To the Graduate Council:

I am submitting herewith a thesis written by Amy Michelle Cole entitled "A Biological Assay for Detection of Toxins Associated with Acremonium-Infected Tall Fescue." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Charles D. Pless

Charles D. Pless, Major Professor

We have read this thesis
and recommend its acceptance:

Kimberly D. Irwin

Neil R. Leonhardt

Accepted for the Council:

Carolyn Winkel

Associate Vice Chancellor
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Amy M. Cole

Date July 23, 1991

A BIOLOGICAL ASSAY FOR DETECTION OF TOXINS ASSOCIATED WITH *ACREMONIUM*-INFECTED TALL FESCUE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Amy Michelle Cole

August 1991

I dedicate this thesis to my parents, Anthony Theodore Cole and Cheryl Theresa Cole. Their support and encouragement has instilled in me the confidence needed to complete this research. Not all are as fortunate as I; for this I am grateful.

ACKNOWLEDGEMENTS

Many individuals played an integral part in this thesis research by contributing labor, ingenuity, and professional expertise. I would like to thank Dr. Charles Pless, my major advisor, for his guidance, understanding, and the confidence he expressed in my abilities. I would also like to thank my committee members, Dr. Kim Gwinn and Dr. Reid Gerhardt, for their support, ideas, and constructive editing. Without their assistance and encouragement the completion of this thesis would not have been possible.

David Chalkley spent many hours helping to calculate concentrations and prepare alkaloid solutions for incorporation into diets. I am very grateful for his time and instruction. I also thank Anita Gavin for administering PAS-ELISA tests for presence of endophyte in tall fescue.

I am especially indebted to Yiyuan Qian who spent most days, including weekends, throughout this project maintaining Drosophila cultures, preparing diets, and counting insects. I would not have been able reach many goals without his effort and dedication.

Most importantly, I wish to express my appreciation to the University of Tennessee Agricultural Experiment Station and the Department of Entomology and Plant Pathology for awarding me a graduate research assistantship and the opportunity to earn the Master of Science degree.

ABSTRACT

The effects of toxic compounds in endophyte-infected (E+) tall fescue on the development of Drosophila melanogaster Meigen, the laboratory fruit fly, were evaluated. D. melanogaster was useful as a model insect for bioassays of insecticidal compounds in E+ tall fescue plant tissues and individual compounds found in these tissues.

Toxins in diets prepared with E+ tall fescue leaf, root, and tiller base powders reduced larval survival significantly. The response induced by these toxins varied with different plant tissues because the concentration of toxins is higher where the endophyte grows (i.e. seed and tiller base).

Ergonovine retarded the growth of Drosophila only slightly; however, two of the ergopeptide alkaloids tested, ergotamine and ergocryptine, reduced larval survival significantly. N-formylloline but not N-acetylloline or loline alkaloid, caused significant larval mortality at the highest concentration assayed.

These studies with Drosophila revealed that the roots of E+ tall fescue contain insecticidal compounds. N-formylloline, which was previously identified in E+ tall fescue roots, is probably a major contributor to toxicity of these plant organs.

TABLE OF CONTENTS

CHAPTER		PAGE
INTRODUCTION		1
I.	LITERATURE REVIEW	3
	TALL FESCUE	3
	FESCUE TOXICOSIS	4
	FESCUE ENDOPHYTE	5
	TOXINS IN TALL FESCUE	6
	THE GRASS/FUNGAL RELATIONSHIP	7
	RESISTANCE TO HERBIVORES	8
	BIOASSAY TECHNIQUES	11
II.	MATERIALS AND METHODS	13
	INSECT CULTURE	13
	Adult Flies	13
	Neonate Larvae	13
	ENDOPHYTE TESTING	14
	ASSAYS INITIATED WITH ADULT FLIES	14
	Leaf and Root Assay I	14
	Leaf and Root Assay II	15
	Leaf and Root Assay III	16
	Oviposition Study	16
	ASSAYS INITIATED WITH NEONATE LARVAE	17
	Leaf, Root, and Tiller Base Assay	17
	Seed Assay	18
	ASSAY OF ALKALOIDS	20
	ERGOT ALKALOIDS	20
	Ergonovine Assay	21
	Ergotamine Assay	21
	Ergocryptine Assay	22
	LOLINE ALKALOIDS	22
	STATISTICAL ANALYSIS	23
III.	RESULTS	24
	ASSAYS INITIATED WITH ADULT FLIES	24
	Leaf and Root Assay I	24
	Leaf and Root Assay II	27
	Leaf and Root Assay III	27
	Oviposition Study	29
	ASSAYS INITIATED WITH NEONATE LARVAE	29
	Leaf, Root, and Tiller Base Assay	29
	Seed Assay	33
	ASSAY OF ALKALOIDS	35

ERGOT ALKALOIDS 35
Ergonovine Assay 35
Ergotamine Assay 35
Ergocryptine Assay 37
LOLINE ALKALOIDS 37

IV. DISCUSSION 41

ASSAYS OF TALL FESCUE 41
ASSAYS OF ALKALOIDS 44
ERGOT ALKALOIDS 45
LOLINE ALKALOIDS 49

V. SUMMARY AND CONCLUSIONS 52

REFERENCES CITED 56

APPENDIX: Preparation of 0.05 M Phosphate Buffer 62

VITA 64

LIST OF FIGURES

FIGURE PAGE

1. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) tall fescue leaf powder 25

2. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) root powder 26

3. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) leaf (L) or root powder (R) 28

4. Number of Drosophila melanogaster which developed from egg to pupa on diets containing powder made from endophyte-infected (E+) or endophyte-free (E-) tall fescue roots collected from the field (F) and greenhouse (GH) 30

5. Number of eggs deposited by adult Drosophila melanogaster onto the surface of diets containing endophyte-infected (E+) or endophyte-free (E-) tall fescue leaf powder (L) or control diet 31

6. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on diets containing powders made from endophyte-infected (E+) or endophyte-free (E-) tall fescue leaves (L) or tiller bases (TB) 32

7. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on control diet and diets containing 6 different ratios of endophyte-infected (E+) : endophyte-free (E-) tall fescue seed powder 34

8. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on control diet and diets containing 4 different dilutions of ergonovine 36

9. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol and tartrate control diets and diets containing 4 different dilutions of ergotamine tartrate 38

10. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol control diet and diets containing 4 different dilutions of ergocryptine 39

11. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol control diet and diets containing 4 different dilutions of N-formylloline 40

12. Ergot alkaloid compounds with similar molecular structure, differing only in the composition of R group structure 47

13. Two loline-type saturated pyrrolizidine alkaloids with similar molecular structure differing only in the composition of the R_2 group 51

INTRODUCTION

Tall fescue, Festuca arundinacea Schreb., is the primary cool-season grass grown in the United States and covers an estimated 12-14 million ha (Siegel et al. 1984). In Tennessee, over 1.4 million ha of tall fescue is produced for turf, erosion control, and, most importantly, forage for livestock (Long and Hilty 1985). Seventy-five to 90 per cent of this grass is infected with an endophytic fungus, Acremonium coenophialum Morgan-Jones and Gams (Shelby and Dalrymple 1987). The tall fescue endophyte toxins that are ingested by herbivores have been implicated as the cause for fescue toxicosis of livestock (Stuedemann and Hoveland 1988). Losses to the livestock industry in Tennessee as a result of fescue toxicosis are estimated to be 85 million dollars annually (Fribourg et al. In press).

Although endophyte-infected (E+) tall fescue can be detrimental to livestock, E+ tall fescue is more vigorous, and more tolerant to adverse environmental factors (e.g. drought). Toxins produced by the fungus may cause deterrence or antibiosis to a number of pests, including foliage feeding insects, soil-borne insects, soil-borne nematodes, and pathogens (Siegel et al. 1987).

Resistance of E+ tall fescue to foliage-feeding insects has been well documented (Clay 1987). Research concerning endophyte-mediated resistance by tall fescue roots to soil

insects, however, is lacking. Maintaining soil insects in an artificial environment is demanding, and often survival is low. Many of these insects also have long life cycles, making it difficult to obtain development and survival data in a short period of time.

This thesis research was conducted primarily to develop an efficient biological assay for toxic compounds in leaves, roots, and seeds of tall fescue using Drosophila melanogaster Meigen, the common laboratory fruit fly. A second objective was to determine which of several known alkaloids produced by E+ tall fescue are insecticidal.

CHAPTER I

LITERATURE REVIEW

TALL FESCUE

In temperate regions of the world tall fescue, Festuca arundinacea (Schreb.), is a predominant cool-season grass. It was introduced into the United States from northern Europe (Cowan 1956) and became an important agricultural crop early in the twentieth century when 'Kentucky 31' (K-31), a tall fescue cultivar, was released (Buckner et al. 1979).

K-31 tall fescue was found established in Menifee County, Kentucky, in 1931. The variety was evaluated and released by the University of Kentucky in 1943. The USDA Soil Conservation Service established fields of K-31 in areas throughout the south central and southeastern states (Tabor 1952). The new cultivar was rapidly accepted by farmers as a dependable high quality forage that prevented erosion and resisted disease.

By the early 1940's, tall fescue grew on approximately 16,000 ha in the United States (Buckner et al. 1979). Presently, it covers an estimated 12-14 million ha from Florida to Canada (Siegel et al. 1984). The majority of this cool season grass grows in the transition zone of the

eastern United States. This wide distribution of tall fescue is due to the plant's adaptability to a wide range of soils and climates as well as its tolerance for environmental stress, such as drought and pest damage (Buckner et al. 1979). Tennessee produces more than 1,420,000 ha of tall fescue for livestock forage (Long and Hilty 1985).

FESCUE TOXICOSIS

As tall fescue acreage for livestock forage increased, poor animal performance and symptoms of illness began to be reported (Stuedemann and Hoveland 1988). Cattle grazing on tall fescue develop a gangrenous condition of the feet referred to as "fescue foot". Another condition, bovine fat necrosis, can develop internally. This condition is designated when hard masses develop in fat tissue surrounding the intestines causing calving problems (Stuedemann and Hoveland 1988). Livestock can develop other disorders that affect animal performance and appearance, including elevated body temperatures, less weight gain, lower milk production, and reduced reproductive rates (Bush and Buckner 1973). Collectively, these conditions are referred to as fescue toxicosis. Fescue toxicosis results in losses exceeding $600 million dollars annually to the livestock industry in the United States (Hoveland, In

press). In Tennessee, $85 million dollars are estimated to be lost as a result of poor animal performance and illness (Fribourg et al., In press).

FESCUE ENDOPHYTE

In 1977, Charles Bacon, a fungal physiologist, determined that livestock which developed fescue toxicosis were grazing on tall fescue infected with an endophytic fungus, Epichloe typhina (Pers.) Tul., (Bacon et al. 1977). Morgan-Jones and Gams (1982) reclassified the endophyte as a new species, Acremonium coenophialum Morgan-Jones and Gams. The genus, Acremonium belongs to the family Clavicipitaceae, which contains many different fungal species that infect grass hosts. A. coenophialum inhabits 75-90% of the tall fescue growing in the United States (Shelby and Dalrymple 1987).

An endophyte is an organism which lives within a plant. A. coenophialum is a seed-borne parasite; hyphae grow between the epidermal cells of the scutellum and endosperm. Shortly after germination of an infected seed, the fungus infects the embryo (Bacon et al. 1986), and within two days the fungus can be observed in the first internode of the emerging shoot (Lyons and Bacon 1985). The first leaf is not infected by the fungus until sheath differentiation occurs. Infection of the leaves begins approximately 8 to

10 days after germination. During the plant's vegetative phase, the mycelium is located primarily in the tiller base. During seed head formation, the endophyte grows through the flowering stem into the seed (Hinton and Bacon 1985).

TOXINS IN TALL FESCUE

Following the discovery of the tall fescue endophyte, scientists observed that livestock grazing on endophyte-free (E-) tall fescue do not have the poor animal performance associated with grazing on endophyte-infected (E+) tall fescue (Hoveland et al. 1980, 1983). Alkaloids found in E+ plants, but not in E- plants, may be the cause of fescue toxicosis (Siegel et al. 1987). Although A. coenophialum mycelium is found only in the leaf sheath, flowering stem, and seed, alkaloids have been found in the leaf blades. Researchers suggest from this finding that these compounds are translocated into different plant parts (Bacon et al. 1986). In addition, concentrations of alkaloids differ in different portions of the plant (Bacon et al. 1986).

E+ tall fescue contains several types of alkaloids. Lyons (1986) established that ergot alkaloids were detected in all infected plants but not in uninfected plant samples. Ten to 50 per cent of the total ergot alkaloids in tall fescue are ergopeptine alkaloids. More than 80 percent of the ergopeptine alkaloid extracted from endophyte-infected

tall fescue is ergovaline (Lyons et al. 1986; Belesky et al. 1988). Ergot alkaloids are most likely produced by the endophyte and not by the plant because they are absent in E- tall fescue (Lyons et al. 1986) and they can be isolated from the fungus in culture (Bacon, 1988). Ergopeptine alkaloids have not been identified in the roots of E+ tall fescue.

Loline alkaloids (mainly N-formyl and N-acetylloline) are also found in high concentrations in E+ tall fescue foliage, roots, and seed, but neither the plant nor the fungus produces loline independently. Production of these compounds appears to be dependent on an infected host (Jones et al. 1983). N-formyl loline has been detected in E+ tall fescue roots (personal communication, Daniel A. Potter, University of Kentucky, Lexington, KY 40546).

THE GRASS/FUNGAL RELATIONSHIP

Acremonium coenophialum is an obligate biotroph. It parasitizes tall fescue without producing symptoms; yet, it cannot survive without nutrients provided by the host (Lewis 1973). Although tall fescue with endophyte is referred to as "infected" the relationship between the plant and the fungus is a mutualistic symbiosis (Arachevaleta et al. 1989). The plant provides a protective environment for the endophyte, and by living intercellularly the endophyte does

not alter host cell structure. The plant provides nutrition, and a means of dissemination for the fungus (Siegel et al. 1987). In turn, the infected plant exhibits improved growth [increased tillering and seed production (Clay 1987)] and is more tolerant to adverse environmental and biotic stress (e.g. drought, insects and disease resistance) (Siegel et al. 1987). These advantages increase survival of E+ fescue, allowing endophyte infestation to increase in a stand over time (Clay et al. 1985, Bacon et al. 1986).

RESISTANCE TO HERBIVORES

Stuedemann and Hoveland (1988) reported that livestock which grazed on E+ tall fescue pastures had lower weight gains than livestock on E- pastures. Steers on infested pastures fed 20 per cent less time than those on E- pastures. Deterrence of grazing by E+ fescue plays a major role in differences of livestock performance on E+ and E- pastures (Schmidt et al. 1982), but toxic substances produced by the endophyte also have a systemic effect (Stuedemann and Hoveland 1988).

Enhanced insect resistance induced by another clavicipitaceaous endophyte, Acremonium loliae Latch, Christensen, and Samuels, was first reported in perennial ryegrass, Lolium perenne L. (Prestige et al. 1982).

Populations of Argentine stem weevil (ASW), Listronotus bonariensis Kuschel, adults were significantly lower on endophyte-infected perennial ryegrass than endophyte-free ryegrass. Ryegrass without endophyte was more susceptible to ovipositional and larval damage (Barker et al. 1984). Endophyte-infected perennial ryegrass also has enhanced resistance to Sphenophorus parvulus (Gyllenhal), the bluegrass billbug, a weevil with feeding habits similar to ASW (Funk et al. 1983). Endophyte-mediated resistance of perennial ryegrass turf to sod webworm, Crambus spp. has also been demonstrated. Negligible webworm damage was observed on plots with high levels of endophyte infestation levels; whereas, plots consisting of E- turf exhibited 70 to 88 per cent webworm damage (Funk et al. 1983).

Adult and nymph survival of hairy chinch bug, Blissus leucopterus hirtus Montandon, was significantly higher on endophyte-free perennial ryegrass tillers than on endophyte-infected tillers. After 21 days, 4 per cent of the adult chinch bugs survived on endophyte-infected plants whereas 93 per cent survived on endophyte-free plants (Mathias et al. 1990).

Acremonium-mediated resistance to insects has also been demonstrated in E+ tall fescue. Endophyte-mediated resistance to insects may be a result of deterrent properties causing insect avoidance, or by antibiosis, by reducing individual growth (Clay, 1989). ASW (Prestidge et

al. 1985); the bird cherry-oat aphid, Rhopalosiphum padi L. (Latch et al. 1985); the large milkweed bug, Oncopeltus fasciatus (Dallas); and the greenbug, Schizaphis graminum Rondani (Johnson et al. 1985) are a few of the insects that have been deterred from feeding or were unable to develop on E+ tall fescue plants, plant tissue, or diets containing alkaloid extracts.

Research on the effects of E+ fescue on soil-dwelling insects is limited due to the difficulty of maintaining artificial populations. The studies at the University of Tennessee involving soil insects survival of Japanese beetle, Popillia japonica Newman, larvae on E- tall fescue was significantly higher than survival on E+ tall fescue (Oliver et al. 1990). Wireworms were found to be more abundant in E- plots than in E+ plots (personal communication, Jason Oliver, University of Tennessee, Knoxville, TN 37901-1071). Lolines have been detected in E+ tall fescue roots (personal communication, Daniel A. Potter) and may be a cause of deterrence or toxicity of E+ fescue roots to soil insects.

Resistance to soil-borne nematodes has also been demonstrated. Pratylenchus scribneri (Steiner) and Tylenchorhynchus acutus (Allen) population densities were higher in soil of E- tall fescue plots than in soil of E+ tall fescue plots (West et al. 1988). P. scribneri is an obligate migratory endoparasite that has been shown to

reduce forage yield of tall fescue. Fifteen weeks after inoculating E- and E+ tall fescue plants with 2,000 P. scribneri nematodes, approximately 63 per cent of the initial nematode inoculation was recovered on E- plants, but a significantly lower number of nematodes was recovered from E+ plants. (Kimmons et al. 1990).

In culture, A. coenophialum exhibited an antibiotic reaction against some fungal plant pathogens and saprophytic fungi (White and Cole 1985). In greenhouse experiments, endophyte-infested seed lots were more resistant to soil-borne fungi causing pre-emergence diseases (Blank et al. 1991). All tall fescue cultivars inoculated with stem rust, Puccinia graminis Pers.:Pers., were suceptible to disease development regardless of endophyte infection (Welty and Barker 1990). Ford and Kirkpatrick (1989) reported incidence of crown rust, Puccinia coronata Corda, on greenhouse plants to be significantly lower on E+ tall fescue than on E- tall fescue. Endophyte-mediated resistance to foliar and soil-borne diseases of tall fescue would enhance turf persistance, vigor and aesthetic value.

BIOASSAY TECHNIQUES

Few biological assays have been developed to determine toxicity of E+ tall fescue or extracts from tall fescue to insects. The large milkweed bug was utilized to determine

ED_{50} values for tall fescue extracts and alkaloids (Yates et al. 1989). A feeding bioassay and an ovipositional bioassay were conducted with the ASW to determine if that insect would be deterred by E+ tall fescue as it is by ryegrasses (Barker et al. 1983). Feeding by ASW was significantly lower on diets containing A. coenophialum mycelium at all concentrations tested (1,5,10 %) than on unmodified diets. Prestige et al. (1985) also conducted bioassays of tall fescue with the ASW to isolate feeding deterrents. Several aphid species were included in preference and no-choice tests of fescue plant parts and potted plants. The bird cherry-oat aphid and greenbug could not survive on E+ tall fescue (Johnson et al. 1985).

All the currently described biological assays have been used for leaves or leaf extract. It has been difficult through biological assays to determine if there are insecticidal compounds in the roots of E+ tall fescue. Japanese beetle larvae feed on the roots of tall fescue, but it is not practical to use them as assay organisms. Japanese beetle mortality is high under experimental conditions. They need a full year to mature, and can be used to assay only living root material. Development of a procedure that can be used to assay for leaf, roots, and seeds quickly and accurately could aid in discovery and identification of insecticidal compounds in different parts of the tall fescue plant.

CHAPTER II

MATERIAL AND METHODS

INSECT CULTURE

Adult flies -

The laboratory fruit fly, Drosophila melanogaster Meigen, (Drosophilidae: Diptera) was used as the assay organism in this research. A culture of wild type D. melanogaster and Ward's Instant Drosophila Medium were obtained from Ward's Natural Science Establishment Inc., Rochester, NY. Stock cultures were maintained on Drosophila Medium in 473 ml glass jars in an environmental chamber (ca. 25°C, 12L:12D photoperiod). Adult flies used in experiments were from these stock cultures.

Neonate Larvae -

Three pieces of filter paper (7.5 cm, Grade 362) were placed into an 8.8 cm by 3.7 cm plastic dish with two screened ventilation windows (0.8 cm in dia.) opposite each other in the vertical wall. A thin layer (ca. 4 g) of Drosophila Medium was spread evenly over the surface of the paper. The medium was moistened with 20 ml of distilled, and deionized water (ddw), and 100 to 150 unsexed flies were introduced. Cultures were maintained in an environmental

chamber set at 25°C, 12L: 12D photoperiod. After a minimum of 7 days, neonate larvae were obtained from the surface of the diet with the aid of a dissecting microscope.

ENDOPHYTE TESTING

Five tillers were collected from each greenhouse and field plot plant used in experiments, and each tiller was tested for endophyte infection with Protein-A sandwich, enzyme-linked immunosorbent assay (PAS-ELISA) (Reddick and Collins 1988). Endophyte infection of seed lots was determined by an in vitro assay (Conger and McDaniel 1983, Gwinn et al. In press) or PAS-ELISA.

ASSAYS INITIATED WITH ADULT FLIES

Leaf and Root Assay I -

Leaves of 2-year-old E- and E+ K-31 tall fescue plants were collected from established field plots at the University of Tennessee Plant Science Farm (UTPSF), Knoxville, TN, in July, 1989. Roots were harvested in October, 1989, from 2-year-old E- and E+ K-31 plants grown in six-inch pots in a greenhouse on the University of Tennessee Agriculture Campus (UTAC). To obtain roots, plants were removed from pots, and the mat of roots which had formed at the bottom of each plant was collected. The

root mat was washed with water and dried. Leaves and roots were frozen at -16°C, lyophilized, then ground separately into a fine powder with a Wiley mill (20 mesh screen). Leaf and root powders were refrozen at -16°C until needed for diet preparation.

Diets were prepared by mixing 1 g of leaf or root powder with 4.5 g of Drosophila Medium and adding 20 ml ddw in a 118 ml (10 cm x 4.4 cm dia.) glass rearing container. Control diet consisted of 5.5 g Drosophila Medium to which 20 ml of ddw were added.

Adult flies of undetermined were immobilized in a freezer (-5°C) for 7 to 10 minutes. The flies were then transferred into a cryolizer, which kept the flies immobile for sexing and collecting. Three male/female pairs of Drosophila were introduced into the rearing containers, which were then closed with foam stoppers. Flies in the rearing containers were monitored until they regained activity (ca. 3 minutes), and those which did not survive anesthesia were replaced. Ten replicates of each treatment were completely randomized and maintained in a laboratory at room temperature (ca. 25°C). Pupae and adults were counted every other day for 18 days after adults were introduced.

Leaf and Root Assay II -

This experiment was conducted by the same methods described in Leaf and Root Assay I except that adult flies

used to infest the rearing containers were less than 4 days old. All adult flies were removed from a stock culture jar three days prior to the initiation of the experiment. Only flies which emerged during the next three days were used in the experiment. In this experiment, and all of the following experiments, the insects were maintained in a environmental chamber set at 25°C and 12L:12D photoperiod.

Leaf and Root Assay III -

Leaf powder material used in Leaf and Root Assays I and II was also used in this experiment. Roots from E- and E+ K-31 tall fescue plants were collected from 2-year-old field plots at the UTPSF and from the UTAC greenhouse in April, 1990. Diets containing root powders made from field or greenhouse plants constituted separate treatments. Assay procedures were the same as those used in Assay II.

Oviposition Study -

Leaves from 2-year-old E- (Forager) and E+ (K-31) tall fescue pastures were collected in May, 1991, from field plots at the Highland Rim Experiment Station, Springfield, TN. E- and E+ leaf powders were prepared by the method described for Leaf and Root Assay I.

Three pieces of filter paper (7.5 cm, Grade 362) were placed into 8.8 cm by 3.7 cm plastic dishes with two screened ventilation windows (0.8 cm dia.). Drosophila

Medium (2.25 g) and 0.5 g of E- or E+ fescue powders were mixed and distributed uniformly onto the filter paper. The mixture and filter paper were moistened with 15 ml of ddw.

Adult flies were immobilized, collected, and sexed following the procedure in Leaf and Root Assay I. Ten 1-to 3-day-old male/female pairs of Drosophila were introduced into each plastic container. Flies in the containers were monitored until they regained activity (ca. 3 minutes), and those which did not survive anesthesia were replaced.

Five replicates of each treatment were completely randomized. Eggs were counted once on the fifth day after adults were introduced.

ASSAYS INITIATED WITH NEONATE LARVAE

Leaf, Root, and Tiller Base Assay -

Leaves, roots, and tiller bases (portions of the plant ca. 1 cm above the soil line to 7-10 cm above the soil line) were collected from E- and E+ plants in field plots at the UTPSF in August, 1990. Roots were washed vigorously to remove clay and soil. Diets containing leaf, root, or tiller base powders were prepared by the procedures used in Assay II. Ten neonate larvae were selected from stock cultures and placed onto the surface of the diets.

Twenty replicates were completely randomized. Beginning five days after larval introduction, adults and

pupae were counted every day for 17 days. In all of the following experiments, insects were counted on this same schedule.

Seed Assay -

Three seed lots were used. E+ seed powder was from a K-31 seed lot with a 65% endophyte infestation level. E- seed powder was obtained from a lot of 'Forager,' an endophyte-free cultivar. For all seed lots, viable endophyte level was determined by embryo culture (Conger and McDaniel 1983, Gwinn et al. In press). Treatment 7 (Table 1) was from a seed lot which had a endophyte infestation level of 85% (seed stain method), but a viable endophyte level of 0. Seeds were frozen and lyophilized, and seed powder diets were prepared in the same manner as leaf and root powder diets. Several treatments with varying ratios of E+:E- seed powders were used (Table 1).

One gram of seed powder was mixed with 4.5 g of Drosophila Medium and prepared by the procedure in Leaf and Root Assay II. A control diet consisted of 5.5 g of Drosophila Medium and 20 ml of ddw. Ten neonates were placed onto each diet and the diet containers were completely randomized. Each treatment was replicated 20 times, except treatment 7, which was replicated 10 times.

Table 1. Amounts of E+, E-, and A seed powders combined with 4.5 g of Drosophila Medium for assay.

Treatment	Grams of Seed Powder		
	E+	E-	A
1	0.0	1.0	—
2	0.125	0.875	—
3	0.25	0.75	—
4	0.50	0.50	—
5	0.75	0.25	—
6	1.00	0.0	—
7	—	—	1.0
8 (control)	—	—	—

ASSAY OF ALKALOIDS

ERGOT ALKALOIDS

Three ergot alkaloids were assayed using Drosophila. These alkaloids either occur in E+ tall fescue or are chemically related to those that do. Ergot alkaloids were obtained from Sigma Chemical Company, St. Louis, MO 63178. All solutions were prepared under reduced light.

Stock suspensions were sonicated for 1 minute, vortexed, and 0.5 ml was pipetted into 4.5 ml of methanol to produce dilution II. A 1:10 dilution was made in methanol from dilution II to create dilution III, and from dilution III to create dilution IV. Aliquots were added to 5.5 g Drosophila Medium in a rearing container (118 ml, 10 cm x 4.4 cm dia.). Volume of the aliquot was dependant upon alkaloid tested. The aliquot of dilution I (stock suspension) contained 4.7 μmoles of ergot alkaloid. Diets containing each dilution served as different treatments.

Chemically-amended diets were infested with 10 neonate larvae per container. Unless noted otherwise, each treatment was replicated 20 times in a completely random design within an environmental chamber.

Ergonovine Assay -

Fifty mg ergonovine (lot no. 94F01451) were added to 3.25 ml methanol. This stock and its dilutions were vortexed for 1 minute before pipetting and aliquots (100 µl) of each were added to 20 ml of ddw, vortexed for 15 seconds, and added to Drosophila Medium. Controls of Drosophila Medium (5.5 g) and ddw (20 ml) and two methanol controls (100 and 200 µl MeOH: 20 ml water) were prepared. Treatments were replicated 17 times.

Ergotamine Assay -

Ergotamine tartrate dihydrate (lot no. 45F-0759) (104 mg) was added to 13 ml of methanol. This stock and its dilutions were vortexed 1 minute, 400 µl were added to 20 ml ddw, vortexed for 15 seconds, and added to Drosophila Medium. A methanol control (20 ml of ddw and 400 µl mixed and added to (5.5 g) Drosophila Medium) and a tartrate control were prepared. To prepare tartrate control, 0.179 mg of tartaric acid was mixed with 100 ml of ddw. From this solution, 2.5 ml were added to 250 ml ddw and vortexed for 15 seconds. Methanol (400 µl) was added to 20 ml volumes of the 250 ml stock which where vortexed for 15 seconds and added to 5.5 g of Drosophila Medium.

Ergocryptine Assay -

Ergocryptine (lot no. 108C-0264) (88 mg) was mixed with 9.75 ml of methanol. This stock and its dilutions were vortexed 1 minute; 300 µl were added to 20 ml ddw, vortexed for 15 seconds, and added to Drosophila Medium. A methanol control (20 ml ddw mixed with 300 µl of methanol and added to 5.5 g Drosophila Medium) was also prepared.

LOLINE ALKALOIDS

Three loline alkaloids that are present in E+ tall fescue foliage and seed were assayed using Drosophila. Lolines were obtained from Richard C. Powell, Bioactive Constituents Research, Peoria, IL, 61604. The stock concentration of lolines was 32.4 mM in methanol.

The loline diets were infested with 10 neonate larvae per container. Each treatment was replicated 20 times in a completely random design within an environmental chamber.

In three separate experiments, 7.3 mg of loline dihydrochloride was dissolved in 1 ml of methanol, 5.9 mg of N-formylloline were dissolved in 1 ml of methanol, and 6.35 mg of N-acetylloline were dissolved in 1 ml of methanol. A 500 µl aliquot was removed from the stock, and vortexed with 24.5 ml of 0.05 M potassium-sodium phosphate buffer (pH 6.4) to produce dilution I of each experiment. In each experiment three serial dilutions (1:10, 1:100, and

1:1000) were made from dilution I in buffer solution. Control dilution contained 500 µl of methanol in 24.5 ml of buffer. Each dilution was vortexed for 15 seconds and 2.2 ml aliquots were pipetted into 0.6 g of diet contained in 2 dram glass vials.

STATISTICAL ANALYSIS

Data collected from all experiments were analyzed with ANOVA and Duncan's Multiple Range Test ($P < 0.05$) (SAS Institute 1987).

CHAPTER III

RESULTS

ASSAYS INITIATED WITH ADULT FLIES

In Leaf and Root Assays I, II, and III Drosophila melanogaster were reared successfully on diets containing E- tall fescue leaf and root powders. E+ tall fescue leaf or root material added to Drosophila Medium hindered development of flies.

Leaf and Root Assay I -

Very few ($\bar{x}$ = 1.4) D. melanogaster reached the pupal stage when fed E+ leaf diet during the 18-day test period; when fed E- leaf diet, a large number ($\bar{x}$ = 140.5) of insects pupated during the same period (Fig. 1). The numbers of pupae which developed on E- and E+ leaf diets were significantly different, beginning on day 6 and continuing until the termination of the experiment. The number of pupae which developed on E- leaf diet was significantly different from the number on the control ($\bar{x}$ = 68.5).

The number of insects which survived to the pupal stage was significantly lower on E+ root diet than on E- root diet, beginning on day 10 of the 18-day test period (Fig. 2). Differences in larval survival on the two root

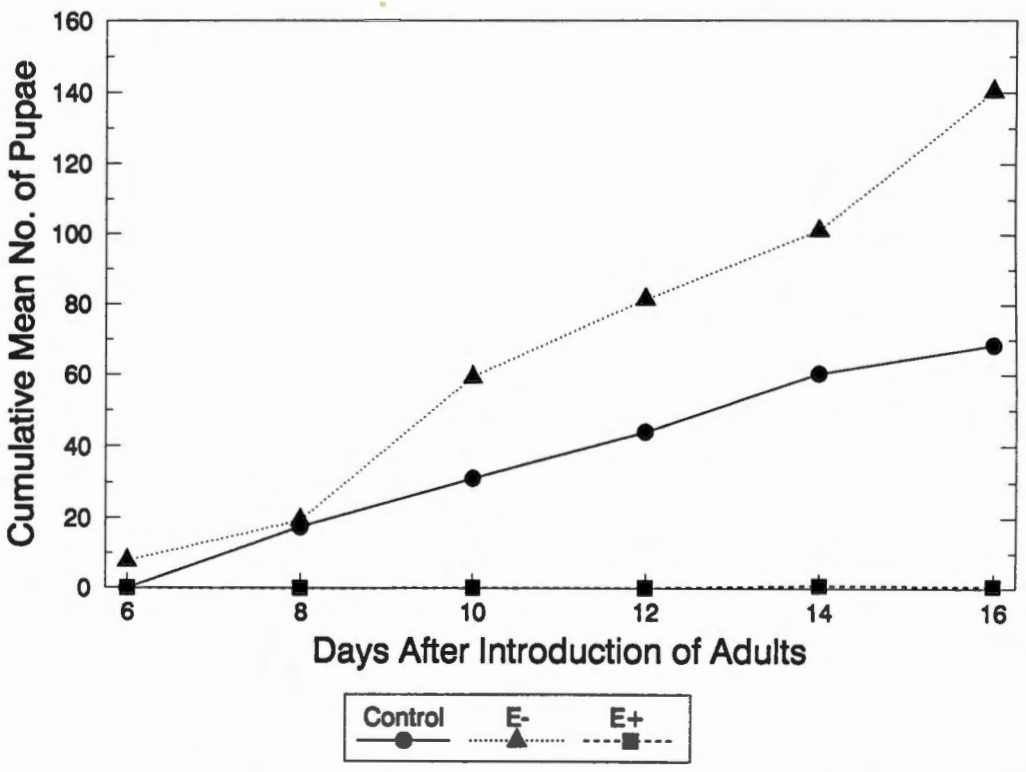

Fig. 1. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) tall fescue leaf powder.

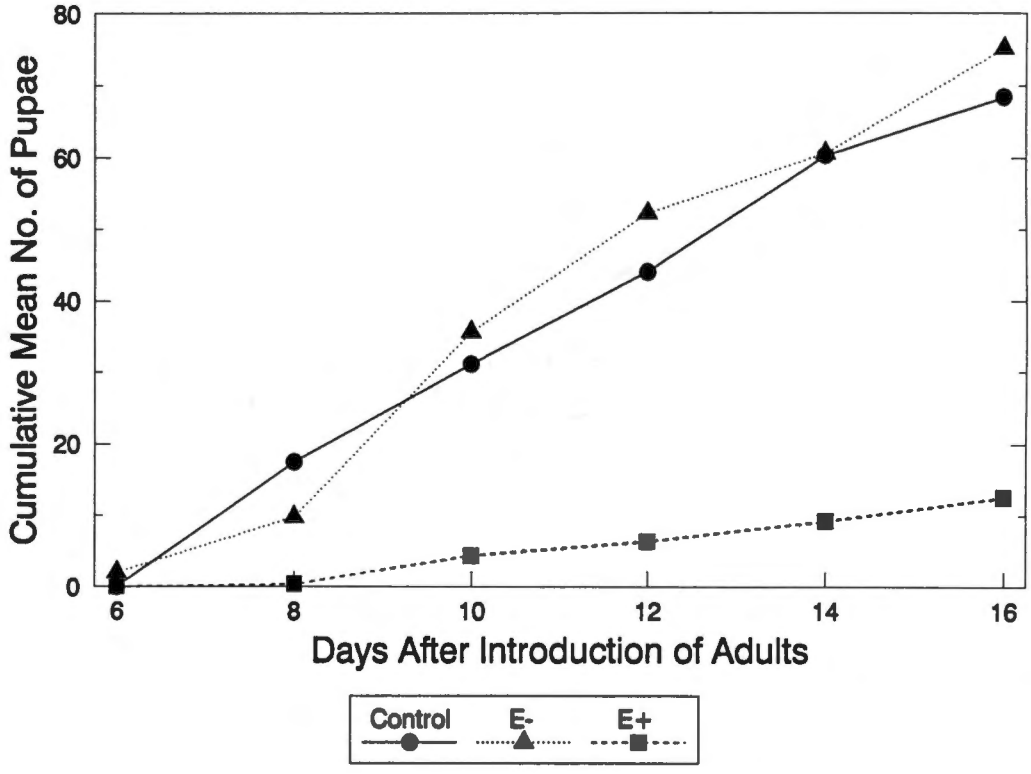

Fig. 2. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) root powder.

diets were not as extreme as on leaf diets; however, by the end of the experiment, survival was more than five times greater on E- root diet ($\bar{x}$ = 78) than on the E+ root diet ($\bar{x}$ = 15).

Survival of P_1 generation flies was not significantly different between E- and E+ treatments. In all experiments, F_1 flies emerged normally from pupae in all treatments, and adult flies appeared normal.

Leaf and Root Assay II -

As in Leaf and Root Assay I, few pupae ($\bar{x}$ = 1.4) developed on E+ leaf diet compared to the significantly higher number of pupae ($\bar{x}$ = 142.9) that developed on E- leaf diet. Significantly fewer pupae ($\bar{x}$ = 80.5) developed on E+ root diet than on E- root diet ($\bar{x}$ = 101.2) (Fig. 3). The number of pupae which developed on E- leaf diet was significantly higher than on the control ($\bar{x}$ = 68.5).

Leaf and Root Assay III -

Survival of larvae to the pupal stage by day 17 was significantly higher ($\bar{x}$ = 110.5) on E- leaf diet than on E+ leaf diet ($\bar{x}$ = 0.1). The numbers of pupae that developed on E+ or E- root diets from the greenhouse or field were not significantly different. Although they were not significantly different, the numbers of pupae that developed on E+ field root diet or E+ greenhouse root diet were

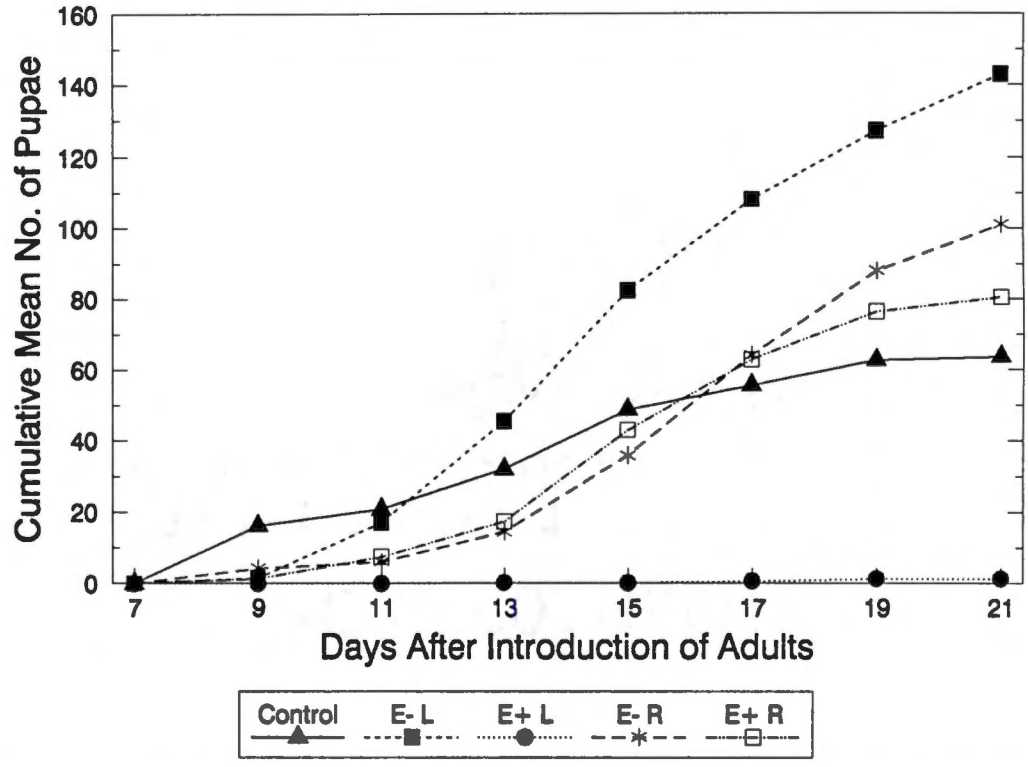

Fig. 3. Number of Drosophila melanogaster which developed from egg to pupa on control diet or diets containing endophyte-infected (E+) or endophyte-free (E-) leaf (L) or root powder (R).

consistantly lower than the number which developed on E- field or greenhouse root diets (Fig. 4).

Ovoposition Study -

D. melanogaster adult females laid significantly more eggs on E- leaf diet ($\bar{x}$ = 483.8) than on E+ leaf diet ($\bar{x}$ = 273.2) or the control ($\bar{x}$ = 212.8) (Fig. 5). The numbers of eggs laid on E+ leaf diet and control diet were not significantly different. The flies were not deterred from laying eggs on E+ leaf diet, but they produced approximately twice as many eggs on E- leaf diet.

ASSAYS INITIATED WITH NEONATE LARVAE

Leaf, Root, and Tiller Base Assay -

Significantly fewer ($\bar{x}$ = 5.3) pupae developed from 10 neonate larvae on E+ leaf diet than on E- leaf diet ($\bar{x}$ = 8.1). Larvae feeding on E+ tiller base diet resulted in significantly lower larval survival ($\bar{x}$ = 3.2) than feeding on E- tiller base diet ($\bar{x}$ = 8.8). The number of pupae which developed on E+ tiller base diet was also significantly lower than the number on E+ leaf diet (Fig. 6).

The numbers of pupae which developed on E- and E+ root diets were not significantly different from each other, contrary to results in Leaf and Root Assays I and II.

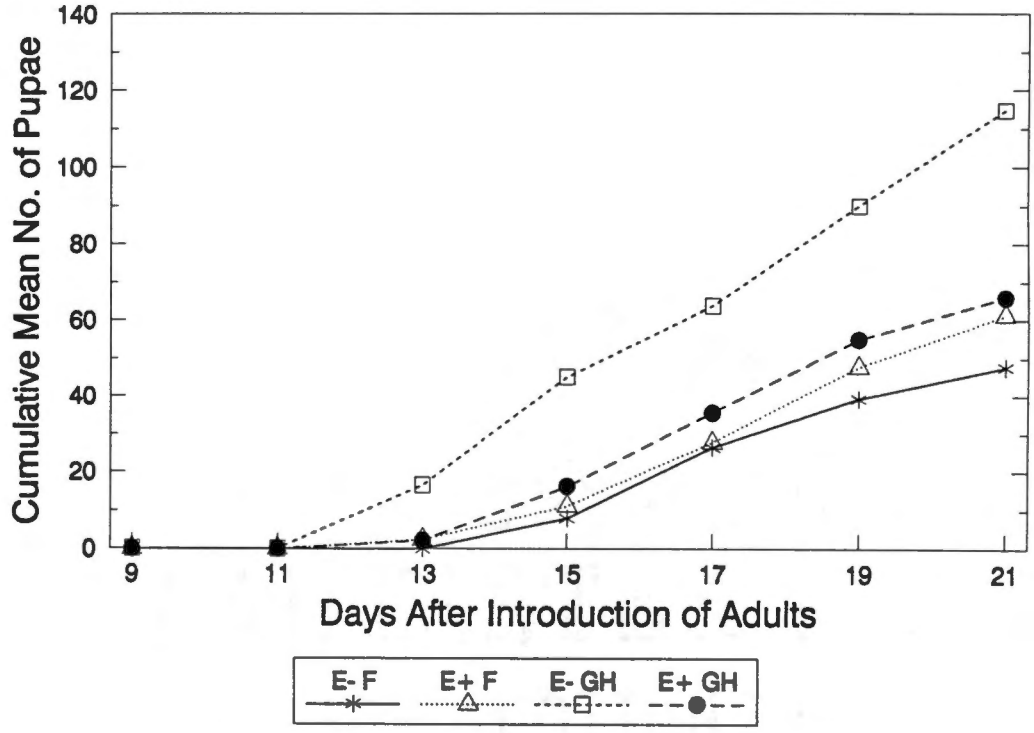

Fig. 4. Number of Drosophila melanogaster which developed from egg to pupa on diets containing powder made from endophyte-infected (E+) or endophyte-free (E-) tall fescue roots collected from the field (F) and greenhouse (GH).

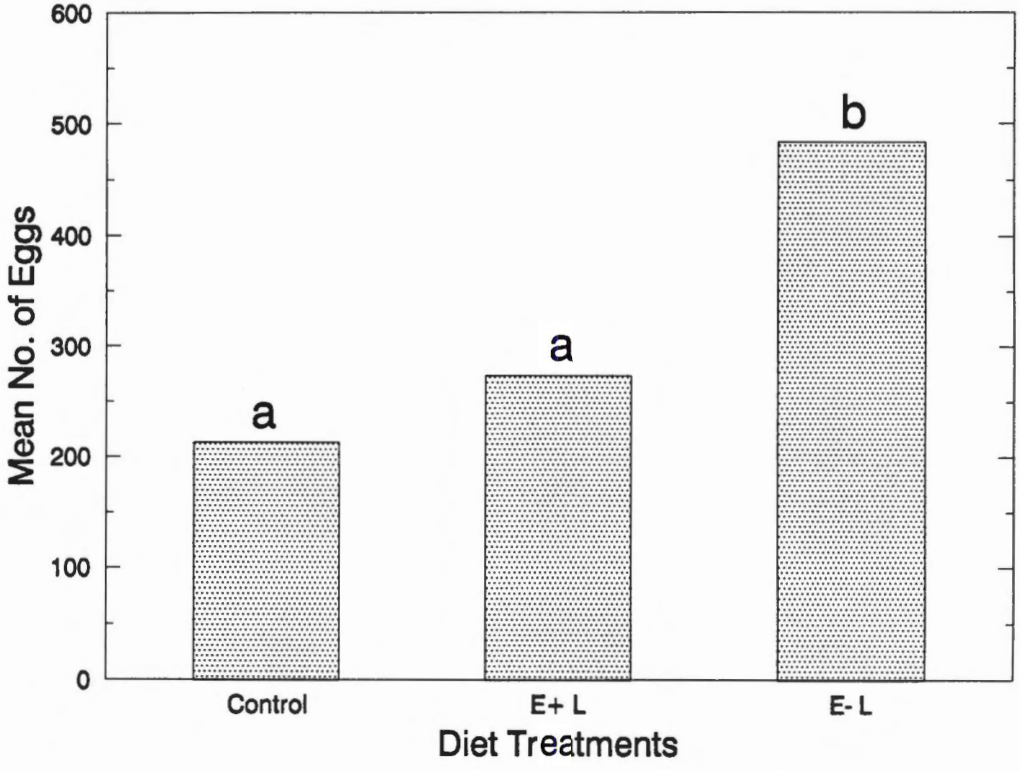

Fig. 5. Number of eggs deposited by adult Drosophila melanogaster onto the surface of diets containing endophyte-infected (E+) or endophyte-free (E-) tall fescue leaf powder (L) or control diet. Treatments with the same letter are not significantly different ($P > 0.05$; Duncan's multiple range test (SAS Institute, 1987)).

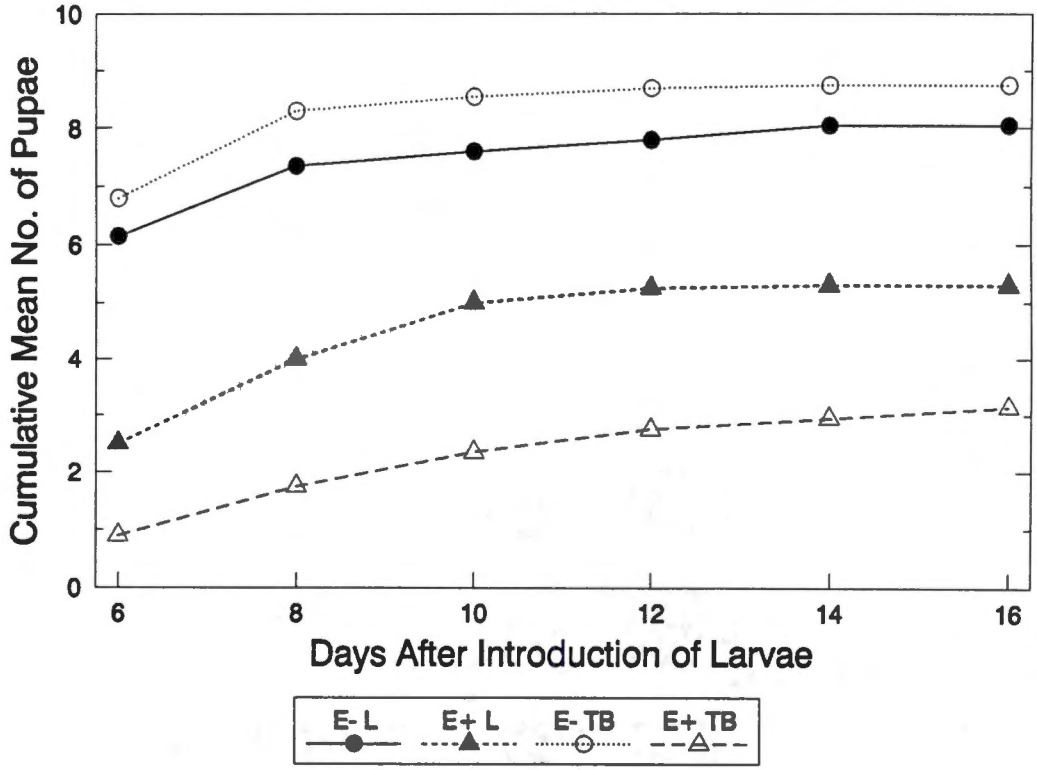

Fig. 6. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on diets containing powders made from endophyte-infected (E+) or endophyte-free (E-) tall fescue leaves (L) or tiller bases (TB).

Lolines, which have been identified from the roots of E+ tall fescue (personal communication, Potter), are water soluble and may have been removed from the roots as a result of vigorous washing. Root material collected from a greenhouse for use in Leaf and Root Assays I and II did not require vigorous washing. It is also possible that toxic compounds in leaves of E+ plants leached into the soil and were absorbed by the roots instead of being translocated into the roots from the tiller base.

Seed Assay -

Survival of larvae to the pupal stage on all diets containing E+ seed powder was significantly lower than survival on diets containing only E- seed powder or on the control diet (Fig. 7). No pupae developed on the diets containing the two highest concentrations of E+ seed powder, and less than 1 per cent of larvae developed to the pupal stage on the diet containing seed powder which was at least 50% E+. By day 16 of this assay only $\bar{x} = 6.25$ and $\bar{x} = 3.5$ pupae developed from 10 neonate larvae on diets containing seed powder which was 12.5% and 25% E+, respectively.

No larvae survived to pupate on diet mixtures which were made from seed containing non-viable Acremonium. Although this seed contained no viable endophyte, toxic compounds associated with endophyte infection were still present in the seed in high concentrations. Based on this

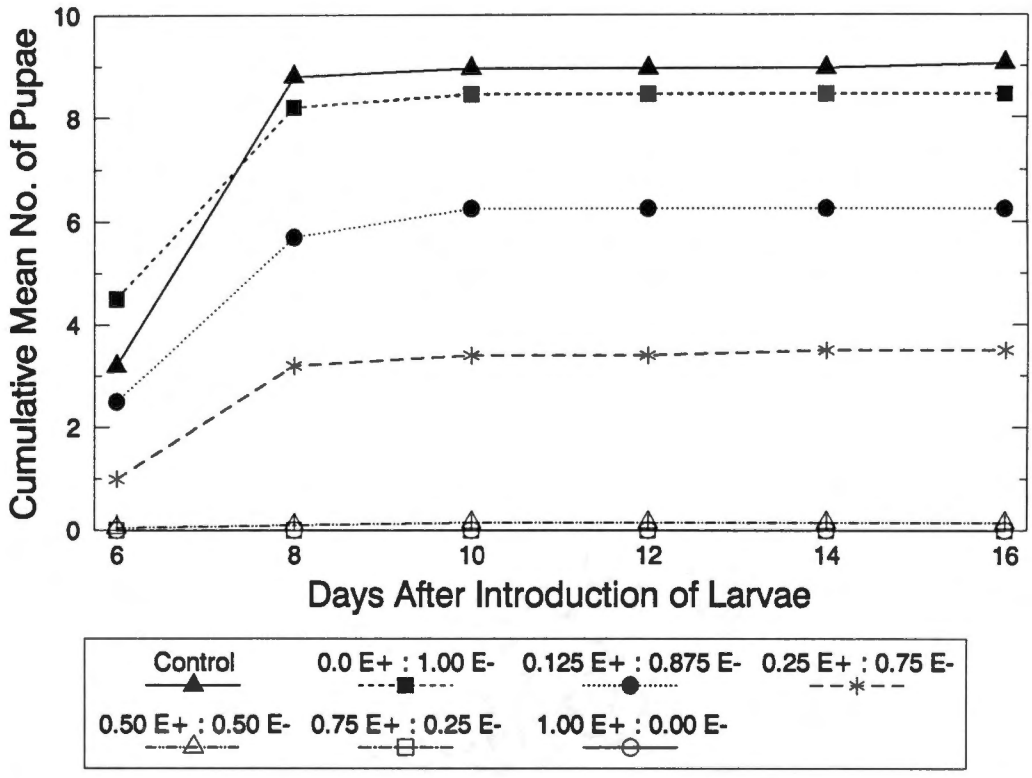

Fig. 7. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on control diet and diets containing 6 different ratios of endophyte-infected (E+) : endophyte-free (E-) tall fescue seed powder.

assay, the toxicity of compounds remaining in diets containing seed powder made from non-viable endophyte seed was equal to or greater than the toxicity of compounds in diets containing 50 to 100 % powder which was made from seed with viable endophyte.

ASSAY OF ALKALOIDS

ERGOT ALKALOIDS

Ergonovine Assay -

On day 6 of this assay, the number of larvae which reached the pupal stage on the highest concentration (dilution I) only, of ergonovine-amended diet was significantly lower than the number which developed on the methanol control (Fig. 8). On day 7 and throughout the remainder of the assay none of the ergonovine dilutions caused significant mortality.

Ergotamine Assay -

Unlike ergonovine, ergotamine caused significant mortality of Drosophila. On day 6 and until termination of this experiment, the number of pupae which developed on all dilutions of ergotamine-amended diet was significantly lower than the number which developed on methanol or tartrate

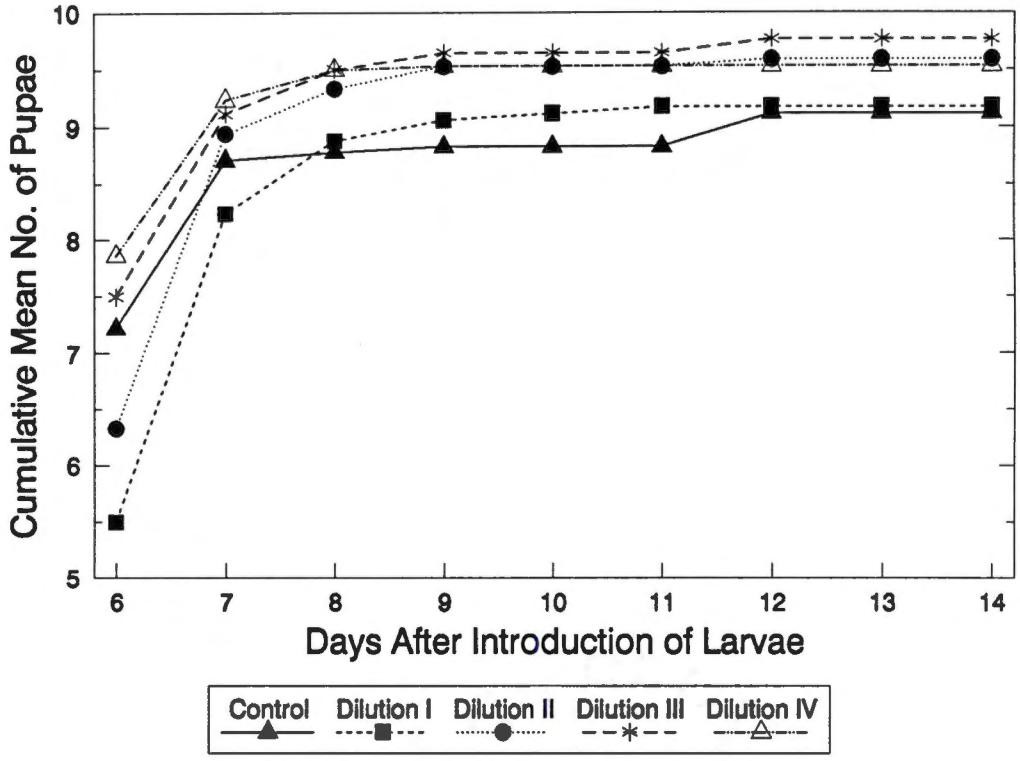

Fig. 8. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on control diet and diets containing 4 different dilutions of ergonovine.

controls (Fig. 9). Survival of larvae to the pupal stage on methanol and tartrate controls did not differ significantly.

Ergocryptine Assay -

At termination of the experiment, survival of larvae to the pupal stage was significantly lower on all dilutions of ergocryptine-amended diets than on the control diet (Fig. 10).

LOLINE ALKALOIDS

None of the N-acetylloline or loline dilutions caused significant larval mortality at the concentrations assayed. The highest concentration (dilution I) of N-formylloline caused significant larval mortality (Fig. 11). No pupae developed on that concentration; however, larval survival on all other dilutions was not significantly different from the control.

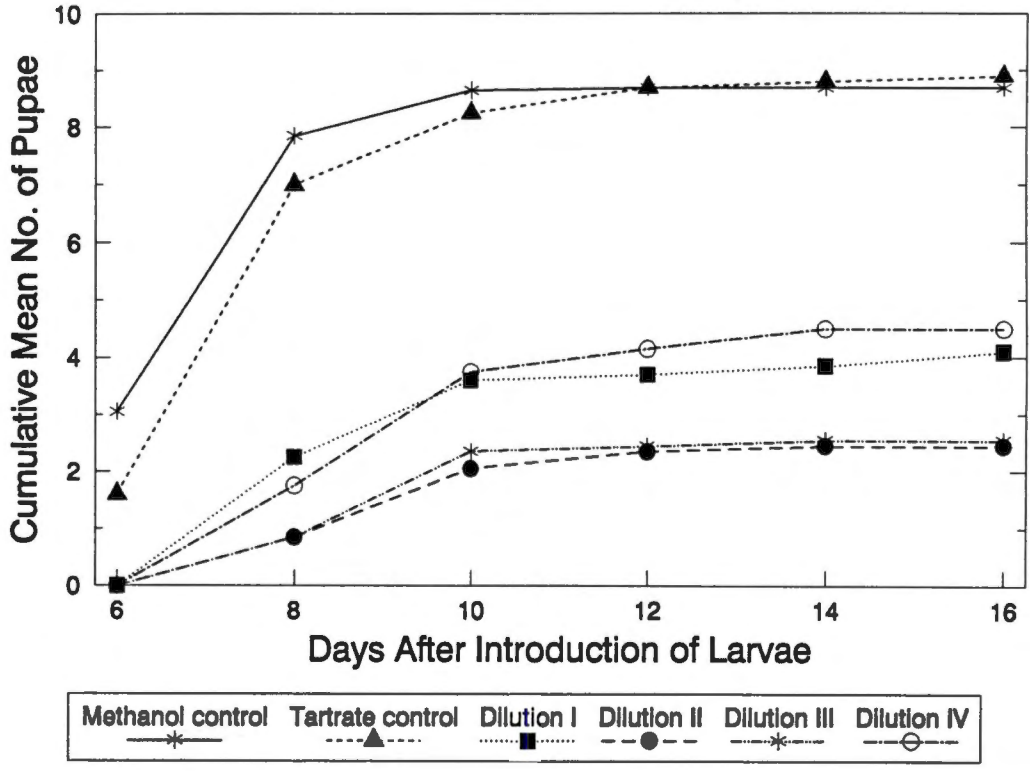

Fig. 9. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol and tartrate control diets and diets containing 4 different dilutions of ergotamine tartrate.

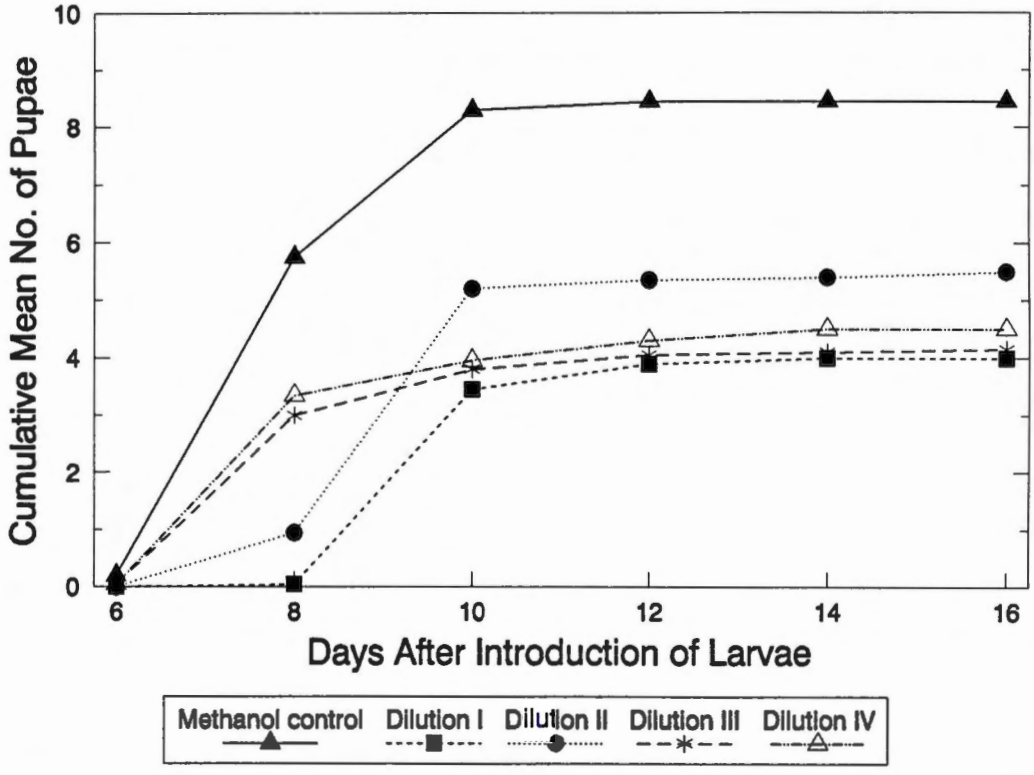

Fig. 10. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol control diet and diets containing 4 different dilutions of ergocryptine.

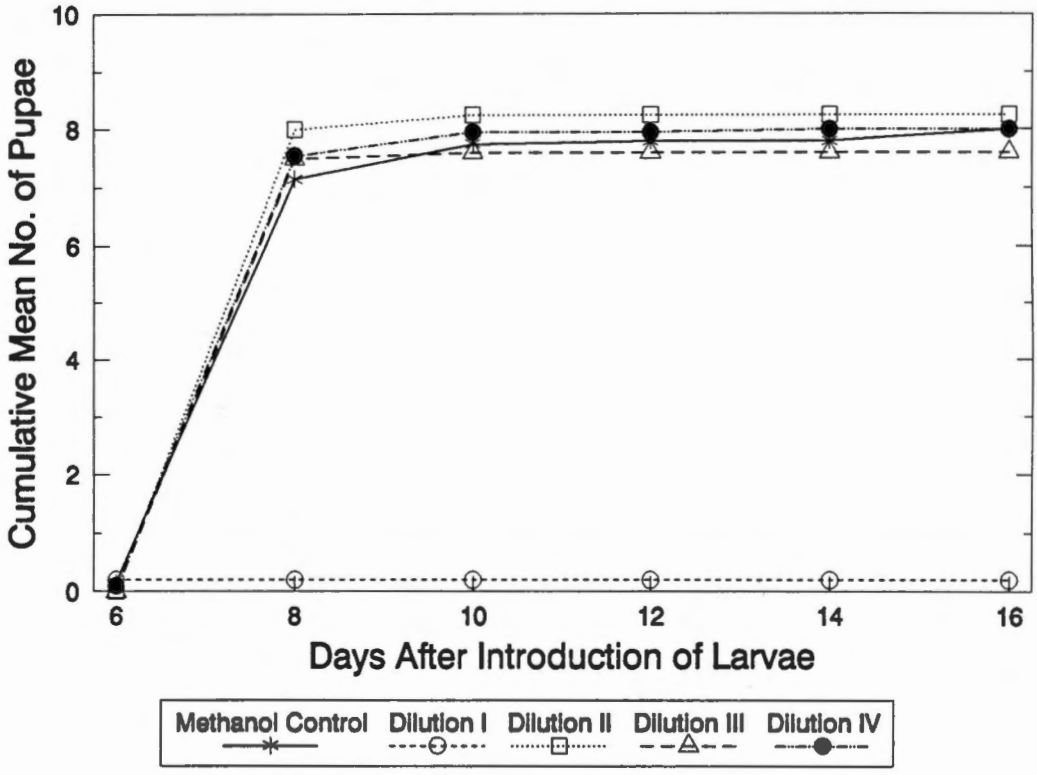

Fig. 11. Number of Drosophila melanogaster which developed from 10 neonate larvae to the pupal stage on methanol control diet and diets containing 4 different dilutions of N-formylloline.

CHAPTER IV

DISCUSSION

ASSAYS OF TALL FESCUE

Acremonium coenophialum in tall fescue is known to produce toxins that are insecticidal to many insects (Clay 1989). These toxins are produced in the tiller base and translocated into the leaves of the plant. Drosophila melanogaster did not survive on diets containing endophyte-infected tall fescue leaves that were collected from field plots in July.

D. melanogaster laid eggs on E+ leaf-amended diet, and the larvae which hatched were forced to inhabit and feed on that diet, resulting in their death. This inability to survive was not a result of reduced nutrition because larvae which fed on E- leaf diet were able to survive, pupate, and emerge normally as adults. Clay and Cheplick (1989) reported that lower larval weight of fall armyworm reared on E+ tall fescue leaves versus E- tall fescue leaves suggests that antibiosis may be the type of resistance expressed by E+ tall fescue to leaf-feeding insects. They also reported that neonate fall armyworm larvae were more sensitive to the presence of A. coenophialum in tall fescue than later instars. In general, older instars tend to be less

sensitive to antibiotic effects of allelochemical compounds (Reese 1983). Survival of D. melanogaster was also inhibited by E+ tall fescue roots collected from greenhouse plants. Although A. coenophialum has not been found in tall fescue roots, loline alkaloids do occur there (personal communication, Potter). Several insects have low survival when exposed to loline alkaloids (Yates 1989, Seigel et al. 1990, Johnson et al. 1985). It has not been determined how toxins produced by A. coenophialum arrive in tall fescue roots. They may be translocated into the roots from upper portions of the plant. It is also possible that toxins leach into the soil from the upper portion of the plant and are absorbed by the roots.

Consistently, in Leaf and Root Assays I, II, and III the number of pupae which developed from eggs in the controls was significantly lower than the number of pupae which developed on E- leaf diet. This indicated that adult Drosophila might be stimulated to lay more eggs on E- tall fescue leaf diet than on control diet. In the oviposition study adult Drosophila laid approximately two times more eggs on E- leaf diet than on the control diet or E+ leaf diet. E- grass-amended diets appear to possess a stronger ovipositional stimulus than do E+ leaf diet or commercial Drosophila diet. To eliminate this variable, the original procedure of introducing adult flies to diets used in Leaf and Root Assays I, II, and III was modified, and known

numbers of neonate larvae was used to infest treatment diets in all subsequent experiments.

There were no significant differences in survival of fruit fly larvae to the pupal stage on E- leaf and control diets when larvae were used. When equal concentrations of leaf, root, or tiller base powder were incorporated into Drosophila Medium and infested with Drosophila larvae, fewer larvae survived on E+ tiller base diet than on E+ leaf diet, which suggests that the concentration of toxic compounds is greater in the portion of the plant where the fungus thrives. Bacon et al. (1986) established that alkaloids are produced by A. coenophialum in the tiller base and are translocated into the leaves, but the concentration of alkaloids is higher in the tiller base. The number of larvae which survived on E+ and E- root diet were not significantly different from each other, as in Leaf and Root Assays I and II. E+ leaves used in Leaf and Root Assays I, II, and III were collected in early July; survival of larvae to the pupal stage on diets made with these leaves was lower than survival of larvae on diets amended with plant materials collected in late August. E+ tall fescue roots were not as toxic to D. melanogaster as E+ tall fescue leaves or tiller bases, which indicates that the toxic factors in leaves are more lethal than in roots. Although concentrations of toxic compounds are lower in tall fescue roots than in leaves, compounds there could contribute to

endophyte-mediated resistance to soil insects that inhabit E+ tall fescue.

When E+ tall fescue is growing vegetatively, the highest concentration of toxic compounds is found in the tiller base where the fungus occurs. On diets containing 1 g of E+ tiller base powder or 0.25 g of E+ seed powder, there was 35% larval mortality. Based on results of the Leaf, Root, and Tiller Base Assay, E+ tiller base had a greater concentration of toxins than did E+ leaves or roots. The results of Seed Assay verify that seeds contain a greater concentration of toxic factors than does the tiller base.

D. melanogaster larvae are very sensitive to low concentrations of endophyte-infected K-31 tall fescue seed powder in their diet. The seed bioassay could be refined and used to determine approximate per cent infestation of seed lots, with the assumption that presence of endophyte in seed results in approximately the same amounts of alkaloid per infected seed.

ASSAYS OF ALKALOIDS

Several leaf-feeding and soil-dwelling insects have been reported to be affected by E+ tall fescue (Johnson et al. 1985, Oliver et al. 1990, Seigel et al. 1990, Yates 1989). Alkaloids which have been identified in E+ tall

fescue may play a major role in endophyte-mediated resistance to these insects. Identification of insecticidal nature of specific compounds which occur in E+ tall fescue leaves and roots was successful using the Drosophila bioassay. The compounds were not assayed in combination, and there is a possibility that if some alkaloids were combined potentiation might occur.

ERGOT ALKALOIDS

The alkaloid dilutions which were incorporated into treatment diets were based on specific concentrations of alkaloids that have been reported to be found in E+ tall fescue foliage. The highest concentration (Dilution I) of ergonovine was 0.28 mg/g of diet. Concentration of both ergotamine and ergocryptine was 0.5 mg/g diet. These concentrations are approximately two orders of magnitude greater than the concentrations of ergot alkaloids reported in E+ tall fescue by Lyons et al. (1986); therefore, the concentration of Dilution III is approximately equal to the concentration of ergot alkaloids in E+ tall fescue plants.

All of the ergot alkaloids used in this study affected the development of D. melanogaster. Clay and Cheplick (1989) reported an antibiotic effect by ergonovine to fall armyworm, which caused significant decline in larval weight at 100 mg/liter of ergonovine without deterring larval

feeding. Ergonovine appears to have an antibiotic effect on D. melanogaster because it extends slightly the time required for development to the pupal stage. At its highest concentration, ergonovine had retarded the development of fruit fly larvae to the pupal stage six days after larval introduction. By day eight, the number of pupae which had developed on treated diet was equal to or greater than on the control, and development was similar in all treatments until termination of the assay. It is possible that larvae were initially deterred from feeding on the ergonovine-amended diets but then continued feeding and were able to complete their pupation at the same time as those on the control.

Ergotamine is a minor constituent of the total ergot alkaloid compounds found in E+ tall fescue; however, its chemical structure differs from ergovaline only in the R_2 group (Fig. 12). Ergovaline was not available for assay, but it is the principal ergot alkaloid in E+ tall fescue. Because these compounds are chemically similar, ergovaline may have similar effects on D. melanogaster. Ergotamine caused significant larval mortality at all dilution levels in the ergotamine-amended diets, and larval mortality was not significantly different between diets containing the highest and lowest concentrations of this compound.

Mode of action for ergotamine against the flies was not determined; both antibiosis and feeding deterrence are

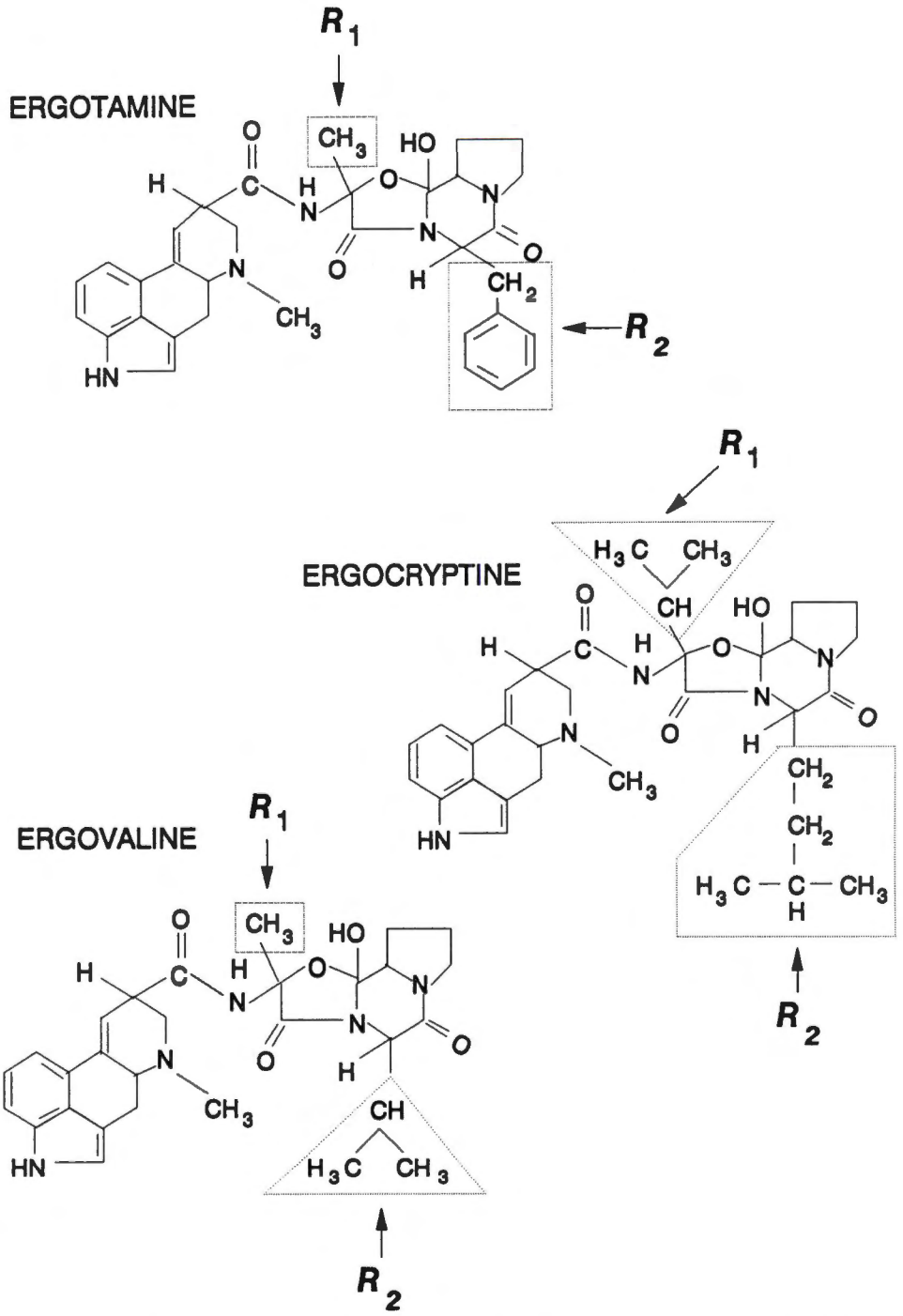

Fig. 12. Ergot alkaloid compounds with similar molecular structure, differing only in the composition of R group structure.

possibilities. Ergotamine has an antibiotic and antifeedant effect on fall armyworm (Clay and Cheplick, 1989). Although ergotamine is a minor constituent in E+ tall fescue, it may plays a role in resistance of E+ tall fescue to insect herbivores.

Ergocryptine does not occur in E+ tall fescue; however, its chemical structure is very similar to that of ergotamine and ergovaline (Fig. 12). By assaying ergocryptine, the portion of ergotamine's chemical structure which contributed to its insecticidal property could be investigated. Ergocryptine caused significant larval mortality at all concentrations as did ergotamine; therefore, the structural differences between the two compounds, as related to their toxicities, are insignificant. It is also probable that ergovaline would cause significant mortality of Drosophila larvae because of the similarities in chemical structure between it and ergocryptine. Yates et al. (1990) used ergocryptine as a substitute for ergovaline for assay by the large milkweed bug since ergovaline was not available commercially.

LOLINE ALKALOIDS

Loline, N-formylloline, and N-acetylloline alkaloids are present in the seed, foliage, and roots of E+ tall fescue. Robbins et al. (1972) reported concentrations of

loline alkaloids present in E+ tall fescue to be 2 mg/g of seed (dry weight) and 1.8 to 5.0 mg/g of foliage (dry weight). Highest concentrations of loline-type alkaloids tested in the Drosophila bioassay were equivalent to 2 mg of loline per g of diet.

Loline and N-acetylloline did not affect development of D. melanogaster at any of the dilutions assayed. N-acetylloline or loline could be toxic to Drosophila at higher concentrations and might contribute to endophyte-mediated resistance to insect herbivores since they can be present in foliage at concentrations twice as high as those assayed.

N-formylloline killed all D. melanogaster larvae at the concentration of 1.8 mg/g diet (dilution I); however, larvae which fed on the three lower concentrations developed normally. Threshold toxicity, therefore, falls between 0.18 mg/g and 1.8 mg/g diet. Like ergotamine, N-formylloline may contribute to endophyte-mediated resistance to insects. The specific threshold could be determined with further experimentation, which is beyond the scope of this research. Yates et al. (1989) reported that N-formylloline was highly toxic to the large milkweed bug. Other loline-type alkaloids (loline and N-acetylloline) were not assayed with large milkweed bug because total isolation of these alkaloids had not been accomplished at that time.

N-formylloline and N-acetylloline have similar chemical structures, differing only in the R_2 component (Fig. 13). The R_2 component of N-formylloline may be the insecticidal factor.

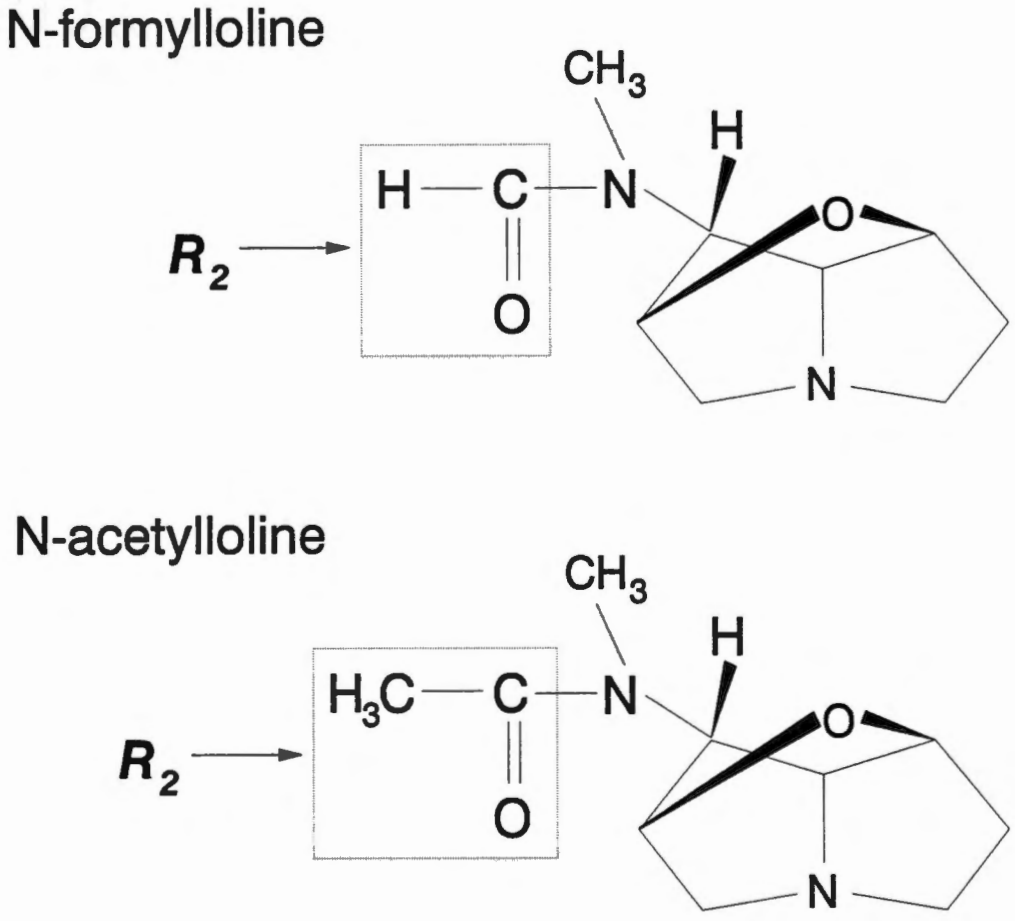

Fig. 13. Two loline-type saturated pyrrolizidine alkaloids with similar molecular structure, differing only in the composition of the R_2 group.

CHAPTER V

SUMMARY AND CONCLUSION

The common laboratory fruit fly, D. melanogaster is one of the most important organisms used in biological research of heredity, cytology, behavior and physiology (Demerec 1965). Drosophila is easy to maintain in an artificial environment, and it has a short life cycle and ability to produce many offspring. The fruit fly can also be anesthetized easily for handling and examination (Shorrocks 1972). For these reasons, D. melanogaster was used as the assay organism in these studies.

Much research has been conducted on endophyte-mediated resistance to leaf-feeding insects; however, resistance of E+ tall fescue to root-feeding insects has not been well documented. Often soil insects are difficult to rear in artificial environments and these insects can be used to assay live plant material only. The Drosophila bioassay was developed primarily for the detection of toxic compounds in roots of E+ tall fescue plants that may have an effect on soil insects.

D. melanogaster was reared successfully on diets amended with powders made from E- tall fescue leaves and roots. Fly larvae were forced to feed and remain in intimate contact with the diet mixtures until pupation. The

presence of toxic compounds in diets containing E+ tall fescue leaf and root powder caused significant mortality. The mechanism of resistance in E+ tall fescue to Drosophila has not been determined; both feeding deterrence and antibiosis have been suspected.

Flies were affected to a greater extent by E+ tiller base powder than E+ leaf powder, and both leaf and tiller base powder were more lethal to Drosophila than root powder. Flies were most sensitive to E+ seed-amended diets. Concentration of toxic compounds in E+ tall fescue is greatest in areas occupied by the fungus (i.e. the tiller base and seed).

The level of alkaloids in Acremonium-infected plants change throughout the year. Plant stress decreases in periods of adequate rainfall and mild temperatures; however, in the hot summer months, plant stress increases and the level of alkaloids increases. D. melanogaster exhibited greater mortality on diets containing E+ tall fescue powder prepared from plants collected from the field in July than from E+ plants collected in May. The seasonal differences of endophyte levels in tall fescue are currently under further investigation.

Many ergot and loline-type alkaloids are found in E+ tall fescue that are not produced in E- tall fescue. These compounds are toxic to several insects, including some species of aphids, large milkweed bugs, fall armyworm, and

European corn borer (Clay and Cheplick 1989, Johnson et al. 1985, Riedell et al. 1991, Yates et al. 1989). The early development of Drosophila larvae was retarded by ergonovine at the highest concentration tested, but did not cause significant mortality. Two ergopeptide alkaloids, ergotamine and ergocryptine caused significant larval mortality at concentrations equal to or less than those found in E+ plants. Although ergocryptine does not occur naturally in E+ tall fescue, its chemical structure is similar to that of ergotamine and ergovaline. Ergovaline was not available for testing, but it is the primary alkaloid in E+ tall fescue and could be a major contributor to endophyte-mediated resistance to insects. It is very likely that ergovaline would have the same effect on D. melanogaster as the other ergopeptide alkaloids tested.

N-formylloline was the only loline-type compound that affected the development of D. melanogaster at the concentrations tested, and it is present in foliage, seed, and roots of E+ tall fescue. The flies were not affected by N-acetylloline, but this loline alkaloid can be found in infected plants at concentrations higher than the concentrations tested. Riedell et al. (1991) reported that N-acetylloline reduced larval weight of fall armyworm and European corn borer larvae without modifying feeding behavior, but each insect was affected differently. Their

work suggests that different insect species may react differently to the same alkaloid.

Both N-formylloline and N-acetylloline have insecticidal activity. Lolines have been identified in the roots of E+ tall fescue (pers. comm., Potter) and probably affect soil insect species that inhabit E+ tall fescue. Unidentified toxins in E+ tall fescue may also be affecting insect populations.

The Drosophila bioassay provides a consistent method to detect toxins associated with Acremonium-infected tall fescue. It could be a reliable assay to screen pastures for endophyte infection and also to determine per cent infestation of Acremonium in seed lots.

REFERENCES CITED

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.

Bacon, C.W. 1988. Procedure for isolating the endophyte from tall fescue and screening isolates for ergot alkaloids. Appl. Environ. Microbiol. 54: 2615-2618.

Bacon, C.W., P.C. Lyons, J.K. Porter, and J.D. Robbins. 1986. Ergot toxicity from endophyte-infected grasses: a review. Agron. J. 78:106-116.

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.

Barker, G.M.,R.P. Pottinger, P.J. Addison, R.A. Prestidge. 1984. Effect of Lolium endophyte fungus infections on behavior of adult Argentine stem weevil. New Zealand J. Agric. Res. 27: 271-277.

Barker, G.M., R.P. Pottinger, and P.J. Addison. 1983. Effect of tall fescue and ryegrass endophytes on Argentine stem weevil. pp. 216-219. In Proceedings, 36th New Zealand Weed Pest Control Conf.

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.

Blank, C.A., K.D. Gwinn, A.M. Gavin. 1991. Acremonium coenophialum infestation levels of tall fescue seed lots affect seedling losses due to Rhizoctonia spp. Phytopathology. (abstr.) (in press).

Buckner, R.C. and L.P. Bush [ed.]. 1979. Tall Fescue. Crop Science Society of America, Inc. Madison, WI. 351 pp.

Bush, L.P. and R.C. Buckner. 1973. Tall fescue toxicity. pp. 99-112. In Anti-quality components of forages. Crop Science Society of America, Inc. Madison, WI. 140 pp.

Clay, K. 1989. Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. Mycol. Res. 92: 1-12.

Clay, K., G.P. Cheplick. 1989. Effect of ergot alkaloids from fungal endophyte-infected grasses on fall armyworm (Spodoptera frugiperda). J. Chem. Ecol. 15: 169-182.

Clay, K. 1987. Effects of fungal endophytes on the seed and seedling biology of Lolium perenne and Festuca arunndinacea. Oecologia (Berlin) 73: 358-362.

Clay, K., T.N. Hardy, and A.M. Hammond, Jr. 1985. Fungal endophytes of grasses and their effects on an insect herbivore. Oecologia (Berlin) 66: 1-5.

Conger, B.W. and J.K. McDaniel. 1983. Use of callus cultures to screen tall fescue seed samples for Acremonium coenophialum. Crop Science 23: 172-174.

Cowan, J.R. 1956. Tall fescue, pp. 283-320. In Advances in Agronomy, Vol. VIII. Academic Press, Inc. Publishers. N.Y. 423 pp.

Demerec, M. 1965. Biology of Drosophila. Hafner Publishing Company, N.Y. and London. 632 pp.

Ford, V.L., and T.L. Kirkpatrick. 1989. Effects of Acremonium coenophialum in tall fescue on host disease and insect resistance and allelopathy to Pinus taeda seedlings. Arkansas Experiment Station Special Report 140. pp. 32.

Fribourg, H.A., C.S. Hoveland, and K.D. Gwinn. 1991. Tall fescue and fungal endophyte -- a review of current knowledge. Tennessee Farm and Home Science. (in press).

Funk, C.R., P.M. Halisky, M.C. Johnson, M.R. Siegel, A.V. Stewart, S. Ahmad, R.H. Hurley, and I.C. Harvey. 1983. An endophytic fungus and resistance to sod webworms: association in Lolium perenne L. Bio/Technol. 1: 189-191.

Gwinn, K.D., R.N. Trigiano, A.M. Gavin, and B.V. Conger. 1991. Control and identification of a bacterium interfering with in vitro assays of tall fescue seeds for Acremonium coenophialum. Crop Science. (in press).

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.

Hoveland, C.S. 1991. Importance and economic significance of the Acremonium endophytes to performance of animals and grass plants. J. Agric. Ecosys. Environ. (in press).

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. 1983. Steer performance and association of Acremonium coenophialum fungal endophyte on tall fescue pasture. Agron. J. 75: 821-824.

Hoveland, C.S., R.L. Haaland, C.C. King, Jr., W.G. Anthony, 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 pastures. Agron. J. 72: 1064-1065.

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

Jones, T.A., R.C. Buckner, P.B. Burrus, II, and L.P. Bush. 1983. Accumulation of pyrrolizidine alkaloids in benomyl-treated tall fescue parents and their untreated progenies. Crop Science 23: 1135-1140.

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

Latch, G.C.M., Christensen, M.J., Gaynor, D.L. 1985. Aphid detection of endophytic infection in tall fescue. New Zealand J. Agric. Res. 28: 129-132.

Lewis, D.H. 1973. Concepts in fungal nutrition and the origin of biotrophy. Biol. Rev. 48: 261-278.

Long, E.A. and J.W. Hilty. 1985. Occurence of Acremonium coenophialum in tall fescue in Tennessee. Plant Disease 69: 467-468.

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

Lyons, P.C., and C.W. Bacon. 1985. Growth of the tall fescue endophyte into seedlings as related to temperature and sheath differentiation. Phytopathology 75: 501.

Mathias, J.K., R.H. Ratcliffe, and J.L. Hellman. 1990. Association of an endophytic fungus in perennial ryegrass and resistance to the hairy chinch bug (Hemiptera: Lygaeidae). J. Econ. Entomol. 83: 1640-1646.

Morgan-Jones, G. and W. Gams. 1982. Notes on hyphomycetes. XLI. Mycotaxon 15: 311-318.

Oliver, J.B., C.D. Pless, and K.D. Gwinn. 1990. Effect of endophyte, Acremonium coenophialum, in 'Kentucky 31' tall fescue, Festuca arundinacea, on survival of Popillia japonica. pp. 173-175. In S.S. Quisenberry and R.E. Joost [eds.], Proceedings, International Symposium on Acremonium/Grass Interactions. Louisiana Agric. Exp. Sta. Baton Rouge.

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 ryegrass and tall fescue. New Zealand J. Agric. Res. 28: 87-92.

Prestidge, R.A., R.P. Pottinger, and G.M. Barker. 1982. An association of Lolium endophyte with ryegrass resistance to Argentine stem weevil. pp. 199-222. In Proceedings of the 35th New Zealand Weed and Pest Control Conference.

Reddick, B.B. and M.H. Collins. 1988. An improved method for detection of Acremonium coenophialum in tall fescue plants. Phytopathology 78: 418-420.

Riedell, W.E., R.E. Kieckhefer, R.J. Petroski, and R.G. Powell. 1991. Naturally-occuring and synthetic loline alkaloid derivatives: insect feeding behavior modification and toxicity. J. of Entomol. Science. 26: 122-129.

Reese, J.C. 1983. Nutrient-allelochemical interactions in host plant resistance. pp. 231-243. In P.A. Hedin [ed.], Plant resistance to insects. American Chemical Society. Wash. D.C.

Robbins, J.D., J.G. Sweeny, S.R. Wilkinson, and D. Burdick. 1972. Volitile alkaloids of Kentucky 31 tall fescue seed (Festuca arundinacea Schreb.). J. Agric. Food Chem. 20: 1040-1043.

SAS Institute. 1987. SAS user's guide: statistics, version 5 ed. SAS Institute, Cary, N.C.

Schmidt, S.P., C.S. Hoveland, E.M. Clark, N.D. Davis, L.A. Smith, H.W. Grimmes, and J.L. Holliman. 1982. Association of an endophytic fungus with fescue toxicity in steers feed Kentucky 31 tall fescue seed or hay. J. Anim. Sci. 55: 1259-1263.

Shelby, R.A., and L.W. Dalrymple. 1987. Incidence and distribution of the tall fescue endophyte in the United States. Plant Disease 71: 783-786.

Shorrocks, B. 1972. Drosophila. Ginn Lmt. London. pp. 144.

Siegel, M.R., G.M.C. 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 acculmulation and aphid response. J. Chem. Ecol. 16: 3301-3315.

Siegel, M.R., G.C.M. Latch, and M.C. Johnson. 1987. Fungal endophytes of grasses. Ann. Rev. Phytopathology 25: 293- 315.

Siegel, M.R., M.C. Johnson, D.R. Varney, W.C. Nesmith, R.C. Buckner, L.P. Bush, P.B. Burrus, II, T.A. Jones, and J.A. Boling. 1984. A fungal endophyte in tall fescue: incidence and dissemination. Phytopathology 74: 932-937.

Stuedemann, J.A. and C.S. Hoveland. 1988. Fescue endophyte: history and impact on animal agriculture. J. Prod. Agric. 1: 49-44.

Tabor, Paul. 1952. Tall fescue grows up. Crop Soils. 4: 9-11.

Welty, R.E. and R.E. Barker. 1990. Reaction of tall fescue cultivars innoculated with stem rust. Phytopathology 80: 1043. (abstr.).

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 and Soil 112: 3-6.

White, J.F. Jr. and G.T. Cole. 1985. Endophyte-host associations in forage grasses. In vitro inhibition of fungi by Acremonium coenophialum. Mycologia 77: 487-498.

Yates, S.G., R.J. Petroski, and R.G. Powell. 1990. Analysis of loline alkaloids in endophyte-infected tall fescue by capillary gas chromatography. J. Agric. Food Chem. 38: 182-185.

Yates, S.G., J.C. Fenster, and R.J. Bartelt. 1989. Assay of tall fescue seed extracts, fractions, and alkaloids using the large milkweed bug. J. Agric. Food Chem. 36: 337-340.

APPENDIX

Preparation of 0.05 M Phosphate Buffer

Into 1 liter of distilled, deionized water, 3.40 g of potassium phosphate monobasic and 6.71 g of sodium phosphate dibasic heptahydrate were added. The pH was adjusted to 6.4 with concentrated hydrocloric acid. The solution was autoclaved at 121°C for 25 minutes.

VITA

Amy Michelle Cole was born in Cincinnati, Ohio, on December 22, 1966. She graduated from Lakota High School located in Westchester, Ohio, in May of 1985. In May, 1989, she received the Bachelor of Science Degree in Biology from Heidelberg College, Tiffin, Ohio. Amy entered the Master's program in the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville, as a graduate research assistant in July 1989. She received the Master of Science degree August 9, 1991.

The author is a member of the Entomological Society of America, Tennessee Entomological Society, Georgia Entomological Society, and Michigan Entomological Society, as well as, Phi Kappa Phi National Academic Honor Society, Gamma Sigma Delta Agricultural Society, Omicron Delta Kappa National Leadership Honor Society, and Beta Beta Beta Biology Honor Society. Miss Cole intends to pursue a Ph. D. degree in Entomology.