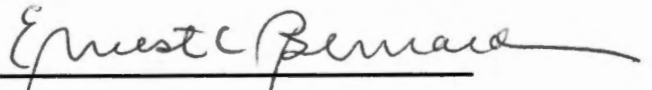
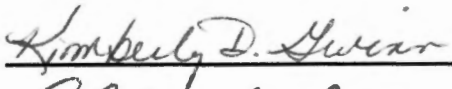
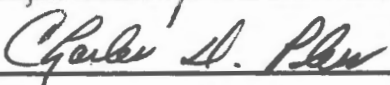
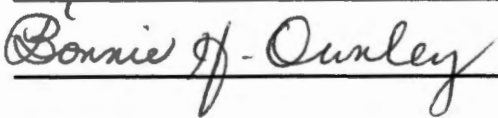


To the Graduate Council:

I am submitting herewith a thesis written by Jeanese Holmes Reiss entitled "Endophyte-mediated resistance of tall fescue to *Pratylenchus* species: population dynamics and refutation of a putative host defense strategy." 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.


Ernest C. Bernard, Major Professor

We have read this thesis and
recommend its acceptance:

Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**ENDOPHYTE-MEDIATED RESISTANCE OF TALL FESCUE
TO *PRATYLENCHUS* SPECIES:
POPULATION DYNAMICS AND REFUTATION OF A
PUTATIVE HOST DEFENSE STRATEGY**

A Thesis
Presented for the
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Jeanese Holmes Reiss
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Abstract

The population dynamics of *Pratylenchus scribneri* and *P. agilis* in endophyte-infected (E+) and endophyte-free (E-) tall fescue were examined with particular attention given to possible reproduction suppression or enzymatic degradation of eggshells. E+ and E- clonal pair tillers of tall fescue were planted in Conetainers and inoculated with 1,000 vermiform *P. scribneri* or *P. agilis* per Conetainer. *P. scribneri* trials were incubated at ambient temperature (ca. 25°C) and included a 14-week short-term study and a 30-week extended study. Short-term *P. agilis* trials were incubated at 30°C or 16.5°C to examine effects of temperature on endophyte-mediated resistance. Roots were harvested at 20, 40, and 60 days after inoculation, and the number of eggs and vermiform nematodes in each root system was counted. The first 50 vermiform nematodes or as many as possible up to 50, were dissected from each root system, mounted, and traced. The life-stage of traced nematodes was determined based on length and presence of a vulva. At all harvest dates during the short-term study, the mean number of vermiform *P. scribneri* in E+ roots was significantly lower than in E- roots. A persistent difference could not be established between *P. scribneri* populations in E+ and E- tall fescue during the extended study. Life-stage distributions during both the short-term and extended studies seemed to indicate a decrease in juvenile development in E+ tall fescue. Adult

P. scribneri females were extracted from two E+/E- root systems for three consecutive harvests, surface-sterilized, and added singly to alfalfa callus cultures to determine reproductive capability. Females from both E+ and E- root systems reproduced in callus culture. *P. scribneri* eggs were exposed to bacterial chitinase solutions and examined with TEM to determine if increased levels of chitinase in E+ tall fescue roots might damage the integrity of eggshells. No difference was found between enzyme-treated eggs and control eggs. There was no significant difference between *P. agilis* populations in E+ and E- tall fescue incubated at 16.5°C. Life-stage distributions were similar and indicated reduced reproduction and development, probably due to the low temperature. Only three tall fescue tillers retained the endophyte after 60 days at 30°C. *P. agilis* populations were lower in the three E+ tillers; however, statistical analyses were not conducted due to the small sample size.

P. scribneri and *P. agilis* populations were negatively impacted by the presence of the endophytic fungus in tall fescue. The reproductive capability of female *P. scribneri* in E+ tall fescue was not permanently disrupted, nor was eggshell integrity compromised by exposure to bacterial chitinase. Slowed juvenile development may be partially responsible for the reduction in *Pratylenchus* populations in E+ tall fescue. Endophyte-mediated resistance is likely a complex of several mechanisms.

Table of Contents

Chapter	Page
1. Introduction.	1
2. Materials and Methods.	11
3. Results and Discussion.	19
4. Conclusion.	44
Bibliography.	48
Appendix.	56
Vita.	59

List of Figures

Figure		Page
1.	Mean number of vermiform <i>P. scribneri</i> per root system in E+/E- tall fescue tillers for the short-term study.	20
2.	Mean number of <i>P. scribneri</i> eggs per root system in E+/E- tall fescue tillers for the short-term study.	20
3.	Gravid female from the short-term E+ tall fescue 20 days after inoculation.	21
4.	<i>P. scribneri</i> life-stage distribution for the short-term study (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.	24
5.	Mean vermiform <i>P. scribneri</i> per root system for Series 1-3 of the extended study.	26
6.	Fluctuations in processed water temperature (provided by Knoxville Utility Board) as compared with fluctuations in	

Figure	Page
<i>P. scribneri</i> populations in E+ and E- tall fescue during the extended study.	28
7. <i>P. scribneri</i> life-stage distributions for Series 1 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.	29
8. <i>P. scribneri</i> life-stage distributions for Series 2 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.	30
9. <i>P. scribneri</i> life-stage distributions for Series 3 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.	31
10. Mean number of vermiform <i>P. scribneri</i> per root system as affected by increased tiller "age" at time of inoculation.	33
11. Transmission electron micrographs of centrifuged <i>P. scribneri</i> eggs	36

Figure	Page
12. Transmission electron micrographs of <i>P. scribneri</i> eggs exposed to (A) chitinase, (B) water, or (C) buffer.	39
13. Mean number of vermiform <i>P. agilis</i> per root system in E+ and E- tall fescue incubated at 16.5°C.	42
14. <i>P. agilis</i> life-stage distributions (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation when incubated at 16.5°C.	43

Chapter 1

Introduction

Plant-parasitic Nematodes

Plant-parasitic nematodes were first observed by Needham in 1743 in blighted wheat kernels. Following this initial discovery, little progress was made in the field of nematology until the latter half of the nineteenth century when nematodes were found in association with several other plant diseases (Dowler, 1984). In the span of 49 years from 1941 to 1990, the number of cited genera of plant-parasitic nematodes increased from 19 to 207 and the number of species increased from 185 to 4,832 (Nickle, 1991). Sigeo (1993) cites nematodes as being second only to fungi in the percent occurrence of agents involved in diseases of select soft fruits, cereals, root crops, and ornamentals. Worldwide losses in life-sustaining and other economically important crops due to nematodes are estimated at 11-14% annually (Agrios, 1997). In the U.S. alone, total annual losses in agricultural production are estimated at 7%, even with the widespread use of various chemical and non-chemical controls (USDA Agriculture Handbook, 1986).

Most plant-parasitic nematodes are grouped in class Secernentea, order Tylenchida: microscopic, root-parasitizing nematodes that feed by means of a hollow stylet inserted into the host cells. Feeding behavior may be characterized as ecto- or endoparasitic (depending on whether the nematode partially or

completely enters the root) and migratory or sedentary (depending on the mobility of the adult female). Physical damage and plant-physiological changes caused by nematodes may result in characteristic root symptoms ranging from necrosis to hypertrophy and hyperplasia. Typically, above-ground symptoms are not obviously different from the symptoms caused by other biotic or abiotic factors and may include stunting, yellowing of the foliage, and reduced yields.

Due to their migratory nature and the complexities of the soil environment, safe, effective, and economically feasible control of nematodes is difficult to achieve. The greatest breakthrough in nematode control occurred in the late 1940s with the introduction of the first commercially available nematicides. The use of nematicides increased dramatically through the 1950s to mid-1970s. However, during the mid to late 1970s, concerns about environmental contamination and mammalian toxicity led to the removal of several nematicides from the market. A series of studies conducted in the 1980s revealed that many shallow-water aquifers as well as numerous individual domestic and municipal wells in the USA were contaminated with nematicidal compounds (Stirling, 1991). In the 1998 Insect and Plant Disease Control Manual published by the University of Tennessee, 12 pesticide chemicals were listed for use against nematodes. Eight of these 12 nematicides were classified with Level 1 toxicity, indicating the lethal oral dosage for an adult human ranges from a few drops to 5 milliliters.

Several nematicides are restricted and require the supervision of a certified applicator to ensure that proper safety and environmental protocols are

followed during application. In addition to safety concerns, the narrow profit margin with which most farmers are confronted restricts widespread and repeated use of chemicals. Alternatively, farmers are urged to integrate the use of biological control agents, resistant hosts, various cultural practices, and crop rotation with limited use of nematicides. Two of the integrated pest management tactics, biological control and host resistance, are being extensively investigated. Both methods have the potential to provide safe, long-term, and economically feasible means of nematode control.

Stirling (1991) defines biological control as:

A reduction of nematode populations which is accomplished through the action of living organisms other than nematode-resistant host plants, which occurs naturally or through the manipulation of the environment or the introduction of antagonists.

Several experiments have shown that the introduction or enhancement of nematode-parasitizing fungi and bacteria can effectively reduce soil-borne populations of plant-parasitic nematodes. Mian et al. (1982) demonstrated that amending soil with chitin reduced the amount of root galling in summer crookneck squash by increasing populations of fungi parasitic to nematode eggs. Timper and Brodie (1994) found that the presence of the nematode-pathogenic fungus *Hirsutella rhossiliensis* Minter & Brady reduced root penetration and population increase of *Pratylenchus penetrans* Cobb in potato. However, no fungus-infected nematodes were observed within the potato roots, indicating that control was limited to

nematodes contacted during migration through the soil. In general, predators and parasites can attack only exposed nematodes. Due to this limitation, endoparasitic nematodes are not easily targeted by biocontrol organisms. *Pasteuria penetrans* (ex Thome) Sayre and Starr is the only parasite known to consistently infect endoparasitic stages of nematodes; however, spores of this parasite must adhere to the nematode before the nematode enters host roots. Migratory endoparasitic nematodes such as *Pratylenchus* spp. may progress through several generations within host roots without an appreciable number of nematodes re-entering the soil, thereby completely avoiding contact with parasites or antagonists (Stirling, 1991).

Nematode populations may also be limited through host plant resistance. Resistance is the active or passive suppression of nematode reproduction by host genes (Trudgill, 1991; Zijlstra et al., 1997). Passive resistance mechanisms affect egg-hatch, survivability, location of food source, or host suitability. Active resistance mechanisms lead to rapid localized cell necrosis and arrest of nematode development (Giebel, 1974; Kaplan and Davis, 1987). Natural host resistance may occur as one of two forms, major gene resistance or quantitative resistance. Major gene resistance prevents replication of avirulent nematode populations. Quantitative resistance, which can be affected by a variety of environmental factors (moisture, temperature, nutrient status of soil), may

allow low rates of multiplication. Major gene or quantitative resistance may be introduced into susceptible cultivars through controlled genetic manipulation (Williamson and Hussey, 1996; Zijlstra et al., 1997).

Typically, plant-parasitic nematodes invade resistant and nonresistant plants indiscriminately with host recognition and defense activation following invasion of a resistant host.

***Pratylenchus* spp.**

The experimental studies addressed in this thesis involve the migratory endoparasites *Pratylenchus scribneri* Steiner and *P. agilis* Thorne & Malek. As of 1991, there were approximately 70 described species within the genus *Pratylenchus*, commonly referred to as root-lesion nematodes due to reddish-brown lesions sometimes formed in infected roots. Members of this genus share a highly uniform morphology. They are stout-bodied nematodes with adult lengths ranging from 0.3 to 0.9 mm. The head is low with heavily sclerotized cephalic framework and is not offset from the neck. Stylets in this genus are short and stout, usually 14-17µm in length, with basal knobs. *Pratylenchus scribneri* and *P. agilis* are closely related species. Members of both species exhibit smooth, broadly rounded tail termini. Males of both species are rare and reproduction usually occurs via parthenogenesis. Eggs are laid singly within infected tissues or in the soil. The eggshell consists of three layers: an outer vitelline layer, a middle chitinous layer, and an inner lipid layer (Wharton, 1980).

In tylenchid nematodes, the vitelline layer is a unit-membrane-like structure composed of lipoprotein. Electron microscopy has revealed strands of particulate material adhered to its outer surface (Bird and McClure, 1976). The function of the vitelline layer is unknown. The middle chitinous layer consists of chitin microfibrils surrounded by a protein matrix. This layer provides structural strength for the eggshell and prevents mechanical damage to the inner lipid layer and developing embryo. The inner lipid layer is composed of lipid and protein and contains a series of lipoprotein membranes. The lipid layer acts as an impermeable barrier to most chemicals and may protect against desiccation (Bird and Bird, 1991; Bird and McClure, 1976; Wharton, 1980). Within the egg, an embryo develops into a first-stage juvenile, then undergoes its first molt prior to hatching as a second-stage juvenile. After hatching, a second-stage juvenile may begin feeding on available host tissue or may migrate through the soil in search of a suitable host. As with most *Pratylenchus* spp., *P. agilis* and *P. scribneri* are polyphagous (Nickle, 1991; Loof, 1978).

Endophyte-mediated Resistance of Tall Fescue to *Pratylenchus* spp.

In 1941, the University of Kentucky released 'Kentucky 31', a long-lived, high-producing, drought-resistant tall fescue (*Festuca arundinacea* Schreb.) cultivar. By 1991, 1.4 million hectares in Tennessee had been seeded to 'Kentucky 31'. As land area seeded to 'Kentucky 31' increased across the southern U.S., so did the occurrence of tall fescue toxicosis- a debilitating

condition in cattle that greatly resembles ergot poisoning. In 1941, a New Zealand journal reported the presence of an endophytic fungus in tall fescue; however, the relationship between endophyte-infection and tall fescue toxicosis was not recognized until the late 1970s (McLaren and Fribourg, 1991). During the 1970s, experiments conducted in Georgia and Alabama confirmed the association between tall fescue toxicosis and consumption of endophyte-infected tall fescue (Fribourg et al., 1991a). Classified as *Neotyphodium coenophialum* (Morgan-Jones & Gams) Glenn, Bacon & Hanlin, this fungus is considered an anamorphic derivative of the family Clavicipitaceae, tribe Balansieae (Glenn et al., 1996). The fungus resides between leaf sheath cells and in seeds of tall fescue. Much like the related *Claviceps*, the tall fescue endophyte produces toxic alkaloids, including ergine and ergovaline, which cause ergotism-like symptoms in cattle grazing on infected pastures (Cornell et al., 1990; Fribourg et al., 1991b). Endophyte-infected tall fescue is drought-tolerant and resistant to a variety of pathogens and pests, including several plant-parasitic nematodes (Clay, 1991; Fribourg et al., 1991a; McLaren and Fribourg, 1991; Oliver et al., 1990; West et al., 1987, 1990).

Naturally occurring soil populations of *P. scribneri* were substantially lower in field plots of endophyte-infected (E+) tall fescue when compared with similar plots of endophyte-free (E-) tall fescue (West et al., 1987, 1990). In two subsequent studies comparing naturally occurring soil populations of *Pratylenchus* spp. in E+ and E- field plots over a 1 to 3-year period, *Pratylenchus*

populations were not significantly reduced by endophyte infection (O'Day et al., 1993; Watson et al., 1995). However, since these observations, greenhouse studies have shown that *Pratylenchus scribneri* populations are severely suppressed in E+ tall fescue (Kimmons et al., 1990; Jones, 1997). Populations of *P. scribneri* were reduced in E+ roots to the point of near extinction (Kimmons et al., 1990). Jones (1997) reported a severe reduction in *P. scribneri* numbers in E+ roots, the absence of eggs, and a distinct lack of juvenile recruitment in E+ roots at 40 and 60 days after inoculation. He attributed the latter effects to either a disruption in egg development within the adult female or disintegration of the eggs following oviposition.

Various chitinases have been identified as PR-proteins involved in plant defense reactions against phytopathogenic nematodes (Zacheo et al., 1997). Chitinase is produced constitutively in the roots and foliage of both E+ and E- tall fescue (Roberts et al., 1992, 1994; Spiers, 1998). Roberts et al. (1992) reported that infection with the plant-parasitic nematode *Meloidogyne marylandi* Jepson & Golden led to a temporary increase in chitinase levels in the roots of E+ seedlings at 10 days after germination. However, by 20 days after germination, there was no difference between chitinase levels in the roots of nematode-infected and uninfected seedlings. Nevertheless, the potential for a chitinase defense strategy targeting nematode reproduction becomes apparent when the middle chitinous layer of the nematode eggshell is considered. Mercer et al. (1992) noted that *Meloidogyne hapla* Chitwood eggs became spherical in shape

following exposure to microbial chitinases; such a shift in form might be indicative of structural changes in the chitinous layer of the eggshell. Jawarsky et al. (cited in Mercer et al., 1992) observed a decreased hatch rate for *M. javanica* (Treub) Chitwood eggs when exposed to microbial chitinase. Jones (1997) also found the hatch rate of undifferentiated *P. scribneri* eggs to be reduced by exposure to microbial chitinase. In contrast, Mercer et al. (1992) demonstrated that exposure of embryonated eggs of *M. hapla* to plant and microbial chitinases enhanced the hatch rate and increased mortality rates in the juveniles released. Mercer has suggested that the developmental stage of the egg (embryonic, J1, or J2) at the time of exposure to chitinase may determine whether hatching is induced or inhibited.

If constitutive or induced chitinases do play a role in nematode resistance by degrading eggshells, the degree to which eggs are exposed to the enzyme would be a determining factor in population impact. Eggs of the sedentary endoparasitic *Meloidogyne* spp. are laid in a gelatinous mass protruding from the root surface and are less likely to be affected by plant chitinases (Punja and Zhang, 1993). Eggs of *Pratylenchus* spp. are laid singly within the root tissue and would be directly exposed to host-plant defense compounds. The structure of the eggshell must be considered as well. While the middle layer of the eggshell does contain chitin, it occurs in the form of chitin microtubules surrounded by a protein matrix and separated from the plant's cellular environment by the outer vitelline layer (Wharton, 1980). It is questionable

whether the chitin would ever be exposed to chitinase without some sort of pre-existing physical damage to the shell.

Research Objectives

Previous greenhouse studies demonstrated a reduction in *P. scribneri* populations within E+ tall fescue over a short two-month period with inoculation occurring shortly (within two weeks) after transplantation of tillers (Kimmons, 1990; Jones, 1997). Jones observed an apparent suppression in *P. scribneri* reproduction in E+ roots that might be due to increased chitinolytic activity affecting the integrity of nematode eggshells. *Pratylenchus scribneri* is the only *Pratylenchus* species for which endophyte-mediated resistance has been documented. The objectives of the current research were to determine:

1. infectivity and survival of *Pratylenchus scribneri* in E+ and E- tall fescue over brief and extended periods of time;
2. reproductive capability of female *P. scribneri* isolated from E+ roots;
3. effects of exposure to proteolytic and chitinolytic enzymes on eggshell integrity of *P. scribneri* eggs;
4. population dynamics for *Pratylenchus agilis* over a brief period of time as affected by high and low ambient temperatures.

Chapter 2

Materials and Methods

Short-term Study of *P. scribneri* Population Dynamics in E+/E- Tall Fescue

A study of *P. scribneri* population dynamics in E+/E- tall fescue was conducted over a period of 14 weeks. Clonal pairs of E+ and E- 'Kentucky 31' tall fescue were obtained from David Trently (University of Tennessee). Individual tillers of each type were separated from grass clumps and planted into separate trays. The grass was trimmed periodically to prevent flowering and seed development. Plants were allowed to grow until each had produced 5 to 10 additional tillers. Random tillers were tested to verify endophyte status. Individual tillers for experiments were separated from the trays and planted into a 4:1 sand:soil mixture in 4-cm-diam. × 14-cm-long Conetainers (Hummert International, Earth City, MO). Conetainers with E+ and E- tillers were paired and arranged in a randomized complete block design. Following transplantation, the tillers were allowed to grow for five weeks, after which each Conetainer was infested with 1,000 vermiform *Pratylenchus scribneri*. For inoculation purposes, vermiform *P. scribneri* were rinsed from corn root explant cultures to produce a tap water solution of 1,000 nematodes/ml. Two 1-cm-deep holes were made in the soil on opposite sides of the tiller, injected with 0.5 ml inoculum, and then loosely filled with sand. Five E+/E- pairs were harvested at 20, 40, and 60 days after inoculation. The roots were rinsed free of soil, cleared in a 20% sodium

sodium hypochlorite solution, stained with acid fuchsin, and stored in acidified glycerin (Byrd et al., 1983). Leaves and leaf sheaths of the harvested tillers were labeled and frozen to allow future determination of endophyte status in case of anomalous results.

Eggs and vermiform *P. scribneri* in each root system were counted using a dissecting microscope. The first 50 vermiform *P. scribneri*, or as many as possible up to 50, were dissected from the roots, mounted on slides, and traced with the aid of a drawing tube. The tracings were then measured with an electronic lineometer (Scalex, Carlsbad, CA). The life-stage (second, third, fourth-stage juvenile, adult) of each nematode was determined based on length and the presence or absence of a vulva. Life-stage distribution for each root system was based on the percent occurrence of each life-stage in the dissected population. Population counts were statistically analyzed with a paired t-test ($P \leq 0.05$) (SYSTAT 7.0, SPSS, Chicago, IL).

Extended Study of *P. scribneri* Population Dynamics in E+/E- Tall Fescue

An extended study of *P. scribneri* population dynamics E+ and E- tall fescue was conducted over a 30-week period. 'Kentucky 31' tall fescue tillers were planted into Conetainers and arranged as described above (page 11). E+/E- pairs were randomly designated as Series 1, Series 2, or Series 3. Series 1 pairs were inoculated with 1,000 vermiform *P. scribneri* three weeks after transplantation. Series 2 pairs were inoculated 12 weeks after transplantation.

Series 3 pairs were inoculated 21 weeks after transplantation. Five E+/E- pairs were harvested 20, 40, and 60 days after inoculation for each trial. Eggs and vermiform *P. scribneri* were counted and life-stage distribution was determined. Statistical analyses were performed as above (page 12).

Reproductive Capability of Female *P. scribneri* Isolated from E+ Tall fescue Roots

Two E+/E- Conetainer pairs from Series 1 of the extended population study were harvested 20, 40, and 60 days after inoculation. After rinsing away the soil, roots were cut into 1-cm segments and placed on mesh screens in a mist chamber to extract live nematodes. The mist chamber timer was programmed to provide a 12-second burst of mist every 6 minutes. Extracted nematodes were collected every 3 or 4 days. Adult female *P. scribneri* were identified by the presence of a vulva and isolated. The isolated females were surface-sterilized by soaking in 0.1% malachite green solution for 15 minutes, followed by 15 minutes in 1.0% streptomycin/penicillin/neomycin solution, then a 10-minute rinse in sterile water (Stefan, 1989). Callus tissue cultured from 'Liberty' alfalfa seedlings (Riedel and Foster, 1970) was transferred onto freshly prepared Riedel's simplified medium (Riedel et al., 1973) in polystyrene culture tubes (Appendix A). Individual females were transferred to polystyrene culture tubes containing alfalfa callus and incubated 45 to 50 days in the dark at 30°C. Following incubation, the lumps of callus tissue were macerated and examined

using a dissecting microscope to determine if reproduction had occurred. The presence of eggs or additional vermiform nematodes was considered positive evidence of reproduction.

Effect of Exposure to Proteolytic and Chitinolytic Enzymes on Eggshell Integrity of *P. scribneri* Eggs

Testing Eggshell Integrity through High-speed Centrifugation

To determine if exposure to proteolytic and chitinolytic enzymes could compromise eggshell integrity, *P. scribneri* eggs were incubated in enzymatic solutions and then centrifuged at high speed. All enzymes were purchased from Sigma (St. Louis, MO). *P. scribneri* eggs were extracted from alfalfa callus cultures and suspended in tap water (Dunn, 1973). A chitinase stock solution (0.217 mg chitinase/ml phosphate buffered saline (PBS), pH 7.3), from *Serratia marcescens*, was used to prepare a series of four 1:10 dilutions. Activity of the chitinase stock solutions ranged from 1.67 units/ml to 0.00167 units/ml. Protease stock solutions included Protease XIII from *Aspergillus saitoi* (2.78 mg protease/ml PBS), Protease XV isolated from *Bacillus polymyxa* (1.85 mg protease/ml PBS), and Protease IX isolated from *Bacillus polymyxa* (3.7 mg protease/ml PBS). The original stock solution for each protease was diluted in series to produce stock solutions with activities of 2.22 units/ml, 0.057 units/ml, 0.0063 units/ml, and 0.00058 units/ml. For each experimental treatment, 250 μ l

of enzymatic stock solution were mixed with 750 μ l of egg suspension (70 eggs) in a microfuge tube. Controls consisted of 250 μ l of PBS or 250 μ l of tap water mixed with 750 μ l of egg suspension. The remaining egg suspension stock was stored at 4°C. Treatments and controls were incubated at room temperature for 16 hours, then centrifuged at 20,000g for 30 minutes. Following centrifugation, egg solutions were examined in Petri dishes using a dissecting microscope for visible damage to eggshells (e. g., fragmentation, loss of embryo, change in shape). Samples were incubated at 30°C for 72 hours and observed again. Following the second observation, 10 eggs in the 4-cell to many-cell stages were extracted from each treatment and control solution and placed in small Petri dishes with fresh water. Uncentrifuged eggs from the egg suspension stock were removed from 4°C storage and placed in Petri dishes for comparison. Eggs separated from the centrifuged samples and uncentrifuged stock solution were incubated at 23°C and observed at 10 and 18 days after centrifugation for developmental progress.

Examination with TEM

Effects of High-speed Centrifugation

P. scribneri eggs were extracted from callus tissue and suspended in tap water. The egg solution was added to microfuge tubes in 1-ml aliquots (100 eggs) and centrifuged at a maximum of 20,000g for 1.5 hours. Following centrifugation, the eggs were concentrated in a single row down the middle of a

15- μ m nylon mesh filter in a vacuum manifold (Millipore, Bedford, MA). Each filter was then inverted onto a thin layer of semi-solidified 2% water agar in a small Petri dish and pressed gently to embed the eggs. The filter was removed and the embedded eggs were covered with another thin layer of 2% water agar for a total thickness of 1 mm. Once solidified, excess agar was removed from each plate leaving only a narrow band containing the eggs. The plates were then flooded with 4% formaldehyde in 0.1M phosphate buffer, pH 7.3 (Bird, 1968). Immediately after immersion, the agar was cut into 1-mm strips to facilitate penetration of the fixative, after which the strips were incubated at room temperature for 24 hours. Following incubation, the strips of agar were rinsed twice with distilled water and post-fixed in 1% OsO₄ for two hours. Fixation was followed by three 10-minute rinses in distilled water. The specimens then were dehydrated through a graded ethanol series to acetone (25%, 50%, 75%, 95%, 100% ethanol, 100% acetone for 15 minutes each). Specimens were infiltrated with Spurr's resin (1:1 acetone:Spurr's for 30 minutes, 100% Spurr's for 2 hours), embedded in fresh Spurr's resin, and polymerized overnight at 70°C. Sections 60 to 90-nm thick were cut with a glass knife on a Sorvall MT-1 ultramicrotome, collected on 400-mesh copper grids, and stained sequentially with aqueous uranyl acetate and lead citrate. Grids were examined with a Hitachi H-600 transmission electron microscope.

Effects of Incubation in Chitinolytic Solution

Pratylenchus scribneri eggs were extracted from callus, concentrated in the vacuum manifold, and resuspended in 4 ml of tap water. A chitinase solution (65 mg chitinase/ml), from *Serratia marcescens* was prepared in PBS buffer to produce an enzymatic activity of 5 units/ml. For the experimental treatment, 300 μ l of the chitinase solution were mixed with 700 μ l of egg suspension (70 eggs), which was then incubated in the dark at 25°C for 24 hours. Two control solutions, prepared by adding 300 μ l of phosphate buffer and 300 μ l of tap water to separate 700- μ l aliquots of egg suspension, were incubated likewise. Specimens were embedded in agar and prepared for TEM as described above with the exception of a more gradual dehydration series (5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% 100% ethanol, 2:1, 1:1, 1:2 ethanol:acetone, 100% acetone for 10 minutes each) and prolonged resin infiltration (3:1, 1:1, 1:3 acetone:Spurr's, 100% Spurr's for 24 hours each, followed by a second incubation for 6 hours in fresh resin).

Short-term Study of *P. agilis* Population Dynamics in E+/E- Tall Fescue as Affected by High and Low Ambient Temperatures

Two sets of E+ and E- tall fescue tillers were planted into Conetainers and arranged as described for the short-term *P. scribneri* study (page 11). Two weeks after transplantation, each tiller was inoculated with 1,000 vermiform *P. agilis*. The Conetainers were incubated at ambient temperature (ca. 25°C) for 1

week. After this period, one set of Conetainers was transferred to an incubator maintained at 16.5°C while the other set was transferred to an incubator maintained at 30°C. Both incubators were programmed for a 12:12 light/dark cycle. To prevent temperature shocks to the experimental system, pitchers of irrigation water were incubated with the Conetainers. Conetainers were watered from above using squeeze bottles to prevent excessive wetting of the foliage. Conetainers in the 16.5°C incubator were watered once daily. Conetainers in the 30°C incubator were watered 2 to 3 times each day to compensate for the higher rate of evaporation. E+/E- Conetainer pairs were harvested 20, 40, and 60 days after inoculation. Populations and life-stage distributions of *P. agilis* were determined and analyzed as described for the short-term *P. scribneri* study (page 12).

Chapter 3

Results and Discussion

Short-term Study of *P. scribneri* Population Dynamics in E+/E- Tall Fescue

Population counts of vermiform *P. scribneri* were significantly lower in E+ tall fescue than E- tall fescue at 20, 40, and 60 days after inoculation (Fig. 1). Fewer *P. scribneri* eggs were found in E+ tall fescue (Fig. 2); however, there was no significant difference between the mean number of eggs per adult in E+ and E- tall fescue. Reproductive systems of adult females from E+ tall fescue appeared fully developed and functional (Fig. 3). The number of vermiform nematodes used to determine life-stage distributions varied depending upon population size (Table 1). Due to the small sample sizes available for the E+ tall fescue populations, life-stage distributions must be considered on the basis of overall trends. The population trend in E- tall fescue is indicative of a healthy population; an initial high rate of reproduction by invading females (evidenced by the large proportion of J2) was followed by maturing among all juvenile stages (Fig. 4A, B, C). The decreased recruitment of J2 by 60 days after inoculation was probably due to decreased fecundity of older adults. Reproduction and juvenile recruitment would be expected to rebound as J4 matured. In E+ tall fescue, the initial high rate of reproduction was followed by a marginal increase in the adult percentage of the population and slow maturing of J2 and J3. The life-stage distribution 60 days after inoculation suggests that feeding on E+ tall

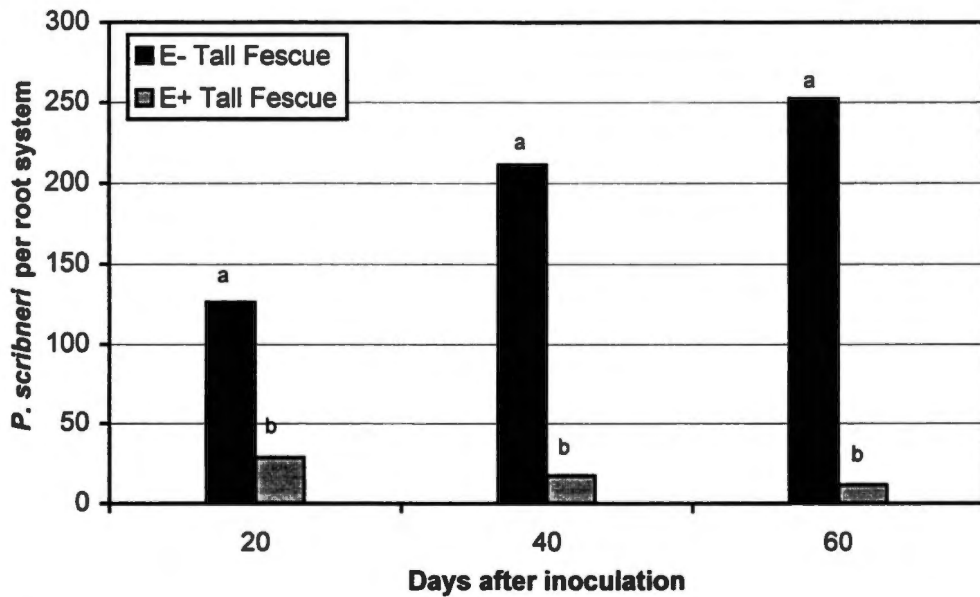


Figure 1. Mean number of vermiform *P. scribneri* per root system in E+/E- tall fescue tillers for the short-term study. Columns in a pair headed by different letters are significantly different when compared by a paired t-test at $P \leq 0.05$.

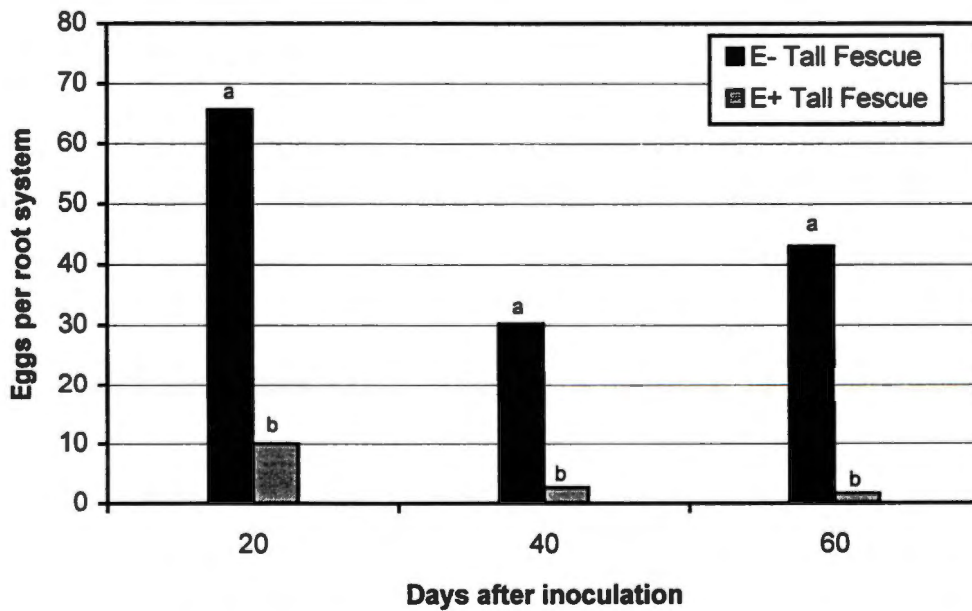


Figure 2. Mean number of *P. scribneri* eggs per root system in E+/E- tall fescue tillers for the short-term study. Columns in a pair headed by different letters are significantly different when compared by a paired t-test at $P \leq 0.05$.

Figure 3. Gravid female from the short-term E+ tall fescue 20 days after inoculation. E=egg.

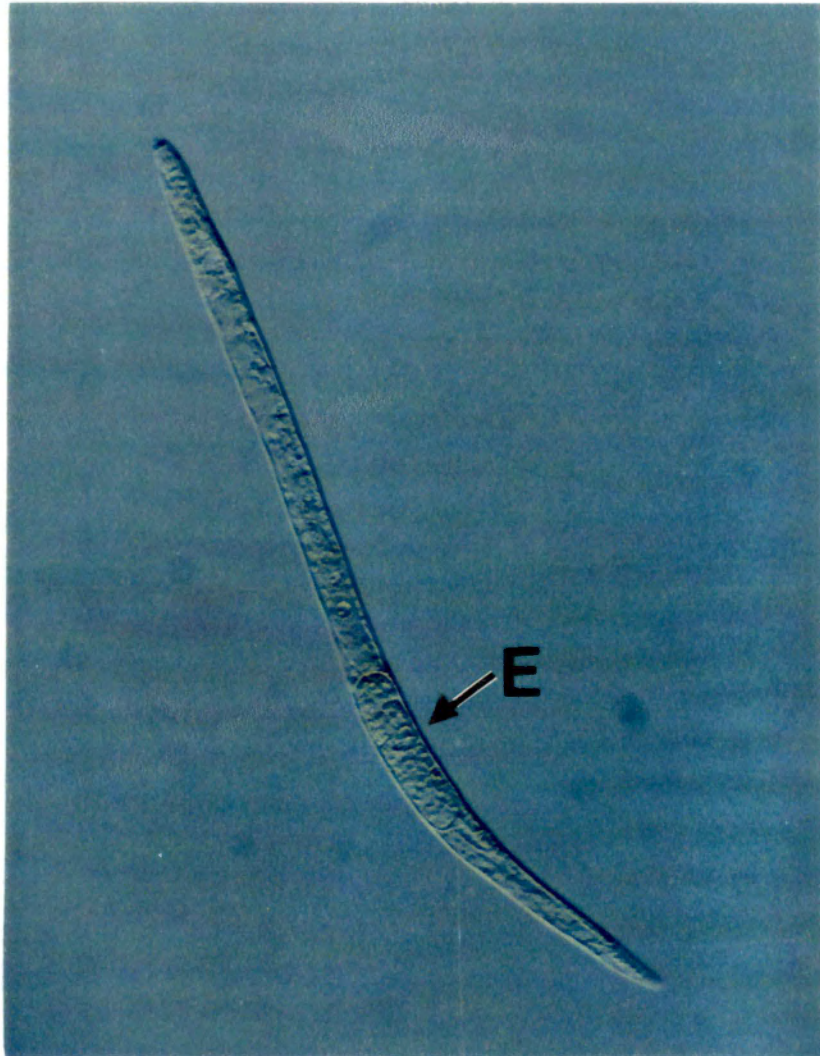


Table 1. Total sample sizes (sum of 5 E+/E- samples per harvest) used to determine *P. scribneri* life-stage distributions.

Days after inoculation	E- Tall Fescue (vermiform nematodes)	E+ Tall Fescue (vermiform nematodes)
Short-term Study		
20	199	92
40	243	68
60	250	49
Extended Study Series 1		
20	250	250
40	250	250
60	235	221
Extended Study Series 2		
20	231	142
40	81	127
60	30	32
Extended Study Series 3		
20	136	172
40	198	111
60	213	43

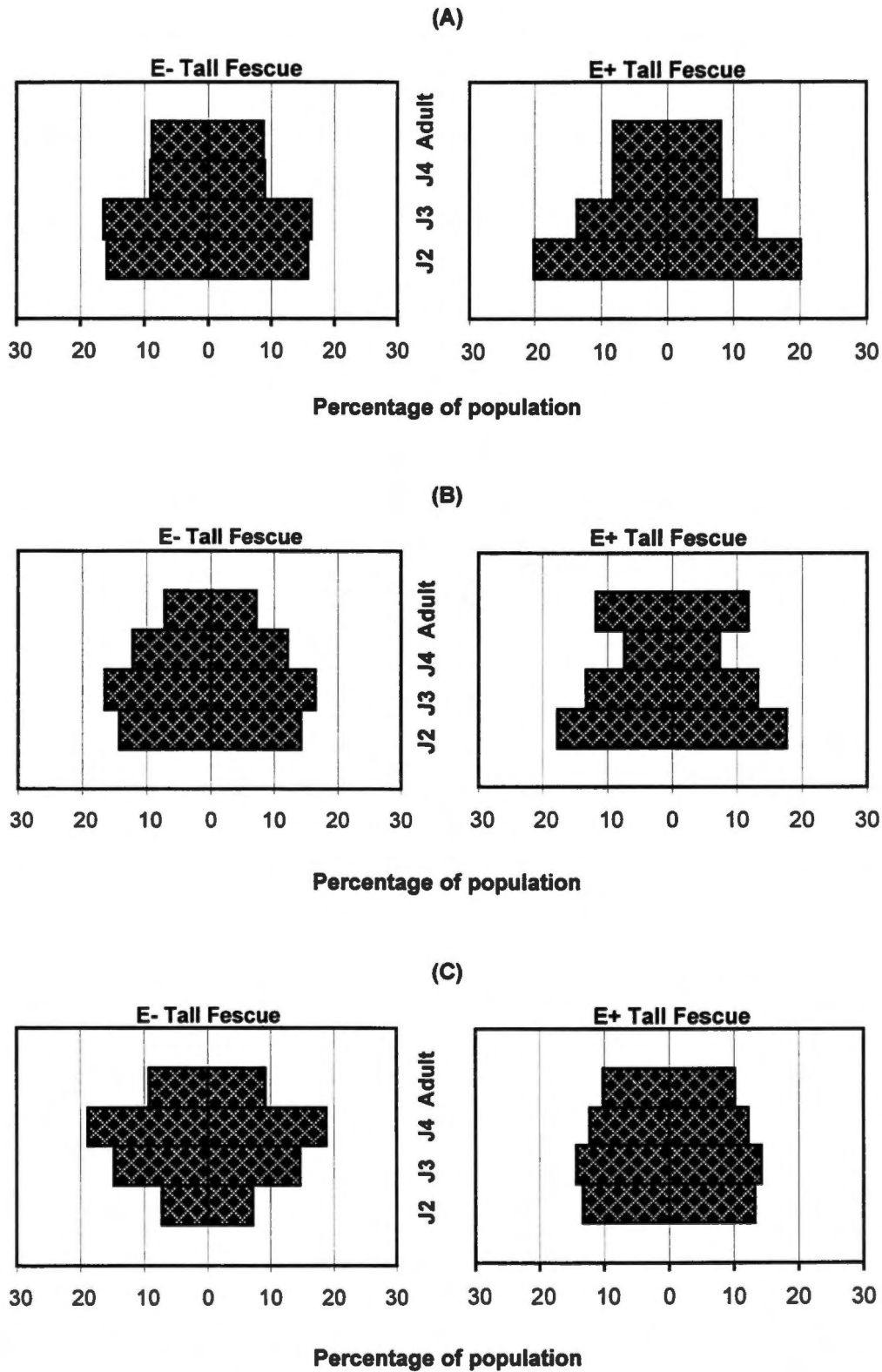


Figure 4. *P. scribneri* life-stage distribution for the short-term study (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.

fescue, or perhaps a lack of feeding, slowed the developmental rate of juveniles. Juveniles and eggs were present in the E+ tall fescue at all harvests. The reduction in E+ populations cannot be attributed to lack of reproduction by existing females.

Populations of *P. scribneri* at 20 days after inoculation were lower than inoculum levels in both E+ and E- tall fescue. Several contaminating species of fungal/bacterial feeding nematodes were found adjacent to or intermingled with *P. scribneri* populations in E+ and E- root tissues. The presence of these contaminating species may have triggered natural host-defense mechanisms prior to *P. scribneri* inoculation, thereby reducing the number of *P. scribneri* successfully infecting the E+ and E- roots. Endophyte-mediated resistance mechanisms may have been triggered as well, accounting for the rapid reduction of *P. scribneri* populations in E+ tall fescue.

Extended Study of *P. scribneri* Population Dynamics in E+/E- Tall Fescue

During the extended study, populations of vermiform *P. scribneri* in E+ and E- tall fescue differed significantly for two of the nine harvests: Series 2, 20 days after inoculation and Series 3, 40 days after inoculation (Fig. 5). *Pratylenchus scribneri* populations in both E+ and E- tall fescue declined dramatically during Series 1 and Series 2, to the point that populations were approaching zero by 60 days after inoculation of Series 2. The decrease in initial numbers of nematodes infecting the roots might be related to increased plant age at time of inoculation. However, the continued decrease in populations of Series 1 and Series 2

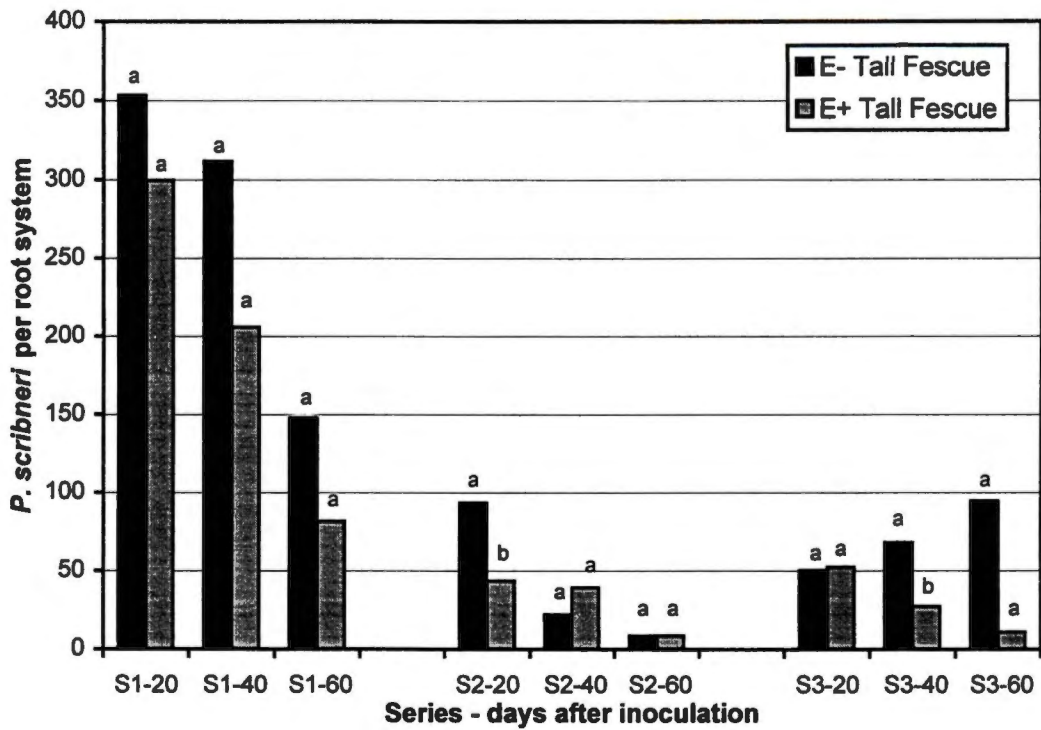


Figure 5. Mean vermiform *P. scribneri* per root system for Series 1-3 of the extended study. Columns in a pair headed by different letters are significantly different when compared by a paired t-test at $P \leq 0.05$.

following infection indicate that environmentally related influences were more likely causes of the overall population decline. The extended experiment was initiated in late autumn and continued through the winter months. While the ambient greenhouse temperature was maintained at ~25°C throughout the experiment, the irrigation water varied in temperature depending upon weather conditions. Daily temperatures of water released for public consumption during the experimental period were provided by the Knoxville Utility Board's Water Processing Plant. Water temperatures at individual spigots may vary from those recorded at the processing plant, however the overall seasonal trend remains the same. Decreasing *P. scribneri* populations during Series 1 and Series 2 were directly correlated to decreasing water temperature ($r = 0.95$) (Fig. 6). Both E- and E+ tall fescue tillers remained healthy throughout the experiment; however, daily temperature shocks during irrigation may have been detrimental to the nematodes or induced a migration from roots to soil.

Life-stage distributions of *P. scribneri* in E+ and E- roots were similar during Series 1 (Fig. 7A, B, C). However, juvenile recruitment and development were greatly reduced as compared to E- populations during the short-term study. Due to the small sample sizes used for Series 2 and Series 3, life-stage distributions must be compared on the basis of overall trends (Table 1). Throughout Series 2 (Fig. 8A, B, C), adults constituted the largest portion of the population in E- tall fescue, whereas J3 and J4 constituted the largest percentage in E+ tall fescue. Little developmental progress was made in either E+ or E- populations during Series 2. During Series 3 (Fig. 9A, B, C), *P. scribneri*

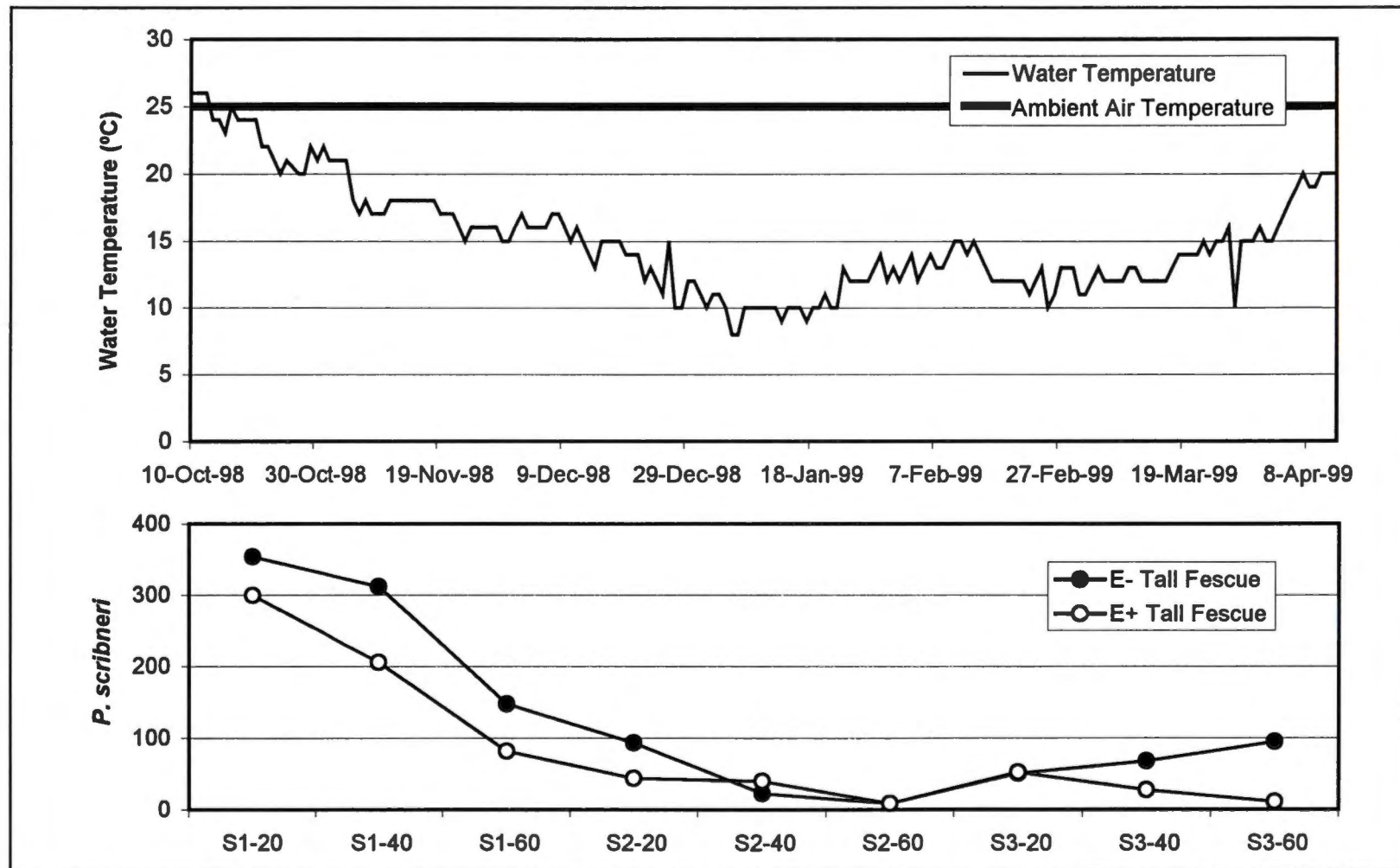


Figure 6. Fluctuations in processed water temperature (provided by Knoxville Utility Board) as compared with fluctuations in *P. scribneri* populations in E+ and E- tall fescue during the extended study.

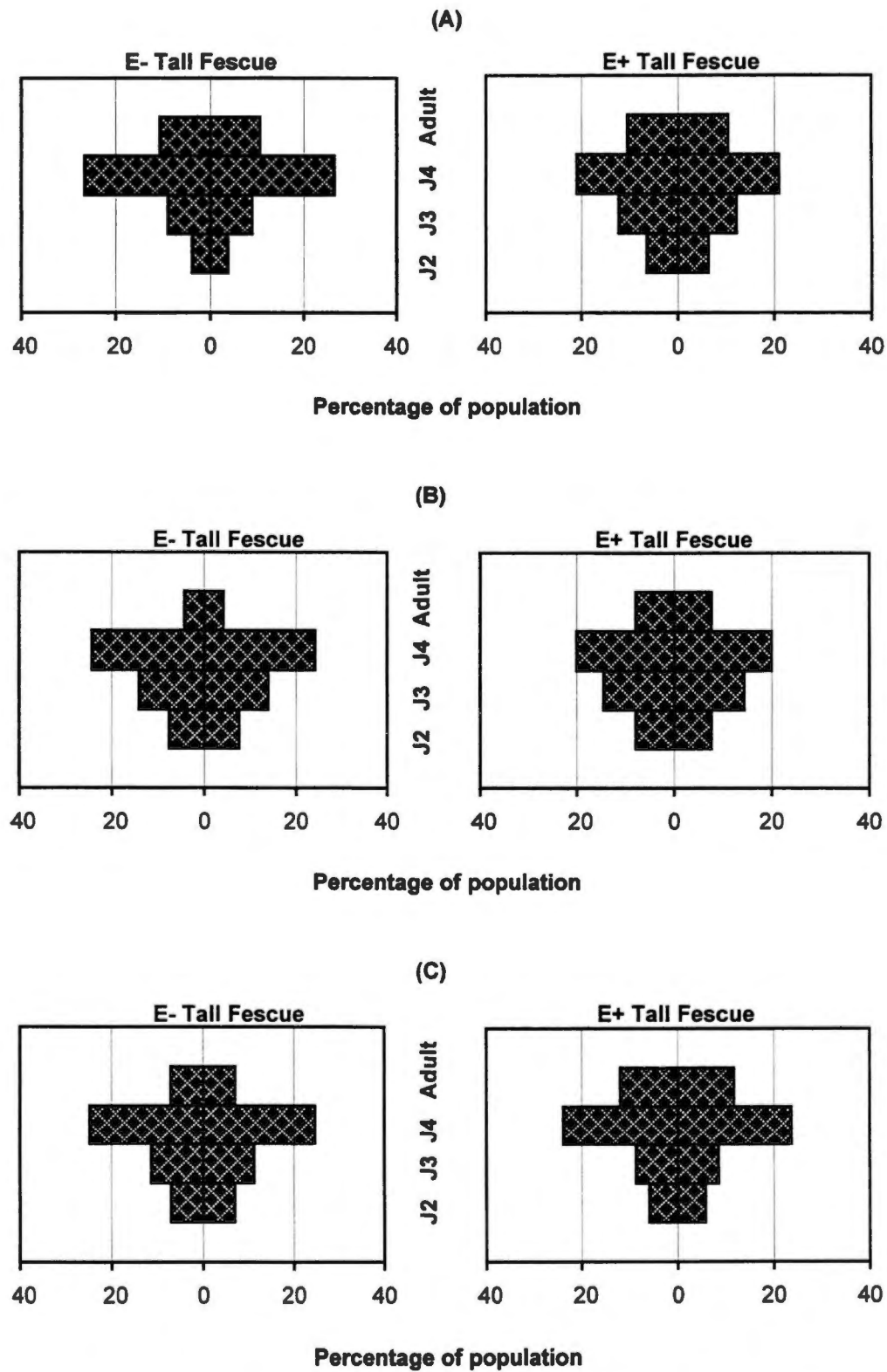


Figure 7. *P. scribneri* life-stage distributions for Series 1 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.

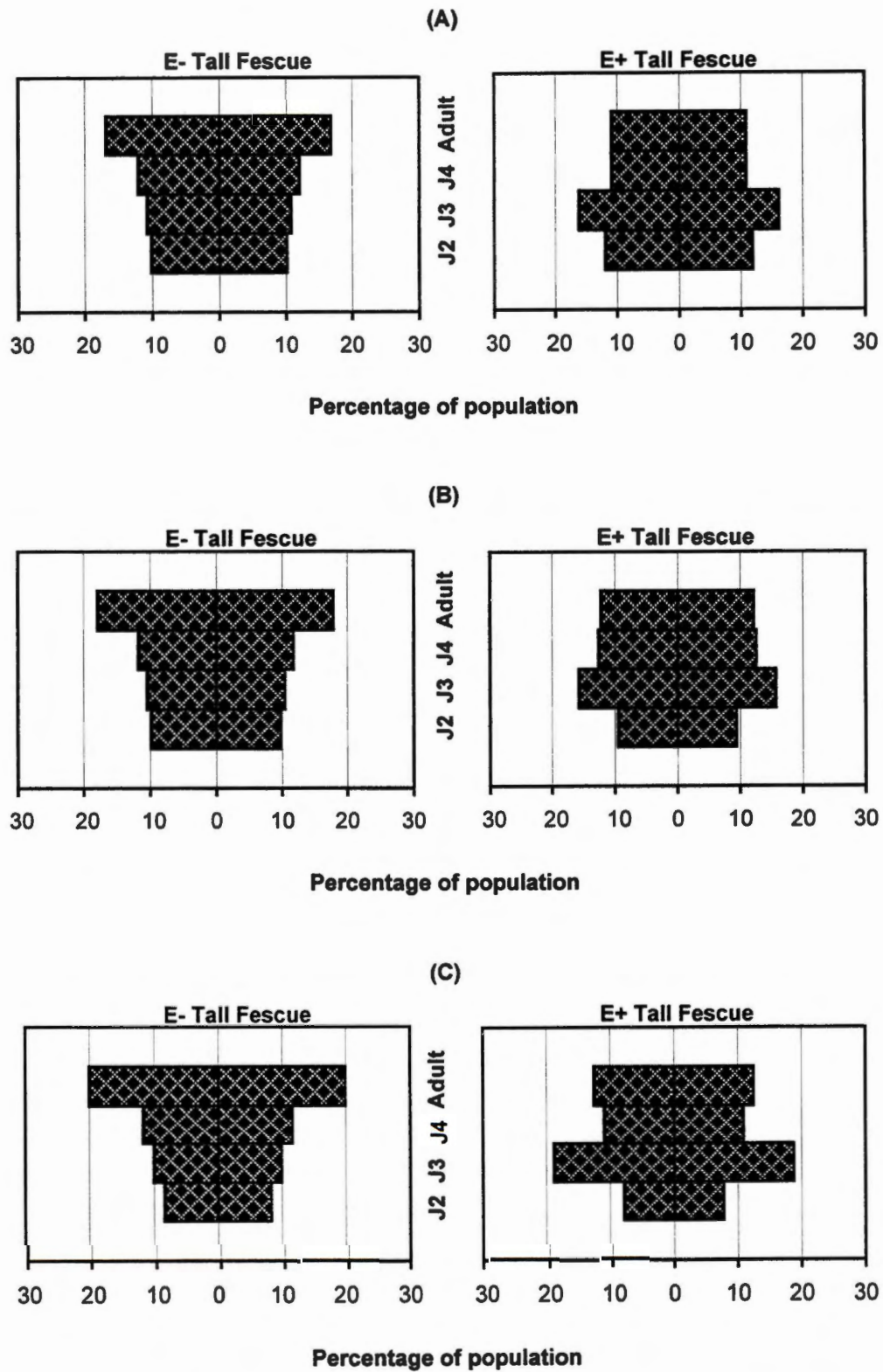


Figure 8. *P. scribneri* life-stage distributions for Series 2 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.

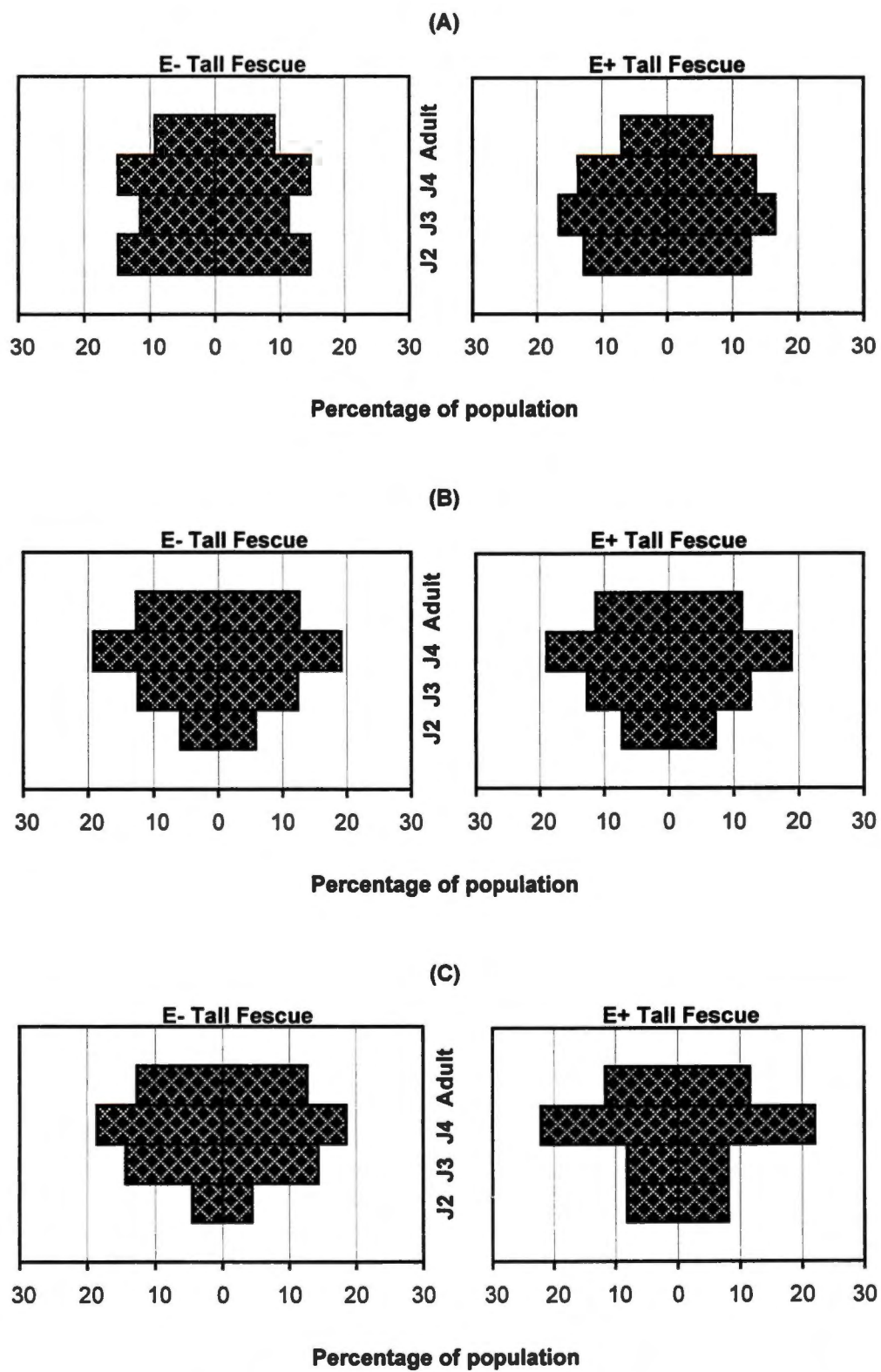


Figure 9. *P. scribneri* life-stage distributions for Series 3 (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation.

populations in both E+ and E- tall fescue were predominated by J3 and J4 with decreasing recruitment of J2 as the trial progressed.

During Series 3, there was no significant difference between the numbers of *P. scribneri* invading E- and E+ roots. Following initial infection, populations increased in E- tall fescue but decreased in E+ tall fescue. Environmental conditions were conducive to survival and reproduction, as evidenced by the steady increase in E- nematode populations; therefore, the reduction in E+ populations may be attributed to endophyte-mediated resistance. As in the short-term study, there was no significant difference between E+ and E- populations in the number of eggs per adult. Eggs and juveniles were present at all harvests.

The initial population of *P. scribneri* infecting E+ and E- roots following inoculation decreased as time after transplantation increased ($r = -0.92$) (Fig. 10). Since populations were affected similarly in E+ and E- tall fescue, the reduction likely is related to plant-associated changes rather than endophyte status. However, the decrease in E+ *P. scribneri* populations following initial infection during Series 3 (Fig. 6) indicates that endophyte-mediated resistance continued to function as tall fescue matured.

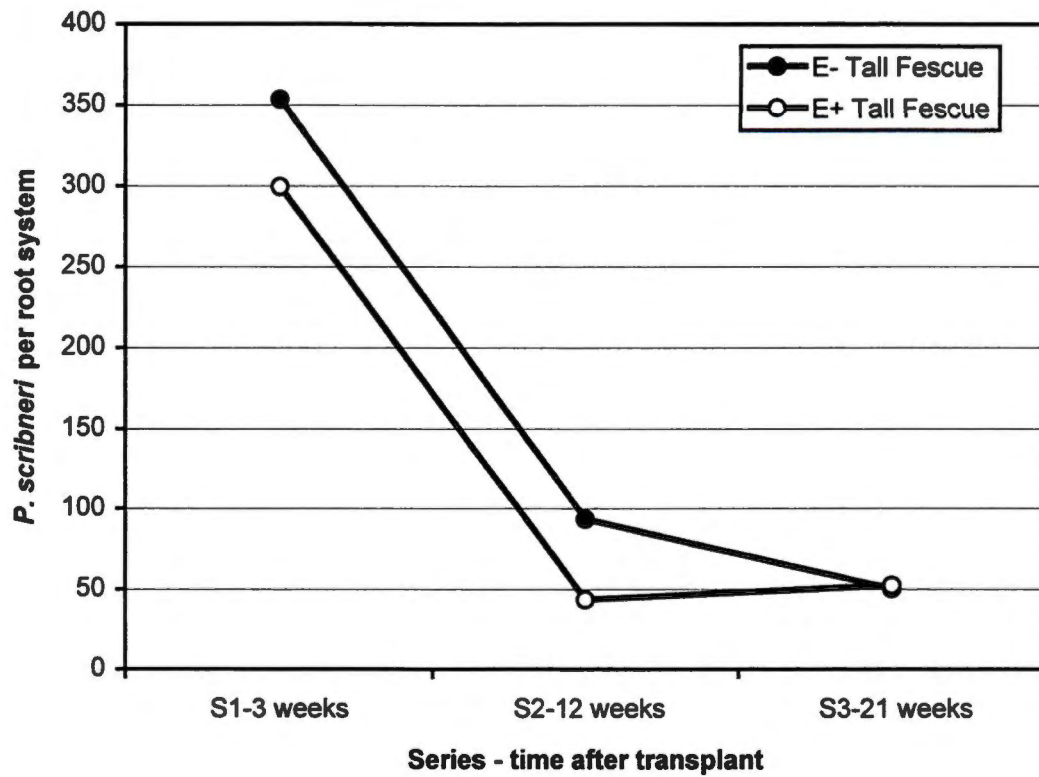


Figure 10. Mean number of vermiform *P. scribneri* per root system as affected by increased tiller "age" at time of inoculation. Series 1 was inoculated 3 weeks after transplanting; Series 2 was inoculated 12 weeks after transplanting; and Series 3 was inoculated 21 weeks after transplanting.

Reproductive Capability of Female *P. scribneri* Isolated from E+ Tall Fescue Roots

Females isolated from Series 1-tall fescue and transferred to alfalfa callus reproduced. Eggs and live juveniles were observed in callus cultures for females from both E- and E+ tall fescue harvested 20 and 40 days after inoculation. No reproduction was observed for females from either E- or E+ tall fescue harvested 60 days after inoculation. Females from the 60-day harvest may have been weakened by temperature shock to the point that they were unable to survive the sterilization process or may have lost fecundity due to age.

Effect of Exposure to Proteolytic and Chitinolytic Enzymes on Eggshell Integrity of *P. scribneri* Eggs

Testing Eggshell Integrity through High-speed Centrifugation

High-speed centrifugation (20,000g for 30 minutes) caused no apparent damage to *P. scribneri* eggshell integrity in the enzyme-treated samples when observed at 16 or 72 hours after centrifugation. Eggs did not appear fragmented or misshapen when examined with a light microscope. Recently hatched active and nonactive juveniles were present after 72 hours; however, development of undifferentiated embryos appeared interrupted. Most eggs were in the early 4-cell stage of development, with some eggs in the 8-cell and many-cell stages, but very few juvenile-stage eggs. In several instances, the cells of 4-cell eggs had been separated into groups of two and spun to opposite poles by centrifugation.

The centrifuged water and buffer controls yielded similar results. *Pratylenchus* eggshells are flexible, allowing eggs to assume shapes dictated by the physical environment. Eggs might be severely distorted during centrifugation but then return to their original shape afterwards. Microscopic fractures in the eggshells due to rapid shape changes could impact further development of the embryos.

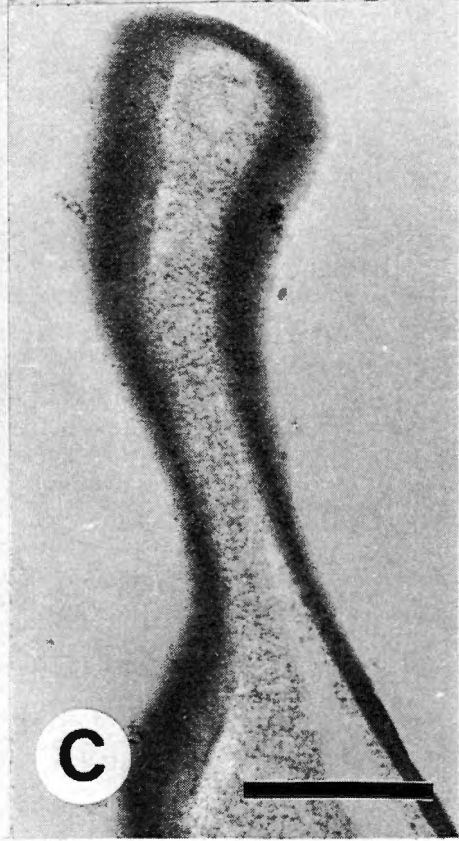
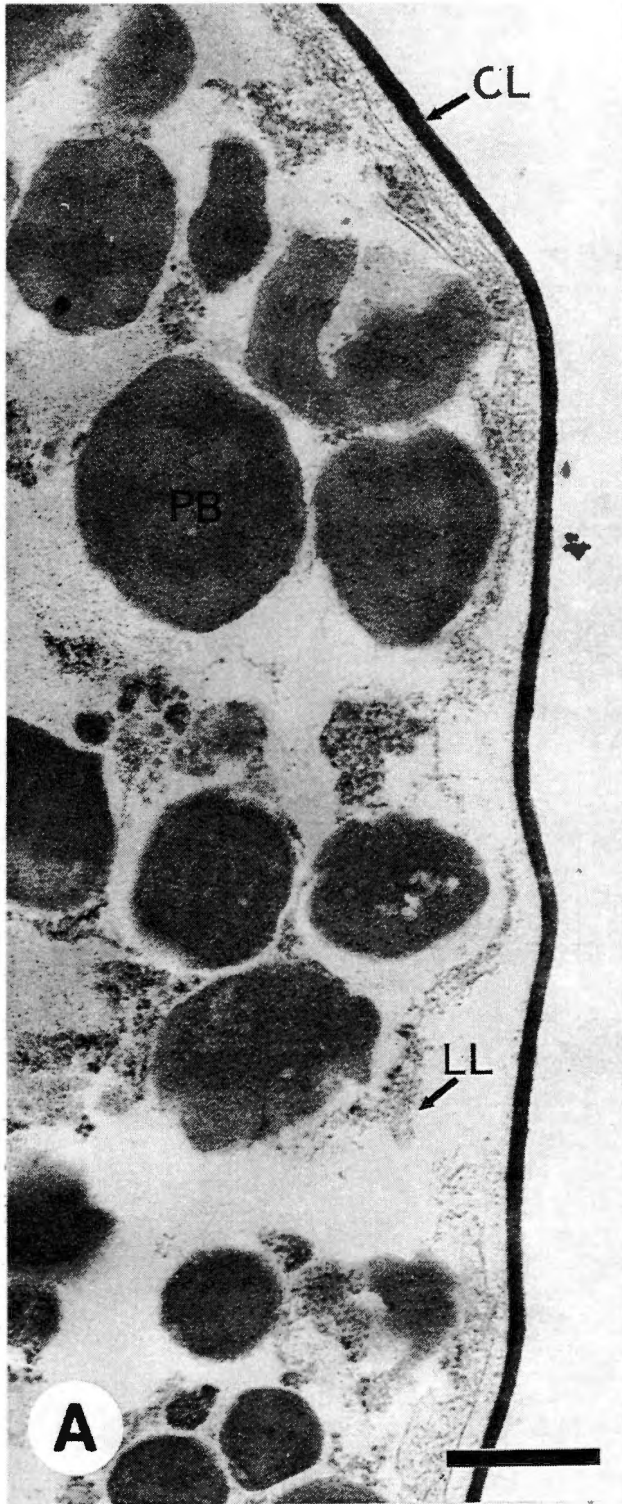
At 10 days after centrifugation, no clear developmental progress had occurred in either the centrifuged or uncentrifuged eggs. At 18 days after centrifugation, no clear development had occurred in the centrifuged eggs; however, all uncentrifuged eggs had progressed beyond the 4-cell stage, including one juvenile-stage egg and two hatched juveniles.

Examination with TEM Using Chemical Fixation and Dehydration

Effects of High-Speed Centrifugation

Several physical disruptions occurred in *P. scribneri* eggshell organization following exposure to high-speed centrifugation (Figure 11A, B, C). The lipid layer was ruptured and extended into the egg proper, and in some areas the entire lipid layer was pulled away from the chitin layer. The double membrane separating the lipid layer and developing embryo was visible but fragmented. In addition, the outer vitelline layer was partially or completely absent in centrifuged eggs. The shape of the eggs was distorted, often with several projections extending away from the body of the egg; however, distortions ranged from minor to severe.

Figure 11. Transmission electron micrographs of centrifuged *P. scribneri* eggs. (A) Centrifuged egg in which the inner lipid layer (LL) of the eggshell is ruptured and no longer continuous with the chitinous layer (CL). Bar = 0.5 μm . (B) Enlargement of eggshell detailing disruption of the lipoprotein membranes (DM) in the lipid layer. Bar = 0.1 μm . (C) Example of projection of eggshell extending away from the main body of the egg. PB = protein body. Bar = 0.5 μm .



Effects of Incubation in Chitinolytic Solution

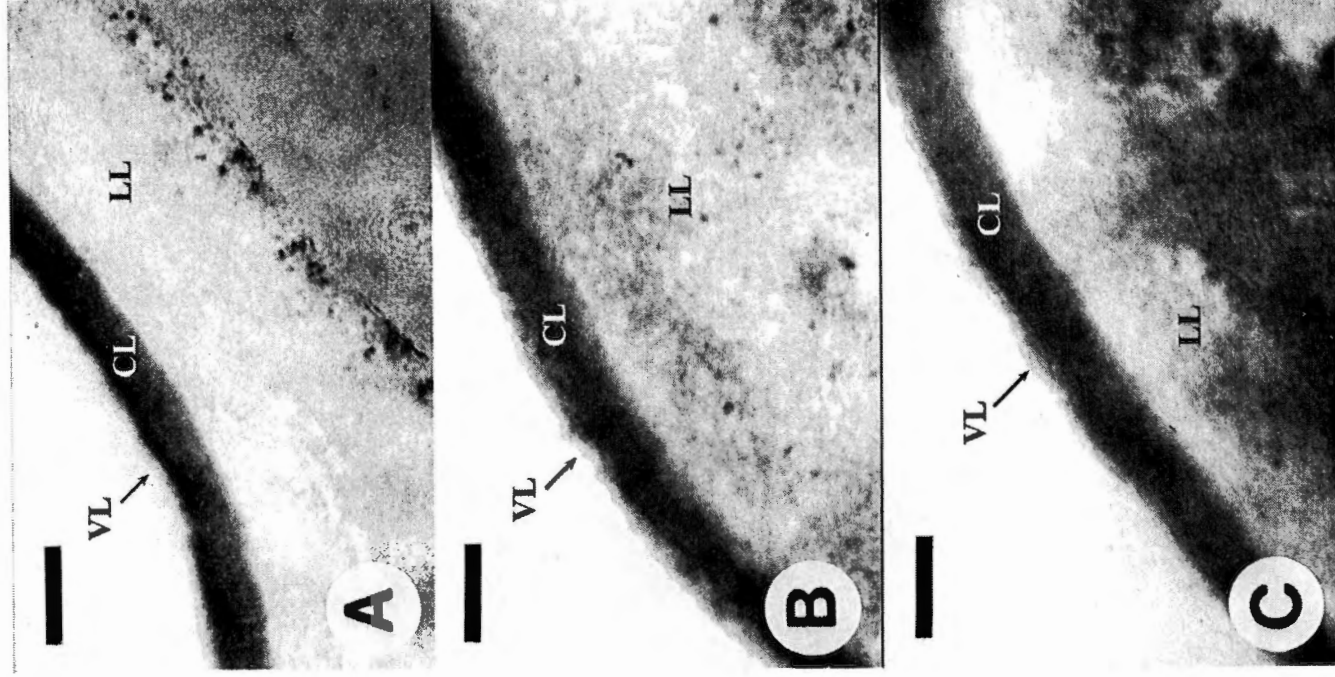
Incubation in a chitinase solution had no apparent affect on the integrity of the chitinous layer in *P. scribneri* eggshells when compared to water- and buffer-incubated control eggs (Fig. 12A, B, C). The lipid layer of noncentrifuged eggs was continuous with the chitin layer. However, lipoprotein membranes were not distinguishable. The vitelline layer was visible as the outer shell layer. As with the centrifuged eggs, the overall shape of the noncentrifuged eggs was distorted by projections extending away from the body of the egg. The presence of distortions in centrifuged as well as noncentrifuged eggs may indicate inadequate fixation, dehydration, or resin infiltration. Prolonged dehydration and resin infiltration did not alleviate distortion. The impermeable nature of the lipid-layer in tylenchid eggshells can prevent adequate penetration of fixatives, dehydrating agents, and resin (Bird, 1991). Disruptions in the lipid and vitilline layers may account for the finer detail of centrifuged eggs. Cryofixation and cryosubstitution may be necessary for preparation of undamaged eggs.

Short-term Study of *P. agilis* Population Dynamics in E+/E- Tall Fescue as Affected by High and Low Ambient Temperatures

ELISA testing revealed that only three “E+” tall fescue samples incubated at 30°C contained the *Neotyphodium*-endophyte at the time of harvest: two samples harvested 20 days after inoculation and one sample harvested 60 days after inoculation. In each of these sample pairs, *P. agilis* populations were higher in the E- tall fescue. Populations of *P. agilis* in E- tall fescue had

Figure 12. Transmission electron micrographs of *P. scribneri* eggs exposed to (A) chitinase, (B) water, or (C) buffer. Note the presence of the outer vitelline layer (VL), the continuity between the chitin layer (CL) and lipid layer (LL), and the lack of a clearly defined double membrane.

Bar = 0.1 μm .



exceeded inoculum level by 40 days after inoculation while populations in the three E+ samples remained below 200 throughout the experiment.

Numbers of eggs and vermiform *P. agilis* in E+ and E- tall fescue incubated at 16.5°C did not differ significantly 20, 40, or 60 days after inoculation. Initial populations of *P. agilis* infecting roots were well below inoculum levels and gradually decreased during the experiment (Fig. 13). Few eggs were found in either E+ or E- tall fescue, yet little change occurred in the percentage of juveniles between harvests. Life-stage distributions were similar in both E- and E+ tall fescue (Fig. 14). Third- and fourth-stage juveniles predominated both populations throughout the experiment. The percentage of adults decreased slightly by 60 days after inoculation, probably due to mortality of old females. The low temperature resulted in reduced reproduction and slowed juvenile development. Resistance mechanisms in the E+ tall fescue may have been depressed by the low temperature, or *P. agilis* may not be affected as strongly as *P. scribneri* by the endophyte's presence.

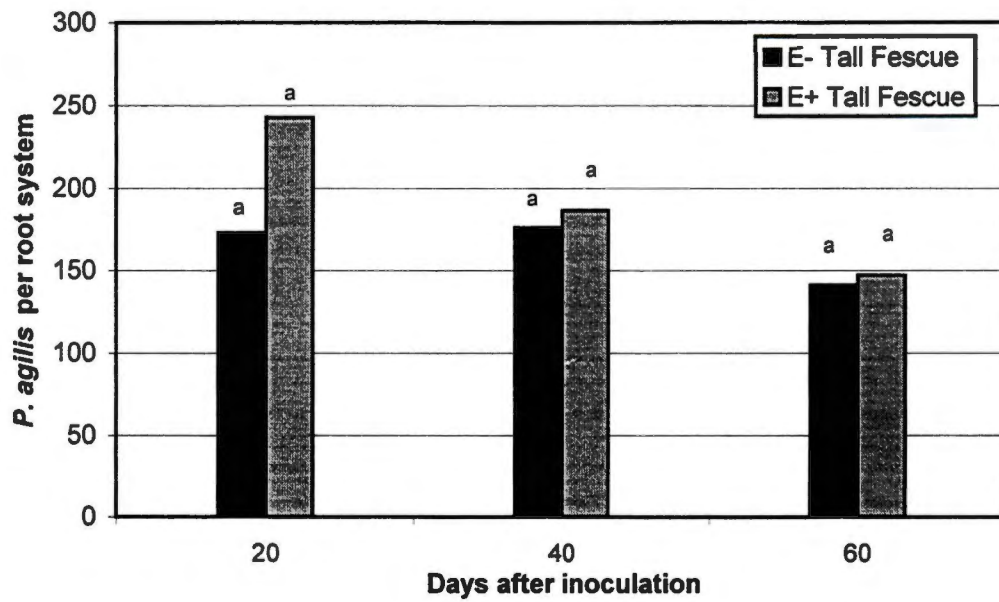


Figure 13. Mean number of vermiform *P. agilis* per root system in E+ and E- tall fescue incubated at 16.5°C. Columns in a pair headed by different letters are significantly different when compared by a paired t-test at $P \leq 0.05$.

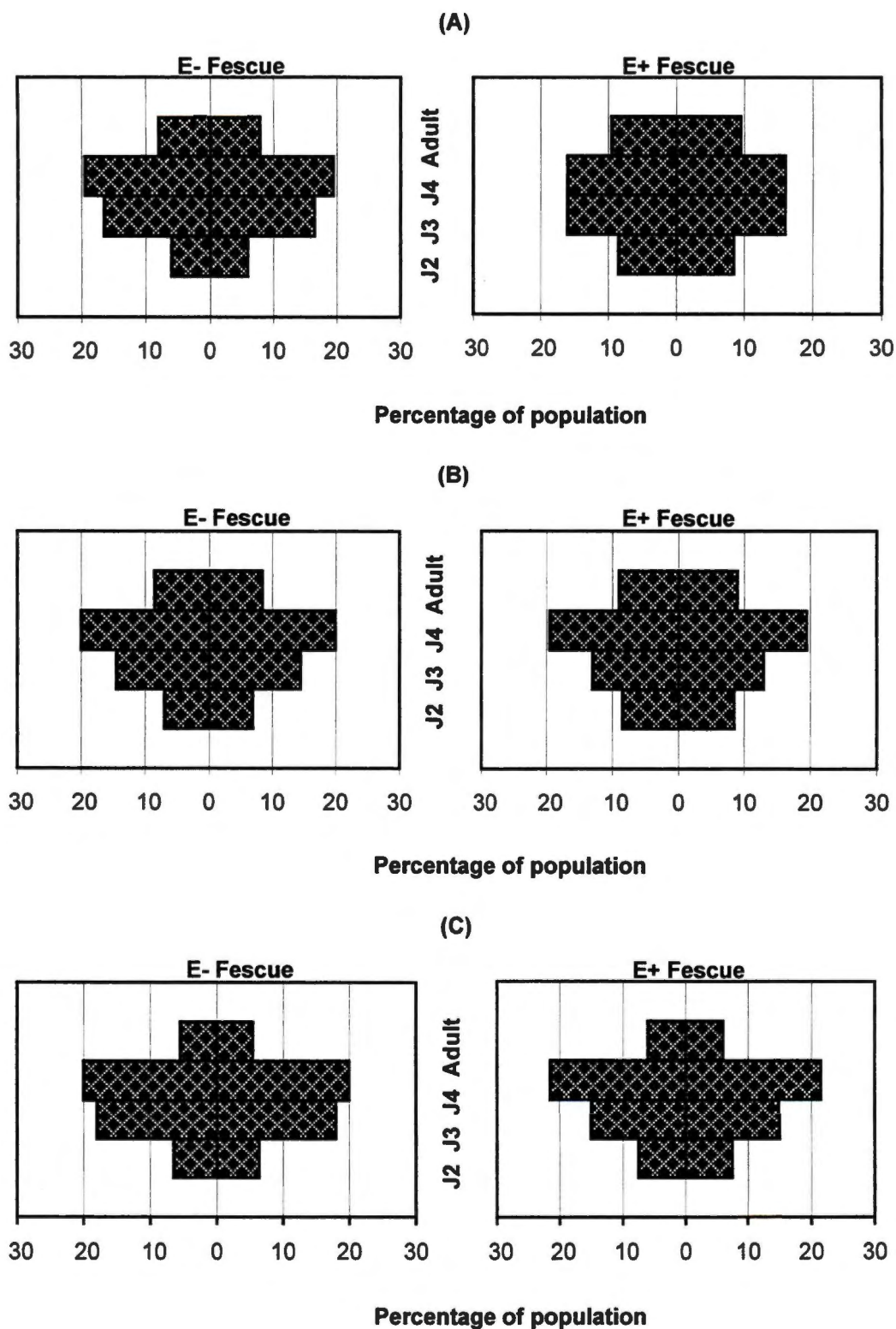


Figure 14. *P. agilis* life-stage distributions (A) 20 days after inoculation, (B) 40 days after inoculation, and (C) 60 days after inoculation when incubated at 16.5°C.

Chapter 4

Conclusion

During the short-term study, populations of *P. scribneri* were significantly reduced in E+ tall fescue. In addition, *P. scribneri* life stage distributions differed between E+ and E- tall fescue. Populations within the E- tall fescue followed a healthy pattern of reproduction and maturation whereas populations in E+ tall fescue underwent limited progressional development. The developmental rate of juveniles appeared slower in E+ tall fescue, resulting in a decrease in adult populations as older females died. The reduction in egg numbers in E+ roots was probably a direct effect of reduction in young females. Juveniles and eggs were present in E+ roots at all harvests; therefore, lack of reproduction was not the primary cause for the decline of *P. scribneri* populations in E+ tall fescue.

Due to the likelihood of environmental influences, a persistent difference could not be established between *P. scribneri* populations during the extended study. Juvenile development was slow in comparison to E- populations of the short-term study, perhaps due to fluctuations in temperature during irrigation. Since all factors except endophyte infection were common to populations in both E+ and E- tillers during the extended study, it can be assumed that differences in life-stage distributions and population size were due to the endophyte's presence. Though the difference between population sizes in Series 3 E+ and E- tall fescue was statistically significant only at 40 days after harvest, the difference in trends was obvious. *Pratylenchus scribneri* populations increased steadily in

the E- tall fescue but decreased in the E+ tall fescue. Decreased developmental rates could contribute to a population decline through lack of fecund adults and a concomitant decrease in juvenile recruitment. However, the rapid population reduction observed during the short-term study suggests that additional factors are involved as well. Females removed from E+ roots were capable of reproduction when placed in a conducive environment; therefore, E+ grass does not affect the development of the reproductive system. Lack of a significant difference in eggs/adult between E+ and E- tall fescue suggests that loss of fecund adults rather than chemical suppression is responsible for any decrease in reproductive rates.

The decrease in initial infection rates during the extended study was negatively correlated to increased tiller age. In addition, populations in both E+ and E- tall fescue may have been affected by the rapid temperature fluctuations caused by irrigation. However, the difference in life-stage distributions and population trends in E+ and E- tall fescue indicates that the endophyte continued to affect *P. scribneri* population dynamics well beyond the seedling stage in tall fescue development.

Incubation in chitinase had no noticeable affect on *P. scribneri* eggshell integrity. These results do not preclude the involvement of chitinase in a resistance mechanism. A number of chitinase isoforms are produced in both E+ and E- tall fescue (Roberts et al., 1994) and it may be that one or more of the particular isoforms produced in E+ tall fescue can affect nematode eggshells. However, the presence of eggs and juveniles throughout the short-term study

suggests that eggshell degradation is not a primary mechanism involved in population reduction.

Due to insufficient sample size, results for *P. agilis* population dynamics in E+ and E- tall fescue incubated at 30°C are inconclusive. Almost all of the “E+” tall fescue tillers incubated at 30°C were endophyte-free at the time of harvest. However, *P. agilis* populations in the three tillers that retained the endophyte were lower than populations in the paired E- tillers. This suggests that *P. agilis* is also affected by endophyte-mediated resistance mechanisms. No significant difference was observed between *P. agilis* populations in E+ and E- tall fescue incubated at 16.5°C. Life-stage distributions indicated a lack of developmental progress in E+ and E- nematode populations. Rapid increase in initial *P. agilis* populations in E- tall fescue incubated at 30°C suggests that tall fescue is a good host for *P. agilis*; therefore, the lack of reproduction and development at 16.5°C was probably temperature-related.

In these studies, *P. scribneri* and *P. agilis* populations were negatively impacted by the presence of the endophytic fungus in tall fescue. However, reproductive capability of female *P. scribneri* in E+ tall fescue was not permanently disrupted, nor was eggshell integrity compromised by exposure to bacterial chitinase. Rather, endophyte-infection appeared to slow the developmental rate of *P. scribneri* juveniles in tall fescue roots. Possibly, chemicals released by the endophyte act as feeding deterrents or even toxins against portions of the nematode population. *Pratylenchus scribneri* and *P. agilis* are considered closely related species; further studies examining the population

dynamics of additional *Pratylenchus* species would be useful for determining the specificity of endophyte-mediated resistance mechanisms in relation to lesion nematodes.

Bibliography

Bibliography

- Agrios, G. N. 1997. Page 51 *in* Plant pathology. 4th ed. San Diego, CA: Academic Press.
- Anonymous. 1998. Insect and plant disease control manual. The University of Tennessee Agricultural Extension Service. Entomology and Plant Pathology Information #67.
- Bird, A. B. and M. A. McLure. 1976. The tylenchid (Nematoda) egg shell: structure, composition and permeability. *Parasitology* 72:19-28.
- Bird, A. F. 1968. Changes associated with parasitism in nematodes. III. Ultra structure of the egg shell, larval cuticle, and contents of the subventral esophageal glands in *Meloidogyne javanica*, with some observations on hatching. *Journal of Parasitology* 54:475-489.
- Bird, A. F. and J. Bird. 1991. The structure of nematodes. 2nd ed. San Diego, CA: Academic Press.
- Boller, T., A. Gehri, F. Mauch and U. Vgeli. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* 157:22-31.
- Byrd, D. W., Jr., T. Kirkpatrick, and K. R. Barker. 1983. An improved technique for clearing and staining plant tissues for detection of nematodes from soil. *Journal of Nematology* 15:142-143.

- Clay, K. 1991. Fungal endophytes, grasses, and herbivores. Pages 199-226 in P. Barbosa, V. A. Krischik, and C. G. Jones, eds. Microbial mediation of plant-herbivore interactions. New York: John Wiley & Sons.
- Cornell, C. N., J. V. Lueker, and G. B. Garner. 1990. Establishing ergovaline levels for fescue toxicosis, with and without endoparasites, under controlled climatic conditions. Page 35 in Program and Abstracts of the International Symposium on *Acremonium*/Grass Interactions, Nov. 5-7, 1990, Baton Rouge, LA.
- Dowler, W. M. and S. D. Van Gundy. 1984. Importance of agricultural plant nematology. Pages 1-12 in W. R. Nickle, ed. Plant and insect nematodes. New York: Marcel Dekker.
- Dunn, R. A. 1973. Extraction of eggs of *Pratylenchus penetrans* from alfalfa callus and relationship between age of culture and yield of eggs. *Journal of Nematology* 5:73-74.
- Endo, B. Y., Zunke, U. and W. P. Wergin. 1997. Ultrastructure of the lesion nematode, *Pratylenchus penetrans* (Nemata: Pratylenchidae). *Journal of Helminthology* 64:59-95.
- Fegan, N., R. I. Webb, A. C. Hayward, and G. R. Stirling. 1993. Cryofixation and cryosubstitution as a method for preparing *Meloidogyne javanica* eggs for electron microscopy. *Fundamental and Applied Nematology* 16:309-313.

- Fribourg, H. A., C. S. Hoveland, and K. D. Gwinn. 1991a. Tall fescue and the fungal endophyte – a review of current knowledge. *Tennessee Farm and Home Science* 160:30-37.
- Fribourg, H. A., K. D. Gwinn, A. B. Chestnut, J. W. Oliver, J. C. Waller, J. H. Reynolds, and B. V. Conger. 1991b. Tall fescue and the fungal endophyte – the Tennessee research program. *Tennessee Farm and Home Science* 160:10-29.
- Giebel, J. 1974. Biochemical mechanisms of plant resistance to nematodes: A review. *Journal of Nematology* 6:175-184.
- Glenn, A. C., C. W. Bacon, R. Price, and R. T. Hanlin. 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* 88:369-383.
- Jones, C. S. 1997. Effect of endophyte-infected tall fescue on reproduction of *Pratylenchus scribneri* and possible relationship to chitinase. M.S. Thesis, The University of Tennessee, Knoxville, Tennessee.
- Kaplan, D. T. and Davis, E. L. 1987. Mechanisms of plant incompatibility with nematodes. Pp. 267-276 in J. A. Veech and C. W. Dickerson, eds. *Vistas on nematology*. Hyattsville, MD: Society of Nematologists.
- 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.

- Kunert, J. 1992. On the mechanism of penetration of ovicidal fungi through eggshells of parasitic nematodes. Decomposition of chitinous and ascaroside layers. *Folia Parasitologica* 39:61-66.
- Loof, P. A. A. 1978. The genus *Pratylenchus* Filipjev, 1936 (Nematoda: Pratylenchidae): A review of its anatomy, morphology, distribution, systematics and identification. Växtskyddsrapporter Jordbruk 5. Swedish University of Agricultural Sciences Research Information Centre.
- Mai, W. F., and P. G. Mullin. 1996. Plant-parasitic nematodes: a pictorial key to genera. 5th ed. Ithaca, NY: Cornell University Press.
- Mansfield, L. S., H. R. Gamble and R. H. Fetterer. 1992. Characterization of the eggshell of *Haemonchus contortus*—I. Structural Components. *Comparative Biochemistry and Physiology* 103B:681-686.
- McLaren, J. B. and H. A. Fribourg. 1991. Early history of tall fescue toxicosis studies in Tennessee. *Tennessee Farm and Home Science* 160:4-9.
- Mercer, C. F., D. R. Greenwood and J. L. Grant. 1992. Effect of plant and microbial chitinases on the eggs and juveniles of *Meloidogyne hapla* Chitwood (Nematoda: Tylenchida). *Nematologica* 38:227-236.
- Mian, I. H., G. Godoy, R. A. Shelby, R. Rodríguez-Kábana and G. Morgan-Jones. 1982. Chitin amendments for control of *Meloidogyne arenaria* in infested soil. *Nematropica* 12:71-84.
- Nickle, W. R. 1991. *Manual of Agricultural Nematology*. New York: Marcel Dekker.

- O'Day, M. H., T. L. Niblack, and W. C. Bailey. 1993. Phytoparasitic nematode populations in *Festuca arundinacea* field plots in southwestern Missouri. *Journal of Nematology* 24:900-906.
- Oliver, J. W., R. D. Linnabary, A. E. Schultze, B. W. Rohrbach, L. K. Abney, and E. M. Bailey. 1998. Reactivity of bovine vasculature to tall fescue toxins. Page 35 in *SERAIEG-8: Tall Fescue Toxicosis Workshop*, Nov. 1-2, 1998, Nashville, TN.
- Oliver, J. W., R. G. Powell, L. K. Abney, R. D. Linnabary, and R. J. Petroski. 1990. N-acetyl loline-induced vasoconstriction of the lateral saphenous vein (cranial branch) of cattle. Page 88 in *Program and Abstracts of the International Symposium on Acremonium/Grass Interactions*, Nov. 5-7, 1990, Baton Rouge, LA.
- Perry, R. N., D. A. Wharton, and A. J. Clarke. 1982. The structure of the egg-shell of *Globodera rostochiensis* (Nematoda: Tylenchida). *International Journal for Parasitology* 12:481-485.
- Punja, Z. K. and Y. Zhang. 1993. Plant chitinases and their roles in resistance to fungal diseases. *Journal of Nematology* 25:526-540.
- Riedel, R. M. and J. G. Foster. 1970. Monoxenic culture of *Ditylenchus dipsaci* and *Pratylenchus penetrans* with modified Krusberg's and White's media. *Plant Disease Reporter* 54:251-254.
- Riedel, R. M., J. G. Foster, and W. F. Mai. 1973. A simplified medium for monoxenic culture of *Pratylenchus penetrans* and *Ditylenchus dipsaci*. *Journal of Nematology* 5:71-72.

- Roberts, C. A., S. M. Marek, T. L. Niblack, and A. L. Karr. 1992. Parasitic *Meloidogyne* and mutualistic *Acremonium* increase chitinase in tall fescue. *Journal of Chemical Ecology* 18:1107-1116.
- Roberts, C. A., S. M. Marek, W. Lei, A. L. Karr. 1994. Determination of chitinase activity in tall fescue by near infrared reflectance spectroscopy: *Crop Science* 34:1070-1073.
- Sigee, D. C. 1993. *Bacterial Plant Pathology: Cell and Molecular Aspects*. Cambridge: Cambridge University Press.
- Spiers, D. 1998. The endophyte affects expression of the fescue chitinases. Page 35 in SERAIEG-8: Tall Fescue Toxicosis Workshop, Nov. 1-2, 1998, Nashville, TN.
- Stephan, S. 1989, unpublished. Dept. of Plant Pathology, University of Missouri, Columbia, MO 65211.
- Stirling, G. R. 1991. *Biological control of plant parasitic nematodes: progress, problems and prospects*. UK: C·A·B International.
- Timper, P. and B. B. Brodie. 1994. Effect of *Hirsutella rhossiliensis* on infection of potato by *Pratylenchus penetrans*. *Journal of Nematology* 26:304-307.
- Trudgill, D. L. 1991. Resistance to and tolerance of plant parasitic nematodes in plants. *Annual Revue of Phytopatholgy* 29:167-192.
- USDA Agriculture Handbook. 1986. *Guidelines for the control of plant diseases and nematodes*. No. 656.
- Watson, R. N., D. E. Hume, N. L. Bell, F. J. Neville, and A. J. Popay. 1995. *Plant-parasitic nematodes associated with perennial ryegrass and tall*

- fescue with and without *Acremonium* endophyte. Pages 199-203 in Proceedings of the Forty Eighth New Zealand Plant Protection Conference, Angus Inn, Hastings, New Zealand, Aug. 8-10.
- West, C. P., E. Izekor, D. M. Oosterhuis and R. T. Robbins. 1987. Endophytic fungus effects on tall fescue tolerance of drought and nematodes. Arkansas Farm Research December-November: 3.
- West, C. P. E. Izekor, R. T. Robbins, and R. Gergerich. 1990. Endophyte effects on infestations of barley yellow dwarf virus and soil-borne nematodes and insects. Page 70 in Program and Abstracts of the International Symposium on *Acremonium*/Grass Interactions, Nov. 5-7, 1990, Baton Rouge, LA.
- Wharton, D. 1980. Nematode egg-shells. Parasitology 81:447-463.
- Williamson, V. M. and R. S. Hussey. 1996. Nematode pathogenesis and resistance in plants. The Plant Cell 8:1735-1745.
- Zacheo, G. T. Blevé-Zacheo, and M. T. Melillo. 1997. Biochemistry of plant defense responses to nematode infection. Pages 201-213 in C. Fenoll, et al., eds. Cellular and molecular aspects of plant-nematode interactions. The Netherlands: Kluwer Academic Publishers.
- Zijlstra, C., V. C. Blok and M. S. Phillips. 1997. Natural resistance: the assessment of variation in virulence in biological and molecular terms. Pages 153-166 in C. Fenoll et al., eds. Cellular and molecular aspects of plant-nematode interactions. The Netherlands: Kluwer Academic Publishers.

Appendix

Appendix

Alfalfa Callus Culturing Procedure

Medium for sprouting alfalfa seeds

Prepare Check agar by mixing 10 g sucrose, 2 g yeast extract, 10 g Difco-nutrient agar, and 1000 ml tap water (Riedel and Foster, 1970). Autoclave for 30 minutes. Pour into 100 × 15 mm sterile Petri plates (50 plates if poured carefully).

Production of alfalfa roots

Sterilize Liberty alfalfa seeds by soaking for 15 minutes in a 30% sodium hypochlorite solution followed by four 5 minutes rinses in sterile water. Using sterilized forceps (dipped in 95% EtOH and flamed) place 15 alfalfa seeds on each plate of Check agar. Wrap the plates with parafilm to prevent contamination. Incubate at room temperature in the dark until sprouted roots are 1 to 2 cm in length.

Medium for callus cultures

Prepare Riedel's simplified medium by mixing 20 g sucrose, 5 g yeast extract, 2.5 mg 2,4-dichlorophenoxy acetic acid, 10 g Difco-Bacto agar, and 1,000 ml distilled water (Riedel et al., 1973). Heat while stirring until all solids are dissolved. Pour into 13 cm × 4 cm glass culture tubes (50 ml per tube, 24 tubes). Cap the tubes and autoclave for 30 minutes. Transfer to incubator at

30°C, incubate overnight or until free water is no longer present on culture tube caps or sides. Better callus growth is achieved if the surface of the agar is free of standing water as well.

Callus culture

Cut alfalfa roots 0.5 cm from root tip and place 3 root tips in each tube of Riedel's simplified medium. Wrap parafilm around bottom of each culture tube cap to prevent contamination and slow dehydration. Incubate at 30°C in the dark. To maintain callus cultures, transfer healthy pieces of callus (0.5 cm diameter) to freshly prepared tubes of Riedel's simplified medium. Pale-colored callus tissue survives transfer better than dark tissue.

Callus cultures can be inoculated with vermiform *P. scribneri* when the callus tissue is approximately 2 cm in diameter. Pipette 0.5 ml inoculum solution over the top of callus tissue (may concentrate inoculum by filtering over #500 mesh screen). Wrap the bottom of the caps with parafilm and incubate in the dark at 30°C.

Vita

Jeanese Holmes Reiss was born in Opp, Alabama on July 30, 1975. She attended Straughn School, a rural public school in Andalusia, Alabama, and graduated in 1993. Following high school graduation, she entered Troy State University, Troy, Alabama, where she obtained a Bachelor of Science in Biology in 1997. She then moved to Knoxville, Tennessee, to enroll in the Master of Science program in Entomology and Plant Pathology at the University of Tennessee. She received the MS degree in August, 1999. She is currently pursuing a PhD in Plant and Soil Sciences at the University of Tennessee.

