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I am submitting herewith a thesis written by Jean Carlson Batzer entitled "Effects of the Tall Fescue Endophyte on Seed Germination and Resistance to Preemergence Damping-off." 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 Entomolgy and Plant Pathology.

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We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

EFFECTS OF THE TALL FESCUE ENDOPHYTE ON SEED GERMINATION AND RESISTANCE TO PREEMERGENCE DAMPING-OFF

A Thesis

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Master of Science

Degree

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Jean Carlson Batzer

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DEDICATION

This thesis is dedicated to the memory of my dad, Harold O. Batzer, who planted in me the love of science and nature; and to my husband, Allen Carlson and children Alex, Nina and Hannah who have supported me in these pursuits; and to my mom, Teddie Batzer, for her encouragement.

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Abstract

Tall fescue (*Festuca arundinaceae*) infected with the endophyte *Neotyphodium coenophialum* is correlated with increased resistance to damping-off . The objective of this research was to determine the mechanism for endophyte-mediated resistance (EMR) to *Pythium aphanidermatum.* In experiments using Kentucky 31 seed differing only in endophyte infestation levels (endophyte-infected (95%) was defined as 95E+ and endophyte-free was defined as 95E-), 95E+ seed were more resistant to damping-off than 95E- seed. However, when seed were presoaked in sterile deionized water (SDW) for 24 h before planting, no resistance was observed. Embryo infection and seed colonization of 95E+ and 95E- seed were compared in *P. aphanidermatum*-infested soilless medium. Seed were incubated in infested medium for 20 h, rinsed, placed on selective medium, and observed at 20 and 30 h. Embryos of 95E- seed developed necrosis at twice the rate of 95E+ seed. However seed coat colonization was not different and did not affect embryo viability. Radicle emergence was assessed from seed incubated in rotating flasks of SDW for 24, 48, 72, or 96 h. At 24 h the germination rate of 95E+ seed was 13% and 95E- seed was 34%; at 96 h germination rates of 95E+ and 95E- seed were equivalent (ca. 80%). To determine the effect of presoaking on germination three seed treatments were compared: 1) nonscarified, 2) scarified, presoaked for 3 h, 3) scarified, presoaked for 24 h. Seeds were planted in soilless mix and incubated for 7 d. No differences in germination were observed between 95E+ and 95E- seed when presoaked for 24 h, but 95E+ shoot lengths were 15% less than those of 95E-. Germination of 95E+ seed was reduced by 30% and 40% when nonscarified or presoaked for 3 h, respectively, but 95E-

seed was not affected by seed treatment. Similar germination rates for all seed treatments were observed when incubated for 3 weeks. Antifungal activity of 95E+ seed against *P. aphanidermatum* mycelium was not observed. The influence of endophyte on the spermosphere was measured using pH, conductivity, carbohydrate content, and a *Pythium* bioassay. Seed were placed in 24-well plates with 1 ml SDW. Using iodine tests of 24 h steep water, it was determined that wells with 95E- seed contained more carbohydrate. Conductivity and pH were measured at 3 and 24 h. Rates of pH decline and conductivity increase were greater in 95E- wells than 95E+ wells. In wells containing 2 seeds/ ml SDW the pH decreased to 4.0 at 3 h. The effects of pH, molarity, and endophyte status on sporangia of *P. aphanidermatum* were tested in sterile sodium citrate buffer with 95E+ and 95E- seed. Buffers tested ranged from pH 4.0 to pH 6.5, and concentrations ranged from 0.05 to 0.001M. Seeds were placed in tissue culture dishes with buffer then a *Pythium*-infested grass piece was placed in each well. Controls with no seeds were included. Cultures were incubated at 28 C with continuous fluorescent light for 24 h and observed for presence of zoospores. Zoospore production was evident in wells with high pH and low molarity. No zoospores were observed in wells with 0.05M buffers; no zoospores were observed in wells with pH of 4.0. At low buffer concentration (0.001M and 0.005 M) and medium pH levels (5.5 and 6.0) zoospore production was less in wells with 95E+ seed than 95E- seed or controls. Thus differences in pH and conductivity in the spermosphere as a result of delayed germination may partially explain EMR. These results were inconsistent with the response of *P. aphanidermatum* propagules to 95E+ and 95E- seed in SDW, where 95E+ seed differentially inhibited zoospore production.

Based on these data, factors other than a reduction in 95E+ seed exudation may be involved in EMR. However, it was concluded that a primary factor of reduced infection of 95E+ seed by *P. aphanidermtum* is believed to be escape through germination delay.

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Part 1: Introduction and Overview

Literature Review

Tall Fescue

Tall fescue (*Festuca arundinacae* Schreb.) is a cool-season perennial grass widely used for hay, pasture and turf. Natural tall fescue populations are found from north Africa to northern Europe (Easton *et al.,* 1994) and may have been introduced to America as a contaminant in grass seed (Hoveland, 1993). The grass can tolerate a wide range of climates and soil types and is therefore well-suited to the transition zone between the northern and southern regions of the eastern United States (Williams *et al.,* 1984). The limits of its range are set by temperature and rainfall (less than 45 cm/year (Easton *et al.,* 1994). The most commonly grown cultivar in the U.S., Kentucky 31, was discovered in an luxuriant pasture in Menifee County, Kentucky. Cultivar performance was highly successful in field testing, and as a result, the commercial variety was distributed to farmers in 1942 (Clay, 1989). Kentucky 31 rapidly became the predominant meadow grass, with over 14 million hectares of tall fescue being cultivated in the eastern U.S. in 1993 (Hoveland, 1993).

The Endophyte

Fescue Toxicosis

Within a few years after the release of Kentucky 31, people became aware that

cattle grazing on this grass often exhibited alarming physical and behavioral disorders with no apparent cause (Pratt and Haynes, 1950). Researchers were puzzled since forage analysis indicated the quality of digestible dry matter, crude protein, amino acid, and mineral content of tall fescue should have provided a high performance diet to grazing animals (Bush and Bruckner, 1973). In 1977 scientists at the University of Georgia discovered that fescue pastures where cattle suffered the most severe symptoms were highly infected with an endophytic fungus that was apparently an anomorph of *Epichloe typhinum* (Fires) Tulsne. (Bacon *et al.*, 1977). The newly discovered endophyte was subsequently identified in grass pastures thoughout the world. A 1987 survey of tall fescue pastures in the southeastern U.S. reported that 95% of the plant samples examined were infected with the *Neotyphodium coenophialum* (Morgan-Jones and W. Gams) Glenn, Bacon & Hanlin comb. nov.(=*Acremonium coenphialum*) (Shelby and Dalrymple, 1987). In New Zealand an estimated 20 to 30% of all grassland is composed of *Neotyphodium lolii* (Latch, Chistensen, and Samuels) Glenn, Bacon & Hanlin comb. nov. (=*A. lolii*) infected perennial ryegrass (*Lolium perenne* L.) (Clay, 1994).

Consumption of tall fescue forage infected with *N. coenophialum* can result in fescue toxicosis. (Patterson *et al.*, 1995; Porter, 1995). Symptoms are greatly influenced by temperature and age of cattle (Hempken *et al.*, 1981). In hot weather, fescue toxicosis of cattle is characterized by decreases in weight gain, reduced milk production, lower conception rate (up to 50%) and suppression of serum prolactin (Patterson *et al.*, 1995). These conditions are manifested by fever, rough hair coat, and behavioral changes. Vascular constriction leading to gangrenous necrosis of tissue extremities occurs during

cold weather (Cross *et al.*, 1995). In older cattle, masses of hard fat in the tissue surrounding the intestines are symptomatic of fescue toxicosis (Bush *et al.*, 1979). An annual loss of more than $609 million in production is attributed to reduced conception rates and decreased calf weight gains caused by the consumption of endophyte-infected fescue (Hoveland, 1993). In Tennessee, annual losses are estimated at $60 million to $85 million (Fribourg *et al.*, 1991).

In addition to the detrimental effects on cattle, endophyte-infected forage causes losses of other livestock and wild animals (Casada, 1991). Horses display similar symptoms of fescue toxicosis to cattle, but do not exhibit increased body temperature or fescue foot. Thickened placentas, high foal mortality, poor milk production, and occasional mare mortality, is a serious effect of fescue toxicosis in pregnant mares (Cross *et al.*, 1995). Consumption of *N. lolii*-infected perennial ryegrass often results in "ryegrass staggers", a neurological disorder of sheep (Fletcher and Harvey, 1981).

Alkaloids in Endophyte-infected Grasses

The toxic effects of endophyte-infected grass on livestock are caused by alkaloids. Endophyte-infected tall fescue contains ergopeptine alkaloids, primarily ergovaline, while endophyte-infected perennial ryegrass contains the indole isoprenoid lolitrem alkaloids, primarily lolitrem B (Porter, 1995). Ergovaline and lolitrem B are believed to be the primary cause of animal toxicosis (Porter, 1995). Ergovaline levels are 5 times or more higher in seed heads than vegetative tissues (Garner *et al.*, 1993). Loline alkaloids, primarily N-acetylloline and N-formylloline, found in infected fescue and the

pyrrolopyrazine alkaloid, peramine, found in infected perennial ryegrass (Rowan, 1993), have been correlated with insect-deterrence, but may augment the animal toxicity of the ergot and lolitrem alkaloids (Porter,1995). Alkaloid accumulation varies with environment and plant age (Bush and Schmidt,1993; Ball *et al.*, 1993). For example, alkaloid content increases with nitrogen fertilization (Garner *et al.*, 1993). The species or strain of endophyte, and the cultivar of grass, affect the alkaloid content of the plant (Kearney *et al.* 1991). Several strains of *N. lolii* that produce the insect-deterrent peramine, but do not produce lolitrem B have been isolated from wild ryegrass. These endophyte strains are being used to infect a number of perennial ryegrass cultivars in order to develop an insect-resistant, animal toxicoses-free forage (Latch, 1994).

Grass Endophyte Taxonomy

Neotyphodium is placed within the Deuteromycota, because it has no sexual stage. Conidiophores and conidia are formed in culture, but have not been observed in endophyte-infected plants (Bacon *et al.,* 1977). Plants grown axenically in agar culture produce conidia and conidiophores on root surfaces (Azevedo and Welty, 1995). Colonies grown in culture are white or yellow, dense and slow growing (Bacon *et al.,* 1977). Endophyte-infected seed placed on agar will develop visible colonies within 4 to 6 weeks (Batzer, personal observation).

Neotyphodium spp. are believed to be anomorphs of the choke disease pathogen *Epichloe*, a member of the family Clavicipitaceae (Hypocreales;Ascomycotina) (Morgan-Jones and Gams, 1982) (Glenn *et al.,* 1996). Until recently, the grass endophytes were

classified in the polyphyletic form genus *Acremonium*, containing distantly related fungi. These endophytes were classified in the *Albo-lanosa* section of the *Acremonium* L. genus, which is in the tribe Balansiae of the family Claviciptaceae of the division Ascomycota because of their *in vitro* colony characteristics (Diehl, 1930). In an effort to place the grass endophytes into a monophyletic group, several *Acremonium* taxa were analyzed using sequences of the nuclear encoded small subunit ribosomal DNA (18S rDNA). As a result of these analyses, grass endophytes were classified into a new taxon *Neotyphodium* (Glenn *et al.,* 1996).

Epichloe and *Neotyphodium* endophytes infect cool season grasses within the Poacaea (White, 1987). White (1988) suggested that *Epichloe*-like endophytes may have evolved from being a parasite to a mutualistic endophyte. While Clay (1993) hypothesized the occurrence of hyphal hybridization within a single host infected with different fungi resulting in nonchoke-inducing *Epichloe* endophytes. Additional evidence supporting the hypothesis that endophytes evolved from *E. typhina* and are not strictly coevolved with their grass hosts include: serological cross-reactivities (Johnson *et al.,* 1982), similar isozyme patterns (Leuchtman and Clay, 1990) and conserved ribosomal RNA nucleotide sequences (Schardl, *et al.* 1991).

Neotyphodium endophytes are often associated with a particular species of grass. Examples of host-endophyte associations are as follows: *N. coenophialum* of tall fescue; *N. lolii* of perennial ryegrass (Latch *et al.,* 1984); and *N. uncinatum* (Gams, Petrini & Schmidt) Glenn, Bacon & Hanlin comb. nov. of meadow fescue (*Festuca pratensis* Huds.). However, several *Neotyphodium* endophytes can infect a single species of grass.

Neotyphodium endophytes are associated with other cool-season turf grasses of the subfamily Pooideae besides those already mentioned. These include the following: strong creeping red fescue (*F. rubra* L. subsp. *rubra*), slender creeping red fescue (*F. rubra* L. subsp. *litoalis* [D.F.W. Meyer] Auquier), Chewings fescue (*F. rubra* L. subsp. *commutata* Gaud.), hard fescue (*F. glauca* La.) and six species of *Poa* (Latch *et al.,* 1984; Sun and Breen, 1993; White and Cole, 1986b). In addition to the *Neotyphodium* endophytes of *Lolium* and *Festuca*, there are *Gliocladium*-like and *Phialophora*-like endophytes (Latch *et al.*, 1984). These endophytes also reside in the intercellular spaces of tiller bases and in the nodes of flowering tillers (Latch *et al.*, 1984). They are easily distinguished from *Neotyphodium* endophytes when hyphae are microscopically observed in leaf sheath and seed sections that are stained with aniline blue in lactic acid (Clark *et al.*, 1983).

***Neotyphodium* Endophyte Life Cycle**

An endophyte is an organism that is spends its entire life cycle living wholly within plant tissues (Cheeke, 1995). Unlike the parasitic *E. typhina*, which infects its host, *Neotyphodium* endophytes have no external state. Their only dissemination in nature is though the seed and vegetative propagules of their hosts (Hinton and Bacon, 1985). Hyphae reside in the intercellular spaces within the tiller bases of the grass, without invading the cell. The localization of the fungus to the meristematic leaf sheath tissues is mediated by the availability of extracellular sucrose, xylose and arabinose while much lower concentrations of sugars are available in the apoplast of the mature leaf

mesophyll (White *et al.*, 1993). Seed transmission occurs when the endophyte mycelium advances with the meristematic cells of the terminal stem which gives rise to the grass inflorescence (White *et al.*, 1993). The fungus colonizes the carpels of the flower and the endosperm cells. As the embryo forms, the ovule is not penetrated by the hyphae (White *et al.*, 1991), but a layer of mycelium is formed beneath the seed coat, contiguous to the scutellum (Hinton and Bacon, 1985). Subsequent infection of the embryo occurs after complete differentiation of the shoot meristem (White *et al.*, 1991). White and Cole (1986a) did not observe hyphae in the coleoptile and root primordia. In a different study Philipson and Christey (1986) observed hyphae within the embryo during the later stage of seed development and reported that hyphae penetrate intercellularly and reside in the leaf and coleoptile primordia.

In either case, upon seed germination, abundant mycelia reside intercellularly in leaf meristem coleoptiles, particularly in the region of the mesocotyl (White and Cole, 1986a). The fungus has not been observed in the roots of mature tall fescue (Bacon and Hinton, 1985). However, the endophyte has been found in the root primordia of 3-week-old tall fescue within spaces created by the separation of the middle lamella in columnar epidermal cells (Azevedo and Welty, 1995). Hyphae growing on the surface of roots have electron dense granules within the cytoplasm that are high in phosphate, similar to mycorrhizae (Azevedo and Welty, 1995).

Survival of the endophyte is dependent upon the success of its host. The fungus is protected, nourished, and disseminated by the plant. The endophyte must disseminate though seed since its host cannot become infected with endophyte in the field. Since a

large proportion of plants are endophyte-infected (Fribourg *et al.*,1991; Latch, 1985), endophyte infection must confer a competitive advantage to its host.

Endophyte-infected Grasses are Better Competitors

Once the endophyte was linked to fescue toxicosis (Bacon *et al.*, 1977), efforts were made to eradicate the fungus. Endophyte-free seed were easily obtained. Endophytic fungi die when seed are stored for 18 month under non-refrigerated conditions (Welty *et al.*, 1987). As endophyte-free fescue and perennial ryegrass were planted, the problem of animal toxicosis was alleviated. However, pasture quality was greatly diminished (Bacon, 1995). Cattle overgrazed endophyte-free tall fescue causing severe defoliation that lead to poor survival of grass plants in the hot, dry summers in the southeastern U.S. (Bacon, 1995).

At first it was assumed that livestock overgrazing was the primary cause of pasture damage. That is, the toxic effects of the endophyte-infected plants had protected the grass plants from excessive defoliation (Siegel, *et al.*, 1987). While overgrazing constitutes part of the problem of poor quality in endophyte-free pastures, several other factors appear to contribute to the improved competitive ability of endophyte-infected grass.

Direct stimulation of plant growth by the endophyte is credited as a factor in improved stand quality (Latch *et al.*, 1985). Endophyte-infected tall fescue produce significantly more seed heads and biomass than uninfected fescue grasses (Hill *et al.*, 1991; Read and Camp, 1986). Improved germination, seedling growth and tillering may

result in greater competitive advantage of endophyte-infected plants over weeds and endophyte-free grasses. Increased seed germination has been associated with endophyte infection (Pinkerton *et al.*, 1990). However Blank (1992) did not find differential germination rates between endophyte-infected and endophyte-free tall fescue seed within the same lot. More tillering in endophyte-infected than in endophyte-free plants may reduce bare spots (Clay, 1987). Higher levels of indole acetic acid in endophyte-infected plants may cause differential tillering of endophyte-infected and endophyte-free grass (DeBattista *et al.*, 1990).

Greater growth and persistence of endophyte-infected grass contributes to enhanced competitiveness and resistance to biotic and abiotic stress factors. For example, in a southern Indiana pasture, infected tall fescue was a better competitor against orchard grass than was uninfected tall fescue; and the competitive advantage was increased in the presence of insect herbivores, relative to herbivore-free controls (Clay *et al*, 1993). Infected tall fescue is a better competitor against perennial ryegrass than uninfected fescue, but endophyte-infected perennial rye grass is more competitive than endophyte-free perennial rye grass when grown in competition with tall fescue (Marks *et al.*, 1991). Since competition is a complex interaction in nature, generalizations that endophyte infection invariably results in enhanced competitive ability should be closely scrutinized with respect to environmental factors such as soil nutrients, drought stress, temperature, species of insect pest, pathogen strain, endophyte strain, and genotype of grass.

Endophyte Enhanced Drought Resistance

Enhanced drought tolerance is one facet of the mutualistic association of the endophyte and its host plant. In the southern regions of the tall fescue range, drought stress is the most limiting factor to pasture persistence (Read and Camp, 1986). During periods of drought, endophyte-free tall fescue pastures are less persistent than endophyte-infected stands (Read and Camp, 1986; West *et al.*, 1993). Endophyte presence apparently results in the elevation of the water-deficit theshold for inducing drought stress in tall fescue, leading to increased tiller and plant survival in endophyte-infected plants (West, 1994). Since endophyte-infected plants have greater tolerance for drought, they are better able to compete with weed species, as well as endophyte-free grass.

Investigations designed to elucidate the mechanisms of endophyte-conferred drought tolerance have revealed the complexity of the endophyte-host interaction. Numerous aspects of drought tolerance mechanisms have been investigated with mixed results and conclusions. Mechanisms that result in reduced transpiration may include the following: higher stomatal resistance (a result of stomatal closure) (Richardson *et al.*, 1993b); delayed leaf rolling (Arachevaleta *et al.*, 1989; White *et al.*, 1992a); higher water efficiency (Bates and Joost, 1990; Richardson, *et al.*, 1990); greater leaf senescence and reduction in leaf expansion rate (Maclean *et al.*, 1993; Belesky, 1989); differential tillering rate (Maclean *et al.*, 1993, Arachevaleta *et al.*, 1989); and endophyte-enhancement of abscisic acid concentration signaling early stomatal closure (Bunyard and McInnis, 1990). Differences in conclusions regarding the mechanism of endophyte-enhanced drought tolerance can be attributed to differences in grass genotype, endophyte

strain and experimental conditions.

While mechanisms leading to enhanced drought tolerance are not completely understood, there is strong evidence that osmotic adjustment is a direct effect of endophyte infection. Osmotic adjustment is a response to water stress, resulting in the accumulation of cell solutes (Turner, 1986). When osmotic potential is reduced, cell turgor pressure is retained and cell metabolism and growth is maintained (West, 1994). Endophyte by-products (hexoses and sugar alcohols) in tiller bases act as osmotica and cause differential osmotic adjustment in the basal meristems of endophyte-infected tall fescue (Elmi *et al.*, 1989; Elmi *et al.*, 1990; White *et al.*, 1992b; Richardson *et al.*, 1992; Richardson, 1993a; West *et al.*, 1990b) leading to increased survival of tillers in severe drought . Thus dessication of endophyte-infected plants is delayed.

Fungal-derived sugar alcohols may also protect plant enzymes and membranes from desiccation (Bacon, 1993) and serve as antioxidants (West and Gwinn, 1993). The endophyte may induce a sort of incipient stress that somehow preconditions or sensitizes the host to drought, thereby permitting the plant to exhibit adaptive responses sooner (West, 1994).

Endophyte-infected Grasses are More Resistant to Insect Pests

Use of endophyte-infected grass is an effective pest management strategy. Endophyte-infected grasses affect over 40 species of insects (Latch, 1994; Rowan and Latch, 1994). Important pests reduced by endophyte infection include the following: grubs (*Popilla japonica* Newman , *Cyclocephala lurida* Arrow), sod web worm

(*Crambus* spp.), billbugs (*Sphenophorus* spp.), chinch bugs (*Blissus* spp.), and fall armyworm (*Spododptera frugiperda* Smith) (Oliver, 1990; Hardy *et al.,* 1986; Johnson *et al.*, 1985; Siegel *et al.*, 1990).

The mechanisms for endophyte-mediated pest tolerance are highly diverse and complex involving survival, behavioral, and ecological effects (Dahlman *et al.*, 1991). Specifically, the Argentine stem weevil (*Listronotus bonariensis* Kuschel) is deterred from laying eggs in infected perennial ryegrass (Rowan and Gaynor, 1986). In general, certain loline and ergopeptine alkaloids have been shown to alter oviposition sites (Prestidge and Ball, 1993), affect feeding choice (Rowan and Gaynor, 1986) and reduce the survival of insect pests (West and Gwinn, 1993). For example, bird-cherry oat aphid (*Rhopalosiphum padii* L.) and green bug (*Schizaphis graminum* Rondani) avoid leaf tissue with high concentrations of loline derivatives (Siegel *et al.*, 1990; Eichenseer *et al.,* 1992). Effects are species-specific and are related to plant age. For example, an initial burst of peramine from germinating *N. lolii*-infected perennial ryegrass increases resistance to Argentine stem weevil, but peramine is nearly absent in the roots of mature ryegrass, which are not resistant to root feeding insects (Ball *et al.,*1993). Endophyte infection has also been adversely linked to survival of insect parasites. For example, larvae of the parasitoid wasp (*Eucplectus comstockii* Howard) reared on fall armyworm larvae, pupate 6 hours sooner when host larvae are fed endophyte-free fescue rather than endophyte-infected fescue (Bultman *et al.,*1993).

The Endophyte Confers Disease Resistance

Viruses. Insect-transmitted viral diseases are reduced in pastures with high endophyte status. Direct resistance to viruses by endophyte-infected grasses has not been demonstrated. However, insect vector deterrence is an important factor. For example, in a 3-year old plot of tall fescue, 73% of endophyte-free plants and 31% of endophyte-infected plants were infected with barley yellow dwarf virus (West *et al*, 1990a). Reduced levels of viral infection were subsequently related to feeding deterrence of the aphid vector (bird-cherry oat aphid) by endophyte-infected tall fescue (Mahmood *et al.*, 1993).

Nematodes. Plant parasitic nematode populations are inversely related to endophyte infestation levels in tall fescue (Elmi *et al.*, 1990; Pedersen *et al.*, 1988; Kimmons *et al.*, 1990). Endophyte-infected tall fescues have enhanced resistance to *Meloidogyne* spp, *Pratylenchus scribneri* Steiner and *Tylenchorynchus acutus* Allen (West *et al.* 1988; Kimmons *et al.,* 1990). Endophyte-mediated nematode resistance is apparently not conferred to perennial ryegrass (Pedersen *et al.*, 1988; Gwinn and Bernard, 1993).

Mechanisms of endophyte-mediated nematode resistance are species-dependent. For example, reproduction of *Meloidogyne graminis* (Sledge and Golden) Whitehead is reduced in endophyte-infected tall fescue roots as a result of fewer numbers of egg masses laid and less eggs deposited per mass (Kirkpatrick *et al.,* 1990). In contrast, juvenile colonization and egg hatching rate of *M. marylandi* Jepson and Golden is

suppressed in endophyte-infected fescue roots (Kimmons *et al.*, 1990). On the other hand, juveniles of *P. scribneri* penetrate roots regardless of endophyte status; after 30 days increases in nematode populations occur on endophyte-free plants only (Gwinn and Bernard, 1993). The endophyte alters the root chemistry and physiology of its host. Root exudates may repel juveniles and suppress hatching rates of some nematode species (Gwinn and Bernard, 1993). Increased presence of chitinase (which could affect the integrity of nematode eggs), and lolines (which have potential toxic effects) have been postulated as possible components of complex host-nematode interactions (Roberts *et al.*, 1992; Siegel *et al.*, 1987)) .

Fungi. Evidence is accumulating that endophyte-infected tall fescue and perennial ryegrass may be more resistant to diseases caused by fungi. These host-pathogen interactions are highly specific. For example, crown rust (*Puccinia coronata* Corda.) severity is reduced in endophyte-infected tall fescue (Ford and Kirkpatrick, 1989). However, endophyte infection does not affect tall fescue resistance to stem rust *P. graminis* Pers:Pers (Welty *et al.*,1991). Pathogenic and beneficial soilborne fungi may be suppressed by endophyte-infected fescue. For example, post-infection colonization and sporulation of *Glomus macrocarpum* Tul. and Tul., a mycorrhizal pathogen of tobacco, is inhibited when tobacco is rotated with endophyte-infected fescue (Hendrix *et al.*, 1992); populations of potentially beneficial mycorrhizae are also suppressed under these conditions (Chu-Chou *et al.*,1992).

Plant age is an important factor in endophyte-mediated resistance (EMR) to soilborne pathogens. For example, endophyte-infected tall fescue seed planted in soil

infested with damping-off pathogens *Rhizoctonia zea* Voorhees (Gwinn and Gavin, 1992), *R. solani* Kuhn or *Pythium aphanidermatum* (Edson) Fitzpatrick (Blank *et al.*, 1993) had higher emergence levels than endophyte-free seed. However, resistance of mature tall fescue to *Rhizoctonia* leaf blight is not affected by endophyte infection (Gwinn and Bernard, 1988; Burpee and Bouton, 1993). Endophyte infection can have beneficial and detrimental effects on resistance to soilborne pathogens. For example, endophyte-infected meadow fescue is more resistant to damping-off caused by *R. cerealis* van der Hoeven, but more susceptible to damping-off caused by *Fusarium culmorum* (Wm.G. Sm.) Sacc. (Schmidt, 1993). The mechanisms associated with colonization and infection of germinating seeds by soilborne pathogens has not been investigated.

Mechanisms for EMR are unknown. Growth of plant pathogenic fungi are suppressed by *N. coenophialum in vitro*. For example, cultures and culture filtrates of *N. coenophialum* inhibit the growth of turf pathogens *R.. cerealis*, *Nigerosporum sphaeritheca* (Sacc.) Mason and *Phoma sorgina* (Sacc.) Boerema, Dorenbosch, and van Tieghem (White and Cole, 1985; Seigel and Latch; 1991). Antifungal activity is affected by endophyte species and strain (Seigel and Latch, 1991). Chistensen *et al.* (1991) observed that antibiotic activity of *N. lolii* was correlated with colony morphology.

The endophyte alters the physiology of its grass host. For example, plant hormones indole acetic acid and abscisic acid are produced by *N. coenophialum in vitro* (Bunyard and McInnis, 1990; De Battista *et al.*, 1990). Sugar alcohol content and nitrogen assimilation patterns are altered in endophyte-infected tall fescue (Richardson, 1992, Lyons *et al*, 1990). Lindstrom *et al.* (1993) detected a novel protease in endophyte-

infected *Poa* species, which was not observed in endophyte-infected varieties of fine or tall fescue. Higher levels of chitinase, a PR-protein, have been observed in endophyte-infected tall fescue (Roberts *et al.,* 1992). These physiological changes have been studied in regard to nematode and drought tolerance, but not fungal disease resistance.

Alkaloid toxicity has been suggested as a mechanism for disease resistance. Guo *et al.* (1991) hypothesized that toxic alkaloids produced by *Neotyphodium (=Acremonium)* in infected tall fescue were responsible for the suppressive effect on mycorrhizal fungi. However, no *in vitro* antifungal activity against *Colletotrichum graminicola* (Ces.) Wilson, *Limonomyces roseipellis* Staplers et Loerakker, *R. zea*, or *R. cerealis* was found using paper disks containing loline, peramine, or the ergot alkaloids ergonovine maleate, ergotamine tartate and ergocryptine (Seigel and Latch, 1991).

Soilborne microbial communities appear to be affected by endophyte status. For example, field soil samples of endophyte-infected fescue plots contain lower populations of *Fusarium* spp, mycorrhizal propagules, and parasitic nematodes *Helicotylenchus* spp. and *Tylenchus* spp. than endophyte-free fescue plots. However, no significant differences in populations of *Pythium* spp. or *Rhizoctonia* spp. have been observed (Chu-Chou *et al.,*1992).

Seed Germination

The Endophyte in Germinating Seed

The fungal endophyte resides in a quiescent hyphal state either within the embryo (Philipson and Christey, 1986) or contiguous to the scutellum (Hinton and Bacon, 1985)

in the mature dry state. The fungus is not dormant and requires nutrients (Hinton and Bacon, 1985). Endophyte viability is lost within one year when seed are stored at room temperature (Siegel *et al.*, 1984). Thus depletion of food reserves may be related to endophyte death. As germination takes place unknown events result in the invasion of the endophyte into the emerging coleoptile (White *et al.*, 1991). The presence of the endophyte during the process of seed germination apparently imparts resistance to some damping-off pathogens. The presence of the endophyte within the seed could alter germinative events and affect resistance to soilborne pathogens.

Food Reserves Within Quiescent Seed

Food reserves are limited at the onset of seed germination. Basic storage reserves (carbohydrates, lipids, and proteins) are laid down during seed development. The dry weight of cereal endosperm consists of approximately 75% carbohydrate (starch), 2% oil, and 10 to 16% protein (Oaks, 1983). Oil is stored predominately in the scutellum (embryonic tissue) of cereals. Although the endosperm is the major source of carbohydrate reserves in cereals, some simple sugars are stored in small quantities within the embryo (Bewley and Black, 1994). Small amounts of sugars and triglycerides are available to the embryo prior to germination. These probably enable respiration during germination. Food reserves within the endosperm are mobilized after germination. In quiescent embryos, macromolecular synthesis and nuclear division have stopped, but these activities are resumed sequentially after the start of imbibition (Georgieva, *et al.*,

1994).

Imbibition of Seed is the Onset of Germination

Nondormant seed, such as *Festuca* spp. and *Lolium* spp., can begin the process of germination when sufficient moisture is present (Bewley and Black, 1994). As dry, viable seed imbibe water, metabolism quickly commences. A series of events is initiated that ultimately results in germination, marked by the emergence of the radicle (Bewley and Black, 1994). Germination is a differentiation process in which a heterotrophic embryo with extremely low metabolic activity changes to a fully active autotrophic organism (Bewley and Black, 1994).

Hydration of the peripheral cells of the seed and the radicle is very rapid (Bewley and Black, 1994). Hence, metabolism can commence within minutes of introduction of the seed to water. Rate of water penetration into the seed is critical to the success of germination. If water uptake is too slow, then germination is reduced because seeds may deteriorate; if water uptake is too rapid, seeds may suffer excess imbibitional damage (Bewley and Black, 1994). Water uptake is more rapid in endophyte-free than endophyte-infected seed, at 4 and 24 h after placement in distilled water (Rice *et al.*1990). Restricted water entry is related to increased germination of endophyte-infected seeds (Rice *et al.* 1990).

Within a few minutes after water enters the cell, respiration rates dramatically increase. Respiratory pathway intermediates (keto acids) are made available from amino acid conjugates (Bewley and Black, 1994). Respiration commences as mitochondrial

enzymes involved in the citric acid cycle and electron transport chain are activated. Mitochondria within freshly imbibed seeds lack cristae and are functionally deficient. However, the impaired organelles can conduct some oxidative phosphorylation, the major source of ATP, via the electron transport system. Mitochondria from early-imbibed seed have only small amounts of cytochome c, thus they oxidize malate or alpha-ketoglutarate poorly, using endogenous NADH (from the citric acid cycle) (Bewley and Black, 1994). Within 2 h of imbibition, corn seed initiates new synthesis of cytochome c oxidase. Other components involved in the synthesis of new mitochondria are synthesized after about 4 h (Georgieva, 1994). During the first few hours to several days after the start of imbibition, the seed channels respirable substrate though the glycolytic pathway to offset the reduced ATP production by mitochondria. Furthermore, to compensate for limited O_2 availability that often accompanies hydration of the seed, pyruvate (the product of glycolysis) can be converted to ethanol. When O_2 is more available and mitochondria become more active, the pentose phosphate pathway predominates as the seeds mobilize nutrient reserves and growth begins. Maize embryonic cells initiate DNA synthesis and cell division at around 14 h after seed imbibition (Georgieva, 1994).

Germination is Complete with the Emergence of the Radicle

Radicle emergence is caused by cell expansion rather than cell division (Bewley and Black, 1994). Growth of the radicle begins with loosening of the cell walls. Abscisic acid prevents radicle emergence by preventing cell wall loosening (Bewley and Black, 1994). Conversely gibberellic acid promotes the action of cell wall hydrolases (releasing

xylose and arabinose, which are endophyte carbon sources) and allows the expansion of the embryo. Abscisic acid and indole acetic acid are produced by *N. coenophialum* in culture (Bunyard and McInnis, 1990; De Battista *et al.* 1990); germinative events may be influenced by these fungal-derived plant hormones.

Mobilization of stored seed reserves mostly occurs after germination (Bewley and Black, 1994). As the radicle elongates, hydrolysis of reserves is first seen in the seedling, subsequently in the scutellum, and finally in the endosperm (Oaks, 1983). The initial stages of storage protein hydrolysis occur as cations (released from phytin) disrupt proteinase-inhibitor complexes, thus producing enzyme activity. Phytin occurs in the aleurone layer and is the primary storage molecule of phosphate. Amino acids liberated from storage proteins (mostly asparagine and glutamine) are utilized for seedling growth (Bewley and Black, 1994). Many essential proteins must be synthesized *de novo* by the embryo and accompany the appearance of enzyme activity (i.e. alpha-amylase, endo-peptidase, carboxypeptidase) (Oaks, 1983). The relative amounts of gibberellins and abscisic acid are important in controlling the induction of these enzymes. Synthesis of gibberellins probably occurs *de novo* in the scutellum (Bewley and Black, 1994). The hormone response is complex and involves both genetic and physiological components (Oaks, 1983). There are two catabolic pathways of starch; one hydrolytic and the other phosphorolytic. Hydrolytic enzymes alpha-amylase, beta-amylase, the debranching enzymes, and alpha-glucosidase degrade starch granules to glucose (Bewley and Black, 1994). Phosphorylase activity is low or negligible in cereals (Bewley and Black, 1994) In the early stages of starch mobilization, production of alpha-amylase occurs in the

region of the scutellum (Bewley and Black, 1994). Later the enzyme is synthesized within the aleurone layers and is secreted into the endosperm (Bewley and Black, 1994). Reliance on stored reserves diminishes as the seedling emerges above the soil and becomes photosynthetically active (Bewley and Black, 1994).

Seed Exudation Occurs During Germination

As seeds start to take up water, there is an immediate release of gas from colloidal adsorption and a rapid loss of sugars, organic acids, amino acids, and proteins (Bewley and Black, 1994). The solutes stimulate the growth of fungi and bacteria in the soil, which can lead to seed rots or preemergence damping-off (Nelson, 1987). During imbibition, organization of cell membranes changes from a gel to a liquid crystal and become temporarily leaky (Bewley and Black, 1994). The stability of these membranes can be maintained with the incorporation of certain sugar molecules in the region of the phospholipid head groups. Presence of the fungal endophyte could influence membrane stability by utilizing certain sugars (sucrose, xylose, and arabinose) (White *et al.*, 1993) as a carbon source or by producing hexose sugars and sugar alcohols (Elmi, 1989; White *et al.*, 1992b). Some seeds leak proteinase inhibitors and lectins that may protect against microbial or insect invasion (Terras *et al.*, 1995). Proteinase inhibitors constitute about 5 to 10% of the soluble proteins of some cereal grains. Their function is not completely understood, but proteinase inhibitors may play a role in storage of protein in cereal grains, control of endogenous protein activity, and the inhibition of proteolytic digestive enzymes of invading organisms (Georgieva, *et al.*, 1994).

Soilborne Pathogens Reduce Seed Establishment

Pythium aphanidermatum

Symptoms. *Pythium* species known to be pathogenic on tall fescue are *P. aphanidermatum*, *P. arrhenomanes* Dreschl. and *P. ultimum* Trow. (van der Plaats-Niterink, 1980). Diseases of cool season grasses caused by *Pythium* spp. include: foliar blights, root and crown rots, preemergence damping-off and seed rots (Saladini, 1995; Nelson and Craft, 1991). Preemergence damping-off occurs when the seed is destroyed after the seed coat is broken (Abad *et al.*, 1994). Postemergence damping-off occurs after germination. Seedlings first become necrotic at the soil surface. As the deterioration progresses up the stem, tissues become water soaked and collapse (Freeman, 1980). Host cells separate from each other because the middle lamella is broken down by pectic and cellulytic enzymes diffused from hyphal tips of the fungus (Kraft *et al.* 1967). The necrotrophic pathogen has no haustoria, rather the fungus kills the host tissue in advance of the mycelium (Hendrix and Campbell, 1973). A characteristic symptom of *Pythium* damping-off is a "patchy" appearance within a vigorously growing stand of turf (Couch, 1995). Patches may extend from a few to several centimeters in diameter. Resistance to *Pythium* damping-off has not been observed among cool season grasses (Freeman, 1980).

Life Cycle. *Pythium* spp. belong to the kingdom Stamenopila and are not considered true fungi (Alexopoulus, 1996). The organism is within the class Oomycetes,

having zoospores with two flagella (one tinsel directed anteriorly and one whiplash directed posteriorly). *Pythium* mycelium is white, slender, non-septate, well-branched, and fast growing. Asexual reproduction is initiated after a period of vegetative growth. Specialized hyphae, sporangiophores, are produced and terminal or intercalary sporangia are formed. Sporangia are filamentous or spherical, depending upon the species (Middleton, 1943).

In nearly all cases, sporangia and oospores of seed- and root-infecting *Pythium* species germinate only after being stimulated by plant-derived molecules present in seed and root exudates (Ruben *et al.* 1980). During germination, the protoplasm of a sporangium migrates out of the sporangium though a thin tube into a bulbous vesicle where it cleaves into zoospores that may number up to 125 (van der Plaats-Niterink, 1980). Zoospores are quickly liberated and swim, propelled by two flagella, in a film of water. When zoospores come to rest, their flagella are absorbed, and the zoospores encyst. Encysted zoospores soon germinate by means of a germ tube to form vegetative hyphae.

Sexual reproduction is initiated by the formation of spherical oogonia and club-shaped antheridia on the same or different branches of mycelium. Fertilization takes place as antheridia contact the oogonium. Male nuclear material is passed into the wall of the oogonium though a tiny fertilization tube. After meiosis, the wall of the fertilized oogonium (now called an oospore) becomes thick. Oospores can survive for extended periods of temperature extremes and drought. Oospores germinate directly by means of a germ tube to form hyphae, or by producing vesicles in which zoospores are formed and

released as described for sporangia (van der Plaats-Niterink, 1980).

Etiology. *Pythium* spp. are rapid colonizers of virgin substrate, such as thatch, and tolerate conditions of poor gas exchange that accompany high soil moisture (Hendrix and Campbell, 1973). *Pythium* spp. are opportunistic pathogens and are more damaging to seed and seedlings than established plants. These pathogens, however, do not compete well with other soil-inhabiting fungi (Webster, 1980). They are not vigorous competitors and grow as saprophytes only when other microbes are absent, or have greatly reduced activity (Lockwood, 1988).

Long term survival is achieved using resistant resting structures rather than mycelium (Hendrix and Campbell, 1973). *Pythium* spp. produce thick-walled oospores and/or sporangia which enable them to survive for extended periods of time (Webster, 1980). Resting structures remain dormant in the soil until seed and root exudates overcome fungistasis in the spermosphere (Lockwood, 1988). Soils suppressive to *Pythium* spp. result from competition from soil microorganisms favored by particular chemical and physical properties of the soil (Fukui *et. al*, 1994). When stimulated by seed and root exudates, resting structures germinate by the production of motile zoospores (Nemec, 1971); oospores may also produce germ tubes at higher temperatures and lower moisture conditions (Alexopolous, 1996). Thus damping-off of seeds and seedlings is usually achieved when infected by germinating sporangia and oospores, rather than infection by short-lived saprophytic mycelium (Kraft *et. al.*, 1967; Miller *et. al.* 1966).

Pythium root rot, damping-off, and seed rot is rapidly spread by zoospores in free flowing surface water (Hendrix and Campbell, 1973). *Pythium* spp. infect the embryos of germinating seeds of wheat within the first 24 h after they are planted in a suitably moist soil (Fukui *et. al*, 1994) Tactic responses by zoospores to host roots contribute to the importance of these pathogens. Zoospores are sensitive to gravity (geotaxis), electrical fields (electrotaxis), and chemical gradients (chemotaxis) including amino acids, sugars, and volatiles such as ethanol and acetaldehyde (Donalson *et al.* 1993; Morris and Gow, 1993; Morris and Ward, 1992; Nelson, 1987; Fukui *et al.*, 1994). Zoospores of *Pythium* spp. are specifically attracted to and infect plant root surfaces (Goldberg *et. al.*, 1989; Longman and Callow, 1987). Soil microbes rely on specific members of their host's array of secreted phenolics to trigger chemotaxis and gene expression appropriate to the rhizosphere environment (Bauer and Caetona-Anolles; 1991). *Pythium* damping-off is most often associated with water-saturated soil, but oospores of some species can germinate into infective mycelium when soil moisture is below saturation.

Factors Affecting Pathogen Virulence. Quality of inoculum can affect the ability of some *Pythium* species to cause damping-off or root rot. Culture age, age of sporangia and oospores, and the nutrient level of culture medium will affect the germination responses of *Pythium* propagules (Nelson and Hsu, 1994). In media containing high levels of nutrients, hyphae and reproductive structures accumulate higher levels of endogenous reserves; when cultures age, endogenous reserves become depleted (Nelson and Hsu, 1994). Propagules that are nutritionally debilitated readily germinate

when stimulated by exogenous nutrients in the spermosphere and rhizosphere (Hsu and Lockwood, 1973).

Several methods have been used to evaluate seed rot and damping-off caused by *Pythium* species. These include placement of seed on agar medium with mycelium (Altier and Thies, 1995), in sand with agar disks of mycelium (Nelson and Craft, 1991); in growing media amended with infested wheat grains or fescue seed (Gwinn and Gavin, 1992; Nelson and Craft, 1991), in soil with grass-leaf cultures containing reproductive structures (Abad *et al.,* 1994) and in soil containing oospores (Hering *et al.*, 1987). Upon evaluating the virulence of *P. vanterpooli* and *P. irregulare* on bentgrass, Abad *et al.* (1994) observed that mycelial plugs of corn meal agar (CMA) were not pathogenic, while pieces of colonized grass inoculum induced 30 to 70% postemergence damping-off of bent grass.

Temperature and soil matric potential can affect the aggressiveness of *Pythium.* For example, disease development on bent grass is greatest at high (28 C) and low (13 C) temperatures for *P. aphanidermatum* (Abad *et al.,* 1994). In contrast, *P. aphanidermatum* is pathogenic on red fescue at 28 C, but not at 13 C (Saladini, 1980). Adequate moisture is required for successful colonization and infection. When soil was saturated (-0.01 MPa matric potential) during the early stages of seed germination, 45% of wheat embryos were infected with *Pythium ultimum* var. *sporangiiferum* and *P. irregulare;* less than 5% of wheat embryos were infected with *Pythium* spp. when soil matric potential was drier than -0.04 MPa (Hering *et al.* 1987).

Nutritional factors can determine whether the fungus is virulent or saprobic (Nelson and Hsu, 1994). Seed and root exudates act as recognition signals that trigger germination and growth of *Pythium* propagules (Nelson, 1987). Mixtures of amino acids and sugars stimulate the germination of sporangia of *P. ultimum* and *P. irregulare*, while mixtures of organic acids and glucose stimulate germination of *P. aphanidermatum* (Nelson, 1990). Sporangia in soil are exogenously dormant and will germinate rapidly in response to introduced nutrient stimuli or upon alleviation of fungistasis (Mandelbaum and Hadar, 1990). Leakage of nutrients from imbibing wheat seed has been detected within 2 h after planting (Nelson, 1987). This may explain why Hering (1987) observed that pregermination of seeds influenced preemergence damping-off and seedling vigor of wheat. Seeds planted directly into *Pythium*-infested soil produced stunted wheat plants (Hering *et al.*, 1987). However, when wheat seeds were pregerminated for 24 h before sowing, differences in emergence and seedling height was less apparent and were nonexistent when seeds were pregerminated for 48 h before sowing. The release of seed exudates at the time of imbibition may have stimulated the oospore inoculum within the soil to germinate and infect the germinating seedlings (Hering *et al.*, 1987).

Factors Affecting Resistance of the Seed and Seedling. Seed age affects the incidence of seed rot and damping-off cuased by *Pythium* spp. In general, older seed are less vigorous, have a slower rate of germination, increased electrolyte leakage and less reserves available to the germinating embryo (Koehler, 1954; Hering *et al.*, 1987; Berjack and Villiers, 1972). As seed ages, ruptured cell membranes reassemble more slowly, allowing more solute leakage during the initial stage of imbibition (Fukui *et al.*,

1994). Thus more nutrients are released into the soil that stimulate the germination of sporangia and oospores of *Pythium* spp.

In summary, *Pythium* infection of endophyte-free seed is determined in the spermosphere of the seed by mainly two parameters: 1) the size of the nutrient supply from the seed (greater in wet than dry soil, owing to the greater diffusivity; and greater with aged seed than new seed, which are leakier) and 2) competition from soil microbes (Fukui *et al*, 1994). In endophyte-infected seed, the endophyte is also increasing in metabolic activity during seed germination and may play an important factor in the etiology of *Pythium* damping-off.

***Rhizoctonia* spp.**

Symptoms. *Rhizoctonia* spp. cause foliar lesions, root disease and damping-off. The most predominate disease in turf and forage grasses is brown patch. Symptoms are circular or irregularly shaped patches of blighted grass up to 30 cm in diameter. Grass in the blighted area is purplish green initially and quickly fades to a light brown (Smiley *et al*. 1992). Symptoms vary greatly depending on turfgrass cultivar, soil condition, environmental conditions, and fungal strain. Symptoms are most prominent during summer and early autumn. Most plants tolerate extensive root damage caused by *Rhizoctonia* spp. and outpace disease by producing new roots (Voorhees, 1934; Couch, 1995; Wiese, 1977). Certain strains of *Rhizoctonia* have been reported to cause a high incidence of preemergence seedling death (Couch, 1995) and often the most damage is to

seedlings (Wiese, 1977). Postemergence symptoms are described as "wire stem". This is a dry necrosis at ground level progressing upward; plants subsequently wither, collapse, and fade into a light brown color (Couch, 1995). *Rhizoctonia* root rot also suppresses tiller development (Smiley and Uddin, 1993).

Host Range. Several *Rhizoctonia* spp. induce foliar, root and seedling disease on a wide range of turfgrass species (Couch, 1995). The most studied of these species is *Rhizoctonia solani* Kuhn [teleomorph *Thanatephorus cucumeris* (Frank) Donk], which induces brown patch on cool- and warm-season turfgrasses (Couch, 1995). *Rhizoctonia solani* has one of the broadest host ranges of all plant pathogens; all Gramineae are susceptible (Wiese, 1977). High temperature and high humidity favor disease caused by *R. solani* (Martin and Lucas, 1983), but the pathogen is also associated with seedling rot of wheat in cool temperatures (Smiley and Uddin, 1993). *Rhizoctonia zeae* and *R. oryzae* Ryker and Gooch (teleomorph *Waitea circinate* Warcup and Talbot) are also associated with high temperatures (32 C) and induce symptoms similar to those caused by *R. solani* (Martin and Lucas, 1983). The binucleate *Rhizoctonia*-like fungus identified as *R. cerealis* [teleomorph *Ceratobasidium cronigerum* (Bourd.) Rogers] induces "yellow patch" associated with cool temperatures (Burpee, 1980). The perfect state is rarely observed in nature and it,s importance in the disease cycle is not known (Martin and Lucas, 1983).

Taxonomy. The hyphae of *Rhizoctonia* spp. frequently form diagnostic branches that leave the parent hyphae at a 90° angle. Branching will often occur near a

constricted dolipore septum across the parent hypha (Smiley *et al.*, 1992). When cultures are grown on laboratory media in the dark, *R. solani* is usually brown; *R. cerealis* is buff-colored; and *R. zeae* and *R. oryzae* are white to salmon pink (Smiley *et al.*, 1992). Sclerotial masses of *R. oryzae* are salmon to orange-colored with indefinite size and shape. Sclerotial masses of *R. zeae* are white to cream-colored when young and become orange, red, and eventually brown at maturity (Ryker and Gooch, 1938; Voorhees, 1934).

Life Cycle and Etiology. Saprophytic mycelia in soil debris and sclerotia are the most important forms of primary inoculum in the *Rhizoctonia* disease cycle (Smiley and Uddin, 1993). Resistant sclerotia that are formed in or on infected tissues is liberated into thatch as tissue decomposes. Sclerotia are capable of survival for extended periods of time. During humid, warm conditions mycelia grow onto moist turf grass leaves and sheaths (Smiley *et al.*, 1992). The necrotrophic pathogen produces a plethora of extracellular enzymes that degrade plant tissues. Cells lose integrity and tissues become water-soaked and dark; as the tissue dries, infected lesions shivel and turn brown (Smiley *et al.*, 1992).

Factors Affecting Resistance of the Seed and Seedling. Optimal temperatures for disease development caused by *Rhizoctonia* spp. vary according to the species and strain of the pathogen. For example, *R. solani* causes more severe root rot of winter wheat at low than high temperatures, but *R. oryzae* causes more root rot at high than low temperatures (Smiley and Uddin, 1993). When stress inducing high temperatures occur during germination of cool-season tall fescue, damping-off caused by

R. solani is favored (Smiley *et al.*, 1995). Abundant nitrogen also increases the frequency and severity of disease (Smiley *et al.*, 1995). Turf grass cultivars resistant to diseases caused by *Rhizoctonia* are not available (Smiley *et al.*, 1995).

Antifungal compounds within the roots of plants have been associated with resistance to root rot caused by *R. solani.* Chitinases and beta-1,3 glucanases are involved in plant defenses against fungal infection (Cordero, 1994). Antifungal enzymes degrade fungal cell wall constituents and inhibit fungal growth (Vierheilig *et al.*, 1993). A transgenic *Nicotiana sylvestris* Speg. with a constitutively expressed vacuolar chitinase from *N. tabacum* L. was resistant to *R. solani* infection (Vierheilig *et al.*, 1993). Similar results were obtained when transgenic tobacco seedlings constitutively expressing a bean chitinase gene showed an increased ability to survive in soil infested with *R. solani* and delayed development of disease symptoms (Broglie *et al.* 1991). A novel *N. typhinum* (=*Acremonium)* proteinase is expressed during endophyte infection of five *Poa* species, but similar activity is not expressed in endophyte-infected tall fescue or perrenial rye grass (Lindstrom and Belanger, 1994). Chitinase is elevated in mature roots and foliage of endophyte-infected plants (Roberts *et al.* 1992). Preliminary experiments indicated that chitinase activity in 3-week-old tall fescue seedlings was higher in endophyte-infected than endophyte-free plants (Gwinn and Trently, unpublished).

Rationale and Objectives

Tall fescue is the most prevalent cool season pasture and turf grass in the southeastern region of the United States (Hoveland, 1993). It is frequently infected with the endophyte. This mutualistic association usually results in the production of compounds toxic to many other organisms including insects, nematodes, and grazing animals (Clay *et al.,* 1989; Siegel *et al.,* 1987; Archavaleta *et al.*, 1989). Endophyte-infected tall fescue also confers resistance to drought and plant disease fungi (West, 1994; Gwinn and Gavin, 1991). This tolerance to abiotic and biotic factors increases the success of endophyte-infected turf and pasture establishment.

Resistance of endophyte-infected plants to soilborne pathogens during stand establishment has been studied. Protection against soilborne pathogens has not been observed in mature plants (Gwinn and Bernard, 1988; Burpee and Bouton, 1993). Endophyte-mediated resistance to soilborne pathogens is limited to young seedlings (Gwinn and Gavin, 1992; Blank *et al.*,1993). However, seedling studies were performed using endophyte-free seed generated from endophyte-infected seed stored at room temperature for one year. Thus seed age may have been a factor in disease susceptibility of endophyte-free seed.

Mechanisms of disease resistance are not known. Toxic compounds found in endophyte-infected plants have deleterious effects on insects and animals, but do not affect the growth of soilborne fungi (Siegel and Latch, 1991). However, pure cultures of *Neotyphodium* spp. and *Phialophora*-like species have antifungal activity (Siegel and Latch, 1991). Fungitoxic compounds have been isolated from the choke disease

pathogen *E. typhina* (Siegel and Latch, 1991), but antifungal compounds have not be isolated from endophytes. Chitinase, an antifungal hydrolase associated with disease resistance, is expressed in endophyte-infected tall fescue (Roberts *et al.*, 1992).

The initial objective of this research was to isolate and identify antifungal compounds responsible for resistance of endophyte-infected seeds to soilborne pathogens. However, as research progressed the hypothesis that toxic compounds were responsible for EMR of seedlings to soilborne fungi could not be supported. Rather differences in the germinative events of endophyte-infected and endophyte-free seed were discovered and these differences could be related to disease resistance.

The general objectives of this research were: 1) to determine the influence of endophyte, pathogen, seed lot and preplant seed treatment on preemergence damping-off of tall fescue; 2) to determine the influence of endophyte-infected seed growth, colonization and infection by soilborne pathogens; and 3) to determine the influence of endophyte on seed germination and spermosphere.

Part 2: Influence of Endophyte, Pathogen, Seed Lot, and Preplant Seed Treatment on Preemergence Damping-off of Tall Fescue

Introduction

Turf and pasture grass establishment is more successful when endophyte-infected tall fescue grass seed are used (Fribourg *et al.* 1991). *Rhizoctonia* and *Pythium* reside on dead plant tissues in most soils. These pathogens can limit the success of seed emergence and subsequent pasture establishment (Couch, 1995). Resistance to soilborne pathogens has been observed in commercially available endophyte-infected Kentucky 31 tall fescue seedlings, but not in mature plants (Gwinn and Gavin, 1992; Blank *et al.*, 1993; Gwinn and Bernard, 1988; Burpee and Bouton, 1993). Further evaluations for EMR in other fescue cultivars resulted in inconsistent results. Many factors contribute to the success of disease resistance. In order for growers to optimally use endophyte-infected seed for turf and pasture management, the parameters of EMR must be identified. It is not known whether EMR is specific to certain germplasm, endophyte species or strain, or pathogen species. The biology of endophyte-infected tall fescue seed during the process of soilborne pathogen colonization and infection has not been studied in detail.

An assay using small growing vessels has been used to evaluate EMR of grass seedlings (Blank, 1992). This assay can be used to study EMR mechanisms. In previous studies, seed evaluated in the assay were scarified, surface sterilized and soaked in water for 24 h. This preplant seed treatment increased the consistency and speed of germination. This procedure had no effect on EMR to *R. zeae.* Therefore the objective of this research was to determine whether germplasm, endophyte species, pathogen species and preplant seed treatment influence EMR.

Materials and Methods

Biological Material

Seed

The primary seed were from genetically similar tall fescue pasture grass (Kentucky 31) harvested in the fall of 1995 differing only in *N. coenophialum* infestation levels [95% (95E+) and 0% (95E-)] (provided by Henry Fribourg, The University of Tennessee, Knoxville). The 95E+ seed lot was periodically checked for endophyte survival and had an 89% infestation level in May, 1997. Seed harvested in 1994 [92% (94E+)] and 1993 [0% (93E-) were also evaluated (94E- and 93E+ seed were not available). Turf grass tall fescue cultivar Pixie was provided by Jacklin Seed Company, Post Falls, ID 83854-94499. The high endophyte Pixie seed lot [75% (PEH)] was harvested in 1993. The low endophyte Pixie seed lot (PEL) had an initial endophyte infestation level of 68% at time of harvest (1992), but at the time of experimental use (Oct 1994) the infestation level was 28%. A high endophyte Pixie seed lot harvested in 1995 (86%) and another low endophyte Pixie seed lot harvested in 1992 (12% at time of experiment) were also included in preliminary studies. Tall fescue pasture grass cultivars Georgia Jessup, Kentucky 31, and Johnstone infected with either or both *N. coenophialum* or *Phialophora*-like endophytes (endophyte infection greater than 85%) were provided by Malcolm Siegel, Lexington, Kentucky.

Tall fescue seed (3 g) were scarified in 60% H_2SO_4 for 30 min on a rotary shaker (80 rpm), rinsed in tap water, surface sterilized in 50% Clorox™ (2.62% NaOCl) for 20

min on a rotary shaker, then rinsed 5 times with sterile deionized water (SDW). Seed were incubated in 30 ml SDW on a rotary shaker at room temperature and continuous light for 24 h except when otherwise stated.

Fungal Pathogens

Cultures of *P. aphanidermatum* (isolate NCS1) and *P. arrhenomanes* were provided by Keith Jones (North Carolina State University). *Pythium* cultures were maintained on CMA. A culture of *R. solani* (isolate RSNC1) was obtained from L.T. Lucas (North Carolina State University). A binucleate *Rhizoctonia*-like species was isolated in the summer of 1995 and included while screening for EMR, but the isolate lost virulence within seven months of initial culture.

Cultures of *Rhizoctonia* were isolated from tall fescue with brown patch symptoms by removing the tissue from the margins of foliar lesions. Grass pieces were placed in 10 ml 10% Clorox™ with 1 ml 70% ethanol for 10 min, rinsed three times in SDW and blotted dry on paper towels. Leaf pieces were placed on 1.5% water agar (WA) and incubated on a laboratory bench overnight. Mycelia were examined for the presence of characteristics of *Rhizoctonia* species. Hyphal-tip transfers were made aseptically to potato-dextrose agar (PDA) acidified with 1 ml/L of 50% lactic acid. Nuclear condition was determined by microscopic examination of mycelium stained with aniline blue. *Rhizoctonia* cultures were maintained on PDA at 4 C.

Pythium aphanidermatum, P. arrhenomanes and *Rhizoctonia* sp. inoculum were prepared as follows. Low endophyte tall fescue cultivar "Stargazer" (20 g) was placed in 250 ml flasks with 40 ml of double deionized water. Flasks were autoclaved for 30 min

on two consecutive days. Three 0.5 cm mycelial cores of 3-day-old fungal cultures were transferred to each flask. Flasks were incubated at room temperature in the darkness for one week. *Rhizoctonia* cultures contained numerous sclerotia. *Pythium* sp. cultures contained numerous reproductive structures. Oospores, sporangia and zoospores were observed when seed cultures were transferred to SDW and incubated under continuous fluorescent light for 48 h.

Greenhouse Studies

Factors Affecting Disease Loss

Soilless medium (Farfard™) was amended with the following treatments: 1) twice autoclaved "Stargazer" seed (50 seeds), 2) *Pythium*-infested "Stargazer" seed (50 seeds) or 3) *Rhizoctonia*- infested "Stargazer" seed (100 seeds). Soilless medium moisture was high; when a handful of medium was squeezed, a few drops of water were expressed. Amended soilless medium was incubated for 48 h at room temperature. Twenty seeds were placed in each growing vessel (Magenta™ box). Growing vessels were maintained in the greenhouse for 3 weeks, then the number of seedlings were counted.

Experiments were performed in a randomized complete block design with five blocks. Each experiment included 95E+ and endophyte-free (E-) treatments and pathogen-free controls. Experiments were repeated three times (n=15). A General Linear Models procedure was used to analyze data (SAS Institute, Cary, NC).

Preplant Treatment Effect on Endophyte-mediated Resistance

Seed (95E+ and 95E-) were planted in soilless medium infested with *P. aphanidermatum*. The experiment was performed and data were analyzed using the preceding methods. Seed (95E+ and 95E-)were either nonscarified, or scarified, surface sterilized and seed incubated in rotating flasks with 30 ml SDW. Scarified seed were incubated for 3, 24, 48, or 72 h at room temperature and continuous fluorescent light.

Results

Factors Affecting Disease Loss

No EMR was observed for the Johnstone, Georgia Jessup or Kentucky 31 seed infected with *Neotyphodium* and *Phialophora*-like endophytes. The binucleate *Rhizoctonia* caused disease in Kentucky 31 and Georgia Jessup seed, but failed to cause disease in Johnstone seed. Virulence of *P. arrhenomanes* was also inconsistent.

Inconsistencies in EMR and disease loss were observed also when comparing Fribourg's Kentucky 31 and Pixie seed lots (Table 1). Endophyte-mediated resistance was observed in the following combinations: 1) Kentucky 31 94E+ vs 93E- infected with binucleate *Rhizoctonia* and 2) Pixie 93EH vs 92EL infected with *P. arrhenomanes.* Pathogen virulence was an important factor in disease loss differences with *P. arrhenomanes* and *Rhizoctonia* species. The *R. solani* culture (RSNC1) aged and lost virulence during initial experiments; in later experiments *P. arrhenomanes* also lost virulence. The binucleate *Rhizoctonia* sp. lost virulence within seven month of

Table 1. Disease loss and endophyte-mediated resistance (EMR) of tall fescue. Tall fescue cultivars Kentucky 31(Fribourg) and Pixie infected with *N. coenophialum* planted in growing media infested with soilborne pathogens.

Pathogen	**Seed Lot**			
	Pixie 93EH vs 92EL	**Pixie 95EH vs 92EL**	**KY 31 95E+ vs 95E-**	**KY 31 94E+ vs 93E-**
P. aphanidermatum	no EMR	no EMR	no EMR	no EMR
P. arrhenomanes	EMR	no EMR	*	no EMR
R. solani (RSNC1)	*	**	**	**
binucleate *Rhizoctonia*	**	*	no EMR	EMR

* no disease

** not tested

Seed were scarified, surface sterilized and soaked in SDW for 24 h. Twenty seeds were placed in growing vessels containing pathogen-infested medium, placed in the greenhouse for 3 weeks and the number of seedlings counted. Experiments were done in a randomized complete block design with five blocks. Each experiment included endophyte-infected and endophyte-free treatments and pathogen-free controls. Experiments were repeated three times (n=15). A General Linear Models procedure was used to analyze data (SAS Institute, Cary, NC). Disease was defined as no significant difference ($P<0.05$) between number of seeds germinated in pathogen-free and pathogen-infested medium. EMR was defined by significant ($P<0.05$) interaction between pathogen and endophyte.

isolation. Variation of EMR in early experiments for these pathogens could not be further investigated because of problems with virulence. *Pythium aphanidermatum* was the only pathogen to cause significant disease loss (P=0.05) in all. Experiments using seed soaked for 24 h resulted in no EMR against *P. aphanidermatum*.

Preplant Treatment Effect on Endophyte-mediated Resistance

Seeds planted in pathogen-free medium had greater germination than *P. aphanidermatum* treatments (P=0.001) regardless of seed lot or preplant treatment. Germination was not affected by the length of time scarified surface sterilized seeds were soaked; in pathogen-free medium the mean germination of 95E+ seed was 84.3% and the mean germination of 95E- was 81.8%. However, nonscarified 95E+ seed had a lower germination rate (67%) than nonscarified 95E- seed (85%) when planted in pathogen-free media.

Significant interaction (EMR) was observed between endophyte-free and endophyte-infected seed for the nonscarified (P=0.034) and scarified, surfaced sterilized and soaked for 3 h (P=0.001) treatment (Figure 1). However, no EMR was observed when seed were soaked for 24 h or longer. Preplant treatments did not affect germination of 95E- seed; however, prolonged soaking (24 h and greater) eliminated EMR to *P. aphanidermatum*.

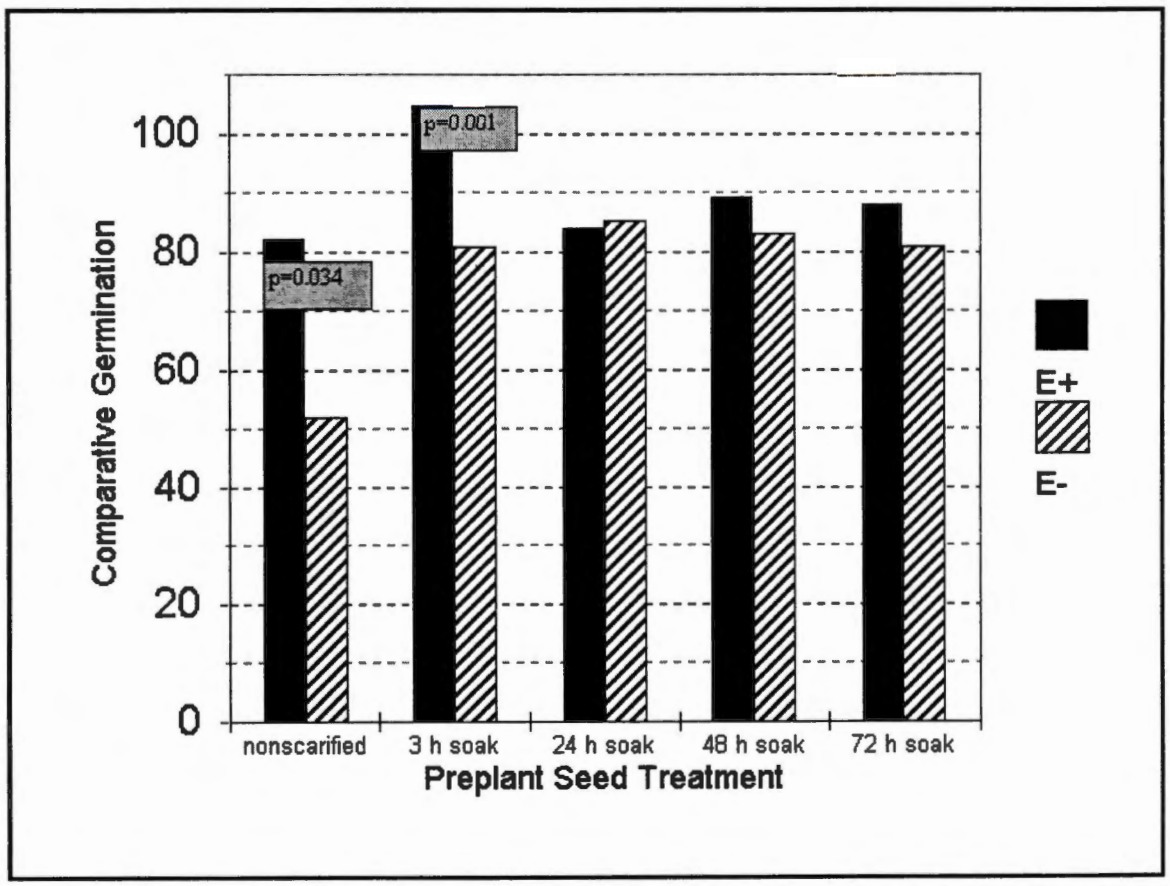


Figure 1. Effect of preplant seed treatments on the germination of endophyte-infected (E+) and endophyte-free (E-) Kentucky 31 planted in soilless medium amended with *P. aphanidermatum.* Preplant seed treatments included nonscarified seed and scarified surface sterilized seed soaked in SDW for 3, 24, 48, 72 h. Comparative germination was calculated by dividing the number of seedlings produced in pathogen-infested medium by the number of seedlings produced in pathogen-free medium. Significance levels were set at $P<0.05$.

Discussion

Endophyte-mediated resistance to *R. zeae* and *P. aphanidermatum* have been observed in previous studies when endophyte-infected seed was compared to endophyte-free seed generated by storing endophyte-infected seed at room temperature for one year. Endophyte-mediated resistance to *R. zeae* and *P. aphanidermatum* was obtained in greenhouse studies with nonscarified seed (Gwinn and Gavin, 1992; Blank, 1992). Resistance to *R. zeae* was obtained from experiments using Magenta box™ growing vessels with endophyte-infected seed that had been scarified, surface sterilized and soaked in SDW for 20 h (Blank, 1992). Therefore, results of these experiments are consistent with previous findings.

Using the EMR assay developed by Blank (1992), useful comparisons were made between germplasm, endophyte species, seed ages, pathogens, and preplanting seed treatments. Loss of pathogen virulence, loss of endophyte viability, and limitations on the quantity of seed severely reduced the amount of useful data obtained from these experiments. The loss of virulence in *P. arrhenomanes* and *Rhizoctonia* species greatly reduced the scope of this study. Further investigation of resistance mechanisms was, therefore, limited to *P. aphanidermatum.* Comparisons between seed germplasm and endophyte species (Table 1) resulting in disease loss did not result in EMR. Since limited quantities of seed were available, these experiments were not repeated with shorter preplant seed soaking times. Therefore, the presence or absence of EMR could not be related to germplasm or endophyte species.

The most interesting result of these assays was that shorter preplant soaking time resulted in EMR to *P. aphanidermatum* in the endophyte-infected seed lot. Although *P. aphanidermatum* consistently caused disease in all seed lots, EMR was observed only when presoaking was eliminated or reduced to 3 h. These results were similar to those obtained with *P. aphanidermatum* in greenhouse studies with nonscarified seed (Blank, 1992). The effect of presoaking of endophyte-infected seed on EMR against *P. aphanidermatum* was not observed for *R. zeae* in previous reports (Blank, 1992). The propagules of *P. aphanidermatum* may respond differently than *R. zeae* when exposed to germinating fescue seed, which may be related to infection and disease. Effect of seed treatment on EMR to *P. aphanidermatum* was discovered after germplasm and pathogen comparisons were completed, using seed imbibed for 24 h. Insufficient quantities of seed and loss of pathogen virulence prevented subsequent EMR assays using other preplant seed treatments. Presoaking did not eliminate EMR in comparisons using *P. arrhenomanes* against PEH vs. PEL and binucleate *Rhizoctonia* against Kentucky 31 94E+ vs. 93E-. Therefore preplant soaking may not eliminate EMR against all soilborne pathogens.

Part 3: Influence of Endophyte-infected Seed on Growth, Colonization and Infection of Tall Fescue by Soilborne Pathogens

Introduction

Endophyte (*Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin comb. nov.) infected tall fescue (*Festuca arundinaceae* Schreb.) is correlated with increased seedling survival when seed are planted in soilless medium infested with *Rhizoctonia zea* Voorhees (Gwinn and Gavin, 1992), *Rhizoctonia solani* Kuhn or *Pythium aphanidermatum* Edson (Fitz.) (Blank *et al.,* 1993). Endophyte-mediated resistance to *P. aphanidermatum* occurred only when endophyte-infected seed were not scarified and when seed were imbibed for 3 h (Batzer *et al.*, 1997). Extended (24 h) preplant soaking eliminated resistance to damping-off.

Cause of EMR is not understood. High concentrations of ergot and loline alkaloids, which deter insects and livestock, are present in endophyte-infected seed (Siegel *et al.*, 1987, Patterson *et al.*, 1995). Diet made from endophyte-infected seed powder is toxic to *Drosophila melangonaster* (Pless *et al.,* 1993). Water soluble loline alkaloids could be released into the spermosphere and influence the colonization and infection of soilborne pathogens. Isolates of *Neotyphodium* sp. have antifungal activity (Siegel and Latch, 1991). However, pure alkaloids derived from endophyte-infected tall fescue and perennial ryegrass do not effect fungal growth (Siegel and Latch, 1991).

Plants respond to pathogen infection with an array of defense mechanisms. Plants synthesize hydrolytic enzymes, such as chitinases and *B*-1,3-glucanases, which inhibit the growth and degrade cell walls of invading *Pythium* spp. (Sharma *et al.*, 1993). Pathogen invasion activates the phenylpropanoid pathway. This results in the production

of nonspecific antifungal compounds, such as polyphenols, and structural reinforcing compounds, such as lignin, that defend against pathogen infection (Nicholson and Hammerschmidt, 1992). Analysis of host and pathogen interaction is required to ascertain the mechanisms of resistance. The ability of plants to resist a pathogen is related to the activation of defense genes, as well as the speed and coordination of defense mechanisms during the course of infection (Isacc, 1992). Plant defenses could be activated as a response to endophyte infection. Based on results presented in Part 2 of this thesis, EMR to *P. aphanidermatum* is believed to be dependent upon synchronized germinative events of the tall fescue seed host, its endophyte, and the pathogen.

Dormant spores of soilborne pathogens germinate in response to root and seed exudates (Garrett, 1956). Zoospores *Pythium* spp. are attracted to, swim toward, and collect on root surfaces (Kraft *et al.*, 1967, Nemec, 1971, Spencer and Cooper, 1967). Soil conditions also affect response of *Pythium* propagules. Available water, oxygen, light, carbon dioxide and soil pH are important factors mediating oospore germination (Qian and Johnson, 1987). Zoospores are attracted to plant roots and display positive chemotaxis toward compounds that may occur at or close to the spermosphere or rhizosphere (i.e. ethanol, amino acids, aldehydes and fatty acids) (Morris and Ward, 1992; Nelson, 1987; Nelson, 1990; Nelson and Hu, 1994). Specific saccharide residues are involved in host recognition by zoospores of *P. aphanidermatum* (Longman and Callow, 1987). Thus lack of host recognition would be an alternative hypothesis for the mechanism of resistance by endophyte-infected seed to *P. aphanidermatum.*

The objectives of this study were to: 1) determine whether endophyte-infected seed has antifungal activity against *R. solani* and *P. aphanidermatum*; 2) determine the influence of the spermosphere of endophyte-infected tall fescue seed on infective propagules of *P. aphanidermatum*; 3) determine the influence of buffer pH and concentration on infective propagules of *P. aphanidermatum;* and 4) to determine the influence of endophyte-infection on seed coat colonization and embryo infection of *P. aphanidermatum* in soilless growing medium.

Materials and Methods

Biological Material

Seed

Genetically similar tall fescue pasture grass (Kentucky 31) seed differing only in *N. coenophialum* infestation levels [95% (95E+) and 0% (95E-)] were used in *P. aphanidermatum* studies. Tall fescue turf grass cultivar Pixie seed was used in the *R. solani* experiments. The seed lot PEH (harvested in 1993) had an initial endophyte infestation level of 75%. Seed lot (PEL) had an initial endophyte infestation level of 68% at time of harvest (1992), but by October, 1994 viable endophyte infection rate was reduced to 28%. Seed (3 g) were scarified in 60% H_2SO_4 for 30 min on a rotary shaker (80 rpm), rinsed, then surface sterilized in 50% Clorox™ (2.62% NaOCl) for 20 min on a rotary shaker and rinsed 5 times with SDW.

Fungal Pathogens

The *P. aphanidermatum* isolate NCS1 and the *R. solani* isolate RSNC1 were used.

Antifungal Bioassays

Rhizoctonia solani

Mycelial Growth. Seed (5 g) were incubated in flasks containing 50 ml SDW on a rotary shaker for 24, 48 or 72 h at room temperature. Fluid was decanted then both seed and steep water were frozen at -20° C. Steep water and seed were thawed at room temperature for 2 h and used to prepare imbibition and macerate media, respectively. Imbibition medium was prepared by mixing 50 ml steep water with 20 ml 3% water agar (WA). Macerate medium was prepared by grinding seeds for 5 min with a sterile mortar and pestle in 30 ml sterile sodium acetate buffer (0.05M, pH 5.0). Macerated seed were mixed with 30 ml of 2% WA. Medium was poured sequentially into 6-well plates to pair endophyte treatments. Macerate and steep water agar were evaluated separately. Three-day-old mycelial cores (0.5 cm) of *R. solani* were placed on each medium and plates were incubated at 28C for 12 h. Colony diameters were measured. The experiment was repeated three times. Data were analyzed using a paired t-test (Abstat, Anderson Bell Inc., Parker, CO).

Pythium aphanidermatum

Mycelial Growth. Three experiments were conducted to determine whether 95E+ seed inhibited mycelial growth of *P. aphanidermatum*. 1) Mycelial cores (0.5 cm) from three-day-old cultures were placed inside 5 cm length of preweighed dialysis tubing autoclaved in deionized water and clamped shut. Tubes were placed in 125 ml flasks containing 5 g scarified, surface sterilized seed with 50 ml SDW. Cultures were incubated for 1 week at 28° C and continuous fluorescent light. Dialysis tubing was dried at 150° C for 2 days and weighed. The experiment was repeated twice, with 3 repetitions, and a SDW control. 2) Thirty seeds were placed along the perimeter of CMA plates. Five plates of 95E+ and 95E- seed were compared. Mycelial cores (0.5 cm) were placed in the center of each plate. Cultures were incubated at 28° C. Colony margins and seed germination were observed at 2 and 7 days. The experiment was repeated twice. 3) Thirty ml of imbibition fluid from 3 g 95E+or 95E- seed soaked in 30 ml SDW for 24 h were lyophilized. Freeze-dried powders were rehydrated in 0.5 ml SDW, dispensed onto sterile filter paper disks (0.05 ml), and placed onto the perimeter of CMA plates. Three imbibition fluid-soaked disks from 95E+ and 95E- seed were compared per plate. Mycelial cores (0.5 cm) were placed in the center of the plate, incubated at 28° C for 2 days, and growth observed. Three plates were compared per experiment. The experiment was repeated twice.

Sporangial Germination. Infective propagules were produced on grass leaves. Endophyte-free tall fescue leaves were cut into approximately 1 cm lengths and

experiments in rotating flasks of 30 ml SDW for 24 h. Fluid was decanted from flasks and diluted with SDW to 5%, 10%, 15%, 20% and 25%. One ml of dilute imbibition fluid or SDW was placed in each well with a colonized grass piece. For all experiments, multiwell dishes were incubated for 24 h at 28 ° C with continuous fluorescent light. Sporangial germination was verified by presence of zoospores. A complete randomized block design was used for all of the experiments, with two replications per plate. Five plates were replicated for each run and experiments were repeated at least three times. Data were analyzed using a General Linear Model procedure (SAS Inst. Cary, NC).

Influence of Altered Steep Water. Thirty ml of steep water, prepared as for the imbibition fluid experiments, were equally divided into 3 parts and placed in centrifuge tubes. The first tube of steep water was set in a beaker of boiling water for 1 h (to denature proteins) then cooled at room temperature; the second tube was placed in a -20 C freezer for three hours, then thawed for 2 h at room temperature; steep water in the third tube was left at room temperature. Fluids were diluted to 5, 10, 15, 20 and 25% then 1.0 ml aliquots were in placed 24-well tissue culture plates, with SDW controls. *Pythium*-colonized grass pieces were placed in each well and plates were incubated at 28 C and constant fluorescent light. Presence of zoospores was determined at 24 h. Ten wells per treatment were evaluated and the experiment was conducted once.

Influence of Buffer pH and Concentration. *Pythium aphanidermatum* was co-cultured with 95E+ and 95E- seed in sterile sodium citrate buffer. Buffer was titrated to pH 4.0, 5.0, 5.5, 6.0 or 6.5 and diluted to 0.05, 0.01, 0.005 or 0.001M. Buffers

boiled for 10 min in double deionized water. Cooled grass pieces (40-50) were placed in Petri plates with SDW water. Three-day-old cultures of *P. aphanidermatum* were cut into 1 cm squares; 8-10 agar pieces were transferred to Petri plates containing boiled grass pieces. Numerous sporangia and oogonia formed after 48 h of incubation at room temperature under continuous fluorescent light. Presence of infective structures was verified by microscopic examination. To stimulate simultaneous Sporangial germination, culture water was decanted, replaced with chilled SDW and incubated at 4 C for 20 min. Oospore germination was not observed.

Three experiments were used to determine the influence of endophyte-infected and endophyte-free spermospheres on sporangia of *P. aphanidermatum.* Experiments were conducted in 24-well tissue culture plates. All seed were scarified and surface sterilized as previously described. 1) "Time zero." Seeds were placed in wells immediately following scarification and surface sterilization. Colonized grass pieces were immediately placed in wells containing 1.0 ml of SDW with 95E+ or 95E- seeds. Each well contained 0, 1, 2, 3, 4 or 5 seeds. 2) "24 h unwashed." Methods used were the same as for "time zero" experiments except seed were incubated in 2.0 ml SDW for 24 h prior to addition of colonized grass piece. 3) "24 h washed." Seeds were incubated on a rotary shaker in flasks containing 30 ml SDW. Seeds were rinsed twice with SDW (to remove the spermosphere) then placed in wells containing 1.0 ml SDW. Colonized grass pieces were transferred to wells after seed placement. Greater numbers of seed (0, 6, 8, 10, 12, or 14 seeds/well) were used in these experiments than in "time zero" or "24 h unwashed." 4) "Imbibition fluid." Seed was incubated as for "24 h unwashed"

were dispensed into 24-well tissue culture dishes. The following conditions were used: one buffer treatment per plate, one ml per well. Eight 95E+ or 95E- seeds were placed in each wells and then a *Pythium*-infested grass piece was added; controls with no seeds were included. Cultures were incubated at 28 C with continuous fluorescent light. After 24 h, wells were scored for presence of zoospores. Eight wells per treatment were compared and the experiment was conducted three times (n=24). Data were analyzed with a mixed models procedure and individual comparisons were based on adjusted Least Squares Means (SAS Inst. Cary, NC).

Seed Coat Colonization and Embryo Infection of Tall Fescue. Seed (1 g subsamples from 4 flasks of prepared 95E+ or 95E- seed) were placed in Magenta™ boxes containing 50 cm^3 of moist, twice autoclaved soilless medium (Farfard™) and 1 g of *Pythium*-infested grass pieces. Sterile grass pieces were included. Four repetitions per endophyte treatment were conducted and the experiment was repeated twice. Boxes were incubated at 28 C with continuous light for 20 h. Soilless medium was removed from seeds by lightly rinsing with SDW. One hundred seeds from each growing vessel were placed on CMA amended with 100 mg/ L pimaricin and 200 mg/L streptomycin sulfate. Plates were incubated at room temperature in darkness. Infection (defined as the presence of fungal growth originating from the embryo) was determined 20 h after seeds were transferred to CMA. Seed coat colonization (defined as the presence of fungal growth originating from the seed coat) was scored 30 h after seeds were transferred to CMA. Values included infected seed observed at 20 h. Seed germination was observed in uninoculated controls at 24 h and 48 h from the onset of imbibition.

Results

Rhizoctonia solani

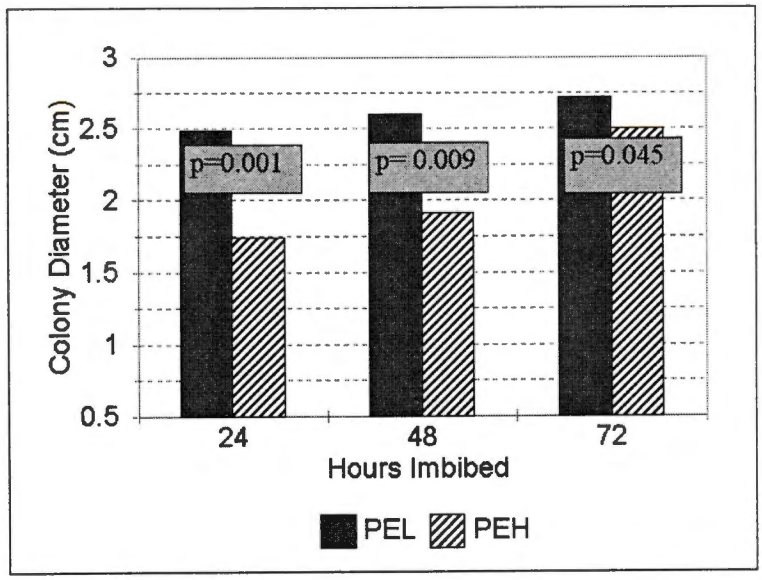
Mycelial Growth

Mycelial growth was influenced by culture medium and length of seed imbibition. Colonies grew more rapidly on imbibition media made from steep water with longer seed soaking times (Fig 2a). Conversely, fungal growth was reduced on macerate media as soaking times increased (Fig 2b). Colony diameters were larger (P>0.05) on PEL than PEH imbibition media, regardless of seed soaking time (Fig 2a). Growth of *R. solani* was greater (P=0.05) on PEL macerate media than PEH macerate media produced from seed that had been soaked for 48 h, but not for 24 and 72 h (Fig 2b).

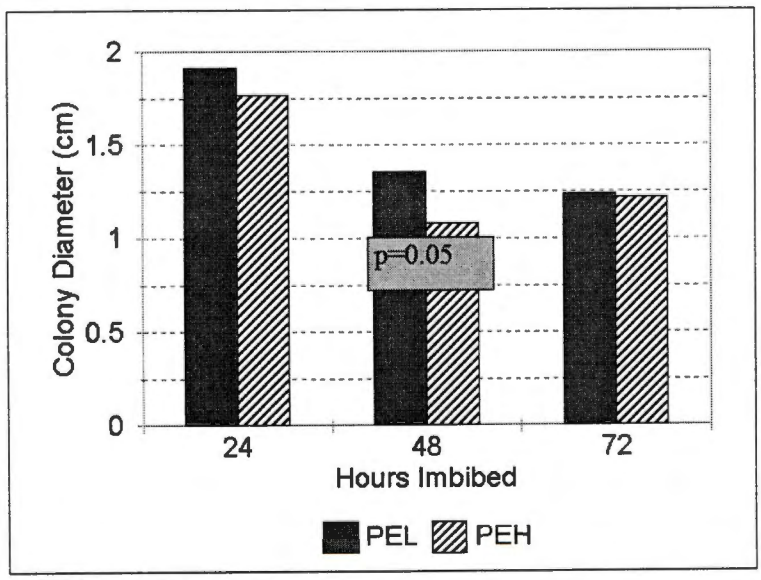
Pythium aphanidermatum

Mycelial Growth

Antifungal activity of 95E+ seed against *P. aphanidermatum* was not observed. No differences of mycelial mass within dialysis tubing placed in flasks of SDW 95E+ or 95E- seed were observed (mean mass for 95E+ and 95E- treatments were 0.072 mg and 0.073 mg, respectively). Mycelial growth of *P. aphanidermatum* was not inhibited when incubated in the presence of lyophilized imbibition fluid or scarified surface sterilized seed. No damping-off or seed rot was observed when tall fescue seed were germinated on CMA, in the presence of *P. aphanidermatum* mycelium.



A. Imbibition media



B. Macerate media

Figure 2. Colony diameters of *R. solani* on A) imbibition media and B) macerate media. Seed from lots with high and low endophyte tall fescue Pixie (PEH and PEL) were soaked in SDW for 24, 48, and 72 h. Seed and steep water were separated then used to make macerate and imbibition media, respectively. Pair difference t-tests were performed to determine whether percentage of endophyte infection was related to growth of *R. solani*. $P < 0.05$ were significantly different.

Sporangial Germination

Zoospore release was suppressed by the addition of seeds to wells. Abundant zoospores were observed in controls within 4 h after transfer to tissue culture dishes; maximum production of zoospores occurred at 12 h and continued until 24 h. Oospore germination was not observed. Seed treatment affected the response of sporangia. The estimated values for the number of seeds per ml required to inhibit 50% of wells from producing zoospores (EC-50) are presented in Figure 3. Values were calculated from the slope and y-intercepts derived from GLM analysis. Slopes of the regression line for the "24 h unwashed" seed was greater (m=-0.345) than the slopes for "24 h washed" (m=-0.071) and the "time zero" (m=-0.086) slopes (Fig.4). Much higher numbers of seed were required to inhibit zoospore production in "24 h washed" treatments.

Sporangial germination by *P. aphanidermatum* was differentially suppressed by 95E+ seed and steep water. Significant differences in sporangial germination were detected between 95E+ and 95E- seed for the "time zero" seed treatment; sporangial germination was 32% higher in 95E- seed than 95E+ seed treatments (Fig. 4a). The 95E- seed resulted in 16% more sporangial germination than the 95E+ seed in "24 h washed" experiments (Figure 4b). The "24 h washed" seed treatments resulted in 17% more wells with zoospores for 95E- than 95E+ seed treatments (Figure 4c).

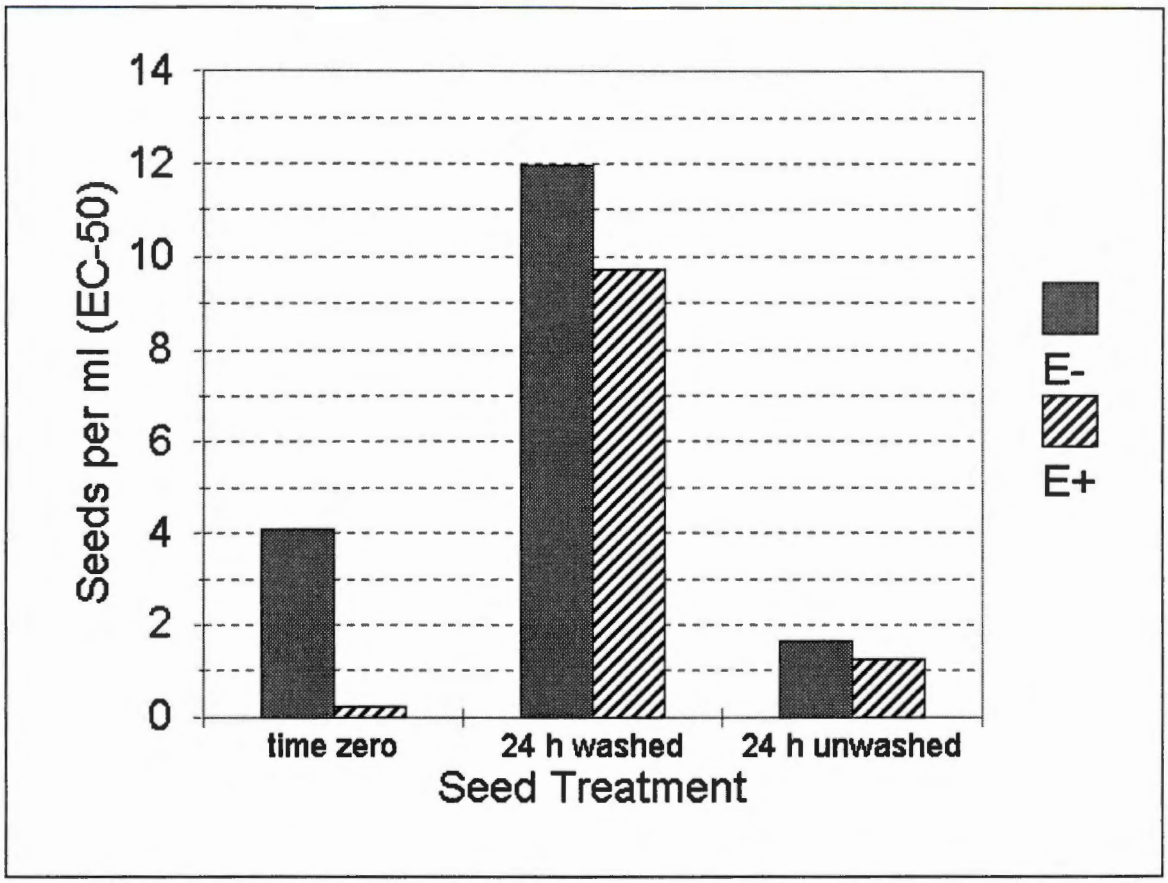


Figure 3. Estimated number of 95E+ or 95E- seeds per ml SDW required to suppress *P. aphanidermatum* production in 50% wells. Seed treatments included: "time zero" where seed was placed in wells with sporangia at onset of imbibition; "24 h washed" where seed soaked for 24 h was rinsed and placed in wells with sporangia; "24 h unwashed" where seed was soaked in wells with SDW for 24 h then sporangia were added. Predicted EC-50 values were derived from GLM analysis.

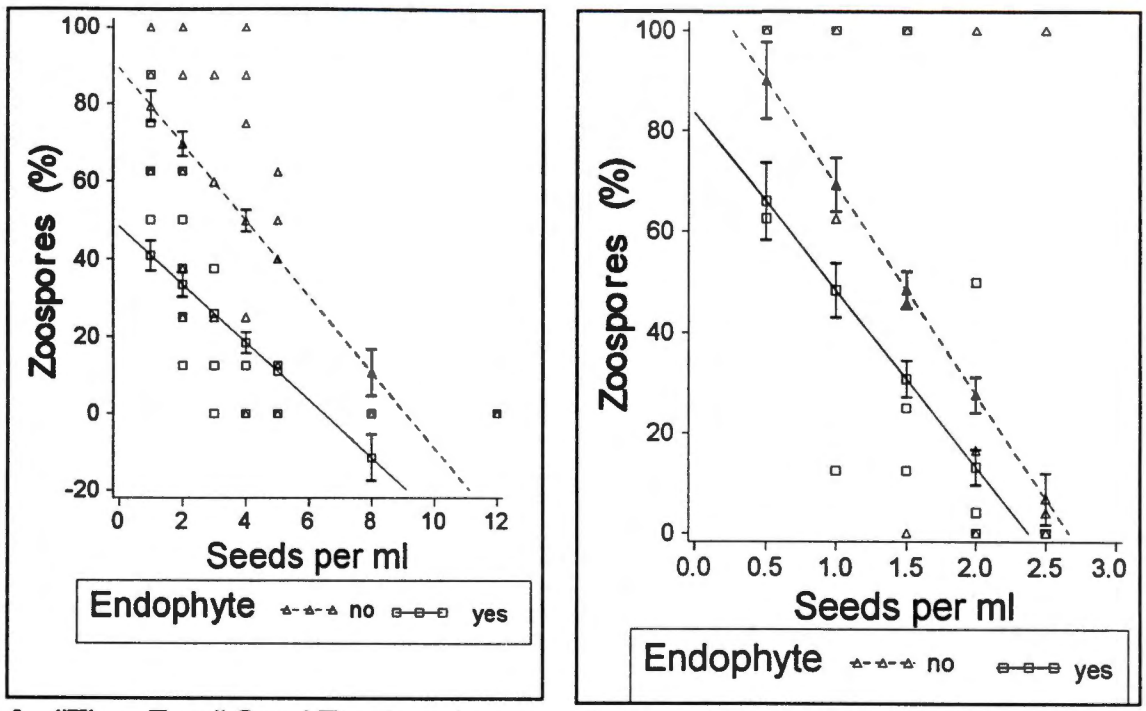
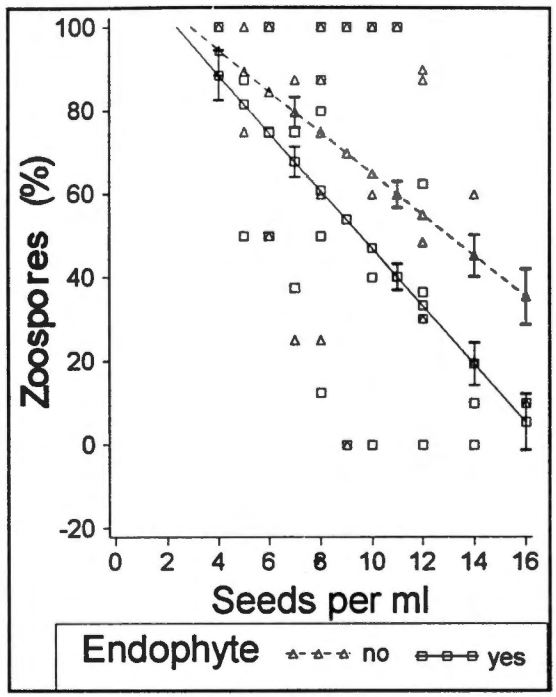


Figure 4. Effect of endophyte-infected seed on zoospore production by *P. aphanidermatum.* Infested grass pieces with sporangia were placed in wells containing 1 ml SDW and fescue seed treated as follows: **A)** "Time zero" seed placed in wells with sporangia at onset of imbibition; **B)** "24 h unwashed" seed placed in wells at onset of imbibition; sporangia introduced after 24 h. Cultures were examined for zoospores after 24 h. Paired data was analyzed with GLM with no interaction ($P<0.05$). Bars represent the standard error of the mean values given. (continued on next page).



C. "24 h Washed" Seed Treatment

Figure 4. (continued)
Effect of 95E+ and 95E- seed on zoospore production by *P. aphanidermatum.* Grass pieces infested with sporangia were placed in wells containing 1 ml SDW and fescue seed treated as follows: C) "24 h washed" seed imbibed for 24 h then rinsed and placed in wells with sporangia. Cultures were examined for zoospores after 24 h. Paired data was analyzed with GLM with no interaction ($P<0.05$). Bars represent the standard error of the mean values given.

There were differences (P=0.05) in zoospore production between imbibition fluids from 95E+ and 95E- seed (Figure 5). Slopes were significantly different, thus the interaction was included in GLM analysisof the endophyte effect. At 5% dilution of 95E+ imbibition fluid, 43% of wells containing infested grass leaves contained zoospores, where as 63% of colonized leaves with 95E- imbibition fluid were observed with zoospores.

Influence of Altered SteepWater

Germination of *P. aphanidermatum* sporangia was not affected by freezing or boiling steep water derived from 95E+ or 95E- tall fescue seed (Table 2). Infected grass pieces placed in SDW as control treatments developed abundant zoospores.

Influence of Buffer pH and Concentration

Pythium-infested grass baits consistently produced zoospores when incubated in sodium citrate buffers (Table 3). No zoospores were observed in buffers of 0.05 M and/or pH of 4.0. Buffer concentration greatly affected zoospore production. However, sporangial germination was not affected by pH in buffer concentrations 0.001 and 0.01. Sporangia in wells with 95E+ seed did not behave differently than control buffers, with one exception (pH 5.5 and 0.001M). In general, 95E- seed had a significant effect on sporangial germination when compared to control buffers with 0.005 and 0.01 concentrations, except at pH 6.5.

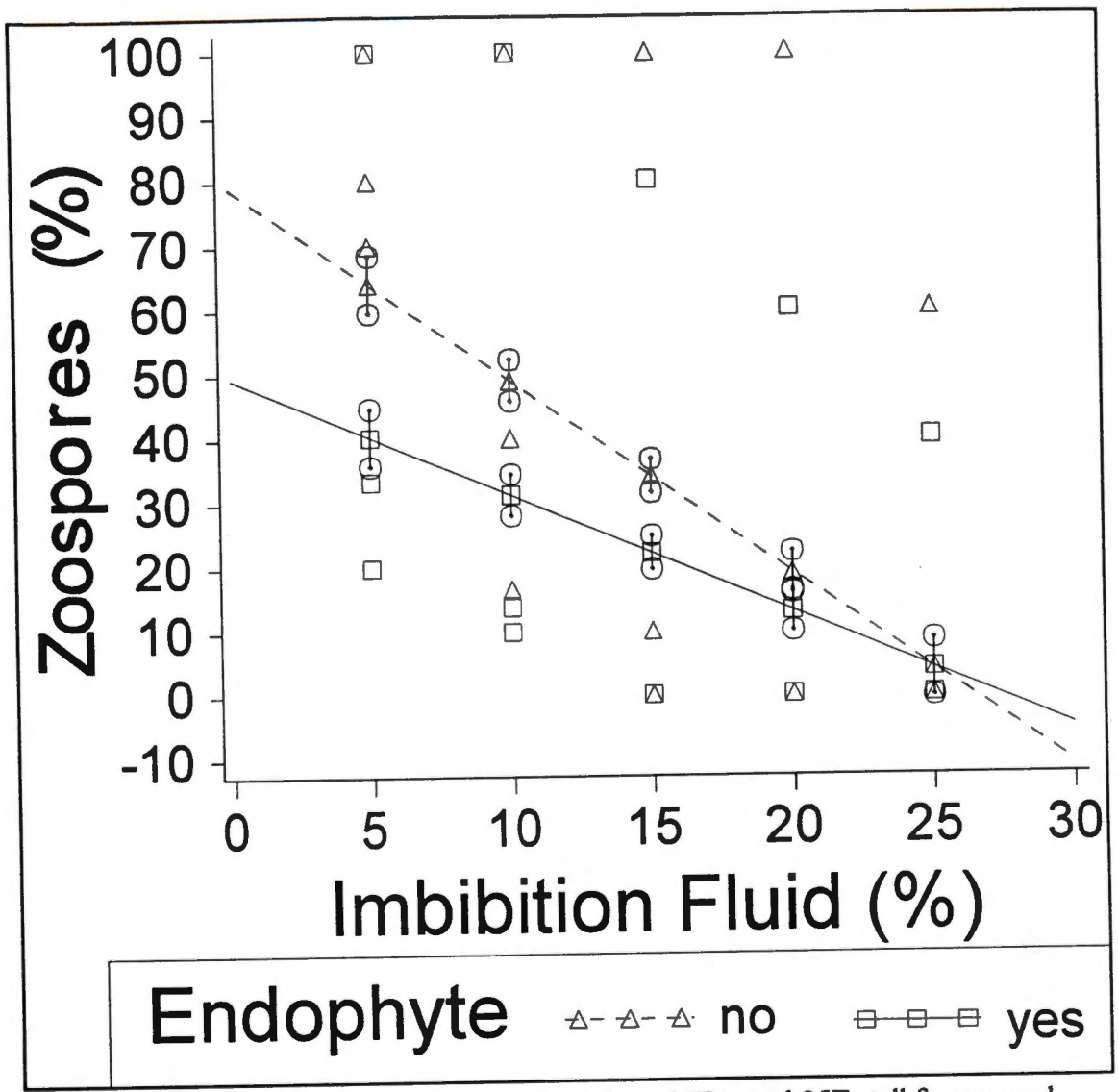


Figure 5. Effect of dilution of imbibition fluid from 95E+ and 95E- tall fescue seed exudates on zoospore production. Leaf pieces infested with *P. aphanidermatum* were incubated with diluted steep water from seed soaked in SDW for 24 h was diluted. Bases on GLM analysis with interaction zoospore production was altered by endophyte status (P=0.05).

Table 2. Effect of boiled, frozen and fresh steep water from endophyte-infected (E+) and endophyte-free (E-) seed on the number of wells with zoospores produced by *P. aphanidermatum.**

	Endophyte Status									
	E+	E-	E	E-	E+	E-	E+	E-	E+	E-
Percentage Steep Water	**5**		**10**		**15**		**20**		**25**	
fresh steep water	6	7	0	3	0	0	0	0	0	0
boiled steep water	3	6	2	0	0	0	0	0	0	0
frozen steep water	5	8	0	4	0	0	0	0	0	0

*Ten wells per treatment were used, experiment was performed once.

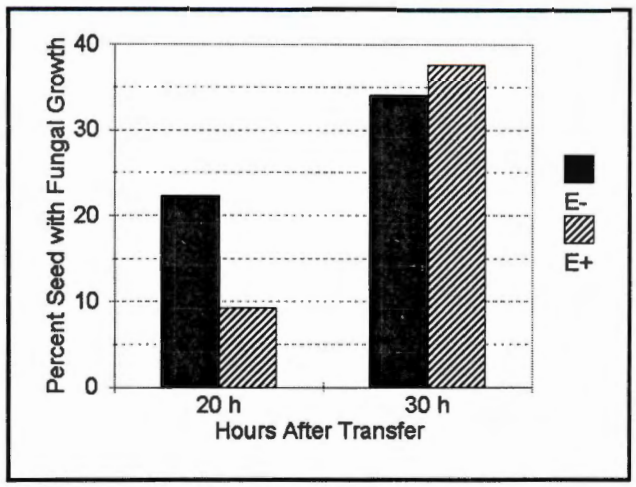
Table 3. Effects of endophyte status and buffer treatment on sporangial germination of *P. aphanidermatum.*

Mol		0			0.005			0.01		
pH	Seed	0	E+	E-	0	E+	E-	0	E+	E-
6.5		8 a	8 a	8 a	6.7 abc	7.3 ab	7.7 a	4 efg	3.3 fgh	2.7 gh
6.0		8 a	7.3 ab	8 a	7.3 ab	7.7 a	4.7 def	3 gh	2.7 gh	2 h
5.5		8 a	4 efg	6 bcd	5.3 cde	6.7 abc	3 gh	3 gh	2 h	0 i
5.0		8 a	8 a	7.7 a	5 de	7 ab	2 h	3 gh	3 gh	0 i

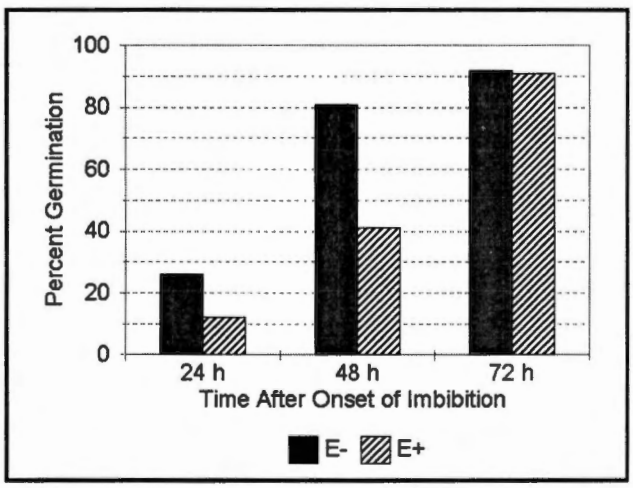
Sporangia of *P. aphanidermatum* were exposed to a series of sodium citrate buffer combinations ranging from pH 5.0 to 6.5 and 0.001M to 0.01M. One ml of buffer was placed in each well. No seeds, E+ or E- seeds (8 per well) and a piece of *Pythium*-infested grass were added. Plates were incubated at 28 C in continuous light for 24 h. Presence of zoospores was determined. Data presented is mean of three experiments, with 8 wells per treatment. Mixed models procedure with Least Squares Mean Separation was used to compare data (SAS, Cary, NC).

Seed Coat Colonization and Embryo Infection of Tall Fescue

Embryo infection of 95E- seed was twice that of 95E+ seed (22% and 9%) (Figure 6a). Embryo infection was assessed at 20 h after transfer to selective medium. Dense mycelium originated exclusively from the embryo. Embryos became watersoaked, necrotic and seeds failed to germinate. The few radicles which pierced the seed coat were dark and lacked root hairs. Presence of fungal growth emanating from seeds assessed at 30 h were not related to endophyte status [95E- (34%) and 95E+ (38%)]. Data collected at 30 h included fungal colonization observed at 20 h. Seed that did not express embryo infection at 20 h, but expressed pathogen colonization at 30 h germinated with no evidence of necrosis. Colonized seeds developed sparse mycelial strands originating from all over the seed coat. Embryos remained white and radicles emerged with numerous root hairs. The time seeds were observed greatly affected results (P=0.0001) and the interaction between endophyte and time assessed was significant (P=0.003). Germination of seeds from uninoculated controls at 24, 48, and 72 h from the onset of imbibition are shown on Figure 6b. Germination rate at 24 h of 95E- and 95E+ seeds in uninoculated controls were 12% and 21%. These data corresponded to the embryo infection rate of inoculated seeds observed at 20 h from transfer. The germination rate of uninoculated controls (95E-=91% and 95E+ =92%) were equivalent at 72 h.



A. Infection and colonization



B. Percent germination

Figure 6. Endophyte effect on **A.)** embryo infection and colonization by *P. aphanidermatum* and **B.)** percent germination. Seeds were incubated with *Pythium*-infected or sterile grass pieces for 20 h and transferred to selective media. Fungal growth was determined at 20 h and 30 h after transfer. Seeds with fungal growth at 20 h (40 h after inoculation) developed embryo necrosis. Fungal growth on seeds at 30 h (50 h from inoculation) that did not have embryo infection was limited to the seed coat and seeds germinated. Measurements at 30 h include 20 h observations. Controls were observed for germination at 24, 48 and 72 h after onset of imbibition. Note embryo infection rate (20 h) on Fig 6a is similar to germination rate at 24 h in Fig 6b for 95E+ and 95E- seed. The time seeds were observed greatly affected results (P=0.0001) and the interaction between endophyte and time assessed was significant (P=0.003).

Discussion

Based on the results of the mycelial growth experiments it is thought that EMR was not related to antifungal activity of endophyte-infected seed. Mycelium of *P. aphanidermatum* was not affected by endophyte status. Although differential growth of *R. solani* was observed between high and low endophyte seed lots, these methods could not be used to determine whether growth differences were related to antifungal activity or availability of nutrients in the growing media. As seed soaking times increased, colony diameter increased on imbibition media and decreased on macerate media; therefore nutrients required for fungal growth were transferred from seed to steep water and may account for differential growth. Differences in seed exudation may have been related to endophyte infection, as subsequent experimental data with *P. aphanidermatum* and Kentucky 31 seed suggest. Conversely, differences within the imbibition fluids may have been the result of aged leakier membranes (PEL was harvested in 1992 and PEH was harvested in 1993). Aging of grass seed results in deterioration of membranes, increased electrolyte permeability, and loss of vigor (Happ *et. al*, 1993). In any case, the RSNC1 isolate of *R. solani* did not produce disease in previous experiments; therefore, no correlation between EMR and *in* vitro fungal growth can be made. During this research, numerous unsuccessful attempts were made to isolate a pathogenic *Rhizoctonia* culture that was sensitive to EMR.

Inhibition of mycelial growth would not be an effective resistance mechanism against *P. aphanidermatum*. No damping-off or seed rots occurred when *P.*

aphanidermatum mycelium was grown on CMA with tall fescue seed. Isolates pathogenic to bent grass when sporangia were used as inoculum, but were not pathogenic as mycelium (Abad *et al.*, 1994). In the soil, mycelium may not be an effective infectious agent of tall fescue seed. Thus, subsequent investigations of resistance mechanisms by endophyte-infected seed were conducted using sporangia.

Results of *in vitro* experiments using SDW as a medium to co-culture 95E+ and 95E- seed with *P. aphanidermatum* sporangia cannot be directly applied to soil. However, these data provided useful insight about the changes in the seed spermosphere during the process of germination. Sporangia of *P. aphanidermatum* are highly sensitive to electrolytes, volatiles, amino acids and sugars (Nelson, 1987). Therefore, sporangial response was an effective bioassay to spermosphere components of 95E+and 95E- seed.

Since sporangia differentially responded to 95E+ and 95E- seed in SDW. All seed inhibited zoospore production, but less 95E+ seed per ml were required to prevent sporangia from producing zoospores than 95E- seed. Interestingly, these differences were not observed when seed were soaked in buffer. Thus, it can be concluded that sporangial responses to the environment are highly complex. Furthermore, sporangia responded differentially to seed treatments. For example, less than 2 seeds per ml prevented sporangia from producing zoospores when the inoculum was placed in wells containing seed soaked in SDW for 24 h. On the other hand, factors that inhibited sporangial germination were apparently removed when seed were rinsed. For example, 10 to 12 seeds per ml inhibited zoospore release when sporangia were added to wells with seed that had been rinsed after soaking in SDW for 24 h. Boiling or freezing imbibiton fluids

in order to denature proteins or otherwise disrupt inhibitory compounds did not result in altering the response of sporangia, compared to fresh steep water. Therefore, sporangial responses were not related to heat and cold sensitive compounds in the spermosphere. Since *Pythium* sporangia are sensitive to pH and osmotic potential (Morris and Gow, 1993), similar experiments were conducted using buffer instead of SDW.

Since sporangia of *P. aphanidermatum* were more sensitive to the effects of seed in SDW than in buffer, pH and electrolyte concentration in the spermosphere were perhaps important factors in sporangial germination in SDW. Other factors in the spermosphere of 95E+ seed probably affect sporangia, since the overall differential effect of seed on sporangia in SDW was reversed in buffers with concentrations of 0.005 and 0.001M. Furthermore pH was not a factor in sporangial germination thus pH may not be the an important factor in the soil. The complexity of the seed-endophyte-pathogen interaction is exemplified by these data.

Based on data obtained from experiments using sodium citrate, it is thoughtbelieved that 95E- seed affected sporangial germination more than 95E+ seed, when using buffers between pH 5.0 and 6.0 with concentrations of 0.005 and 0.001 M. This may be related to osmotic pressure on sporangial germination. The 95E- seed may have contributed more electrolytes to the solution than 95E+ seed, resulting in lower osmotic pressure between sporangia and their surroundings. Reduced osmotic pressure may have inhibited sporangial germination. Subtle differences in the spermospheres of 95E+ and 95E- seeds were detect ed using this *in vitro* bioassay because sporangia are highly sensitive to pH and molarity. Based on these data, it was concluded that 95E+

seed was less leaky than 95E- seed. The aberrant data point (well containing pH 5.5 0.001M buffer) was probably caused by contamination of laboratory tools and exemplifies the highly sensitive nature of the bioassay .

Results obtained from experiments where seed were exposed to *P. aphanidermatum* sporangia in soilless medium were consistent with data derived from *in vitro* experiments and results obtained from screening for EMR in Part 2 of this thesis. That is, early events during germination greatly affect infective propagules and EMR. Embryo infection by *P. aphanidermatum* occurred during the first 24 h of germination. Mycelium growing from embryos was dense, fast growing, and associated with tissue necrosis; where as mycelium originating from the seed coat was sparse, slow growing, and associated with apparently healthy tissue. These different growth habits indicate the fungus was occupying two different niches: embryo pathogen and seed coat saprobe. Nutrient source (derived from seed exudates) can be a mediating factor in determining whether *Pythium* behaves as a saprobe or a pathogen (Nelson and Hu, 1994; Hering, 1987; Mandelbaum and Hadan, 1990). Based on these data, it is suggested that the nature of the nutrient source was different between 95E+ and 95E- seed. Since embryo infection was so closely related to germination rate of 95E+ and 95E- seed, it is likely that EMR was related to events that occurred during seed germination. Interestingly, the rate of embryo infection and germination for 95E- seed was twice that of 95E+ . Therefore subsequent experiments were done to quantify the nature of 95E+ and 95E- seed exudates and germination.

Part 4: Influence of Endophyte on Seed Germination and Spermosphere

Introduction

Neotyphodium coenophialum is transmitted only though seed. Since the endophyte is present in the seed during germination, the question arises as to whether the endophyte has an effect on the germination process. Pinkerton *et al.* (1990) indicated that endophyte-infected seeds had a higher germination percentage than endophyte-free seed. They also observed that endophyte infection generally increased the number of days to half maximal germination, but this was variable across genotype. Germination experiments reported by Rice *et al.* (1990) observed that during the initial process of water uptake, endophyte infection reduced relative water gain of lines tested. However, endophyte-infected seed had greater germination percentages than endophyte-free lines. The authors suggested that the endophyte-free embryos may have been damaged from rapid water uptake that would result in reduced germination.

Endophyte-mediated response to *P. aphanidermatum* was negated when tall fescue seed were soaked for 24 h or longer (Part 2 of this thesis). Thus the events associated with the early stages of germination are important in resistance mechanisms. Results of experiments reported in Part 3 indicated the endophyte did not produce antifungal products active against mycelium of *P. aphanidermatum*. However, based on *in vitro* tests using *P. aphanidermatum* sporangia, the endophyte is believed to have a complex effect on the spermosphere. When compared to endophyte-free treatments, zoospore production of *P. aphanidermatum* was reduced in the presence of endophyte-infected seed soaked in SDW, but not when endophyte-infected seed were soaked in

sodium citrate. When seeds were rinsed the influence of the spermosphere on sporangial germination was reduced. Furthermore, steep water from tall fescue seed reduced zoospore production. Finally, embryo infection was significantly higher in endophyte-free seeds than endophyte-infected seeds incubated in soilless medium, but colonization of the spermosphere was not different when exposed to extremely high numbers of fungal propagules. Percentage of embryo infection closely correlated to percentage germination of uninoculated controls. Therefore, it was concluded that spermosphere components play an important role in EMR.

The following experimental hypothesis was tested: endophyte infection differentially affects tall fescue seed germination and spermosphere. The objectives of this study were 1) to determine the effects of endophyte infection on seed germination; 2) to determine the effects of presoaking on seed germination; 3) to analyze differences that the endophyte confers on the spermosphere of germinating seed.

Analysis of the spermosphere can be done using a variety of tests. Vigor testing of ryegrass seed is commonly determined by conductivity readings, shoot height and germination percentage (Happ *et al.*, 1993). Higher vigor is associated with greater shoot height. Higher germination percentage is associated with lower steep water conductivity. Increased electrolytes in leachate of seed is attributed to the degradation of cellular membranes and increased cellular permeability (Hap *et al*, 1993). Therefore steep water of endophyte-infected and endophyte-free seed were compared using conductivity and pH determination to detect differences in leakiness during germinating. Spermosphere pH (less than 5.0) and buffer concentration influenced the behavior of *P.*

aphanidermatum sporangia (Part 3 this thesis). Propagules of *P. aphanidermatum* were inhibited by low pH and low osmotic pressure (Ruben *et al.,* 1980). Conversely, chemoattraction of zoospores is stimulated by sugars and amino acids (Nelson, 1987). Thus exudates from embryo tissue may have been responsible for increased embryo infection when seed were exposed to zoospores of *P. aphanidermatum.* Steep water was analyzed for carbohydrate content using an iodine test. Protein analysis was performed.

Materials and Methods

Seed

Genetically similar tall fescue pasture grass seed (Kentucky 31) differing only in *N. coenophialum* infestation levels [95% (95E+) and 0% (95E-)] were used. Seed (3 g) were scarified in 60% H_2SO_4 for 30 min on a rotary shaker (80 rpm), rinsed in tap water, surface sterilized in 50% Clorox™ (2.62% NaOCl) for 20 min on a rotary shaker and rinsed 5 times with sterile distilled water (SDW).

Germination in Water

Seed (3 g) were placed in flasks with 30 ml SDW and incubated on a rotary shaker (80 rpm) with continuous fluorescent light for 24, 48, 72, or 96 h at room temperature. Randomly selected seeds (200) were microscopically examined for radicle emergence (germination). The experiment was conducted three times. Comparisons between endophyte status were analyzed using a Students t-test (Quattro Pro, Borland International, Scotts Valley, CA).

Germination in Soilless Medium

Three seed treatments were compared: 1) nonscarified, 2) scarified, surface sterilized and soaked in SDW on rotary shaker for 3 h at room temperature, 3) scarified, surface sterilized and soaked in SDW on rotary shaker for 24 h at room temperature. Seeds (20 seeds per pot, 5 pots per treatment) were planted into soilless mix (Farfard™) and incubated for 7 d at 28 C in continuous light. Seeds were removed from media; germination rates and shoot length were determined. The experiment was repeated twice. Comparisons between endophyte status and preplant seed treatement were analyzed with a Students t-test (Quattro Pro, Borland International, Scotts Valley, CA).

Characterization of Steep Water

Protein Analysis

Three g of 95E+ and 95E- seed were placed in flasks containing 30 ml SDW and incubated for 24 h on a rotary table (80 rpm) in continuous light at room temperature. Steep water was decanted, lyophilized, and analyzed with SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Lyophilized steep water was diluted with 1 ml loading buffer centrifuged at 1500 rpm for 3 min and 5 or10 *u*l were loaded on to a 12% Tris-glycine gel with a 4% stacking gel (pH 8.8). Gels were stained with colloidal coomasie. The procedure was repeated twice. A total protein analysis of steep water was conducted using a Bio-rad Protein Assay™; microtiter plate protocols supplied by the manufacturer were used. The assay was conducted twice with different batches of

steep water using 1) an ovalbumin standard and 2) a bovine serum albumin standard.

Conductivity, pH and Carbohydrate Analysis

Seeds (1, 2, 3, 4, or 5) were placed in 24-well tissue culture plates (30 plates per experiment; 6 plates per treatment) with 1 ml SDW. Wells were paired, 95E+ and 95E-, and SDW controls were included on each plate. Plates were incubated at room temperature with continuous light for 3 or 24 h. Steep water was transferred into glass vials (95E+, 95E-, SDW) then electrolytic conductance and pH were assessed. The value of the SDW control was subtracted from the readings to obtain comparative conductance. One ml of each 24 h sample was subsequently dispensed into a test tube. A crystal of iodine was added to each test tube then samples were heated to a boil for 30 seconds in a microwave. Color change (indicating the presence of carbohydrates) was quantified using a spectrophotometric plate reader (450nm). Percent germination of seed soaked for 24 h was determined for comparison. The experiment was repeated twice.

Results

Germination in Water

Endophyte-infected seed germinated more slowly than endophyte-free seed. At 24 h mean germination of 95E+ and 95E- seed were 11.7% and 30.3%, respectively (P=0.009). Differences between germination percentage of 95E+ and 95E- gradually became less (P values increased) as time from onset of germination increased. At 96 h germination percentages of 95E+ and 95E- seed were not different (P=0.322) (Fig.7).

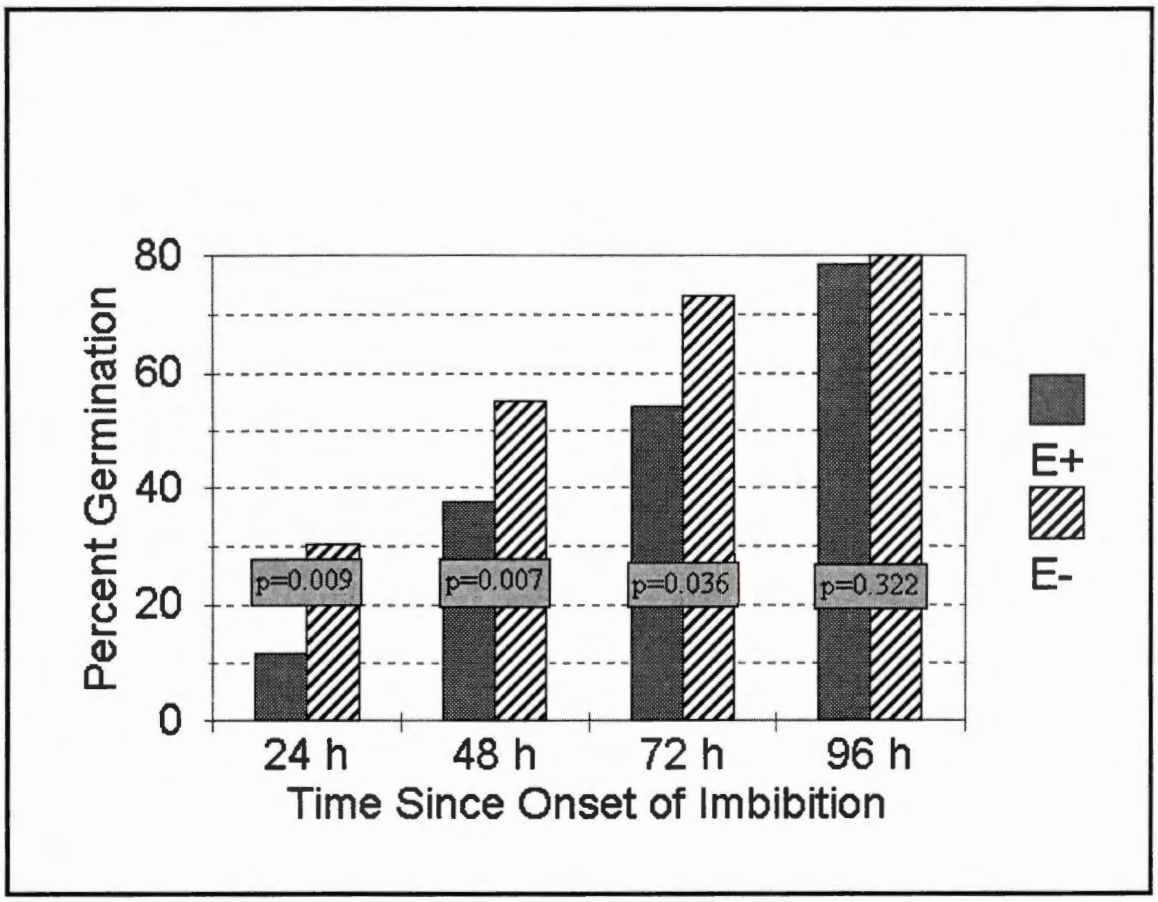


Figure 7. Percent germination of endophyte-infected (E+) and endophyte-free (E-) seed imbibed in SDW. Three g seed were incubated in flasks with 30 ml SDW on a rotary shaker (80 rpm) with continuous fluorescent light for various times. Randomly selected seeds (200) were microscopically examined for radicle emergence (germination). The experiment was conducted three times. Based on paired t-test analysis, germination of E+ was less than E- until seed was imbibed for 96 h. ($P<0.05$).

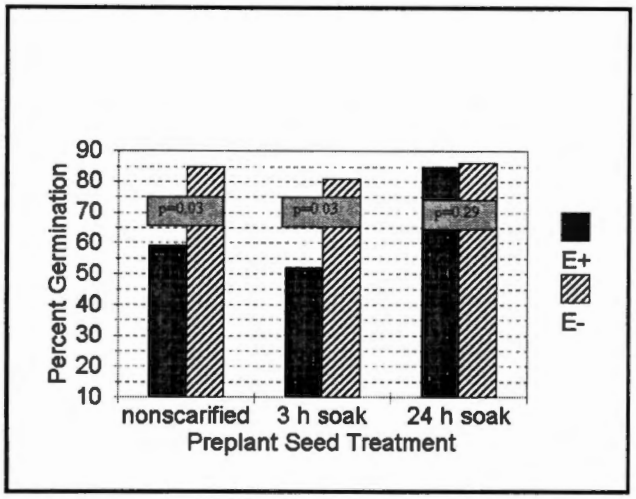
Germination in Soilless Medium

Seven days after planting, no significant differences in germination percentage were observed between 95E+ and 95E- seed when presoaked for 24 h (Fig. 8a), but 95E+ shoot length were 15% less than those of 95E- (Fig. 8b). Preplant seed treatment did not significantly affect germination of 95E- seed. The 24 h soak preplant seed treatment affected percent germination of 95E+ seed (3 h soak vs 24 h soak, P=0.04; nonscarifed vs 24 h soak, P=0.05); however germination percentages of 95E+ seed with nonscarified and 3 h soaking preplant treatments were not significantly different. Preplant seed treatment greatly influenced shoot length on of 95E- and 95E+ seed (P<0.001) (Fig. 8a). Compared to 24 h presoaked 95E+ seed, germination of nonscarified and 3 h presoaked 95E+ seed was reduced by 30% and 40%, respectively (Fig. 8a). Shoot length of 95E+ seedlings were also less than shoot length of 95E- seedlings regarldless of preplant seed treatment (Fig. 8b).

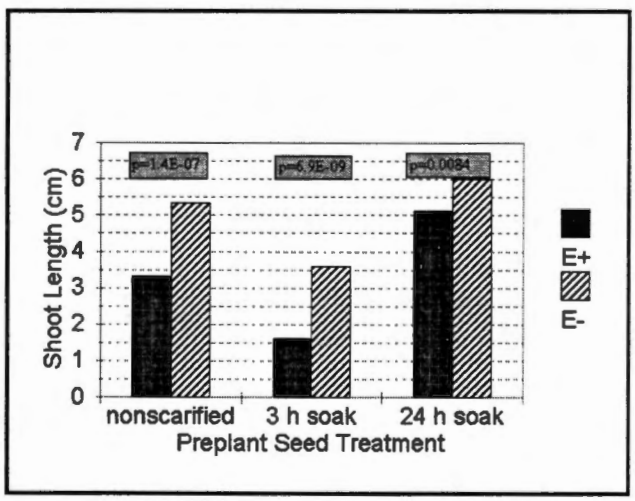
Characterization of Steep Water

Protein Analysis

No protein bands larger than 7000 kilodaltons (the smallest limits of the gel) were observed in lyophilized steep water from 95E+ and 95E- seed. Based on Bio-Rad protein assays, 95E- steep water contained more protein than 95E+ steep water. Protein content of 95E- and 95E+ steep water was 7.36 *u*g/ml and 1.65 *u*g/ml, respectively, using the ovalbumin standard. Protein content of 95E- and 95E+ steep water was 3.83 *u*g/ml and 0 *u*g/ml, respectively, using the bovine serum albumin standard.



A. Percent Germination



B. Shoot length

Figure 8. Endophyte and preplant seed treatment effects on **A)** percentage germination and **B)** seedling shoot length. Effects of endophyte status were compared using three seed treatments: 1) nonscarified, 2) scarified and soaked in sterile distilled water (SDW) for 3 h, 3) scarified and soaked in SDW for 24 h. Seeds (20 seeds per pot, 5 pots per treatment) were planted into Farfard ™ mix and incubated for 7 days at 28C in continuous light. Seeds were removed from media; germination and shoot length were determined. Data were analyzed using a Students t-test. Preplant treatment affected seed germination and shoot length were affected of endophyte-infected (E+) seed. However, percent germination of endophyte-free seed (E-) was not influenced by seed treatment. Comparison of E+ and E- seed were made for each seed treatment. $P < 0.05$ were significantly different.

Conductivity, pH and Carbohydrate Analysis

Germination of seed soaked in wells with SDW for 24 h was slightly lower than seed incubated in rotating flasks with SDW. However, overall mean germination of 95E- seed (17.4%) was consistently twice as high as the overall mean germination of 95E+ seed (9%).

Steep water pH from 95E+ and 95E- seed decreased quadratically (P=0.0008) to pH 4.0 with 4 seeds/ml(Fig 9a and 9b); pH data from 3 and 24 h samples were different (P=0.005). Steep water from 3 or more seeds per ml was similar in pH at 3 h and 24 h, while steep water from less than 3 seeds per ml continued to decrease with time. Data from preliminary experiments using 11 seeds/ml did not result in pH readings below 3.8. Steep water pH from 95E+ seed was higher than steep water pH from 95E- seed (P=0.001). Conductivity increased linearly (P=0.001) as seeds per ml increased (Fig 9c and 9d); conductivity from 3 and 24 h samples were different (P=0.006). Values continued to increase with time and number of seeds and conductivity did not reach an equilibrium during the experiment. Conductivity of 95E- steep water was higher than 95E+ steep water (P=0.001). Based on iodine tests using 24 h steep water, carbohydrate content was greater in 95E- than 95E+ treatments when paired samples were compared (p=0.015) (Fig 10). However, there was no linear increase in absorbance with increasing seeds per ml and there were large differences between plates. A color change occurred in all treatments with seeds, thus carbohydrate was present in all 24 h steep water samples. Color change was not observed in controls.

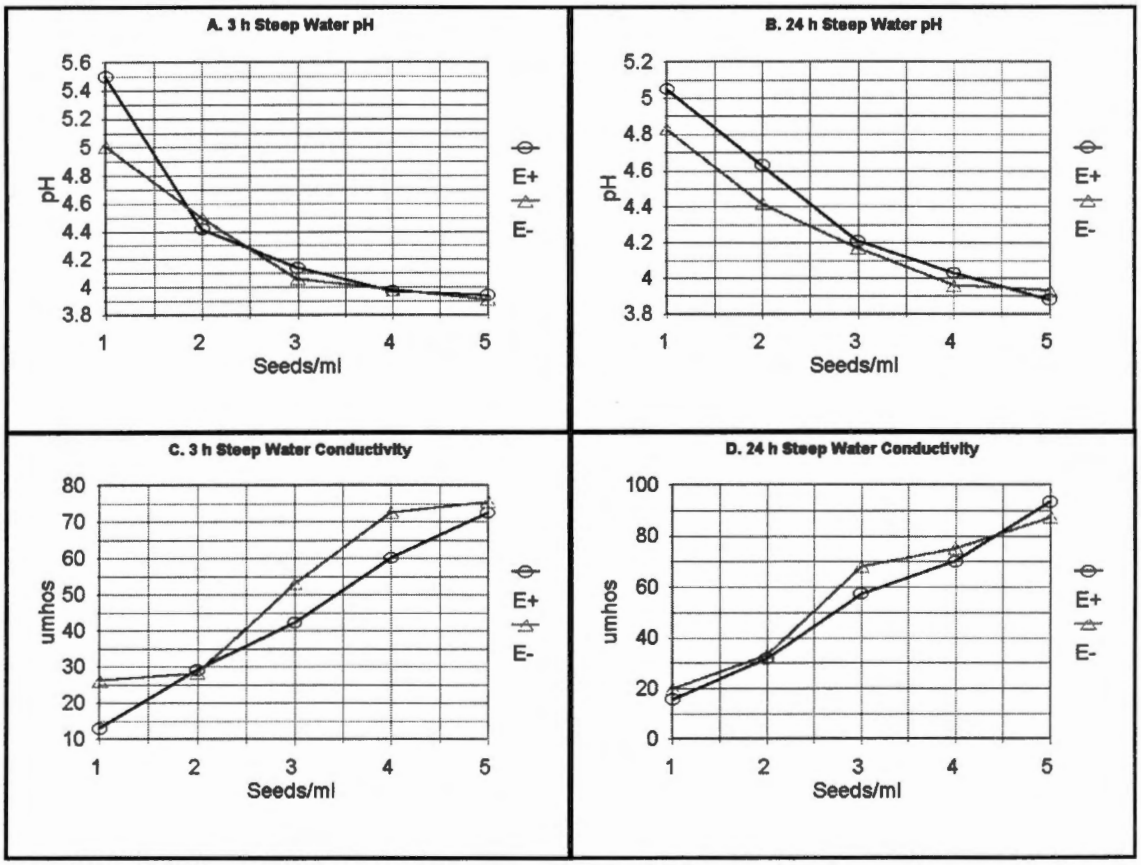


Figure 9. Conductivity and pH of steep water from endophyte-infected (E+) and endophyte-free (E-) seeds soaked for 3 or 24 h in SWD. Seeds (1, 2, 3, 4, or 5) were placed in 24-well tissue culture plates (30 plates per experiment; 6 plates per treatment) with 1 ml SDW. Wells were paired, E+ and E-, and SDW controls. Plates were incubated at room temperature with continuous light for 3 or 24 h. Steep water (E+, E-, SDW) from each plate was pipetted into 3 glass vials then pH and electrolytic conductance were assessed. The value of the SDW control was subtracted from the readings to obtain comparative conductance. Steep water pH from E+ and E- seed decreased quadratically (P=0.0008) to pH 4.0 with 4 seeds/ml; pH data from 3 and 24 h samples were different (P=0.005). Steep water pH from E+ seed was higher than steep water pH from E- seed (P=0.001). Conductivity increased linearly (P=0.001) as seeds per ml increased; conductivity from 3 and 24 h samples were different (P=0.006). Conductivity of E- steep water was higher than E+ steep water (P=0.001).

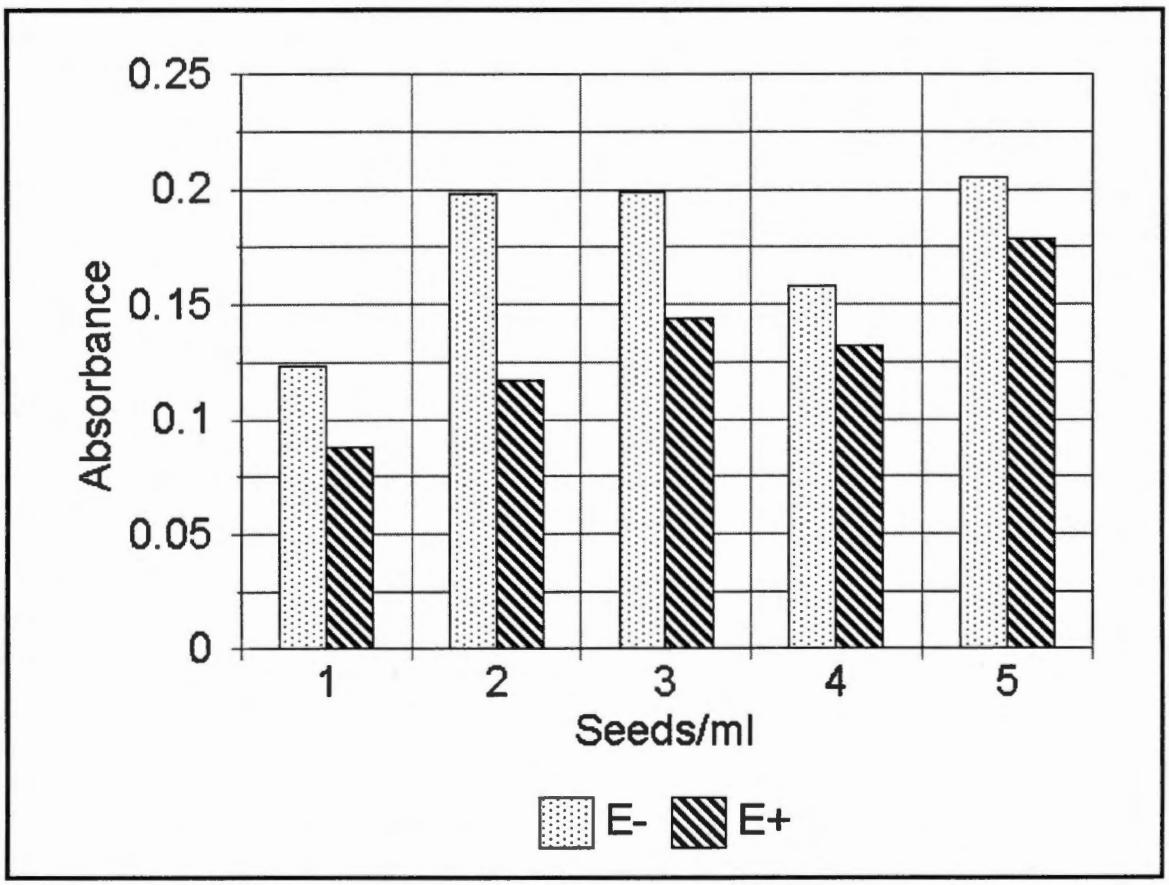


Figure 10. Average absorbance of 24 h steep water from endophyte-infected (E+) and endophyte-free (E-) exposed to iodine. Seeds (1, 2, 3, 4, or 5) were placed in 24-well tissue culture plates with 1 ml SDW. Wells were paired, E+ , E-, and SDW controls. Plates were incubated at room temperature with continuous light for 24 h. Steep water treatments from each plate was combined into glass vials then 1 ml of each 24 h sample was dispensed into a test tube. A crystal of iodine was added to each test tube then samples were heated to a boil in a microwave for 30 sec. Subsamples (200ul) were placed in 96 multiwell plates. Color change (indicating the presence of carbohydrates) was quantified using a spectrophotometric plate reader (450nm). The experiment was repeated twice. Based on paired t-tests, it was concluded carbohydrate content of steep water from E+ and E- seed were different (P=0.05).

Discussion

Based on data obtained from germinating seed in rotating flasks of water, germination of 95E+ seed was delayed by about 50% at 24 h, but by 96 h total germination percent of 95E+ seed was similar to 95E- seed. These data conflict with findings of Pinkerton *et al.* (1990) and Rice *et al.* (1990) who screened several endophyte-infected and endophyte-free seed lots for germination rate. They observed higher percentage of germination in most endophyte-infected seed lots. However Pinkerton *et al* (1990) and Rice *et al* (1990) observed slower imbibition of endophyte-infected seed and higher numbers of days to half maximal germination, similar to the findings of this report. Blank (1993) observed no correlation with endophyte status to the rate of seedling emergence from soil. These inconsistencies may be accounted for by the differences in experimental methods. That is, a 24 h delay in radicle emergence may not have been detected during daily observations for leaf emergence. Furthermore, differences between studies may be caused by the effects of seed germplasm, growing conditions, seed age, or whether endophyte-free seed contains dead endophyte. In this study a Kentucky 31 seedlot differing only in endophyte status was used (95E+ and 95E-). The germination rate of the Kentucky 31 1996 harvest seed lot (96E+ and 96E-) from the same pastures was also determined. Seed quality was poor. However, at 24 h twice the number of endophyte-infected seeds had germinated (germination rates at 24 h were 15% for 96E+ and 28% for 96E-), and germination rates at 96 h were similar (59% for 96E+ and 61% for 96E-). Therefore the effects of germination delay appear to be

consistent for this germplasm. A more thorough investigation with numerous germplasm is needed. A standard seed blotter germination test with scarified and nonscarified seed would reduce unwanted anaerobic effects and elucidate the effects of scarification. Rotating flasks of water were also used to incubate seed in this experiment in order to investigate the effect of preplant treatment soaking on EMR observed in experiments reported (Part 2, this thesis). This method was not ideal for determining the effect of endophyte on germination.

Based on data obtained from observing germinating seeds in soilless medium, endophyte status and preplant treatment affected germination rate. Endophyte-infected seed germinated more slowly and 7 d seedling length was reduced. The destructive data collection method prevented monitoring further plant growth and germination. However, 95E+ and 95E- control plants grown for 3 weeks in the greenhouse had similar germination percentages (95E+, 84.3%; 95E-, 81.8%) and differences in seedling length were not observed (Part2, this thesis). Presoaking in SDW for 24 h reduced the percentage difference in germination between 95E+ and 95E- seed. The effect of prolonged presoaking on 95E+ seed may be explain the loss of EMR to *P. aphanidermatum* observed in previous experiments reported in Part 2.

According to laboratory analysis, spermospheres of endophyte-free seed were richer in nutrients than endophyte-infected seed. Total protein content of lyophilized steep water from 95E- seed was higher than 95E+ seed. However, based on electrophoresis data, proteins were small. This data is consistent with reports that sugars, organic acids, amino acids and proteins are released into the spermosphere as seeds start

to take up water (Bewley and Black, 1994). The iodine test had limited quantitative value. The test was not sensitive enough to detect increasing levels of carbohydrate with higher seed number, thus quantitative data may not be valid. However, when paired data were analyzed, significant differences between 95E+ and 95E- steep water were detected. An alternate form of carbohydrate analysis is required to verify these data. A preliminary experiment to detect pectin content was performed as described in the Fanny Farmer Cookbook (Farmer, 1984). One part absolute ethanol and one part steep water were combined. The 95E- " jelly" had a firm, highly viscous texture while the 95E+ "jelly" was runny with no gelatinous characters. Although these methods were crude, differences were observed between 95E+ and 95E- steep water. Sporangia of *P. aphanidermatum* are sensitive to osmotic pressure (Ruben *et al.*,1980). Osmotic pressure is defined as the force of nonelectrolytes such as urea or sucrose across a semipermeable membrane (Lewis, 1971). Therefore the response of *P. aphanidermatum* to buffer containing fescue seeds, reported in Part 3, may be related to the release of nonelectrolytic molecules exuded from the imbibing seed into solution. Higher concentrations of sugars and urea in steep water would affect the osmotic pressure of the sporangia and thus influence zoospore germination.

Based on large changes in pH and conductivity in 3 h steep water, it is believed that very rapid changes occurred in the spermosphere and these changes occurred more rapidly in 95E- seed than 95E+ seed. The pH of steep water plummeted to 4.0 in wells with 3 or more seeds within 3 h. This may account for the inhibition of sporangial germination (Part 3, this thesis). Zoospores were not produced in wells with several

seeds and zoospore production was inhibited when exposed to pH 4.0 buffer. Conductivity readings detect the presence of electrolytic substances such as acids, bases, and salts (Lewis, 1971). Ions were released at a greater rate in 95E- seeds than 95E+ seeds. The 95E- seed were leakier thus creating a spermosphere higher in nutrients. Simple sugars stored in small quantities in the embryo are immediately available to imbibing seed. (Bewley and Black, 1994). The major source of energy is stored in the endosperm as carbohydrates (Oakes, 1983). These food reserves become available to the embryo only after the onset of germination (Oakes, 1983). Therfore linear increases in conductivity data in 95E+ and 95E- steep water may have been the result of exudates from the seed, which continues to mobilize nutrients from the endosperm as germination progressed. Results of the spermosphere analysis, indicating lower levels of electrolytic molecules in 95E+ seed, can be directly attributed to delayed germinative events.

The relationship between endophyte infection and seed germination has not been considered. Presoaking for 24 h increased the rate of 95E+ germination, but had no effect on 95E- germination. Since radicle elongation is the result of hydration and enlargement of existing embryo cells (Bewley and Black, 1994), differential cell membrane permeability may be related to germination rate differences. Another possible cause for germination delay of 95E+ seed may be related to metabolic activity of the endophyte while invading the embryo. The endophyte resides contiguous to the scutellum (Hinton and Bacon, 1985) or within the embryo (Philipson and Christy, 1986) prior to germination. Small amounts of simple sugars are available to the embryo before germination and carbohydrate reserves are mobilized from the endosperm only after

germination (Oaks, 1983). As the fungus invades the coleoptile during germination, the developing embryo may be temporarily deprived of nutrients causing a slight delay in radicle emergence and subsequent growth. On the other hand, germination delay of 95E+ seed could be related to plant hormones released by the endophyte. Germination is orchestrated by the balance of gibberellins and abscisic acid (Bewley and Black, 1994). Plant pathogenic fungi are known to produce a broad range of plant hormones (Isaac, 1992). The endophyte produces the plant hormone 3-indole acetic acid and abscisic in culture (DeBattista *et al.*,1990) it may be capable of producing other plant hormones as well. Increases in abscisic acid would inhibit the production of enzymes responsible for the hydrolysis of starch and reduce the food supply to the embryo, delaying germination. Further investigation on the mechanism of germination delay in 95E+ seed may be warranted if these data are consistent with large scale germination experiments using numerous germplasm.

Part 5: Summary

Conclusions

Primitive, unspecialized *Pythium* species are regular members of the soil flora, have well-developed saprophytic activity, and infect seedlings and juvenile root tissues (Garrett, 1956). In the absence of the plant host, the propagules of the pathogen survive in the soil in a quiescent, inactive form known as fungistatis (Lockwood, 1988). This phenomenon is a result of deprivation of the propagules of essential nutrients (Lockwood, 1988). The rhizosphere and spermoshere is the arena in which fungistasis is nullified. When a propagule is in the vicinity of root and seed exudates, the pathogen is activated though a series of successive signals causing it to interact with the host tissues. The germinating propagules reach the infection court, interact with the host plant, produce infection structures, and penetrate the host tissues (Lockwood, 1988). Root and seed exudates contain many nutrients (sugars and amino acids) that enhance the activities of soil microorganisms. Volatile seed exudates (e.g., acetaldehyde, ethanol, ethane, acetone, and methanol) stimulate the germination of sporangia of *Pythium* species (Nelson, 1987). *Pythium aphanidermatum* sporangia are stimulated to form zoospores in the presence of glucose (Nelson, 1990).

The slight delay of endophyte-infected seed germination and reduced leakiness, compared with endophyte-free seed, could result in a spermosphere with fewer factors to stimulate the germination of sporangia and chemoattraction of zoospores to embryo tissues by *P. aphanidermatum.* Exudates released by the seed during imbibition of water support the growth of *Pythium* spp. and antagonistic bacteria . Competition of

carbohydrate was the primary factor affecting antagonism in the spermosphere by strains of *Pseudomonas* spp. on sugar beet against *Pythium ultimum* (Fukui *et al.*, 1994).

The influence of the endophyte on the seed during the early stages of germination apparently alters the window of opportunity for *P. aphanidermatum* infection. This would explain EMR that was observed in 95E+ seed which were nonscarified or soaked for 3 h before planting. Loss of EMR in seed soaked for 24 h could be attributed to a stimulatory effect of prolonged soaking on seed germination or to disruption of cell membranes that may occur in excessive water. This hypothesis is supported by additional data; seed (95E+) soaked for 24 h had higher germination rates and longer shoot length at 7 days than 95E+ seed that were nonscarified or scarified and soaked for 3 h before planting. Interestingly, these results differ from Fukui *et al.* (1994) who reported wheat seeds soaked in water had less embryo infection by *Pythium* spp. There were no differences in damping-off of 95E- seeds when comparing soaking times.

The behavior of sporangia indicate that multiple changes occurred in the spermosphere as a result of endophyte infection. The sporangia bioassay developed in this study served as a highly sensitive indicator of spermosphere changes. Caution should be used when relating this data directly to interactions in the soil. Even so, based on data obtained from these bioassays and laboratory analyses, it is believed that 95E+ and 95E- spermosphere are different in several ways, including pH, electrolytic molecules, sugars, proteins.

Reduced leakage of exudates may attract less inoculum to endophyte-infected seed. A delayed germination rate could also benefit endophyte-infected seed by means of

inoculum reduction. Earlier germination of 95E- seed could stimulate the germination of pathogen propagules and chemotactically attract zoospores. This could reduce the number of *P. aphanidermatum* propagules in the spermosphere and minimize the invasion of late germinating endophyte-infected seeds. The endophyte-imparted advantage of inoculum escape and enhanced drought tolerance may contribute to the success of endophyte-infected stand establishment. An extensive analysis of the spermosphere of several endophyte-infected germplasm and its affect on various soilborne pathogens may clarify the mechanisms that cause EMR.

In conclusion, reduced preemergence damping-off of endophyte-infected seed by zoospores of *P. aphanidermatum* is thought to be primarily caused by escape of the host from the pathogen. Traditional antifungal resistance mechanisms have not be observed. Therefore the term "endophyte-mediated resistance" may not be an appropriate application to the benefit the endophyte imparts to germinating tall fescue.

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