single tall fescue ramet that possessed a different chemical profile, or 2) we used a *P. scribneri* isolate whose viability lended itself to increased reproduction levels in soils of endophyte-infected plants.

Our work demonstrates that *N. coenophialum* – infected tall fescue produces nematotoxic compounds (alkaloids and polyphenolics) that are potentiating and/or synergistic *in vitro*. While individually exhibiting adverse effects against nematode motility, these allelochemicals working in unison have additive effects that have acute toxicity to *P. scribneri*. Nevertheless, the modes

of action by which these compounds are active against nematodes in general are unknown. In order to make determinations of what is affected internally in plant-parasitic nematodes one must undoubtedly make close comparisons to the free-living nematode, *Caenorhabditis elegans*. The *C. elegans* genome has been completely sequenced and has revealed 97Mb of sequencing that encodes for nearly 19,099 genes that are highly homologous to other nematode species and humans (62). In particular, BLAST searches have revealed that the nervous system of *C. elegans*, more importantly their neurotransmitters, are highly conserved across free-living and plant-parasitic nematode species (62).

The neurotransmitters dopamine, serotonin, and acetylcholine have been detected in *C. elegans* and play a significant role in nematode behavior. Dopamine has also been detected in *Meloidogyne incognita* and cyst nematodes (*Heterodera* sp. and *Globodera* sp.) (93,100). *C. elegans* exposure to exogenous dopamine causes numerous negative effects including paralysis, as well as inhibition of egg laying and defecation (17,87,111). Acetylcholine is involved in locomotion, controlling their wave initiation during movement, egg laying, pharyngeal pumping, and in male mating behavior (27,35). Serotonin is also involved in various nematode behaviors such as locomotion, egg laying, and pharyngeal pumping (54,87,111). Treatment of *C. elegans* to exogenous serotonin caused paralysis, stimulation of pharyngeal pumping in the absence of food, and stimulation of premature egg laying (39,92,111). When exposed to the drug Methiothepin, an antagonist to the serotonin receptor, there appears to be an increased hypersensitivity to the nematicide aldicarb, inducing paralysis (65).

The use of nematicides is a common practice in the treatment of plants under plant-parasitic nematode attack. Nematicides are divided into the categories of fumigants (chloropicrin and methyl-bromide) and non-fumigants (for example, Temik and Vydate). The

mode of action involved in the efficacy of fumigants as a nematicide is not well understood. However, it is known that ingestion of the chemical is not necessary and direct penetration of the nematode cuticle is needed to interfere with nematode respiration that leads to death (116). Non-fumigants are organophosphate and carbamate compounds, which also penetrate the cuticle, but go further by interfering with the nematodes nervous system and act as acetylcholinesterase inhibitors (99,116). Opperman and Chang stated that when acetylcholinesterase is inhibited there is a build up of acetylcholine that causes hyperactivity followed by relaxation of the muscle receptors, which leads to paralysis (66).

Since this project did not focus on the mechanisms of action the alkaloids and polyphenolics play on the nematodes internal systems, some conclusions on mode of action can be based on the chemicals effect of known nematicides and relate this to molecular structure similarities of the toxin such as the ergopeptide alkaloids,. The ergoline ring system of ergot alkaloids are structural homologues to the neurotransmitters epinephrine, norepinephrine, dopamine, and serotonin. The structural similarities may explain why some ergot alkaloids act as agonists or antagonists on neurotransmitter receptor sites (31). When studying the structures alone, the biogenic amines dopamine and serotonin stand out as most similar to the ergots ergoline ring. In livestock, ergot alkaloids mimic dopamine and exert an agonistic interaction with the D2 dopamine receptor to inhibit acetylcholine and dopamine release, depress chemosensory activity, inhibit norepinephrine release, and reduce prolactin secretions (21). N-formyllolline and N-acetylloline did not express any binding affinity to the D2 dopamine receptor (51). However, there are studies that have demonstrated that lolines do bind to acetylcholine and/or serotonin receptors (60). Thus, the mode of action may be related to receptor sites in nematodes, which are expected to vary according to nematode species. If this is

correct, this might account for species specificity observed for the tall fescue and nematode reaction.

Endophytes protect plants from biotic and abiotic stresses, and little information about how they suppress nematode populations are documented. The data from this project along with knowledge of the structure-activity relationships of the toxins tested leads us to conclude that there is a nematicidal effect from secondary metabolites of fungal and plant origin. The polyphenolic compounds in the root extracts and the alkaloids (ergonovine, ergocornine, and NFL) had a reversible effect on the lesion nematode similar to that of the carbamate type of non-fumigant nematicides. This nematicidal comparison became evident during *in vitro* bioassays where total root extracts and the alkaloids were tested individually against *P. scribneri*. During the chemical treatment nematodes were paralyzed and following fresh water replacement a percentage of subjects in both studies regained motility. This reversible narcotic effect is a key characteristic of the carbamate non-fumigant nematicides. Earlier studies reported that lower concentrations of the carbamate carbofuran did impair orientation and locomotion of *Pratylenchus* species. However, the more important findings were that root attraction was inhibited and those that did eventually reach host roots did not feed (116). The non-fumigant nature of polyphenolics produced in endophyte-infected tall fescue, as well as the alkaloids (NFL, ergocornine, and ergonovine) should not be overlooked as potential nematicides because their effects could aid in the decrease of nematode populations. They can prevent invasion and feeding of nematodes by interfering with their nervous and sensory systems. Nematodes subjected to these compounds will have to use up their food and energy stores inefficiently to migrate toward roots, but can not complete the foraging and feeding aspects of their life cycle, which leads to their eventual demise. The end result is still a reduced nematode population.

Unlike the carbamates, the organophosphate non-fumigant nematicides exert a mode of action on nematodes that leads to the irreversible binding of acetylcholinesterase. This feature is important because it allows this class of chemical to still remain non-phytotoxic and effective at low doses just as carbamates, while effectively decreasing nematode populations. The ergot (α-ergocryptine and ergovaline) and loline alkaloids modes of action were similar to those displayed by the organophosphate nematicides. There was an initial paralysis suggesting that there was interference with the nematodes nervous system, followed by a rapid death. Fresh water replacement did not give rise to regained motility; the effect was toxic.

The secondary metabolites occur in endophyte-infected tall fescue as mixtures and not individually, tests of alkaloid combinations revealed that their interactions have a potentiating and/or synergistic effect on this lesion nematode species. When paired with α-ergocryptine or loline, ergocornine began to affect nematode mortality at 50µg/ml. Whereas individually, ergocornine treatments at high concentrations could not cause dramatic decreases in nematode motility. The nematicidal effects of loline or α-ergocryptine were neither enhanced nor inhibited with the addition of ergocornine. Testing all three (loline, ergocornine, and α-ergocryptine) alkaloids collectively lends to the theory that their interactions are potentiating because a nematicidal effect was attained at the lowest concentration of 5µg/ml.

We must consider and factor into the toxicity dialogue the information presented by Malinowski et al. that there are chemical changes on the root surface of tall fescue in response to endophyte infection (59). These changes are expressed as altered membrane changes and translocation processes reflective of increased releases of phenolics and reducing activity (59). We must also account for the nematode feeding behavior that must increase the downward flow of metabolites to the roots where an increased accumulation at feeding sites is expected to occur.

This suggests that nematode infected or antagonized planting of tall fescue could have higher levels of ergot alkaloids and other compounds than non-nematode parasitized endophyte-infected plants.

The ecological benefits of the secondary metabolites tested here are uncertain but they should assist in extending the role of grass endophytes, while offering some probable mechanisms of action against plant-parasitic nematodes. These mechanisms are suggested from the chemical evidence provided and from the structure-activity associations detailed here and in references cited. The larger questions of ecological benefits derived from the association are complex and probably will remain complex. The complexity is confounded by the intended use of symbiotic grasses, which as suggested here are varied mixtures of ecotypes, some of which may relate to the data examined and discussed here. These natural symbiota have all the merits of allelopathic agents, complete with additional benefits, although some are problematic, especially to grazing livestock. The unknown fractions, polyphenolic and alkaloid compounds tested in this study should be screened further for their efficacy as nematicides against other nematodes with differing feeding styles to truly assess the spectrum of activity. Of practical and ecological concerns are the effects on the beneficial free-living nematodes and nematophagus fungi in soils, and not only soils planted with symbiotic tall fescue but with soil exposed to ground water runoff from fescue plantings. Further studies of these associations, details of their bioactive chemicals and targets of action will clarify their overall ecological role and any importance to and among forage and range grass situations.

LITERATURE CITED

1. Adcock, R. A., N. S. Hill, J. H. Bouton, H. R. Boerma, and G. O. Ware. 1997. Symbiont Regulation and Reducing Ergot Alkaloid Concentration by Breeding Endophyte-Infected Tall Fescue. J. Chem. Ecol. 23:691-704.

2. Agee, C. S. and N. S. Hill. 1992. Variability in progeny from high- and low-ergovaline producing tall fescue parents, p. 82. *In*: Agronomy Abstracts. American Society of Agronomy, Madison, WI.

3. Agee, C. S. and N. S. Hill. 1994. Ergovaline variability in *Acremonium*-infected tall fescue due to environment and plant genotype. Crop Sci. 34:221-226.

4. Arachevaleta, M., C. W. Bacon, C. S. Hoveland, and D. E. Radcliffe. 1989. Effect of the tall fescue endophyte on plant response to environmental stress. Agron. J. 81:83-90.

5. Azevedo, M. D. and R. E. Welty. 1995. A study of the fungal endophyte *Acremonium coenophialum* in the roots of tall fescue seedlings. Mycologia 87:289-297.

6. Bacon, C. W., J. K. Porter, J. D. Robbins, and E. S. Luttrell. 1977. *Epichloe typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34:576-581.

7. Bacon, C. W. and M. R. Siegel. 1988. The endophyte of tall fescue. J. Prod. Agric. 1:45-55.

8. Baldridge, G. D., N. R. O'Neill, and D. A. Samac. 1998. Alfalfa (*Medicago sativa* L.) resistance to the root-lesion nematode, Pratylenchus penetrans: defense-response gene mRNA and isoflavonoid phytoalexin levels in roots. Plant Mol. Biol. 38:999-1010.

9. Belesky, D. P., J. A. Stuedemann, R. D. Plattner, and S. R. Wilkinson. 1988. Ergopeptine alkaloids in grazed tall fescue. Agron. J. 80:209-212.

10. Bernard, E. C., K. D. Gwinn, and G. D. Griffin. 1998. Forage Grasses, p. 427-454. *In*: K. R. Barker, G. A. Pederson, and G. L. Windham (eds.), Plant and Nematode Interactions. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison.

11. Boling, J. A., L. D. Bunting, G. M. Davenport, J. L. Van der Veen, K. M. Meekins, N. W. Bradley, and R. E. Kohls. 1989. Physiological responses of cattle consuming tall fescue to environmetal temperature and supplemental phenothiazine. J. Anim. Sci. 67:2377-2385.

12. Bouton, J. H. 2000. The use of endophytic fungi for pasture improvement in the USA, p. 163-168. *In*: The Grassland Conference 2000. Proc. 4th Int. Neotyphodium/Grass Interactions Symposium. Soest, Germany.

13. Bultman, T. L., K. L. Borowicz, R. M. Schneble, T. A. COudron, and L. P. Bush. 1997. Effect of a Fungal Endophyte on the Growth and Survival of Two Euplectrus Parasitoids. Oikos 78:170-176.

14. Burpee, L. L. and J. H. Bouton. 1993. Effect of eradication of the endophyte *Acremonium coenophialum* on epidemics of Rhizoctonia blight in Tall Fescue. Plant Dis. 77:157-159.

15. Bush, L. P., F. F. Fannin, M. R. Siegel, D. L. Dahlman, and H. R. Burton. 1993. Chemistry, Occurrence and Biological Effects of Saturated Pyrrolizidine Alkaloids Associated with Endophyte-grass Interactions. Agriculture, Ecosystems and Environment 44:81-102.

16. Bush, L. P., H. H. Wilkinson, and C. L. Schardl. 1997. Bioprotective alkaloids of grass-fyngal endophyte symbioses. Plant Physiol. 114:1-7.

17. Chase, D. L., J. S. Pepper, and M. R. Koelle. 2004. Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. Nat. Neurosci. 7:1096-1103.

18. Cheplick, G. P. and K. Clay. 1988. Acquired chemical defenses in grasses: The role of endophytes. Oikos 52:309-319.

19. Clay, K. 1988. Fungal Endophytes of Grasses: A Defensive Mutualism between Plants and Fungi. Ecology 69:10-16.

20. Creek, R. and G. L. Wade. 1985. Excretion of phenolic compounds from the roots of *Festuca arundinacea*, *Eragrostis carvula*, and *Lespedeza striata*. Trans.Kentucky Acad. Sci. 46:51-55.

21. Cross, D. L. 1997. Fescue Toxicosis in Horses, p. 289-309. *In*: C. W. Bacon and N. S. Hill (eds.), Neotyphodium/Grass Interactions. Plenum Press, New York.

22. De Battista, J. P., J. H. Bouton, C. W. Bacon, and M. R. Siegel. 1990. Rhizome and herbage production of endophyte-removed tall fescue clones and populations. Agron. J. 82:651-654.

23. Dickerson, O. J., H. M. Darling, and G. D. Griffin. 1964. Pathogenicity and population trends of *Pratylenchus penetrans* on potato and corn. Phytopath. 54:317-322.

24. Diehl, W. W. 1950. *Balansia* and the *Balansiae* in America, p. 1-82. *In*: USDA Agriculture Monograph. U.S. Government Print Office, Washington, DC.

25. Dyer, D. C. 1993. Evidence that ergovaline acts on sarotonin receptors. Life Science 53:223-228.

26. Elliott, A. P. and G. W. Bird. 1985. Pathogenicity of *Pratylenchus penetrans* to navy bean (*Phaseolus vulgaris L.*). Nematology 17:81-85.

27. Garcia, L. R., P. Mehta, and P. W. Sternberg. 2001. Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating. Cell 107:777-788.

28. Ghorbani, G. R., J. A. Jackson, and R. W. Hemken. 1989. Performance of Holstein calves fed varying additions of endophyte-infected tall fescue. Nut. Rep. Intern. 40:931-938.

29. Giebel, J. 1970. Phenolic content in roots of some Solanaceae and its influence on IAA-oxidase activity as an indicator of resistance to Heterodera rostochiensis. Nematologica 16:22-32.

30. Glenn, A. E., C. W. Bacon, R. Price, and R. T. Hanlin. 1996. Molecular Phylogeny of *Acremonium* and its Taxonomic Implications. Mycologia 88:369-383.

31. Groger, D. and H. G. Floss. 1998. Biochemistry of Ergot Alkaloids, p. 171-218. *In*: G. A. Cordell (ed.), The Alkaloids: Chemistry and Biology. Academic Press.

32. Gwinn, K. D. and Bernard, E. C. Interactions of endophyte-infected grasses with the nematodes Meloidogyne marylandi and Pratylenchus scribneri. Hume, D. E. and Latch, G. C. M. 156-160. 1993. Palmerston North, New Zealand, AgResearch. Proceedings of the 2nd international symposium on Acremonium/grass interactions.

33. Gwinn, K. D., E. C. Bernard, and C. D. Pless. 1992. Pathogen and pest resistance in endophyte-infected tall fescue, p. 139-145. *In*: P. M. Gresshoff (ed.), Plant Biotechnology and Development. CRC Press, Boca Raton, FL.

34. Halisky, P. M. and R. F. Myers. 1989. Effect of *Acremonium* endophytes on parasitic nematodes in *Festuca* and *Lolium* grown under turf maintenance, p. 124-131. *In*: Rutgers Turfgrass Proceedings. Rutgers University, New Brunswick, NJ.

35. Hallam, S., E. Singer, D. Waring, and Y. S. Yin. 2000. The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification. Development 127:4239-4252.

36. Hardy, T. N., K. Clay, and A. M. Hammond. 1986. Leaf age and related factors affecting endophyte mediated resistance to fall armyworm (*Lepidoptera: Noctuidae*) in tall fescue. Environ. Entomol. 15:1083-1089.

37. Hill, N. S., F. N. Thompson, J. A. Stuedemann, G. E. Rottinghaus, H. J. Ju, D. L. Dawe, and E. E. Hiatt, III. 2001. Ergot alkaloid transport across ruminant gastric tissues. J. Anim. Sci. 79:542-549.

38. Hinton, D. M. and C. W. Bacon. 1985. The distribution and ultrastructure of the endophyte of toxic tall fescue. Can. J. Bot. 63:36-42.

39. Horvitz, H. R., M. Chalfie, C. Trent, J. E. Sulston, and P. D. Evans. 1982. Serotonin and octopamine in the nematode *Caenorhabditis elegans*. Science 216:1012-1014.

40. Hoveland, C. S. 1993. Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plants. Agric. Eco. Environ. 44:3-12.

41. Hoveland, C. S., R. Rodriguez-Kabana, and C. D. Berry. 1975. Phalaris and Tall fescue forage production as affected by nematodes in the filed. Agron. J. 67:714-717.

42. Hoveland, C. S., S. P. Schmidt, C. C. King, and E. M. Clark. 1983. Summer Syndrome on Tall Fescue, p. 5-10. *In*: Proc. Tall Fescue Toxicosis Workshop Atlanta, GA. University of Georgia Extension, Athens, GA.

43. Hoveland, C. S., S. P. Schmidt, C. C. King, Jr., J. W. Odom, E. M. Clark, J. A. McGuire, L. A. Smith, H. W. Grimes, and J. L. Holliman. 1980. Association of *Epichloe typhina* fungus and steer performance on tall fescue pasture. Agron. J. 72:1064-1065.

44. Hung, C. and R. A. Rohde. 1973. Phenol accumulation related to resistance in tomato infection by root-knot and lesion nematodes. Nematology 5:253-258.

45. Jenkins, W. R. 1964. A Rapid Centrifugational Flotation Technique for Separating Nematodes from Soil. Plant Disease Reporter 48:692.

46. Johnson, M. C., D. L. Dahlman, M. R. Siegel, L. P. Bush, G. C. M. Latch, D. A. Potter, and D. R. Varney. 1985. Insect feeding deterents in endophyte-infected tall fescue. Appl. Environ. Microbiol. 49:568-571.

47. Kimmons, C. A., K. D. Gwinn, and E. C. Bernard. 1990. Nematode reproduction on endophyte-infected and endophyte-free tall fescue. Plant Dis. 74:757-761.

48. Kirfman, G. W., R. L. Brandenburg, and G. B. Garner. 1986. Relationship between insect abundance and endophyte infestation level in tall fescue in Missouri. J. Kansas Entomol. Soc. 59:552-554.

49. Koulman, A., G. A. Lane, M. J. Christensen, K. Fraser, and B. A. Tapper. 2007. Peramine and other Fungal Alkaloids are Exuded in the Guttation Fluid of Endophyte-infected Grasses. Phytochemistry 68:355-360.

50. Lane, G. A., M. J. Christensen, and C. O. Miles. 2000. Coevolution of Fungal Endophytes with Grasses: The Significance of Secondary Metabolites, p. 341-387. *In*: C. W. Bacon and J. F. White, Jr. (eds.), Microbial Endophytes.

51. Larson, B. T., D. L. Harmon, E. L. Piper, L. M. Griffis, and L. P. Bush. 1999. Alkaloid binding and activation of D2 dopamine receptors in cell culture. J. Anim. Sci. 77:942-947.

52. Larson, B. T., M. D. Samford, J. M. Camden, E. L. Piper, M. S. Kerley, J. A. Paterson, and J. T. Turner. 1995. Ergovaline binding and activation of D_2 dopamine receptors in GH4ZR7 cells. J. Anim. Sci. 73:1396-1400.

53. Latch, G. C. M., M. J. Christensen, and D. L. Gaynor. 1985. Aphid detection of endophyte infection in tall fescue. N. Z. J. Agric. Res. 28:129-132.

54. Loer, C. M. and C. Kenyon. 1993. Serotonin-deficient mutants and male mating behavior in the nematode *Caenorhabditis elegans*. Neurosci. 13:5407-5417.

55. Long, E. A. and J. W. Hilty. 1985. Occurrence of *Acremonium coenophialum* in Tall fescue in Tennessee. Plant Dis. 69:467-468.

56. Loof, P. A. A. 1991. The Famile *Pratylenchidae* Thorne, 1949, p. 363-422. *In*: W. R. Nickle (ed.), Manual of Agricultural Nematology. Marcel Dekker, Inc, New York.

57. Lyons, P. C., R. D. Plattner, and C. W. Bacon. 1986. Occurence of peptide and clavine ergot alkaloids in tall fescue. Science 232:487-489.

58. Mackeen, M. M., A. M. Ali, M. A. Abdullah, R. M. Nasir, N. B. Mat, A. R. Razak, and K. Kawazu. 1997. Antinematodal activity of some malaysian plant extracts against the pine wood nematode, Bursaphelenchus xylophilus. Pest. Sci. 51:165-170.

59. Malinowski, D. P., G. A. Alloush, and D. P. Belesky. 1998. Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte *Neotyphodium coenophialum*. Plant Soil 205:1-12.

60. Mattocks, A. R. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. Academic Press, New York.

61. McGlohon, N. E., J. N. Sasser, and R. T. Sherwood. 1961. Investigations of plant-parasitic nematodes associated with forage crops in North Carolina. North Carolina Agricultural Experiment station Technical Bulletin 148:1-39.

62. McVeigh, P., S. Leech, G. R. Mair, N. J. Marks, T. G. Geary, and A. G. Maule. 2005. Analysis of FMRFamide-like peptide (FLP) diversity in phylum Nematode. Int. J. Para. 35:1043-1060.

63. Meyer, S. L. F., R. N. Huettel, and R. M. Sayre. 1990. Isolation of fungi from *Heterodera glycines* and *in vitro* bioassays for their antagonism to eggs. Nematology 22:532-537.

64. Moubarak, A. S., E. L. Piper, C. P. West, and Z. B. Johnson. 1993. Interaction of purified ergovaline from endophyte-infected tall fescue with synaptosomal ATPase enzyme system. J. Agric. Food Chem. 41:407-409.

65. Nurrish, S., L. Segalat, and J. M. Kaplan. 1999. Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron. 24:231-242.

66. Opperman, C. H. and S. Chang. 1992. Nematode acetylcholinesterases: Molecular forms and their potential role in nematode behavior. Parasitology Today 8:406-411.

67. Panaccione, D. G. and C. M. Coyle. 2005. Abundant Respirable Ergot Alkaloids from the Common Airborne Fungus *Aspergillus fumigatus*. Appl. Environ. Microbiol. 71:3106-3111.

68. Panaccione, D. G., R. D. Johnson, J. H. Wang, C. A. Young, P. Damrongkool, B. Scott, and C. L. Schardl. 2001. Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte, p. 12820-12825. *In*: Proc. Nat. Acad. Sci. USA.

69. Panaccione, D. G., J. B. Kotcon, C. L. Schardl, R. D. Johnson, and J. B. Morton. 2006. Ergot alkaloids are not essential for endophytic fungus-associated population suppression of the lesion nematode, *Pratylenchus scribneri*, on perennial ryegrass. Nematology 8:583-590.

70. Patterson, D. J., D. A. Potter, and F. F. Fannin. 1991. Feeding deterrency of alkaloids of endophyte-infected grasses to Japanese beetle grubs. Entomologia Experimentalis et Applicata 61:285-289.

71. Pedersen, J. F. and R. Rodriguez-Kabana. 1984. Differences among nematode populations in tall fescue pastures in north, central, and south Alabama. Crop Sci. 24:819-821.

72. Pedersen, J. F., R. Rodriguez-Kabana, and R. A. Shelby. 1988. Ryegrass cultivars and endophyte in tall fescue affect nematodes in grass and succeeding Soybean. Agron. J. 80:811-814.

73. Peters, C. W., K. N. Grigsby, C. G. Aldrich, J. A. Paterson, R. J. Lipsey, M. S. Kerley, and G. B. Garner. 1992. Performance, forage utilization, and ergovaline consumption by beef cows grazing endophyte fungus-infected tall fescue, endophyte fungus-free tall fescue, or orchardgrass pastures. J. Anim. Sci. 70:1550-1561.

74. Petroski, R. J., D. L. Dornbos, and R. G. Powell. 1990. Germination and Growth Inhibition of Annual Ryegrass (Lolium multiflorum) and Alfalfa (Medicago sativa L.) by Loline Alkaloids and Synthetic N-Acylloline Derivatives. J. Agric. Food Chem. 38:1716-1718.

75. Plowright, R. A., R. J. Grayer, J. R. Gill, M. L. Rahman, and J. B. Harborne. 1996. The induction of phenolic compounds in rice after infection by the stem nematode Ditylenchus angustus. Nematologica 42:564-578.

76. Porter, J. K. 1994. Chemical constituents of grass endophytes, p. 103-123. *In*: J. K. Porter (ed.), Biotechnology of Endophytic fungi of grasses. CRC Press, Boca Raton, FL.

77. Powell, R. G. and R. J. Petroski. 1992. Alkaloid Toxins in Endophyte-infected Grasses. Natural Toxins 1:163-170.

78. Prestidge, R. A., D. R. Lauren, S. G. van der Zijpp, and M. E. Di Menna. 1985. Isolation of feeding deterrents to Argentine stem weevil in cultures of endophytes of perennial reygrass and tall fescue. N. Z. J. Agric. Res. 28:87-92.

79. Read, J. C. and B. J. Camp. 1986. The effect of the fungal endophyte *Acremonium coenophialum* in tall fescue on animal performance, toxicity, and stand maintenance. Agron. J. 78:848-850.

80. Rebois, R. V. and R. N. Huettel. 1986. Population dynamics, root penetration, and feeding behavior of Pratylenchus agilis in monoxenic root cultures of corn, tomato, and soybean. Nematology 18:392-397.

81. Rhodes, M. T., J. A. Paterson, M. S. Kerley, H. E. Garner, and M. H. Laughlin. 1991. Reduced blood flow to peripheral and core body tissues in sheep and cattle induced by endophyte-infected tall fescue. J. Anim. Sci. 69:2033-2043.

82. Rice, J. S., B. W. Pinkerton, W. C. Stringer, and D. J. Undersander. 1990. Seed production in tall fescue as affected by fungal endophyte. Crop Sci. 30:1303-1305.

83. Rowan, D. D. 1993. Lolitrems, Peramine and Paxilline: Mycotoxins of the Ryegrass/Endophyte Interaction. Agric. Eco. Environ. 44:103-122.

84. Rowan, D. D. and D. L. Gaynor. 1986. Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte. J. Chem. Ecol. 12:647-658.

85. Rowan, D. D., M. B. Hunt, and D. L. Gaynor. 1986. Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. J. Chem. Soc.935-936.

86. Roylance, J. T., N. S. Hill, and C. S. Agee. 1994. Ergovaline and Peramine production in endophyte-infected tall fescue: independent regulation and effects of plant and endophyte genotype. J. Chem. Ecol. 20:2171-2183.

87. Schafer, W. R. and C. Kenyon. 1995. A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*. Nature 375:73-78.

88. Schardl, C. L., R. B. Grossman, P. Nagabhyru, J. R. Faulkner, and U. P. Mallik. 2007. Loline Alkaloids: Currencies of Mutualism. Phytochemistry 68:980-996.

89. Schardl, C. L. and C. D. Moon. 2003. Processes of Species Evolution in *Epichloë/Neotyphodium* Endophytes of Grasses, p. 273-310. *In*: J. F. White, Jr., C. W. Bacon, N. L. Hywel-Jones, and J. W. Spatafora (eds.), Clavicipitalean Fungi. Marcel Dekker, Inc., New York.

90. Scott, B. 2001. Epichloe endophytes: fungal symbionts of grasses. Current Opinion in Microbiology 4:393-398.

91. Scott, B. and C. L. Schardl. 1993. Fungal symbionts of grasses: evolutionary insights and agricultural potential. Trends Microbiol. 1:196-200.

92. Segalat, L., D. A. Elkes, and J. M. Kaplan. 1995. Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. Science 267:1648-1651.

93. Sharpe, M. J. and H. J. Atkinson. 1980. Improved visualization of dopaminergic-neurins in nematodes using the glycoxilic-acid fluorescence method. Zoology 190:273-284.

94. Siegel, M. R. and L. P. Bush. 1996. Defensive Chemicals in Grass-Fungal Endophyte Associations, p. 81-119. *In*: J. T. Romeo, J. A. Saunders, and P. Barbosa (eds.), Phytochemical Diversity and Redundancy in Ecological Interactions. Plenum Press, New York.

95. Siegel, M. R. and L. P. Bush. 1997. Toxin Production in Grass/Endophyte Associations, p. 185-207. *In*: G. Carroll and P. Tudzynski (eds.), The Mycota V Part A:Plant Relationships. Springer, Berlin.

96. Siegel, M. R., D. L. Dahlman, and L. P. Bush. 1989. The role of endophytic fungi in grasses: New approaches to biological control of pests, p. 169-186. *In*: A. R. Leslie and R. L. Metcalf (eds.), Integregated pest management for turfgrass and ornamentals. US-EPA, Washington, DC.

97. Siegel, M. R., G. C. M. Latch, L. P. Bush, N. F. Fammin, D. D. Rowan, B. A. Tapper, C. W. Bacon, and M. C. Johnson. 1991. Alkaloids and insecticidal activity of grasses infected with fungal endophytes. J. Chem. Ecol. 16:3301-3315.

98. Siegel, M. R., G. C. M. Latch, L. P. Bush, F. F. Fannin, D. D. Rowan, B. A. Tapper, C. W. Bacon, and M. C. Johnson. 1990. Fungal endophyte-infected grasses:alkaloid accumulation and aphid response. J. Chem. Ecol. 16:3301-3315.

99. Spurr, H. W. Jr. 1985. Mode of action of nematicides, p. 269-276. *In*: J. N. Sasser and C. C. Carter (eds.), An Advanced Treatise on Meloidogyne. Volume I: Biology and Control. NCSU Graphics, Raleigh.

100. Stewart, G. R., R. N. Perry, and D. J. Wright. 2001. Occurrence of dopamine in *Panagrellus redivivus* and *Meloidogyne incognita*. Nematology 3:843-858.

101. Stone, J. K., C. W. Bacon, and J. F. White, Jr. 2000. An Overview of Endophytic Microbes: Endophytism Defined, p. 3-29. *In*: C. W. Bacon and J. F. White, Jr. (eds.), Microbial Endophytes. Marcel Dekker, Inc., New York.

102. Strickland, J. R., D. L. Cross, G. P. Birrenkott, and L. W. Grimes. 1994. Effect of ergovaline,loline, and dopamine antagonists on rat pituitary cell prolactin release in vitro. Am. J. Vet. Res. 55:716-721.

103. Stuedemann, J. A. and C. S. Hoveland. 1988. Fescue Endophyte: History and Impact on Animal Agriculture. J. Prod. Agric. 1:39-44.

104. Tan, R. X. and W. X. Zou. 2001. Enodphytes: A Rich Source of Functional Metabolites. Nat. Prod. Rep. 18:448-459.

105. Tepaske, M. R. and R. G. Powell. 1993. Analyses of selected endophyte-infected grasses for presence of loline-type and ergot-type alkaloids. J. Agric. Food Chem. 41:2299-2303.

106. Thies, J. A., A. D. Petersen, and D. K. Barnes. 1995. Host suitablility of forage grasses and legumes for root-lesion nematode *Pratylenchus penetrans*. Crop Sci. 35:1647-1651.

107. Thompson, F. N. and J. A. Stuedemann. 1993. Pathophysiology of fexcue toxicosis. Agriculture, Ecosystems and Environment 44:263-281.

108. Timper, P., R. N. Gates, and J. H. Bouton. 2005. Response of Pratylenchus spp. in tall fescue infected with different strains of the fungal endophyte Neotyphodium coenophialum. Nematology 7:105-110.

109. Townshend, J. L. 1963. The pathogenicity of *Pratylenchus penetrans* to strawberry. Can. J. Plant Sci. 43:75-78.

110. Tudzynski, P., T. Correia, and U. Keller. 2001. Biotechnology and Genetics of Ergot Alkaloids. Appl. Microbiol. Biotechnol. 57:593-605.

111. Weinshenker, D. Z., G. Garriga, and J. H. Thomas. 1995. Genetic and pharmalogical analysis of neurotransmitters controlling egg laying in *C. elegans*. Neuroscience 15:6975-6985.

112. West, C. P., E. Izekor, D. M. Oosterhuis, and R. T. Robbins. 1988. The effect of *Acremonium coenophialum* on the growth and nematode infestation of tall fescue. Plant Soil 112:3-6.

113. White, J. F., Jr. 1987. The widespread distribution of endophytes in the Poaceae. Am. J. Vet. Res. 71:340-342.

114. White, J. F., Jr., C. W. Bacon, N. L. Hywel-Jones, and J. W. Spatafora. 2003. HIstorical perspectives: Human interactions with *CLaviciptalean* fungi, p. 1-16. *In*: J. F. White, Jr., C. W. Bacon, N. L. Hywel-Jones, and J. W. Spatafora (eds.), *Claviciptalean* Fungi: Evolutionary Biology, Chemistry, Biocontrol, and Cultural Impacts. Marcel Dekker, Inc, New York.

115. Wilkinson, H. H., M. R. Siegel, J. D. Blankenship, A. C. Mallory, L. P. Bush, and C. L. Schardl. 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Mol. Plant Microbe Interact. 13:1027-1033.

116. Wright, D. J. 1981. Nematicides: Mode of action and new approaches to chemical control, p. 421-449. *In*: B. M. Zuckerman and R. A. Rohde (eds.), Plant Parasitic Nematodes Vol. III. Academic Press, New York.

117. Wuyts, N., R. Swennen, and D. De Waele. 2006. Effects of plant phenylpropanoid pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes Radopholus similis, Pratylenchus penetrans, and Meloidogyne incognita. Nematology 8:89-101.

118. Yates, S. G. 1983. Tall Fescue Toxins, p. 249-273. *In*: M. Recheigel (ed.), Handbook of naturally occurring food toxicants. CRC Press, Boca Raton.

119. Yates, S. G., R. D. Plattner, and G. B. Garner. 1985. Detection of ergopeptine alkaloids in endophyte infected, toxic Ky-31 tall fescue by mass spectrometry. J. Agric. Food Chem. 33:719-722.

120. Yates, S. G. and R. G. Powell. 1988. Analysis of Ergopeptine Alkaloids in Endophyte-infected Tall fescue. J. Agric. Food Chem. 36:337-340.

121. Zhang, H. W., Y. Y. Soong, and R. X. Tan. 2006. Biology and Chemistry of Endophytes. Nat. Prod. Rep. 23:753-771.

122. Zhang, Q., D. E. Spiers, G. E. Rottinghaus, and G. B. Garner. 1994. Thermoregulatory effects of ergovaline isolated from endophyte- infected tall fescue seed on rats. J. Agric. Food Chem. 42:954-958.

123. Zinov'eva, S. V., Zh. V. Udalova, I. S. Vasil'eva, S. A. Vanyushkin, and V. A. Paseshnichenko. 2001. Role of isoprenoid compounds in plant adaptation to biogenic stress caused by parasitic nematodes. Prikl. Biokhim. Mikrobiol. 37:533-541.

124. Zunke, U. 1990. Ectoparasitic behavior of the root lesion nematode, *Pratylenchus penetrans*, on root hairs of different host plants. Rev. Nematol. 13:331-337.

125. Zunke, U. 1990. Observations on the invasion and endoparsitic bahvior of the root lesion nematode *Pratylenchus penetrans*. Nematology 22:309-320.