Biological control of turfgrass pests by fungal endophytes /

BIOLOGICAL CONTROL OF TURFGRASS PESTS

BY FUNGAL ENDOPHYTES

A Thesis Presented

by

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ABSTRACT

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BY FUNGAL ENDOPHYTES

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Grasses infected with a fungal endophyte may have a negative effect not only on foliar feeding insects but on root feeding turfgrass pests. The goal of this work was to evaluate the effectiveness of endophyte-infected (EI) grasses for the biological control of Japanese beetle larvae. In the first field season, larvae were implanted at densities of 0,10,20,40,60, and 90 grubs/ft^2 into cores on field plots of EI and endophyte-free (EF) cultivars of a variety of grass species. High variation resulted in all comparisons being nonsignificant. In the second year, second instar larvae were introduced at densities of 0,20,40, and 60 grubs/ft^2 into EI and EF tall fescue, and Kentucky bluegrass planted in pots submerged in the ground. Grubs feeding on infected grasses exhibited significantly lower survival and weight, suggesting a possible deterrence or antibiosis effect. Tolerance of grasses was assessed through comparision of clipping yields. The study is consistant with no-choice pot studies by Potter et.al. (1991) and Oliver (1990) showing an effect of EI grasses on Japanese beetle larvae.

It is in contrast to results of an almost identical study at U.R.I. (R.A. Casagrande, personal communication) Thus under natural conditions, other factors may influence the effect of EI grasses on root feeders.

A greater understanding of the relationship between endophyte and host grass can be achieved more easily in _in vitro_ experiments. Endophyte was isolated from various grasses and cultures were used to inoculate mycelium into seed and seedling grasses as well as embryogenic, initiating, and mature callus of EF grasses. Failure to detect endophyte in inoculated grasses may relate to isolate variability which operates at many levels and affects cross-compatability of host and endophyte. Variation was exemplified in the present study where _A. coenophialum_ isolated from _Poa autumnalis_ grew faster than isolates from cultivars of tall fescue. Successful inoculation, especially into Kentucky bluegrass, of endophyte varying in characteristics such as alkaloid production and antifungal activities, might yield grasses with enhanced agronomic qualities and would thus increase the potential for use of EI grasses in Integrated Pest Management (IPM) of turfgrasses.

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CHAPTER I

LITERATURE REVIEW

Significance

An increasing amount of attention is being given to the mutualistic relationship between fungal endophytes and host that exists in over 80 genera and in hundreds of species of grasses used for food, forage, and turf [Clay 1988, 1989, 1990; Bacon and DeBattista 1991; Funk and Clarke 1989; Funk et al. 1989; Siegel et al. 1984a, 1985, 1987a, 1989; White and Morgan Jones 1987; White et al. 1987;]. Lost productivity, resulting from fescue toxicosis in cattle and ryegrass staggers in sheep, associated with grazing of livestock on endophyte-infected (EI) pastures, has led to the introduction of new endophyte-free (EF) cultivars [Bacon et al. 1977; Funk and Clarke 1989]. At the same time, greater insect resistance, stress tolerance, and increased overall vigor have been shown for EI plants versus comparable EF individuals [Clay 1984, 1989; Latch et al. 1985]. Thus the presence of endophytes may be a useful method of biological control in grasses, reducing the need for expensive, potentially hazardous pesticides and herbicides [Bacon et al. 1986; Clay 1989; Siegel et al. 1985, 1987, 1989]. There us further potential in the possibility of the use of endophytes as vectors for the insertion of traits to enhance host characteristics [Clay 1989; Funk et al. 1989].

Ecology

'Endophyte' describes a range of taxonomic and geographically-distributed fungi living intercellularly within host plant tissue. Endophytes exist in a variety of host plant species and are often related to protection from herbivores and from other environmental stresses [Carrol 1988; Clay 1986, 1987]. Decreased herbivory may result from infection of marine green algae, Douglas Fir (Abies sp.), and Dutch elm (Ulmus hollandica) with their associated endophytes [Carrol 1988; Clay 1987b]. Fungal endophytes occur most frequently among grasses, where they are present in all but four tribes and are known to infect over 125 species [Bacon et al. 1986]. Of ecological interest is the persistance in evolution of the grass-endophyte interaction which relies on the selection of advantageous characteristics conferred on the host plant by the mutual relationship.

The mutualistic relationship (type 2 or type 3) between fungi of the genus Epichloe and festucoid grasses is thought to have coevolved from a pathogenic association (type 1) [White 1988]. Alkaloids produced by certain individuals in a grass population were effective in suppressing the fungal stomata resulting in selection against sexual reproduction. At the same time, alkaloids produced by the fungus proved to be an advantageous defense mechanism for the plant. Benefit to the fungus is derived from receipt of nutrients, photosynthate, and a means of dissemination through association with the host plant [Smith et al. 1985].

Classification

Endophytic fungi of grasses are predominantly Ascomycetes in the family Clavicipitaceae and are known to infect the host families Poaceae, Cyperaceae, and Junaceae [Clay 1989]. Within this broad classification is the tribe Balansiae which comprises the genera Balansiopsis, Atkinsonella, Myriogenospora, Balansia, and Epichloe, distinguished from each other on the basis of differences in conidial fructification [Clay 1986a; Siegel et al. 1987]. Of these, Balansia contains the greatest number of fungi. The majority of the 17 species occur in the U.S. on varieties of Graminaceous weeds [Bacon et al. 1986; Clay 1988; Leuchtmann and Clay 1988]. Epichloe typhina is most commonly responsible for infection of important genera of turfgrasses (Agrostis, Dactylis, Festuca, Hordeum, and Lolium) [Bacon et al. 1977]. All genera except Myriogenospora, produce systemic infection [Leuchtmann and Clay 1988; Luttrell and Bacon 1977].

Endophytes of tall fescue and perennial ryegrass, originally classified as Sphacelia typhina, were later placed in a new genus, Acremonium [Latch et al. 1984; Morgan-Jones and Gams 1982; White and Cole 1985a,b]. Acremonium endophytes are related to the teleomorphic state of Epichloe typhina, but are anamorphic. They cause no visible symptoms on the host and reproduce through maternal transmission of host seed [Morgan-Jones and Gams 1982; Rykard et al. 1985; Siegal et al. 1987]. Similar reactivities of E. typhina tested against the antiserum of A. coenophialum from tall fescue (Festuca arundinacea Shreber) and A. lolii from perennial ryegrass (Lolium perenne) suggest that these imperfect fungi may be considered biotypes of E. typhina [Clay 1986a].

Incidence and Spread

Spread of the fungus occurs either through production of conidia and ascospores, as is typical of most endophytes, or, as for Acremonium species, through internal reproduction where dissemination occurs via the host seed [Clay 1986a, 1988, 1989; Siegel et al. 1987]. During vegetative growth, systemic hyphae of the endophyte run parallel to the longitudinal axis of host epidermal cells. In spore-producing endophytes, hyphae reaching external areas of the host broaden to form stromata which, at the time of host flowering, are the site of spore production. Mycelium of Acremonium grow intercellularly into the ovule of the host and are dispersed via the host seed [Bacon et al. 1986 Siegel et al. 1987]. During dormancy, the endophyte is associated with the aleurone layer of the seed. Upon germination, mycelium invades the endosperm and infects the developing leaf sheath [Philipson and Christy 1986; Siegel et al. 1985, 1987]. The reproduction of fungus and host thus occur simultaneously. While asexual or sexual spores are short-lived, viability of the endophyte in seeds may be a year or more, depending upon environmental or storage conditions [Bacon et al. 1986; Rolston et al. 1986; Welty et al. 1983].

A majority of Lolium and Festuca grasslands throughout Europe with an endophytic fungus where 53 of 64 cultivars of Lolium tested contained an endophyte [Latch et al. 1987]. In New Zealand almost all of the perennial ryegrass pastures are host to A. lolii [Latch and Christensen 1982; Latch et al. 1984,1987]. Over 90% of the pasturelands in the U.S. are infected with a variety of Acremonium [Siegal et al. 1985, 1987]. In a survey of 26 states in the U.S., 58% of the over 14 million

hectares of old pasture were found to be infected with endophyte [Shelby and Dalrymple 1987; White and Cole 1985a]. Germplasm of Lolium at the Western Regional Plant Introduction Station was found to contain endophyte [Wilson et al. 1991]. In addition, non-choke-inducing endophyte may be found in hard fescue (Festuca longifolia), Chewings fescue (F. rubra L. ssp. commutata Gaud), creeping red fescue (F. rubra L. ssp. rubra), and in other species of fine fescue [Saha et al. 1987]. A number of woodland grass species are also infected with endophytic fungi [Clay and Leuchtmann 1989].

Alkaloid Production

Production of alkaloids is thought to be directly related to many of the agronomic characteristics of the host-endophyte association. A variety of ergot alkaloids, such as ergovaline and ergotamine, are found in tall fescues. It is these compounds that are largely reponsible for symptoms of mammal toxicosis. In addition, six ergot alkaloids caused antifeeding and antibiosis effects on the fall armyworm (Spodoptera frugiperda) [Clay and Cheplick 1989]. Ergot alkaloids are produced by the cultures of isolated fungi growing on specific media [Bacon 1985, 1988]. Pyrrolizidine alkaloids, including N-formyl and N-acetyl loline occur in both tall fescue and perennial ryegrass and may be produced as part of the interaction of the two organisms. Natural and semi-synthetic loline alkaloid derivatives caused reduced feeding and weight gain on fall armyworm, European cornborer (Ostrinia nubilalis), and a variety of other insects [Riedell et al. 1990]. The majority of the alkaloids are present in the leaf sheath where the endophyte resides [Bacon et al. 1986]. Only 10-15% of the loline alkaloids in the plant

are found in plant roots [Siegel et al. 1989]. Other secondary compounds include peramine and lolitrem B.

Presence of alkaloids can be detected in a variety of ways. Levels of loline alkaloids may be tested in bioassays with the oat cherry aphid Rhopalosiphum padi or with the milkweed bug Oncopeltus fasciatus [Siegel et al. 1989]. The greenbug aphid, Schizaphus graminum, is sensitive to loline and other alkaloids. Quantitative methods of determining alkaloid types and levels include thin layer chromatography (TLC) [Bush and Jeffreys 1975], nuclear magnetic radiation spectral analyses [Petroski et al. 1989], and capillary gas chromatography [Yates et al. 1990]. Differences exist between alkaloid types and levels found in specific grass-endophyte combinations. In addition, plant characteristics such as age and location [Eichenseer et al. 1989; Jones et al. 1983] as well as environmental factors such as drought and heat [Arachevaleta et al. 1989; Kennedy and Bush 1983] influence levels of alkaloids.

Mammal Toxicosis

Interest in endophytes was initiated largely because of their association with syndromes known as fescue foot and ryegrass staggers resulting from livestock feeding on infected grass [Bacon et al. 1977]. Symptoms include decreased weight gain, muscle spasms, lowered milk production, gangrene, vasoconstriction, and abortions which are similar to those associated with ergot poisoning [Bacon et al 1977, 1986; Clay et al. 1985; Hoveland et al. 1983; Seman et al. 1990]. A number of ergot alkaloids are produced in cultures of A. coenophialum [Bacon 1985, 1988; Bacon et al. 1981, 1986; Lyons et al. 1986; Porter et al. 1985;

Yates et al. 1985]. Perloline and perlolidine inhibit cellulose digestion and produce volatile fatty acid both in-vivo and in-vitro [Bush et al. 1982; Kennedy and Bush 1983]. The alkaloids reach the highest concentration in the plant during the late spring and summer corresponding with high incidence of cattle syndromes. Ryegrass staggers result mainly from the neurotoxin lolitrem, in particular lolitrem B, a lipophilic complex substituted by an indole group [Gallagher et al. 1981, 1984, 1985]. Feeding trials have verified the relationship between symptoms of animal toxicity in EI, mixed, and EF grasses [Fribourg et al. 1990; Hoveland et al. 1983].

Resistance to Insects

Endophyte-enhanced resistance to foliar feeding insects has been well documented [Cheplick and Clay 1988; Potter and Braman 1991; Siegel et al. 1989]. Evidence has been collected from experiments using mainly three approaches: 1) Correlation in the field of percentages of endophyte infection with diversity and number of insects, 2) Laboratory no-choice feeding experiments, and 3) Laboratory choice experiments [Clay 1989]. The negative effects of endophyte infection on insects were first observed in the field in New Zealand where infected ryegrass was less susceptible to attack by Argentine stem weevil (Listronotus bonariensis) in comparison with EF varieties, especially during hot, dry periods [Prestige et al. 1985]. Reduced survival and weight gain have been found for fall armyworm (Spodoptera frugiperda), hairy chinch bug (Blissus leucopterus hirtus) and four species of billbug (Sphenophorus sp.) feeding on EI grasses [Cheplick and Clay 1988; Clay et al. 1985; Johnson-Cicalese and White 1990; Rowan and Gaynor 1986]. Given a

choice, hairy chinch bugs and other insects often select the EF variety or avoid the sheath where the majority of endophyte is concentrated [Mathias et al. 1990]. In contrast, Johnson-Cicalese and White [1990] found no significant differences in feeding preferences for billbugs [Johnson-Cicalese and White 1990]. In addition, studies by Potter et al. [1991] and Oliver [1990] have shown some resistance of EI grasses on root feeding larvae of masked chafer (Cyclocephala lurida) and Japanese beetle (Popilla japonica). Reduced numbers of certain nematodes as well as other soil-inhabiting organisms were found beneath grasses containing endophyte [Kimmons et al. 1990; Kirpatrick et al. 1990; O'Day et al. 1990; Pedersen et al. 1988; West et al. 1988, 1990]

Resistance to insects by endophytic grasses may be a result of deterrence, antibiosis effects, or tolerance of damage in EI grasses. As mentioned previously, alkaloids are thought to play a role in all of these mechanisms of resistance. In general, the bitter taste of the alkaloid may act as a deterrent to insect feeding [Clay 1988]. Ergot alkaloids acted as feeding deterrents to fall armyworm [Cheplick and Clay 1989]. Various combinations and types of loline alkaloids resulted in reduced weight gain as well as altered feeding behaviors of fall armyworm and European corn borer [Riedell et al. 1991]. Resistance to sod webworms and chinch bugs has been related to both non-preference as well as direct toxin effects [Hellman and Mathias 1990]. Levels of N-formyl loline in the grass may be determined by assaying the sensitivity of the large milkweed bug or the oat-birdcherry aphid to the alkaloids [Siegal et al. 1989, 1990]. Alkaloid levels vary with the specific endophyte-host combination, plant age, and environmental conditions,

thus influencing the relative resistance of EI plants to insects [Eichenseer et al. 1991; Siegel et al. 1989].

Antifungal Activity

In *in vitro* experiments, EI grasses exhibit antifungal activity against a number of plant pathogens. White and Cole [1985] found that isolates of *A. coenophialum* from tall fescue inhibited growth of cultures of *Nigrospora sphaerica*, *Phoma sorghina*, and *Rhizoctonia cerealis*. More recently, agar bioassays of various endophytes and of different isolates of the same endophyte inhibited twelve grass pathogens [Siegel and Latch 1991]. Filter paper disks soaked with loline, peramine, or ergot alkaloids had no effect on the pathogen cultures. The researchers found, however, that there was variation both in the level and spectrum of antifungal activity produced by the specific endophyte isolate.

Growth, Vigor, and Competition

Benefits to the grass include those arising from reduced herbivory as well as those from direct effects of the endophyte on growth and survival. Controlled environment studies comparing identical or similar host genotypes with and without the endophyte serve as an effective means of examining the direct effect of the endophyte on host fitness [Clay 1987b]. Maternal transmission of *Acremonium* through the host seed is dependent upon host fitness and on the ability of the host to compete with other species in the field [Kelley and Clay 1987; Latch et al. 1985b].

Host fitness and vigor have been measured on the basis of a variety of criteria: Increased seed set and germination have been shown for

Lolium and Festuca hosts containing Acremonium endophyte [Clay 1987a; Pinkerton et al. 1990; Rice and Undersander 1990]. Despite reduced inflorescence and fecundity due to flower abortion by Atkinsonella hypoxylon, field trials revealed greater vegetative growth of the host grass. Similar data were obtained for greenhouse trials on the effect of the endophyte on the growth and reproduction of purple nutsedge (Cyperus rotundus L.) [Stovall and Clay 1988]. The plant produced smaller tubers, but in larger numbers, which may compensate for a loss of inflorescence and seed. Evaluation of characteristics as production of tillers, dry matter, and non-structural carbohydrates reveals the overall greater biomass of EI grasses [Belesky et al. 1989a; Hill et al. 1990].

Researchers have shown the ability of EI grasses to compete with EF grass under varying environmental conditions [Marks et al. 1991]. Arachevaleta et al. [1989] considered the thicker, narrower blades, leaf rolling behavior and difference in stomatal resistance of infected clones of tall fescue to reflect an adaptive advantage under drought stress [Arachevaleta et al. 1989; Belesky et al. 1987]. A number of studies have been designed to examine the physiological response including stomatal resistance, osmotic adjustment, alkaloid concentrations for EI versus EF grasses under water stress [Bates and Joost 1990; Belesky et al. 1989a,b; Elmi et al. 1990]. Greater efficiency in nitrogen utilization was proposed to explain an increase in tillering with nitrogen fertilization for EI versus EF plants [Lyons et al. 1986,1990]. Increased regrowth was thought to be related to changes in carbohydrate metabolism.

Physiological differences between EI and EF grasses may explain the enhanced growth and vigor in the former, especially under adverse environmental conditions. Hyphae around the meristematic regions of the host may influence hormonal regulation of cell differentiation and development leading to increased vegetative growth. Arachevaleta et al. [1989] suggests that the difference in leaf morphology in the EI grasses may be due to the endophyte's interaction with a host growth regulator. Enhanced re-growth could be related to mobilization and translocation of carbohydrates, generally a result of the action of auxins. A TLC analysis revealed production of 3-indole acetic acid, 3-indole ethanol, 3-indole acetamide, and methyl-3-indole carboxylate by B. epichloe grown on tryptophan media [Bacon 1985; Porter et al. 1985]. The biosynthesis of both ergot and 3-substituted indolyl alkaloids are also dependent upon tryptophan metabolism. Growth, then, may be regulated by catabolism of host tryptophan [Porter et al. 1985]. In addition, metabolic changes due to endophyte-induced synthesis of secondary products, as well as fungal use of saccharide reserves that affect source-sink dynamics, may be responsible for a lowered net rate of photosynthesis in EI plants [Belesky et al. 1987; Smith et al. 1985].

Modification and Study

The advantage of EI grasses for pasture and EI grasses for turf and conservation areas is evident. Replanting of pastures with EF varieties of grass will reduce losses due to animal toxicity. Inoculation of endophytes into uninfected cultivars will result in increased tiller density, and increased herbivore and stress tolerance reducing the input and maintenance requirements for turfgrasses.

In both processes, removal or inoculation, there is a need for detection of the endophyte. Current methods include the following: 1) Observation under the microscope of stained mycelium in the inner epidermis of the leaf sheath or in seed [Clarke et al. 1983; Saha et al. 1988]. 2) Production of antisera to cultures and subsequent detection of fungal antigens by analysis with an enzyme-linked-immunosorbent assay (ELISA) [Johnson et al. 1982, 1985]; 3) Culture of endophytic fungi from the leaf sheath, nodes, or stem pith of the host [Davis et al. 1986; Kulkarni and Nielson 1986; Welty et al. 1986]; 4) Insect bioassays [Funk et al. 1983; Johnson et al. 1985a; Latch et al. 1985b; Prestige et al. 1985]; 5) Gas chromatography or high pressure liquid chromatography methods to detect alkaloids produced by the endophyte-grass interaction [Gallagher et al. 1985; Jeffreys, 1975; Siegal et al. 1985]. By taking advantage of variation in isolates [Christensen and Latch 1991; Christensen et al. 1991; Leuchtmann and Clay 1989, 1990] fungi can be selected for incorporation into EF grasses.

Chemical and nonchemical techniques have been used to remove endophyte infection. Viability of the endophyte decreases with length of storage of the seed, as well as with increasing temperatures [Latch and Christensen 1982; Rolston et al. 1986; Welty et al. 1983;]. At low temperatures (0-5 C) and low humidity (25-50%) endophytes remained viable when stored for fifteen years [Rolston et al. 1986]. Heat treatment of seeds decreases viability of the endophyte as well as germination of the seed [Siegal et al. 1984b]. Chemical methods rely on the use of fungicides such as propiconazole, prochloraz and triadimefon which are benzimidazoles and ergosterol biosynthesis inhibitors [Siegal

et al. 1984b, 1985, 1987a]. Fungicides applied to the seed are more effective than foliar sprays or granular forms used on whole plants [Siegal et al. 1984]. The best control is removal of the endophytes from the seed, creating a plant that can be used as the new mother stock. Grass cultivars such as 'Forager', 'Johnstone', and 'Kenhy' are sold as EI varieties [Bacon 1988; Siegal et al. 1985].

Funk et al. [1989] suggest that in order to develop new endophyte-enhanced varieties of grass, it is necessary to 1) isolate, 2) genetically breed, and 3) incorporate the endophyte into high-quality EF cultivars via breeding or inoculation. Attempts at producing infected grasses have resulted in varying degrees of success. Latch and Christensen [1985b] were mostly successful (except infection of Lolium perenne with A. coenophialum) in artificially infecting the host grasses Festuca arundinaceae and L. perenne with five types of fungi by placing the endophyte mycelium into the coleoptile tissue of sterile seedlings grown on water agar. Leuchtmann and Clay [1988b] inserted the mycelium and conidia of Atkinsonella hypoxylon and Balansia cyperi above the meristem of aseptic seedling of a sedge and two types of grasses. Regeneration of synthetically-infected tall fescue occurred for 17% of the tall fescue host callus inoculated with mycelium of A. coenophialum, but was not successful for ryegrass, bluegrass, or orchardgrass [Johnson et al. 1986; Kearney et al. 1991]. Naturally-existing infected plants may be propagated clonally [Shelby and Dalyrmple 1987]. In 1988, over 7 million kilograms of endophyte-containing turf-type ryegrass seed (i.e.'All Star,''Citation II,''Commander') were bred via repeated backcrossing of EI plants with EF varieties [Clay 1989; Funk and Clarke

1989]. At present there is an increasing number of endophyte-enhanced varieties of tall fescue, perennial ryegrass, Chewings fescue and hard fescue, alone, and in mixtures [Funk et al. 1989; Miller 1991; Saha et al. 1987].

Biological Control

In a recent review, Clay [1989] described the use of Clavicipitaceous endophytes of grasses as a method of biological control. According to his criteria, a biological control agent should possess the ability to decrease pest damage, maintain a low range of activity, thereby minimizing other environmental impacts, and be competitive with application and maintenance costs under alternate methods of control. In confirmation, he argues that grass endophytes affect a wide range of insects but affect only those that feed directly on the plant. Once established in the host, imperfect endophytes are transmitted maternally, eliminating the need for reapplication. Stromata-producing endophytes that do sterilize hosts may be used for long-lived, spreading grasses [Clay 1989]. Thus, with the development of new techniques in inoculation of endophyte into EF grasses, there is a great deal of potential for using endophytes for biological control and IPM in grasses.

Potential

While current research is aimed at manipulation of the grass-endophyte mutualism, it is necessary to be mindful of maintaining a balance between the economics of livestock and that of insects. In New Zealand, newly planted EF ryegrass suffered from insect herbivory and competition from weeds. Enhanced biomass and competitive effects of the

EI plant may result in the grass itself becoming a pest. Infection of endophytes into certain varieties of grasses may shift insect preferences to new plant species [Clay 1989]. Advances in tissue culture methods for transfer and regeneration increases the potential of EI varieties of high-quality cultivars of endophytic turfgrasses [Kearney et al. 1991]. The use of endophytes as vectors for enhancing additional host traits, as well as the prospects of utilizing selective-alkaloid mutants of the fungus, will significantly enhance future, widespread use of EI grasses.

CHAPTER II

FIELD STUDIES

Introduction

Resistance to foliar-feeding insects, in both the field and lab, [Clay 1989; Johnson-Cicalese and White 1990; Mathias et al. 1990; Siegel et al. 1989] has been associated with the presence of mycelium of Acremonium coenophialum or A. lolii in the leaf sheaths of tall fescue or perennial ryegrass hosts. Toxins, particularly loline alkaloids, produced by the fungus and/or by a plant-fungus interaction may be responsible for decreased survival and antibiosis in no-choice experiments and for deterrence and antixenosis in choice experiments [Clay 1989; Mathias et al. 1990]. Resistance conferred by the relationship is especially prominant during periods of stress when physiological differences give endophyte-infected (EI) plants greater advantage over endophyte-free (EF) grasses [Funk et al. 1989]. While there is, at present, sufficient evidence to recommend use of EI varieties of turfgrass against foliar feeding insects, there is increasing evidence suggesting that endophyte-infection may affect root feeders. However, effects on root feeding nematodes [Kimmons et al. 1990; Pedersen et al. 1988; West et al. 1988], annual white grub (Cyclocephala), collembola, and Japanese beetle larvae [Oliver 1990; Potter et al. 1991] tend to be variable and yield inconsistant results, particularly in field trials. Additional studies are needed in order to

provide sufficient evidence to determine the potential use of infected grasses as a method of control.

The Japanese beetle larvae is considered to be a major pest of home lawns and golf courses, as well as most areas of fine turf [Fleming 1972; Tashiro 1987; Vittum 1986]. Damage at high densities is caused by the feeding of larvae which results in excessive root loss by the grass, with maximum plant mortality under dry conditions. In the Northeast, the adult beetle lays eggs in turf mainly between mid-July and mid-August. Damage peaks in the Fall when the third instar larvae feed extensively on grass roots. Larvae again feed prior to pupation the following spring. Therefore, turf is susceptible to attack in Autumn and late Spring.

A variety of control measures have been attempted on Japanese beetle larvae. Conventional methods include use of organophosphates and carbamates [Tashiro 1987]. Chemicals are targetted against second instar larvae in the late Summer and third instars in the Spring. They must be applied with a liberal amount of water in order to penetrate the turf, increasing the risk of leaching. Biological control agents such as milky spore (Bacillus popilliae), microsporidia [Hanula 1990] and nematodes [Forschler and Gardener 1991] have led to inconsistent levels of reduction.

Recent work by Potter et al. [1991], Oliver [1990], and Casagrande [personal communication] suggest that EI grasses might also be used to reduce Japanese beetle grub damage. Although only 10-15% of the loline alkaloids in the plant are found in the root [Siegel et al. 1989], reduced survival and weight gain have been noted in pot studies where

grubs fed on EI roots [Casagrande, personal communication; Oliver 1990; Potter et al.1991]. Further work is necessary to verify possible resistance in the field.

This report presents results of two seasons of field work designed to investigate the effect of EI grasses on Japanese beetle larvae. More specifically, the aim of this work was to compare EI and EF grass species and cultivars with regard to survival and weight gain of Japanese beetle larvae implanted at varying densities. In addition, grasses were assessed for their tolerance to grub damage by consideration of root and shoot yields. Both studies were sponsored by a Low Input Sustainable Agricultue (LISA) grant at the University of Rhode Island where comparable field plots were set up.

Materials and Methods

Field Season 1990

Plant Source and Seeding

In mid-May, a 45′ X 30′ area at the University of Massachusetts turf field plots in South Deerfield, Ma. was treated with glyphosate (Round up) and was then prepared for seeding. At the end of May, turfgrass species and cultivars were randomized and seeded in 3′ X 6′ areas. There were five blocks containing each of the following: 'Titan' endophytic (E+) tall fescue (8lbs seed/1000 ft^2), 'Chieftan' non-endophytic (E-) tall fescue (8lbs seed/1000 ft^2), 'Repell'(E+) and 'Tara' (E-) perennial ryegrass (6lbs seed/1000ft^2), 'Jamestown II' (E+) and 'Jamestown'(E-) Chewings fescue (4lbs seed/1000 ft^2), 'SR3000' (E+) and Crystal' (E-) hard fescue, 'Nassau' Kentucky bluegrass, 'Titan' mixed with 2%, 5%, or 10% Kentucky bluegrass, and 'Chieftan' mixed with

2%, 5%, or 10% 'Nassau' Kentucky bluegrass. Grasses were irrigated regularly (1.5 in./week) and fertilized with Scott's 19-26-5 starter fertilizer (1lb N/1000 ft^2). Half strength Triadimefon (Bayleton) was applied after seedling establishment. The entire plot was mown at least once a week at a height of 2 1/2 inches.

Larval Densities

In mid-July, cores 6 inches in diameter X 8 inches deep were obtained by driving plastic tubes into the ground with a front-end loader. The cores were placed in "bags" made from lumite mesh and were reinserted into the ground. In this way, grubs could be confined to the core volumes.

At the beginning of July, a 12' X 3' X 6' cage covered with insect netting was set up over an area of mixed tall fescue-colonial bentgrass. Adult Japanese beetles were released into the cage which was kept stocked with grape vines. In mid August, the area was dug up and second instar larvae were identified by head capsule size and collected for implantation into the cores at densities of 0, 10, 20, 40, 60, 90, and 120 grubs/ft^2. Larvae were also collected from The International Golf Course in Bolton, Ma. and from under turf at the S. Deerfield plots.

Data Collection

After 8 weeks, cores were dug out of the ground. A saw was used to cut through the core at the base of the shoots. Roots and shoots were then washed free of soil with a high pressure hose and placed in plastic bags. Samples were brought back to the lab and blotted dry before weighing fresh. Shoot samples were then dried for 3 days. Root samples were frozen for later alkaloid analysis. Grubs collected from each core

at the time of washing were put into plastic bags with soil and kept in the refrigerator until counted and weighed.

Experimental Design and Statistical Analysis

The experiment was comprised of five replications of fifteen cultivars or mixtures in a completely randomized block design. All plots (15 X 5= 75) contained cores with initial densities of 20 grubs/ft^2 and were considered the 'Single Density experiment'. The eight plots in each block (8 X 5 = 40) planted with EI or EF tall fescue alone and mixed with 2%, 5%, or 10% Kentucky bluegrass were grouped as the 'Mixtures experiment'. The 'Density experiment' included tall fescue with and without endophyte, and EI perennial ryegrass, Chewings fescue and hard fescue, all of which contained six cores of differing larval densities. Data were analyzed using BMDP P7D which ran Levene's test for equality of variance as well as the Welch and the Brown-Forsythe ANOVA in which variances are not assumed to be equal (BMDP Statistical Software, 1990).

Field Season 1991

Plant Source and Seeding

Seed of 'Shenandoah' EI tall fescue, 'PE7', a genetically similar non-endophytic tall fescue, and 'Nassau' Kentucky bluegrass were used in the experiment. Plastic pots 8 inches wide X 8 1/2 inches deep were filled with screened Hadley silt loam (coarse, silty, mixed, mesic type, Udifluvent) (Appendix D) from the University of Massachusetts turf facility in South Deerfield, Ma. On 30 May, tall fescue was seeded at a rate of 8 lbs seed/1000ft^2 and Kentucky bluegrass at a rate of 2 lbs seed/1000ft^2. Seed was covered with a thin layer of peat moss and were

kept moist until germination and early seedling development. Pots were then placed in the ground up to approximately the top four inches. Scott's Proturf starter fertilizer (16-21-3) with pre-emergence weed control (A.I. Siduron [1-(2-Methylcyclohexyl)-phenylurea 3.10%) was used to apply 1 pound of nitrogen to each pot.

Moisture Regime

Beginning 2 months after seed establishment, plants were maintained under one of two moisture regimes; adequate water or water deficient. For the first, 1/2 inch of irrigation was applied every other day (1 1/2 -2 inches of water per week) in order to maintain fairly saturated soil conditions. Pots recieving the water deficient treatment were allowed to dry to near-wilting at which point they were irrigated with 1/2 inch of water. Visual determinations of moisture levels were confirmed by use of gypsum blocks and with a moisture meter with a range of 1-10. Gypsum blocks were inserted into nine extra pots which were treated in the same way as the dry treatments. Readings were taken using a voltmeter and a 1-megaohm resistor which had been calibrated for that soil at saturation, .3 bar and at 2 bar. A "rainout" shelter was constructed by spreading clear plastic over the plots in a tent-like fashion: The plastic was supported by 2 1/2 ft. wooden beams down the center of the plots and was tied at the corners so that it was approximately 6 inches from pots along the edges. The plots were covered for as close to the duration of the rainstorm as possible. Plots were left covered through the night after late afternoon rain. In addition, when prediction of rain remained high, plots were kept covered

even during the day. Moisture regimes were upheld through the remainder of the field season.

Larval Densities

In order to exclude egg laying by natural populations of beetles, the area surrounding the pots was completely enclosed with tobacco netting from 30 June through 20 August. On 15 August, second instar larvae collected from Amherst Golf Course (Amherst, Ma.) were implanted into pots of blocks 1-6 at densities of 0, 20, 40, and 60 grubs/ft^2 (7, 14, 21 grubs/pot). Grubs collected from the University of Rhode Island turf plots were kept on ice for two days and implanted in blocks 7-10 on August 18th.

Data Collection

Visual quality of grasses in each pot was rated weekly throughout the field season on a scale of 1-9 (1- unacceptable 5-acceptable 9-outstanding quality turf). Each week from the onset of the moisture treatments, clippings from individual pots were dried and weighed. In the first week of October 1 1/2 months after implantation of the grubs, all grasses were harvested. Roots and shoots were separated and washed as for the 1990 season. Root and shoot samples were then dried for 4 days prior to weighing. Grubs were collected from each pot during washing and kept in plastic bags with soil in the refrigerator before weighing in the lab.

Experimental Design and Statistical Analysis

The experimental design was a completely randomized block. There were 24 treatments (3 grasses X 4 densities X 2 moisture treatments) randomized in each of 10 blocks (total 240 pots). Data were analyzed

using SAS General Linear Models Procedures (GLM) (SAS Institute 1987). A GLM Repeated Measures procedure was used for clipping data which was collected over time. Treatment means for the variables were separated using Duncan's Multiple Range Test (SAS Institute 1987).

Natural Infestation Experiment

In the spring of 1991, areas of the 1990 field plot where cores had been removed were reseeded. At the beginning of July, about the time of adult Japanese beetle emergence, floral attractants removed from Japanese beetle traps were positioned inside of the experimental area at a height of approximately one foot above the ground. Placement of the lures was rotated following weekly mowings from late June through mid August. At the end of September, a 1' X 3' section of turf was cut from each of the treatment areas and all larvae were counted. The data were analyzed by SAS Analysis of Variance procedure and means of counts for the grasses were compared using Duncan's Multiple Range Test.

Results

Field Season 1990

Single Density Experiment

No differences existed among the grasses for the density of grubs recovered at the end of the season (Table A.1). The mean final grub density was 17.5 grubs/ft^2. Density of third instars alone was 14.08. Mean weight was 140 miligrams for third instars and .047 for second instars. Values for per cent recovery appear similar for tall fescue and Chewings fescue and lower for EI versus EF cultivars, but

differences are nonsignificant (Figure 2.1). The opposite was true for perennial ryegrass. In all cases, high variation precluded any statistical significance in the trends.

Comparison of Mixtures

The analyses run for 'Titan' and 'Chieftan' alone and with 2%, 5%, and 10% 'Nassau' Kentucky bluegrass revealed no differences in recovery of grubs (Table A.2). Overall mean recovery was 86.2% for all grubs recovered and 69.2% for recovery of only third instars.

Density Experiment

a) Recovery of grubs. Variance in grub recovery was not homogeneous across the initial densities according to Levene's test (Table 2.1.). The Welch and Brown-Forsythe ANOVAs were thus used to reveal differences with density (Table A.3.). Separation of means for the densities combined over the grasses revealed four groups; 0 grubs/ft^2, 10,20,40 grubs/ft^2, 60 grubs/ft^2, and 90 grubs/ft^2. The trend was similar for recovery of third instars alone (Table A.4). Variance was very high in both cases making any differences between grasses nonsignificant. Weight of third instar larvae averaged 141 miligrams (Table A.5).

b) Root fresh weight. There was a large amount of variation associated with root fresh weights. The Levene's test for equal variances was violated for grass, density, and their interaction. No significance was attained with the Welch or Brown-Forsythe ANOVAs (Table A.6).

c) Shoot wet weight. Shoot wet weight varied for the grasses according to the Welch and Brown-Forsythe ANOVA (Table A.7). Weights

were lowest for 'Jamestown II' and highest for 'Titan' and 'Chieftan' tall fescues.

Table 2.1 Density of larvae (grubs/ft^2) recovered from plots at the end of the 1990 field season. Means and standard errors are shown for each of the grasses at each of the initial densities. Densities were significantly different (p=.01) while grasses were not.

	Initial Density (grubs/ft^2)					
	0	10	20	40	60	90
Grass						
'Titan'	6.0±2.4	11.2±2.9	12.0±4.9	18.0±4.6	24.0±10	47.0±5.1
'Chieftan	11.2±5.2	19.0±7.5	17.0±4.4	24.0±9.2	45.0±11	53.0±7.8
'Nassau'	9.0±6.6	10.0±3.5	20.0±2.0	18.0±8.9	41.2±3.1	47.0±2.0
'Repel'	8.0±4.9	7.0±4.6	26.0±9.4	16.0±7.8	42.0±17	40.0±4.2
'JamestownII'	8.0±5.6	15.0±5.9	12.0±4.1	12.0±8.7	33.0±10	41.2±9.4

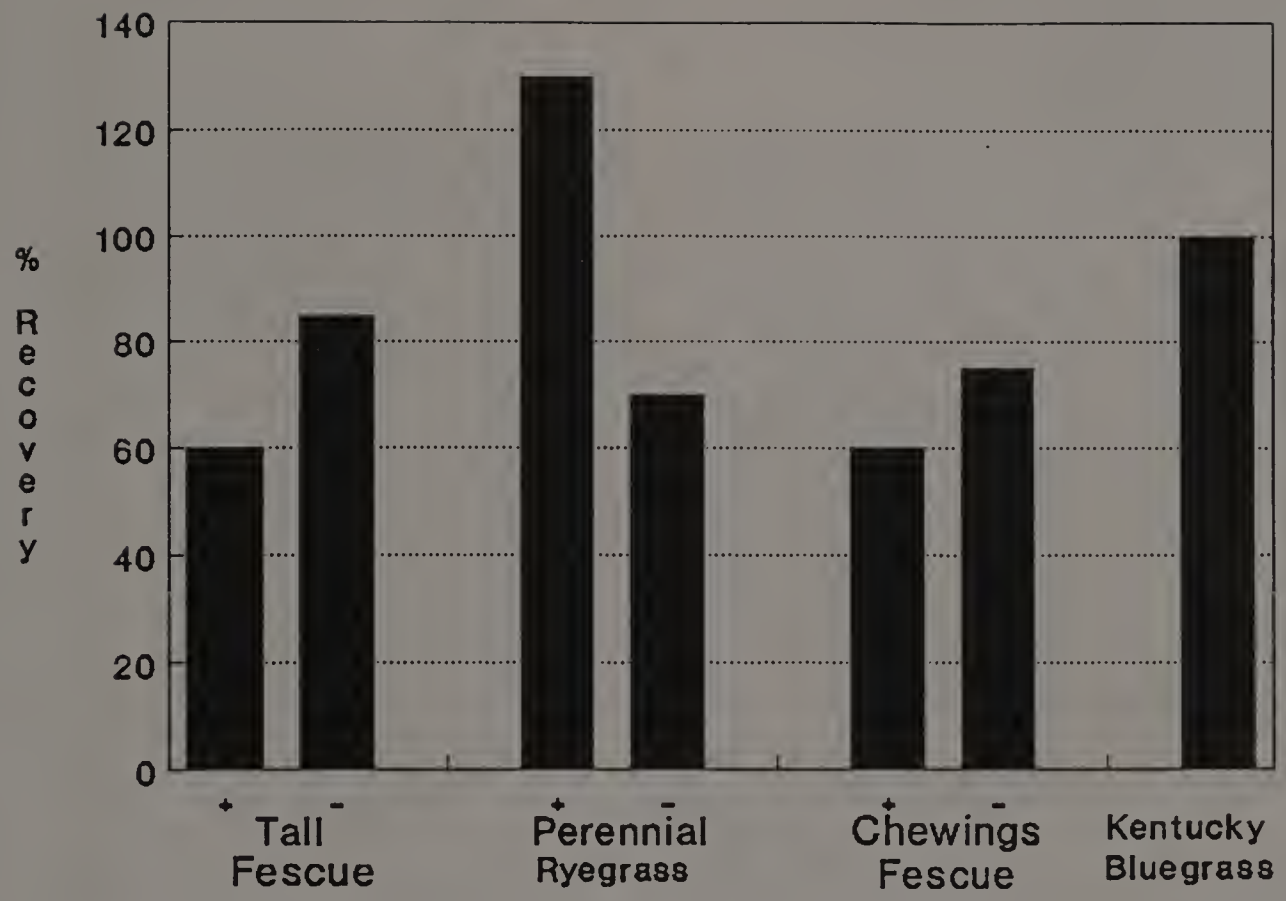


Figure 2.1. Mean recovery (%) of larvae from cores in the ground (initial density 20 grubs/ft^2) at the end of the 1990 field season. Endophyte-infected and endophyte-free cultivars are shown for each grass species. Differences are not significant.

Field Season 1991

Seedling Establishment and Growth

Emergence of 'Shenandoah' was evident after one week in contrast to approximately two weeks for 'PE7'and 'Nassau'. Seed germination was highest for 'Shenandoah'. Establishment and early growth was faster for 'Shenandoah' than for the other grasses.

Recovery of Larvae

A three-way ANOVA using grass, density (excluding 0 density), and moisture treatments as the main effects revealed differences in the number of larvae (2nd and 3rd instars in grubs/ft^2) recovered from each of the pots to be due to variation in grass ($p=.01$), density ($p=.01$), and moisture treatments ($p=.05$) (Table A.8). Final density increases with initial density (Figure 2.2) but the rate of increase tends to decrease at increasing densities. While final density at the 40 grub/ft^2 inoculation rate is roughly double that at the 20 grubs/ft^2 level, the change is less between 40 grubs/ft^2 and 60 grubs/ft^2 (Table 2.2). When averaged over all grasses, final densities differed at each of the initial densities ($p=.05$ Duncan's Multiple Range Test) (Table 2.3.).

Larval recovery for 'Shenandoah' was, in all cases (except for 20 grubs/ft^2 in the dry treatment), different than that of 'PE7' and 'Nassau' (Table 2.4)(Figure 2.2.). Average number of grubs combined over the initial densities, was 2.8 grubs per pot for 'Shenandoah', 4.3 grubs per pot for 'PE7', and 4.7 grubs per pot for 'Nassau'. Overall final density was 8, 12.2, and 13.6 grubs/ft^2 for 'Shenandoah', 'PE7', and 'Nassau', respectively.

Percent survival, density recovered divided by initial density, changed little across the densities for all grasses. Survival was highest on 'Nassau' and lowest on 'Shenandoah' (Figure 2.3). Averaged over the densities, grub survival was 21% for 'Shenandoah', 31% for 'PE7', and 35% for 'Nassau'.

Differences with moisture treatment reflect a variation in pattern of recovery (Figure 2.2). While recovery peaked at 40 grubs/ft^2 on 'Nassau' in the dry treatment, density on 'Shenandoah' increased less steeply. Increasing recovery with increasing densities was more consistant in the wet treatment (Table 2.2).

Development of Larvae

The ratio of second to third instar larvae was considered to be an indication of developmental state of grubs feeding on roots of the grasses. Total numbers of second and third instar larvae were compared between the grasses by means of a X^2 test. Numbers of seconds and thirds recovered from 'PE7' (73:664) were assumed to be the fixed ratio against which 'Shenandoah' and 'Nassau' were tested. The ratio 54:383 from 'Shenandoah' was similar to that of 'PE7' (X^2=.752). A greater number of third instars versus seconds (45:778) were collected from 'Nassau' (X^2=130.02 p=.01). Thus the larvae confined on 'Nassau' Kentucky bluegrass apparently developed more rapidly than those on the tall fescues.

Table 2.2. Density of larvae recovered from pots in the field 7 weeks after implantation in the 1991 season. Means and standard errors are shown for grasses for the moisture treatments at each initial density.

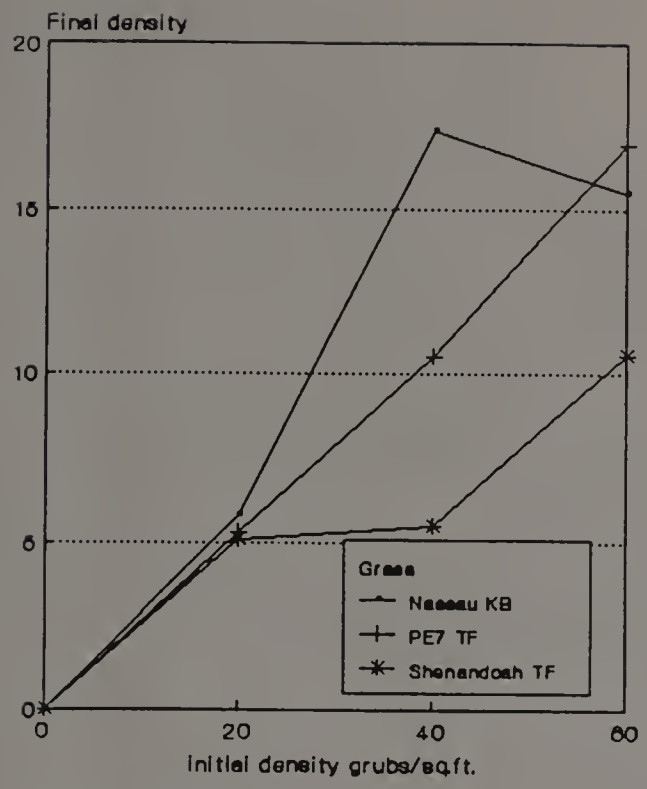
	Initial Density (grubs/ft^2							
	0		20		40		60	
Moisture	W	D	Wet	Dry	Wet	Dry	Wet	Dry
Grass								
'Shenandoah'	0	0	4.7±1.4	5.1±1.4	10.4±1.3	5.5±1.4	11.8±2.6	10.6±1.9
'PE7'	0	0	7.9±1.4	5.3±1.2	14.4±1.5	10.5±1.5	18.2±2.6	17.0±2.8
'Nassau'	0	0	9.3±1.7	5.8±1.2	15.6±1.2	17.4±1.8	17.7±1.9	15.5±2.6

Table 2.3. Separation of means by initial density for the variables number of larvae recovered, weight of larvae (g), root weight (g), and shoot weight (g) collected from pots at the end of the 1991 field season. Means with the same letters are not different at the .05 level of Duncan's Multiple Range Test.

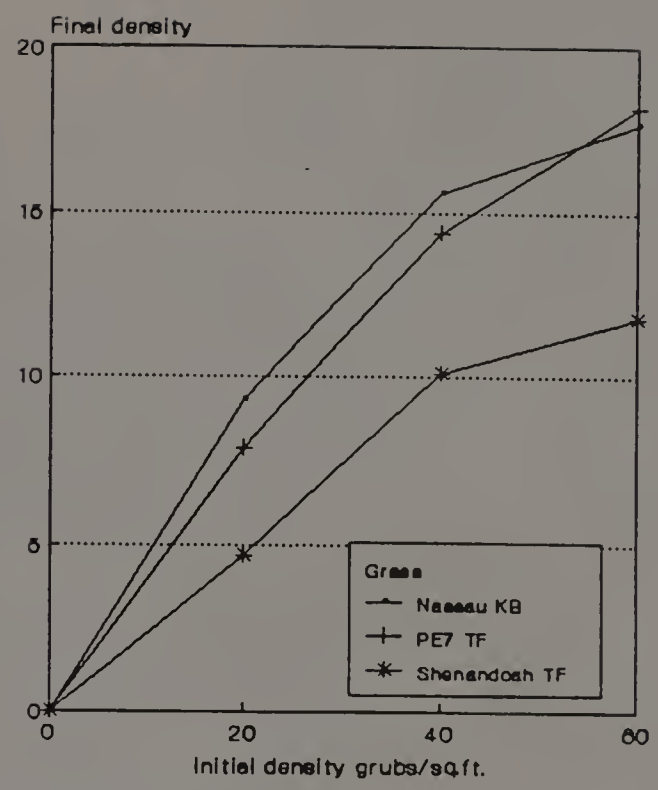
Initial Density (grubs/ft^2)	Number of Larvae Recovered	Larval Weight (g)	Root Weight (g)	Shoot Weight (g)
0	0	-	13.97 a	18.96 a
20	2.22 c	.128 a	11.19 b	16.88 b
40	4.28 b	.131 ab	10.81 b	16.11 b
60	5.28 a	.118 b	9.67 b	15.38 b

Table 2.4. Separation of means by grass for the variables number of larvae recovered, weight of larvae (g), root weight (g), and shoot weight collected from pots at the end of the 1991 field experiment. Means with the same letters are not significantly different at the .05 level according to Duncan's Multiple Range Test.

Grass	Number of Larvae Recovered	Larval Weight (g)	Root Weight (g)	Shoot Weight (g)
'Shenandoah'	2.8 b	.112 b	19.09 b	17.33 a
'PE7'	4.3 a	.133 a	9.90 a	16.83 a
'Nassau'	4.7 a	.132 a	5.00 c	16.40 a



a) Dry treatment



b) Wet treatment

Figure 2.2. Final density of larvae (grubs/ft^2) recovered from potted grasses in the field 7 weeks after implantation in the 1991 season. Means for the grasses at each density are shown for wet and dry moisture treatments. 'Shenandoah' was significantly lower for moisture and densities combined (p=.05 Duncan's Multiple Range Test.

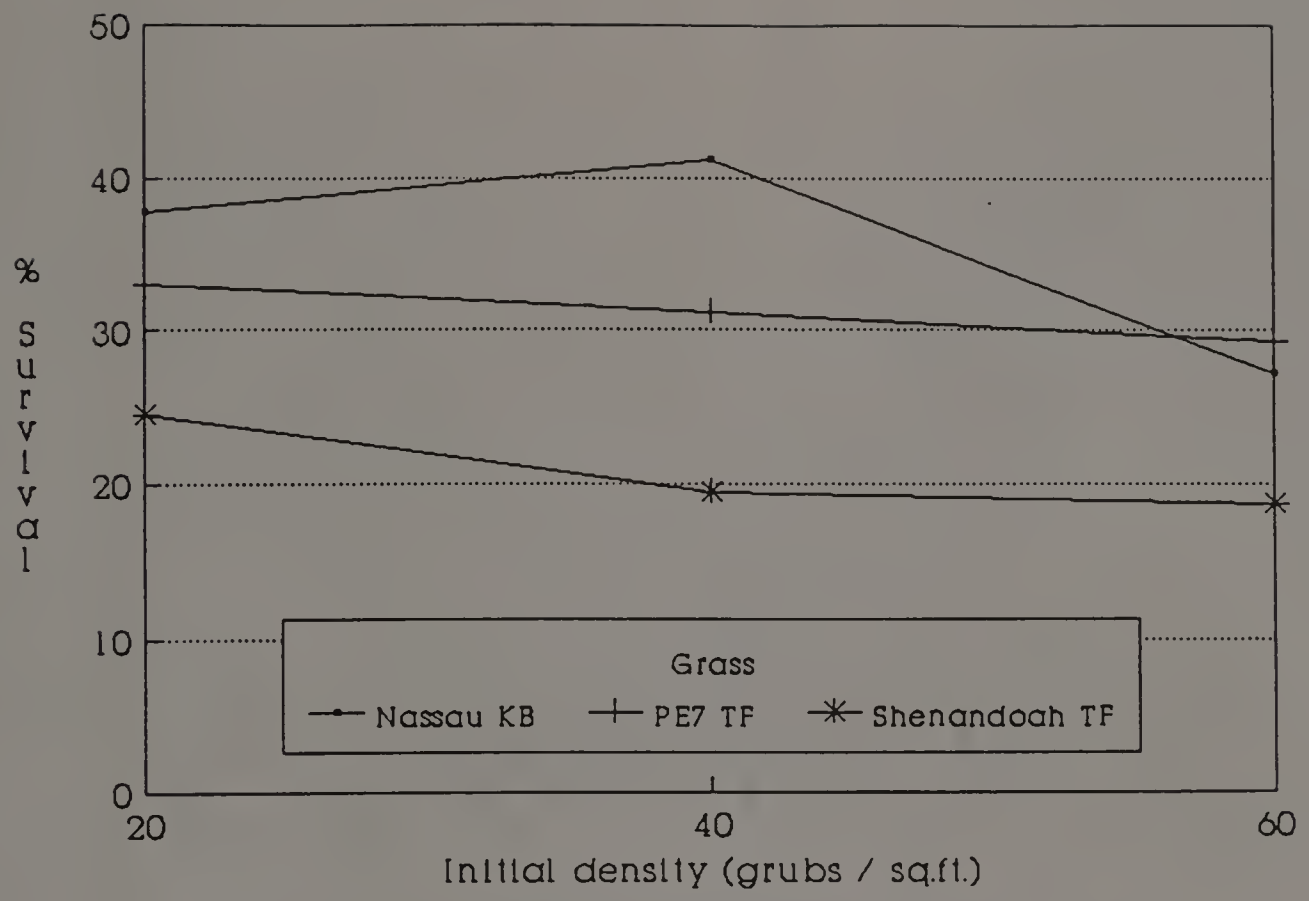


Figure 2.3. Survival of larvae (%) based on recovery from potted grasses in the field 7 weeks after implantation in the 1991 season. Means for the grasses at each initial density are combined for the moisture treatments.

Larval Weight

Weight of larvae differed between the grasses and between the densities (p=.05) (Table A.9). There were no differences between wet and dry treatments and there were no significant interactions between grass, density, or water treatments. Larval weights were lower for 'Shenandoah' at all of the densities (Table 2.5) but especially at 60grubs/ft^2 (Figure 2.4). For the combined densities, weight of larvae on 'Shenandoah' (.112) was different at the .05 level from 'PE7' (.133) and 'Nassau' (.132) (Table 2.4). Weight remained fairly constant over the densities for 'Nasau' and 'PE7' but was lower for 'Shenandoah' at 60 grubs/ft^2 resulting in significant variation in density (Table 2.3).

Table 2.5. Weight of third instar larvae recovered from pots in the field 7 weeks after implantation in the 1991 season. Means and standard errors are shown for grasses at each density for the moisture treatments combined.

	Initial Density (grubs/ft^2)			
Grass	0	20	40	60
'Shenandoah'	-	.127 ± .008	.127 ± .005	.104 ± .008
'PE7'	-	.141 ± .007	.138 ± .005	.137 ± .004
'Nassau'	-	.136 ±.003	.138 ± .02	.130 ± .004

Root Weights

Root dry weights were different (Table A.11) for all three grasses (Figure 2.5). 'Shenandoah' had the greatest root mass and 'Nassau' the least at each of the initial densities (Table 2.6) and over all

densities (Table 2.4). There were no differences in root weights between the wet and dry treatments. Root weights at all densities differed from the control (Table 2.3), but there were no differences among density levels.

Table 2.6. Root weights collected from pots of grasses at the end of the 1991 field season. Means and standard errors are shown for the combined moisture treatments.

	Initial Density (grubs/ft^2)			
Grass	0	20	40	60
'Shenandoah'	21.80 ± 2.3	19.98 ± 2.0	17.40 ± 1.8	16.97 ± 1.5
'PE7'	12.71 ± 1.1	8.66 ± .91	10.70 ± 1.2	7.38 ± .89
'Nassau'	6.98 ± .64	5.06 ± .50	3.59 ± .37	4.21 ± .52

Reduction in root weight was used as a variable to determine the effect of grub feeding on root mass. The variable was calculated by subtracting final root weight from the zero density weight for that grass type and dividing by the zero density weight. Zero density (0%) differed from 22%, 27%, and 34% reduction for the 20, 40, and 60 grubs/ft^2 densities, respectively, for the grasses combined. Percent root reduction for the grasses was 12.5% for 'Shenandoah', 21.9% for 'PE7', and 28% for 'Nassau' over all of the densities.

Shoot Weights

The grasses had similar shoot weights (Table A.12) which averaged 16.8 grams (Table 2.7). Shoot weights did not change significantly with moisture level, density had a significant effect (Figure 2.6) for

'Shenandoah' and 'Nassau' were higher at the 0 grubs/ft^2 density (Figure 2.6) making density a significant source of variation.

Table 2.7. Shoot weights collected from pots of grasses at the end of the 1991 field season. Means and standard errors are shown for the combined moisture treatments.

	Initial Density (grubs/ft^2)			
Grass	0	20	40	60
'Shenandoah'	19.86 ± 3.0	16.66 ± .97	16.18 ± 1.1	16.43 ± .88
'PE7'	17.10 ± .64	17.15 ± .89	17.17 ± 3.4	15.89 ± .81
'Nassau'	19.91 ± 1.4	16.80 ± 1.3	14.90 ± .67	13.93 ± 1.0

Turf Quality

Quality ratings of turf in individual pots remained fairly consistent each week. Fluctuations seemed correlated with weather conditions. Quality for dry treatments was lower just prior to watering, but recovery was rapid. In general, weekly ratings were 5.5 - 7.5. The bluegrass ratings were generally 5.5-6 while tall fescues tended to be 6.5-7.

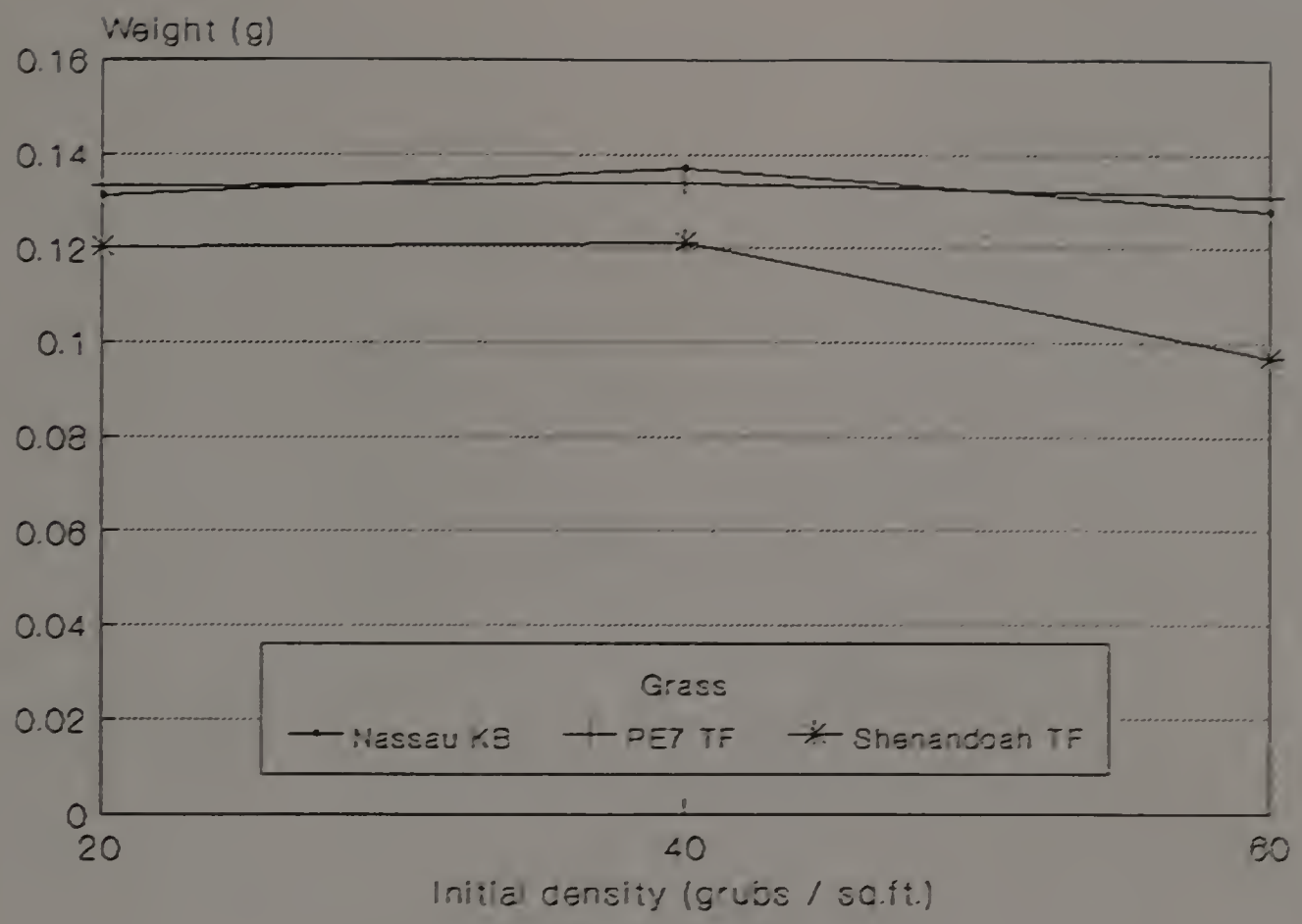


Figure 2.4. Weight of larvae (g) recovered from potted grasses in the field 7 weeks after implantation in the 1991 season. Means are combined for the moisture treatments. Weights are lower for 'Shenandoah' (p=.05 Duncan's Multiple Range Test).

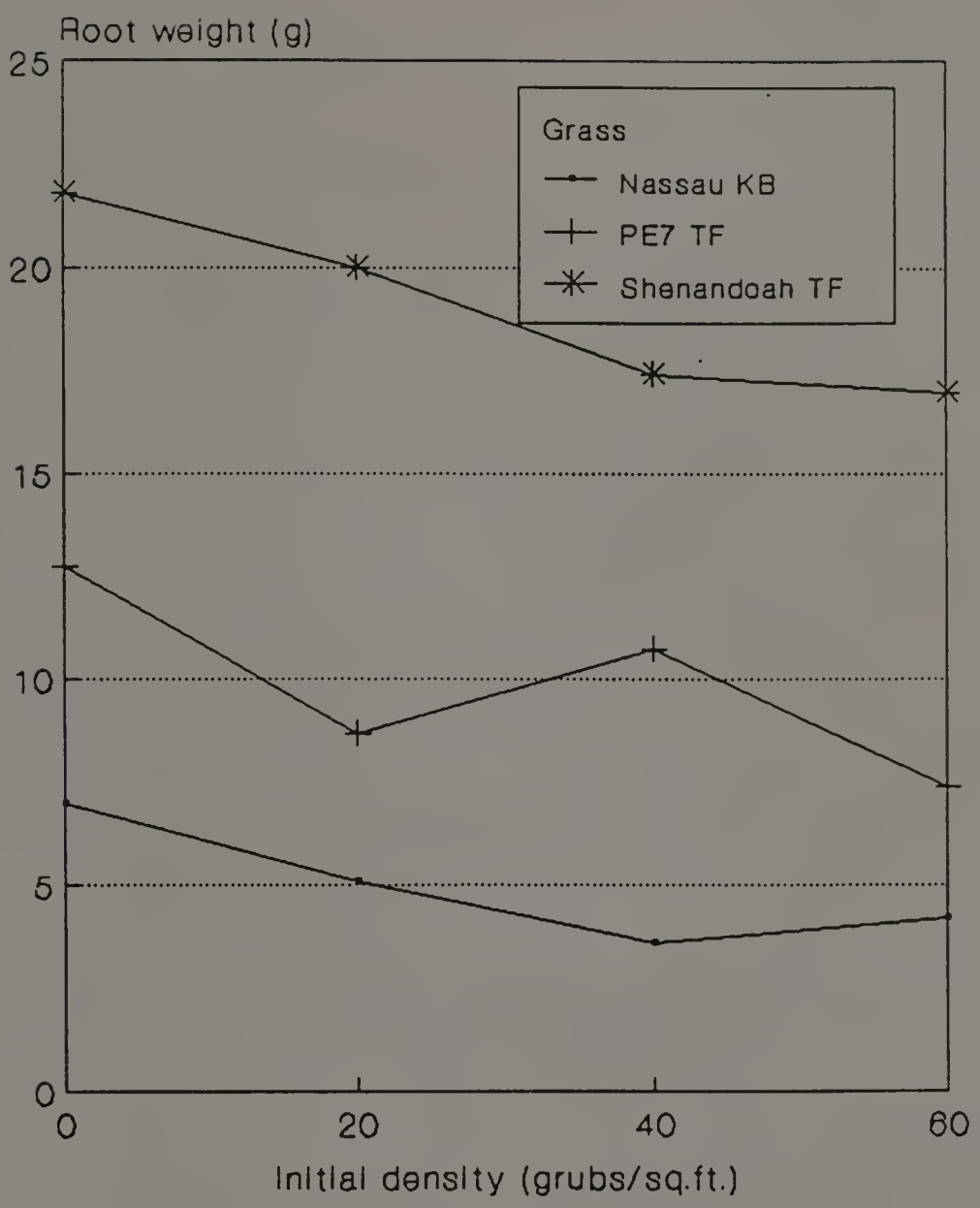


Figure 2.5. Dry root weight (g) from potted plants at the end of the 1991 field season. Means are combined for the moisture treatments. Weight is different for each grass when combined over densities (p=.05 Duncan's Multiple Range Test).

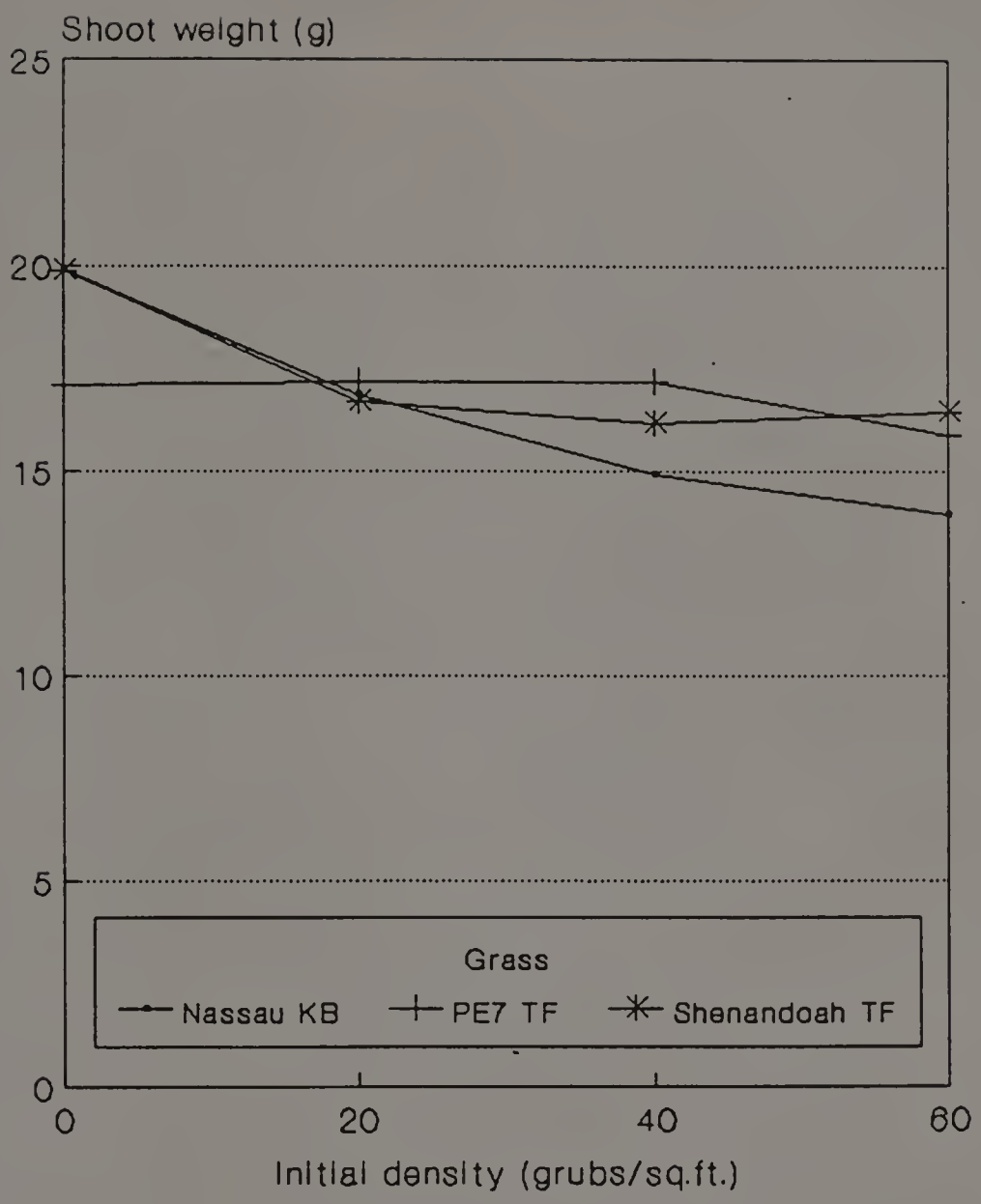


Figure 2.6. Dry shoot weight (g) from potted plants at the end of the 1991 field season. Means are combined for the moisture treatments. Weights are similar for all grasses but differ significantly for the densities (p=.01).

Weekly Grass Clippings

Repeated measures analysis was used to compare clippings taken at weekly intervals. There were significant differences between grasses over time (weeks 1-4) and between grasses and densities over time (weeks 5-10), prior to and after implantation of grubs (Table A.14). Yields were not affected by the moisture treatment. Blocks also differed on a number of the weeks. There were no interactions in between-subject effects. Despite weekly fluctuations, correlated in part with weather, 'PE7' maintained the highest and 'Nassau' the lowest yields throughout the experiment and over all of the densities before and after the introduction of grubs (Table 2.8). Yields at densities of 40 and 60 grubs/ft^2 were always lower than the 0 density control, but differences were not always significant (Table 2.9). There was an increase in yield for the tall fescues in weeks 1-5 and then a decline following implantation of grubs (Figure 2.7). The decrease with feeding was fairly parallel for all grasses at each of the initial densities (Figure 2.7). Cumulative yields over the 6 weeks following implantation of grubs appeared fairly consistent (Figure 2.9) despite density being a significant source of variation (Table 2.9). Yields were always highest from 'PE7' and lowest from 'Nassau' (Table 2.8) before and after grubs were introduced into the pots (Table A.10).

Natural Infestation

Total number of grubs removed from beneath grasses was approximately 18 for all grass species and for EI or EF grasses. An average of 13.2 grubs were recovered from Kentucky bluegrass areas. Average quality rating was 5.8 except for the EI hard fescue and

Kentucky bluegrass which were 3.8. Endophyte status did not affect total larvae or quality even when the grass species were grouped and compared as EI versus EF. There were highly significant differences in density of grubs between the replicates (Table A.13) as well as observed quality differences.

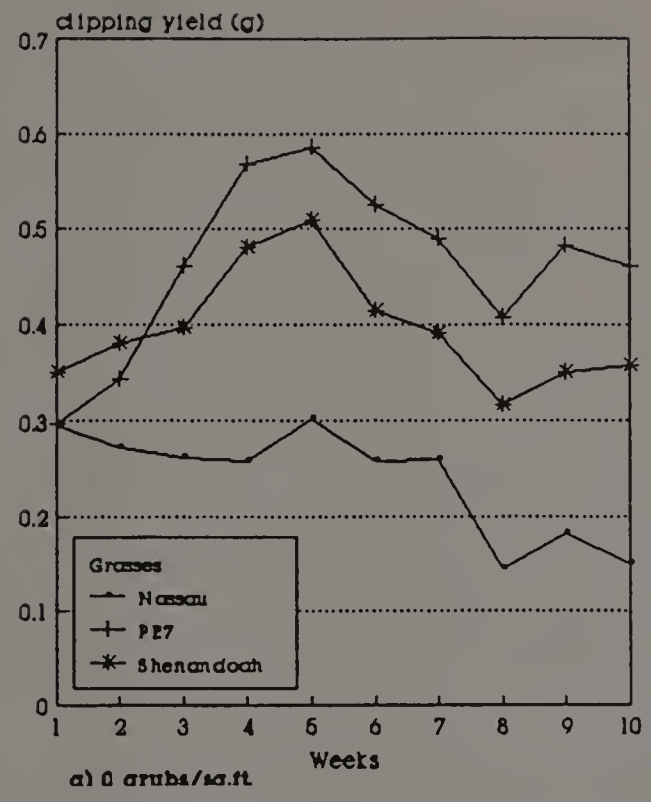
Table 2.8. Individual and cumulative (CUM) clipping yields (g) for potted grasses in the field collected in weeks 1-4 and 5-10, prior to and after implantation of larvae in the 1991 season. Mean weights are combined over density and moisture treatments. Means with the same letter are not significantly different at the .05 level of Duncan's Multiple Range Test.

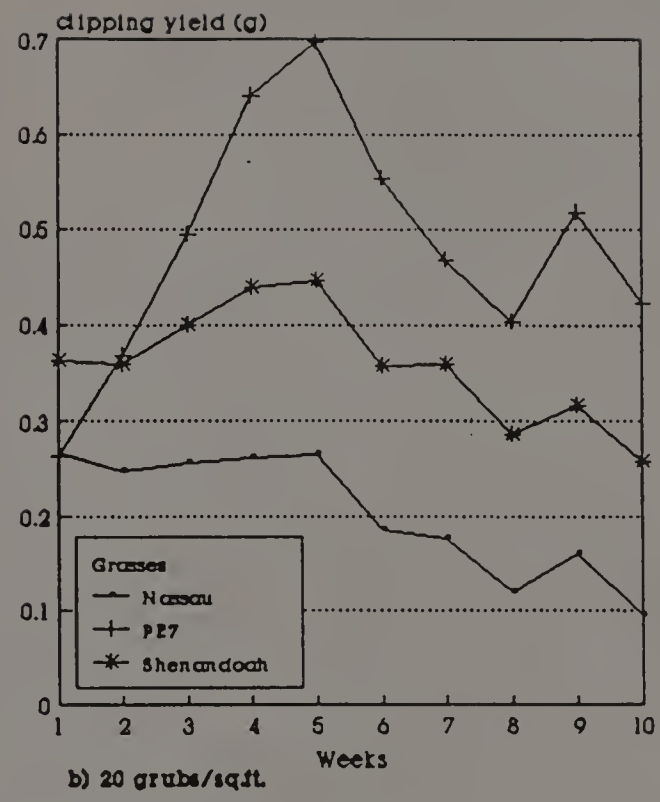
	Week				
Grass	1	2	3	4	CUM
'Shenandoah'	.358a	.361a	.370b	.451b	1.36b
'PE7'	.296b	.364a	.493a	.614a	1.54a
'Nassau'	.284b	.260b	.244c	.249c	.885c

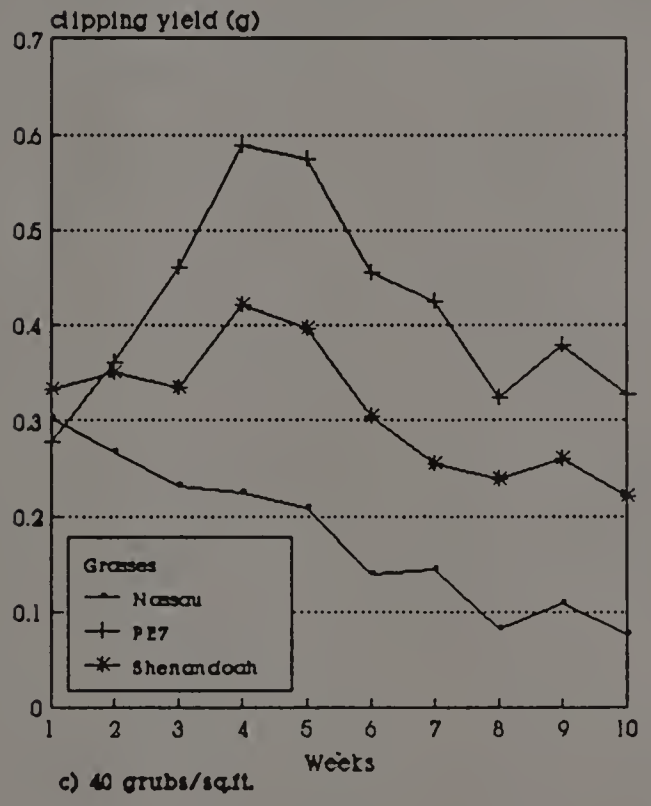
	Week						
Grass	5	6	7	8	9	10	CUM
'Shenandoah'	.450b	.447b	.353b	.334b	.269b	.305b	1.99b
'PE7'	.613a	.621a	.488a	.452a	.358a	.448a	2.76a
'Nassau'	.249c	.249c	.181c	.183c	.108c	.141c	.961c

Table 2.9. Individual and cumulative (CUM) clipping yields (g) collected from weeks 5 through 10, after implantation of grubs into potted grasses in the field in 1991. Mean weights are separated by larval density and combined for the grasses and moisture treatments. Means with the same letter are not significantly different at the .05 level of Duncan's multiple range test.

	WEEK						
DENSITY	5	6	7	8	9	10	CUM
0	.435a	.466a	.399a	.380a	.290a	.338a	2.20a
20	.448a	.469a	.366a	.333ab	.269a	.331a	2.02a
40	.412a	.396b	.301b	.303b	.213b	.249c	1.74b
60	.450a	.426ab	.296b	.275b	.207b	.273bc	1.65b







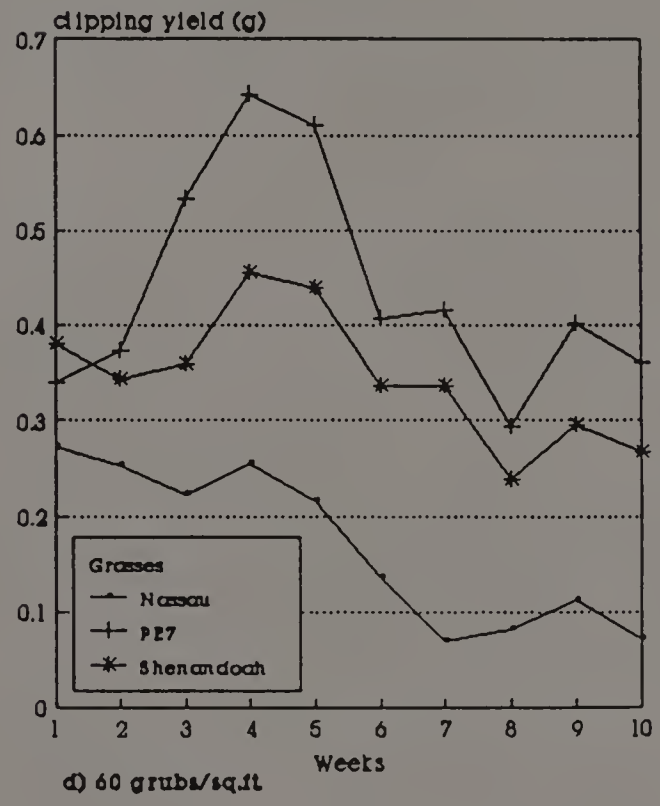
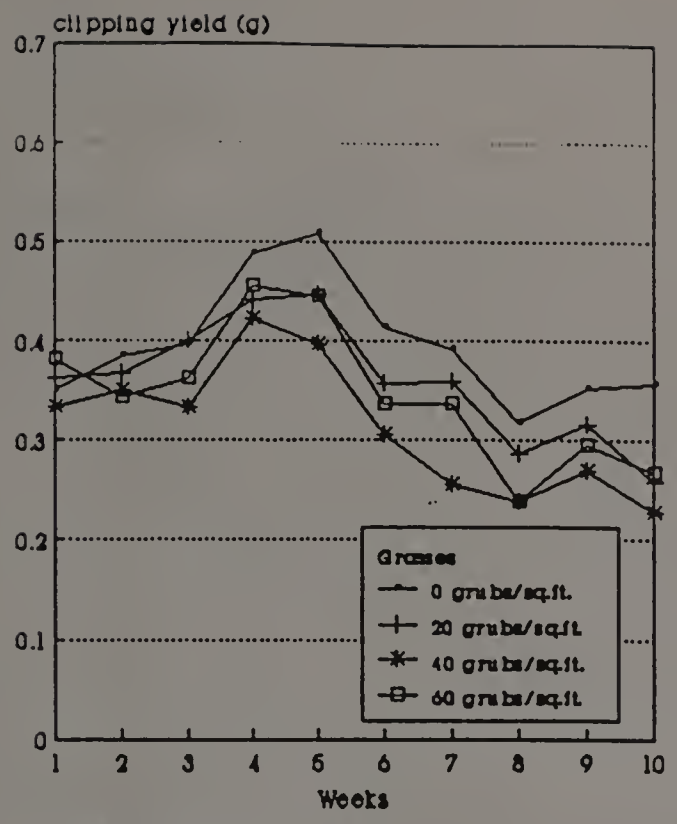
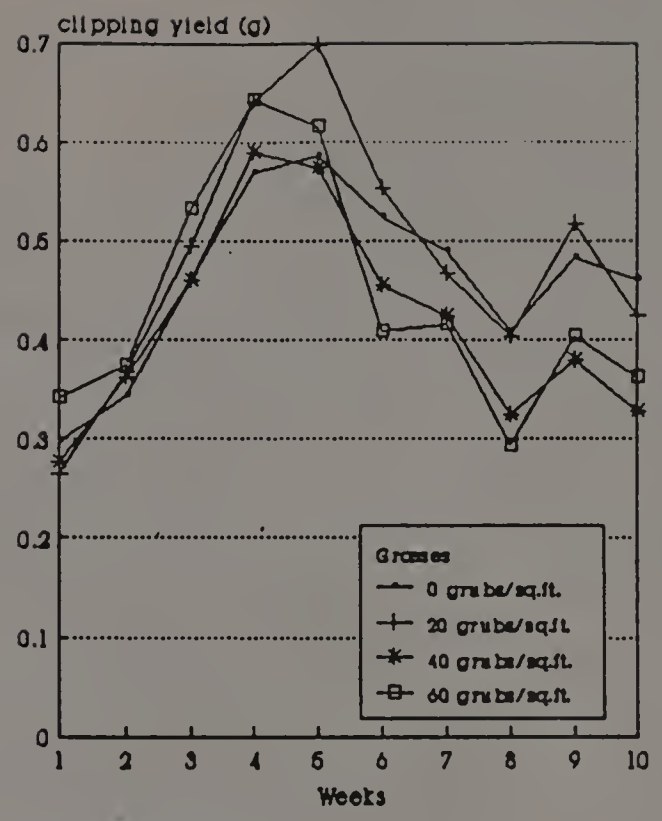


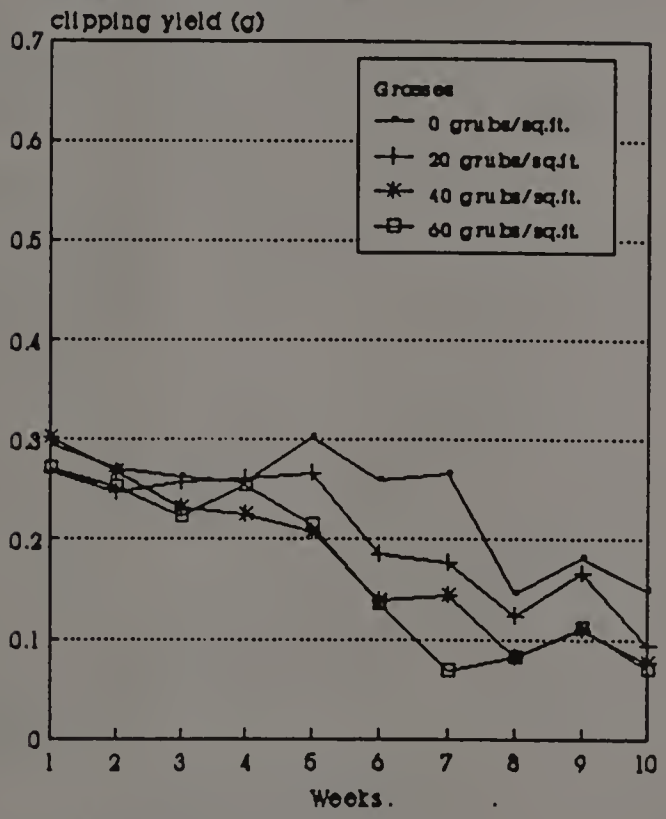
Figure 2.7. Yields of weekly clippings (10 weeks) for grasses at each of the initial densities in 1991. Means for the grasses are shown separately for each of the initial densities and are combined for the moisture treatments. Grasses grew differently from each other throughout the period and at all densities (p=.01). Larvae were implanted between weeks 4 and 5.



a) 'Shenandoah' hill fescue



b) 'FE7' hill fescue



c) 'Nassau' Kentucky bluegrass

Figure 2.8. Yields of weekly clippings (10 weeks) for individual grasses at each of the initial densities in 1991. Means for each grass are shown at each of the initial densities and are combined for the moisture treatments. Grasses grew differently from each other throughout the period and at all densities (p=.01). Larvae were implanted between weeks 4 and 5.

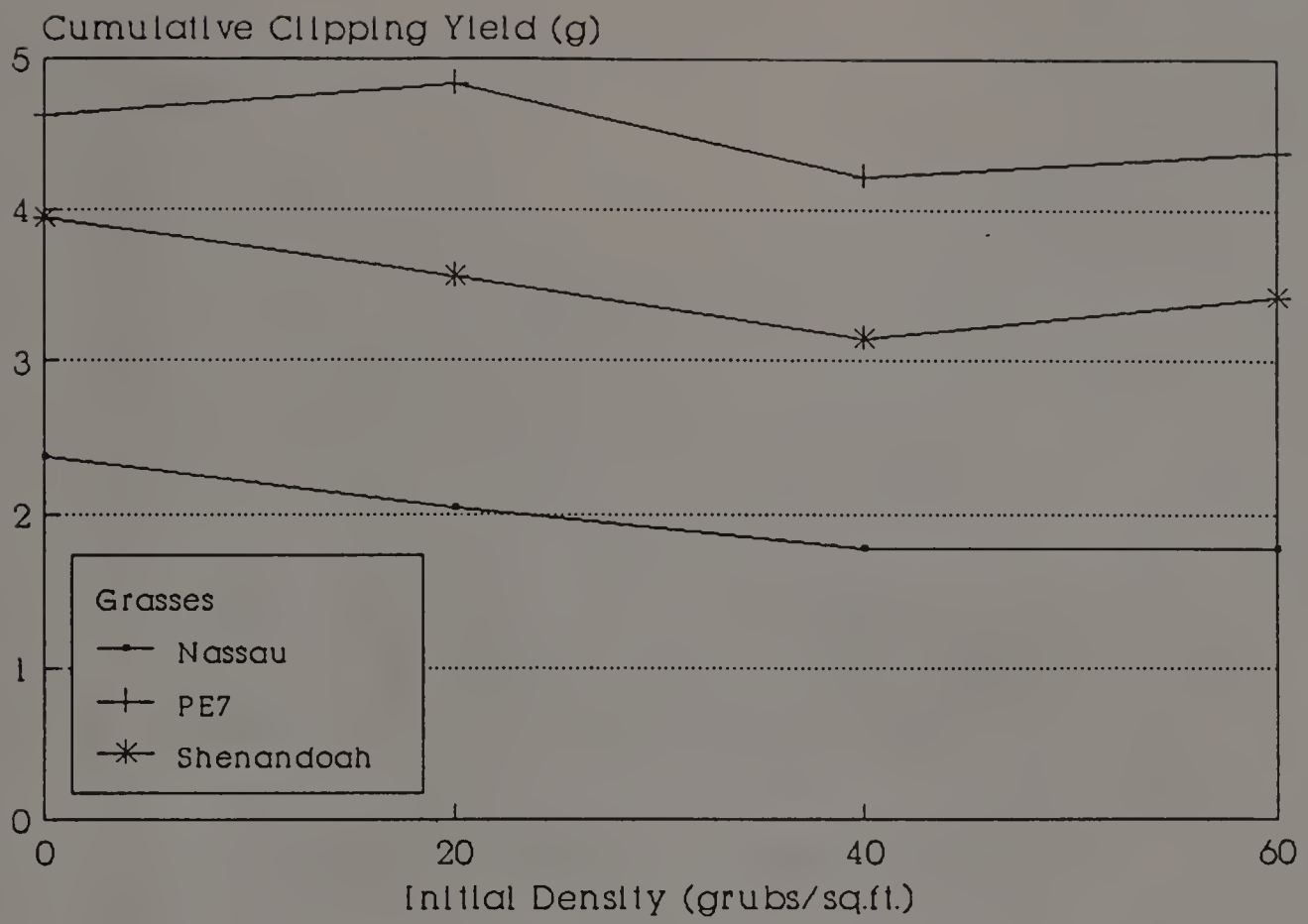


Figure 2.9. Cumulative clipping yields from weeks 5-10 for grasses in the 1991 field season. Means are combined for the moisture treatments. Grasses and densities are significantly different (p=.01).

Discussion

EI grasses affect root-feeding arthropods such as nematodes [Pedersen et al. 1988; West et al. 1988], collembola [Oliver 1990], and white grubs [Oliver 1990; Patterson et al. 1991; Potter et al. 1991]. Pot studies have implicated xenobiosis, antibiosis, and tolerance as mechanisms of resistance against first and third instar Japanese beetle larvae [Oliver 1990; Potter et al. 1991]. Field studies, however, have yielded a large amount of variation and have proved inconclusive [Oliver 1990; Potter et al. 1991]. The present work was aimed at providing further data on the effect of EI tall fescue on Japanese beetle larvae in the field.

Field Study 1990

The aim of the Single Density experiment was to compare grass species and endophyte status in terms of susceptibility to or effect on larval feeding at 20 grubs/ft^2. Due to high variation within grasses, no distinctions could be made. Nonsignificant trends revealed similar per cent recovery on infected tall fescue and Chewings fescue which were lower than for Kentucky bluegrass and perennial ryegrass and lower recovery for EI tall fescue and chewings fescue as compared with the EF varieties of these species (Figure 2.1).

Potter et al. [1991] compared feeding of Japanese beetle on hard fescue, perennial ryegrass, Chewings fescue, and EI and EF tall fescue. Despite differences in weight gain and survival of *C.lurida* on grasses, pot studies revealed no significant effects on grubs of *P. japonica* amongst grass species or between EF or infected tall fescue. Increases in total amino acids may occur in the leaf sheath of infected grasses

[Lyons et al. 1990] and nitrogen content of leaves may be higher for infected versus EF perennial ryegrass [Ahmad et al. 1987]. However, Kjeldahl analysis of the roots showed that nitrogen content was similar for all of the grasses [Potter et al. 1991]. Seemingly, endophyte status does not affect sugar content in the root which also might act as a phagostimulant or deterrent to grubs [Ladd 1988]. In sum, all cool season grasses serve as a suitable food source for Japanese beetle grubs [Fleming 1972; Potter et al. 1991; Tashiro 1987]. Variation in tolerance to damage is the most likely cause of differences in resistance of grass species [Potter et al. 1991]. Chemical feeding deterrents may account for differences in EI versus EF grasses and will be discussed later in the text.

Although tall fescue appears to be most tolerant of white grub feeding [Beard 1973], it may not be desirable from an agronomic standpoint. Infected tall fescue in combination with Kentucky bluegrass could potentially give insect protection as well as a high quality, well knit sod. It seems unlikely to expect differences between the mixtures since, according to field studies in the literature [Oliver 1990; Potter et al. 1991] there is no strong endophyte influence even for EI tall fescue alone. Oliver [1990] found that a greater number of third instar larvae were recovered from 80% EF tall fescue three months after implantation into pots in the field than for 80% and 100% EI tall fescue. However, there were fewer larvae on 100% EF tall fescue [Oliver 1990]. Clay and Cheplick [1989] found that larval weights and consumption were higher for fall armyworm feeding on leaf discs with low levels of ergot alkaloids versus controls. They cite other examples in

which low concentration of insecticides stimulate insect growth. Thus, it may be unrealistic to hypothesize that, mixed with greater amounts of Kentucky bluegrass, endophyte effects might be diluted in a linear fashion.

The goal of the Density experiment was to determine economic and aesthetic thresholds for the EI grasses 'Titan' tall fescue, 'Repel' perennial ryegrass, 'Jamestown II' Chewings fescue, and 'SR3000' hard fescue, as well as 'Nassau' Kentucky bluegrass. The initial densities 10 and 20 grubs/ft^2 are in the range of what is considered damaging to most turf [Tashiro 1987]. Higher densities were also used in an effort to achieve a leveling-off effect. Natural egg laying contributed to the high variation in the data and proved to be random across plots. Thus, there was no control for the experiment. In addition, due to grub mortality during the trial, final densities were not equal to initial densities. Densities were not all different but fell into four groupings. Estimates of the variables based on initial density may not then provide accurate information. Furthermore, it was impossible to tell at what point mortality occurred. It may, therefore, be more accurate to determine thresholds based on number of larvae recovered at each density averaged over the grasses. There were no differences in quality ratings among the densities, yet assessment of aesthetic threshold by observation of 6 inch core areas may not be entirely representative.

The hypothesis that EI grasses negatively affect, or better tolerate Japanese beetle grubs was not confirmed in the 1990 field season. Recoveries of greater than 100% due to random egg laying by

adult beetles tended to obscure even weak trends in the data. Roots and shoots were weighed fresh which resulted in variation considerably higher than the norm. Even in the analyses where variation was not assumed to be equal, there was no significant diffence between grasses or between EI and EF tall fescue. The use of lumite mesh around cores seemed to be an effective means of containing grubs while allowing for natural root growth and water movement.

Field Season 1991

Economic thresholds tend to be higher and feeding damage is often not observed on well irrigated turf [Ladd and Buriff 1979; Potter 1982; Tashiro 1979]. In addition, EI grasses tend to perform better than EF varieties under stressful conditions such as drought [Arachevaleta et al. 1989; Belesky et al. 1989a,b; Kennedy and Bush 1983]. Thus, in the hopes of observing damage as well as differences between infected and non-infected grass, a moisture treatment was introduced into the design of the second year's experiment. In addition, in an effort to increase the degree of control within a field situation, grasses were planted in pots which were submerged into a fallow area of the field. Endophytic tall fescue was the only infected grass type tested, as it is known to have high levels of alkaloids and is toxic to mammals and other insects. By comparing genetically similar EI tall fescue, differences are more likely to be due to an endophyte effect.

Mortality of grubs was high in all of the initial densities. Averaged for all of the grasses, final larval density never exceeded 8, 12, and 15 grubs/ft^2 for 'Shenandoah', 'PE7', and 'Nassau', respectively. As such, final density never exceeded what Tashiro [1987]

considers to be a damaging limit, especially under dry conditions. The highest level of grub survival was 41% for Kentucky bluegrass. It is possible that handling contributed to grub mortality. However, similar handling in the 1990 resulted in much higher survival. Despite being submerged in the ground, soil temperatures in the black plastic pots may have been higher than optimum [Regniere et al. 1981a]. This may explain some of the difference between the two seasons. Blocking of larvae by weight [Potter et al. 1991] may have indicated a relationship between larval weight and mortality. Larvae were blocked by location of collection but no differences were observed between blocks. No signs of milky spore or fungi that might influence grub mortality were observed on recovered grubs. Oliver [1990] also found a high level of mortality in field studies despite high egg hatch in the lab.

Although differences in final larval density were significant for wet and dry treatments, the results appear to be due not so much to reduced survival in dry soil as to a greater amount of variation in pattern of survival on the dry soil (Figure 2.2). Density of grubs continued to increase even up to 60 grubs/ft^2. No levelling-off effect was observed. Nor was there a decline in number at the higher densities that might have resulted from cannibalism. Survival appears fairly steady at each of the densities (Figure 2.3). In pot studies conducted by Ladd and Buriff [1979] survival of Japanese beetle larvae implanted 4-7 weeks prior to recovery decreased similarly with increasing density for moisture levels of both 60% and 90% field capacity.

Final grub density was significantly lower on the EI versus the EF tall fescue and Kentucky bluegrass. The results are most similar to those occurring in no-choice pot experiments in which survival of many foliar feeding insects is reduced when forced to feed on EI grasses [Clay 1989; Mathias et al. 1990; Siegel et al. 1989]. Similar effects have been shown for root feeding nematodes in the field [Kimmons et al. 1990; Pedersen et al. 1988; West et al. 1988] and for C. lurida [Potter et al. 1991], annual white grubs (Cyclocephala spp.), and certain families of collembola [Oliver 1990]. Survival was lower for first and third instar Japanese beetle grubs implanted into pots containing EI or EF tall fescue plants [Potter et al. 1991] or in soil in which the plants had previously been growing [Oliver 1990]. Loline and ergot alkaloids present in the above-ground tissue seem to be responsible for much of the feeding inhibition observed for insects feeding on the foliage of infected grasses [Clay and Cheplick 1991; Reidell et al. 1991]. Smaller concentrations (10-15ug/g) of these compounds may be translocated to the roots [Siegal et al. 1989]. In addition, leaching of alkaloids from seed and leaf tissue may also inhibit feeding by soil insects.

More specifically, resistance seems to be due to pyrrolizidine and, to a lesser extent, ergot alkaloids. Quantitative studies using alkaloids as diet supplements support the hypotheses that they may be the deterrent compound [Clay and Cheplick 1989; Patterson et al. 1991; Riedell et al. 1991]. Corn leaf disks soaked with various ergot alkaloids caused reduced feeding by fall armyworm but at concentrations higher than those found in the plant [Clay and Cheplick 1989]. The

aphid Rhopalisiphum padi is sensitive to plants containing loline alkaloids [Eichenseer et al. 1991]. Extracts of natural and semi-synthetic lolines influenced feeding behavior of fall armyworm larvae and, to a lesser extent, European corn borer larvae [Riedell et al. 1991]. In no-choice experiments, mortality was particularly high in diets with N-formyl loline. A number of the loline compounds were comparable with nicotine sulfate in their action as contact insecticides against greenbug adults [Riedell et al. 1991].

Patterson et al. [1991] added ergot and loline alkaloids to an agar cellulose medium on which Japanese beetle larvae were feeding. Ergot, ergovaline, and ergocryptine caused reduced feeding but at levels higher than those normally found in roots whereas extracts of N-formyl and N-acetyl loline significantly reduced feeding of third instar Japanese beetle larvae at concentrations similar to those found in the root. However, in a diet of washed tall fescue roots, feeding was not deterred [Potter et al. 1991]. In highly controlled experiments, then, feeding deterrence, and ultimate mortality, has been shown to be directly affected by alkaloid concentrations. Results of field studies have been less clear. Patterson et al. [1991] questions whether the stimulus complex in the tall fescue root might alter deterrence by alkaloids. No analysis of loline alkaloids was performed in the present study. Thus it can only be suggested that loline alkaloids in 'Shenandoah' might have inhibited feeding of grubs and contributed to higher mortality in the EI tall fescue.

Changes in larval weight have also been observed for insects feeding on EI grasses [Clay 1989; Siegel et al. 1989]. Antifeeding

behavior (antixenosis) explains many of the decreases in weight gain noted in the literature [Clay 1989; Mathias et al. 1990]. However, alkaloids may also act by affecting metabolism [Riedell et al. 1991). Larvae of the southern armyworm initially gained weight when feeding on EI perennial ryegrass. This phenomenon was thought to be due either to allelochemical stimulation causing increases in water weight of grubs, or to feeding on EI grasses that contained higher nitrogen levels [Ahmad et al. 1987]. Weight gain and development of armyworm in the previously mentioned study then decreased drastically as larvae fed directly on leaf sheath from EI grasses [Ahmad et al. 1987]. Weight of fall armyworm declined when fed pure loline on leaf discs [Reidell et al. 1991]. Both suggest that alkaloids may be responsible for the antibiosis effect. If a similar phenomenom occurred in the present study it could not be observed, because larvae were weighed only at the end of 7 weeks of feeding.

That larvae feeding on enodphyte-infected 'Shenandoah' weighed less than on 'PE7' may be a result of deterrence of feeding or due to antibiosis. Higher mortalities suggest that feeding may have been inhibited. Lower weights on 'Shenandoah' might be associated with lack of maturity. Growth reduction occurred for C. lurida but not Japanese beetle larvae feeding on EI grasses grown in pots [Potter et al. 1991] but did not occur in the field. Despite reduction in survival, Oliver [1990] found few differences with respect to weight for Japanese beetle grubs feeding on EI tall fescue. In the present study there was a sudden drop in weight at 60 grubs/ft^2 on 'Shenandoah'. This is perhaps due to the combined stress of competition for food and space as well as

an actual toxin effect. Potter [1982] showed decreased weights at higher densities for the southern masked chafer. In general, antibiosis, as evidenced by weight loss, has been inconsistent in studies with Japanese beetle larvae [Potter et al. 1991].

Resistance to root feeders may be due less to toxins than to tolerance of the grass [Potter et al. 1991]. While most cool season grasses are nutritionally suitable as larval diet [Potter et al. 1991], it may be the specific growth characteristics of the grass i.e. root to shoot ratios, that are responsible for variation in susceptibility or tolerance of root damage. Tolerance and economic or aesthetic thresholds are most often assessed on the basis of visual quality ratings and clipping yields [Potter 1982]. In the present study, final root and shoot weights were also used to compare foliar yield with root damage.

Tolerance of grasses to larval feeding is affected by soil moisture. Both dry soil conditions and high density of root feeders cause a decrease in yield [Ladd and Buriff 1979; Potter 1982]. The combined effects may cause a significant decline in quality. Potter [1982] and Ladd and Buriff [1979] showed significant decreases in quality as well as yields of grasses with feeding by southern masked chafer and Japanese beetle larvae, especially at high larval densities and in dry soil. In addition, endophyte status tends to mediate tolerance of reduced moisture levels [Belesky et al. 1989a,b; Funk et al. 1989]. In general, endophyte-enhanced varieties are able to maintain a greater biomass over EF grasses during drought stress [Arachevaleta et al. 1988; Belesky et al. 1989a]. In this study, no

significant differences in quality were observed for moisture treatments either between the densities or between EI or EF grasses. It was difficult to monitor moisture levels even in pots in the field. Thus, although soils looked "wet" and "dry" when plants were removed from pots at the end of the season, there was a large amount of variation in the level of "dry", perhaps making the overall effect nonsignificant. Wet and dry treatments were therefore combined and only endophyte status and density compared in the following measurements of tolerance.

Based on visual ratings, all grasses appeared fairly tolerant of feeding at the final densities that occurred in the study. Aesthetic thresholds, then, were not established. Regardless of larval densities, the tall fescues maintained a fairly constant average rating of 7. Quality of 'Nassau' was lower (6). Weekly fluctuations in quality were observed for all grasses and seemed most closely related to weather conditions. The sensitivity of Kentucky bluegrass to summer heat was reflected in the ratings. Although a color change and the beginnings of leaf rolling were noted for dry treatments prior to watering, recovery seemed rapid as wet and dry treatments did not differ with respect to overall ratings. The findings are in contrast to observations in similar studies by Potter [1982] and Ladd and Buriff [1979] where quality of Kentucky bluegrass declined with increasing density of southern masked chafer or Japanese beetle grubs, particularly on non-irrigated turf.

Root weights differed for all three grasses (Figure 2.5). In no case were plants pot bound, so roots were not limited in achieving their full potential for the season. Weights of Kentucky bluegrass were much

lower than for the tall fescues, presumably a result of differences in the natural growth characteristics of the grass species. However, root weights for 'PE7' were almost half of those for 'Shenandoah' at all of the densities. Numerous studies have shown differences in growth for EI versus EF grasses [Belesky et al. 1989a; Hill et al. 1990; Stovall and Clay 1988]. The more extensive root system could contribute to endophyte-enhanced tolerance of dry soil moistures as well as damage by root feeders. Individual root weights declined with increasing larval densities (Table 2.6) as for Ladd and Buriff [1979] but, in this study, over all grasses, densities differed only from the control. Per cent reduction of root weight revealed that reduction occurred to a lesser extent for 'Shenandoah'. However, it must be kept in mind that although root weight for 'Shenandoah' was reduced by an average over the densities of 12.5% in contrast with 21.9% for 'PE7', the average final grub densities were 8 and 12 grubs/ft^2, respectively. Relative to numbers of grubs feeding, tolerance of damage is fairly comparable, if not somewhat higher, for the EF tall fescue.

Final dry shoot weights were similar for all of the grasses (Figure 2.6). Although the Kentucky bluegrass appeared less vigorous throughout the season, the added weight of some of the rhizomes may have made top growth weights comparable to those of the tall fescues. Weights were not affected by individual densities but combined densities were lower than those of the control. Individual values reveal that shoot weight of Kentucky bluegrass did decline fairly consistantly with increasing larval density while the tall fescues seemed relatively unaffected (Table 2.7).

Above-ground growth is better compared by weekly clipping yields (Figures 2.7-2.9). Yields of 'Shenandoah' and 'PE7' increased during weeks 1-5, perhaps owing to weather that favored growth of tall fescues versus Kentucky bluegrass (Table 2.7). Yields, in general, seemed relatively unaffected by density. At all of the densities, yields declined somewhat after implantation of grubs between weeks 4 and 5. In all cases, the response is seen most dramatically between weeks 5 and 6 as the grubs had perhaps resumed feeding after the stress of transfer. Weekly and cumulative yields from densities of 40 and 60 grubs/ft^2 were lower than those of 0 and 20 grubs/ ft^2 (Figure 2.8,2.9). Despite weekly fluctuations, yields at week 10 were always below those at week 5, suggesting that damage due to feeding is additive [Ladd and Buriff 1979; Potter 1982]. In general, EI grasses tend to produce greater biomass in tillers [Hill et al.1990] yet yield of EF 'PE7' was always above that of 'Shenandoah' and had the highest yields over the season both prior to and after implantation of grubs.

It is difficult to evaluate tolerance of grasses adequately under the experimental conditions. In order to do so, characteristics such as quality, yield, and root and shoot weights must be based on a standardized level of density. Variables perhaps should be expressed based on the final densities of 8, 12, and 15 grubs/ft^2 for 'Shenandoah', 'PE7', and 'Nassau', respectively, although it is uncertain at what point mortality occurred. Root feeding did reduce clipping yields significantly at the higher densitites. The level of decline seemed fairly parallel in all grasses despite differences in number of grubs and root weights. If tolerance is defined as a root to

shoot ratio, the greater root mass with more modified shoot growth on 'Shenandoah' may be adaptive for tolerance of drought and damage by root feeders. However, where tolerance is evaluated by quality and clipping yields relative to root damage, 'PE7' may be more tolerant than 'Shenandoah'. Quality remained relatively high throughout the season and thus tolerance was not evaluated under stressful conditions. Perhaps differences would have been clearer at more damaging densities or with greater distinctions between densities.

Although results of this field study suggest a certain degree of resistance for 'Shenandoah', comparable studies at the University of Rhode Island (U.R.I.) reveal the opposite. In the Rhode Island study, final densities were highest on 'Shenandoah' and lowest on 'Nassau'. The same pattern occurred both when third instars were implanted into pots in the ground in the Spring and when second instar larvae were implanted in the Summer [Casagrande, personal communication]. Potter et al. [1991] also found that tall fescue was preferable as a food source for Japanese beetle grubs. However, these differences were not observed when second instars were implanted into cores in the field [Potter et al.1991]. In both Rhode Island studies and in Potter et al.'s work, grubs were implanted into cores of established turf. In this study grasses were started from seed in the same pots in which grubs were implanted 2 1/2 months later. In contrast to mature turf, alkaloids tend to be higher in young grasses or may have leached from seed thus influencing larval feeding. It is impossible, however, to separate this factor from others as physical, chemical, and biological properties of the soil, regional weather conditions, and grub populations.

It is impossible to replicate an experiment. Grubs may be more affected by soil properties and organic matter than nutrient sources of the grass, the former of which tends to be more variable. Thus, if repeated, the results of the present study might not be the same. All variables act both singly and in combination resulting in between-site and within-site variation and conflicting outcomes.

Under completely natural conditions, there are numerous interacting factors which seem to influence grub survival and grass tolerance to a greater extent than does endophyte-infection. Natural infestation experiments have failed to show differences between EI and EF grasses, with respect to numbers of Japanese beetle larvae. Patchiness of grub populations makes adequate sampling within a field plot difficult [Potter et al. 1991]. Regardless of endophyte infection, factors affecting survival of Japanese beetle larvae include soil moisture, temperature, pH, texture and organic matter [Regniere 1979, 1981a,b; Vittum 1990]. Vertical movement by grubs is mediated largely by soil moisture and temperature [Villani and Wright 1988]. In moist turf horizontal movement of grubs is limited [Fleming 1972]. Choice of oviposition site of the adult beetle, in moist, lush turf, may thus be responsible for much of the larval distribution. Grub density in the present natural infestation study was most related to turf quality, which in turn is dependent upon soil moisture, nutrients, pH, etc. The interaction of all factors acts at the level of grass, fungi, and insect and in this way mediates the overall effect.

In general, the results of the present work are consistent with those laboratory and field studies that show effects of EI grasses on

Japanese beetle larvae. Data from the first year's work point to the high variation under natural conditions that has obscured results in other field studies [Oliver 1990; Potter et al. 1991], making it difficult to assess the actual impact of endophyte infection on Japanese beetle larvae under non-experimental conditions. In the second field season, survival of Japanese beetle larvae and weight of third instars was lower on 'Shenandoah' tall fescue suggesting possible deterrence and antibiosis of grubs feeding on EI tall fescue. Yet the effects may vary with developmental stage of the grass, or larvae, and on environmental conditions. Egg viability is not affected by infected grasses [Oliver 1990] while early instars may be controlled by feeding on seedling turf [Casagrande, personal communication]. Deterrence and antibiosis are thought to be due to pyrrolizidine and ergot alkaloids which are altered by such factors as age of plant, clipping frequency, and environmental stresses [Kennedy and Bush 1983] resulting in variability or inconsistencies in level of resistance. Many other factors may also interact under field conditions and confound any of these effects. While EI grasses may affect Japanese beetle larvae, the resistance is highly variable in the field.

CHAPTER III

INOCULATION STUDIES

Introduction

Enhanced performance of endophyte-infected (EI) turfgrass cultivars has been documented [Funk and Clark 1989; Saha and Johnson-Cicalese 1987]. Clavicipitaceous endophytic fungi have been detected in a wide range of host species, particularly in the genus Poaceae [Clay 1989]. The non-sporulating Acremonium endophytes, A. coenophialum and A. lolii, occur naturally in tall fescue and perennial ryegrass, respectively. The market is expanding for endophytic cultivars of tall fescue ('Shenandoah', 'Titan'), perennial rye ('Repell', 'SR4200'), and for low maintenance grass mixtures containing perennial ryegrass, Chewings fescue, and hard fescue. Recently, A. coenophialum has been identified in Poa autumnalis and P. ampla but not in P. pratensis.

The development of methods of artificial infection will lead to a greater understanding of the mutualistic relationship that has evolved between grass and fungus. In learning the contribution of each organism, it may be possible to take advantage of certain host-endophyte combinations. For example, host grasses may be infected with strains of endophyte that confer high resistance and competitiveness but contain low levels of alkaloids. In addition, the ability to infect such non-host species as Kentucky bluegrass would be economically valuable to the turfgrass industry.

Various attempts have been made to infect endophyte-free (EF) grasses with endophytic fungi. Latch and Christensen [1985] were successful in infecting EF host grasses by placing mycelium from cultures of their respective endophyte into the coleoptile tissue of seedlings growing on water agar. Leuchtmann and Clay [1988] had mixed success when inserting mycelium and conidia of Atkinsonella hypoxylon and Balansia cyperi into host grasses and sedges. The outcome led them to suggest the notion of host races. About 17% of the plants were infected in attempts by Johnson and Siegel [1986] to inoculate A. coenophialum into callus of EF tall fescue. Inoculation of somatic embryos of tall fescue also resulted in EI plants [Kearney et al. 1991].

In this study, endophyte isolated from a variety of sources was inoculated into enodphyte-free grasses through use of five methods: 1) inoculation of seed; 2) inoculation of seedlings; 3) inoculation of mature callus; 4)inoculation of initiating callus; 5) inoculation of embryogenic callus. Infection was determined through examination of leaf peels under the microscope.

Materials and Methods

Isolation of Endophyte

Seed or leaf sheath tissue was used to initiate cultures of the following endophytes from their respective sources: Acremonium coenophialum from 'Rebel II' and 'Titan' tall fescue as well as from Poa autumnalis; Epichloe typhina from 'SR3000' hard fescue and 'SR5000' chewings fescue; and A. lolii from 'Repell' and 'SR4200' perennial rye (Table 3.1).

Table 3.1. Sources of isolates for the fungal endophytes A. coenophialum, A. lolii, and Epichloe typhina used in inoculations.

Endophyte	Source
Acremonium coenophialum	'Rebel II' tall fescue 'Titan' tall fescue Poa autumnalis
Epichloe typhina	'SR5000' chewings fescue 'SR3000' hard Fescue
Acremonium lolii	'Repell' perennial ryegrass

Isolation from Seed

Seed was deglumed by stirring in 50% sulfuric acid for approximately 10-15 minutes, rinsed with water, and surface sterilized for 20 minutes in 50% sodium hypochlorite containing several drops of Tween 20 [Bacon 1988]. Seeds were then rinsed with sterile distilled water and put on petri plates containing Potato Dextrose agar (PDA Difco #0013-01-4) or Corn Meal Malt agar (CMM) (17g/l Corn meal agar, 20g/l malt extract, 2g/l yeast extract in 1000mls distilled water)[Bacon 1988,1990].

Isolation from Leaf Sheaths

Leaf sheaths from endophytic plants maintained in the greenhouse were surface sterilized with full strength sodium hypochlorite for 5 minutes and then rinsed with distilled water [Bacon 1988]. Tissue was teased apart before placing in petri dishes with CMM. All plates were sealed with parafilm and placed in the dark at approximately 25 C. In general, cultures from seeds were evident in about 6-8 weeks while mycelium visibly extended from leaf sheaths in about 4 weeks.

Grasses

The cultivars of EF grasses used for inoculation trials were the following:

Grass species	cultivar
Festuca arundinaceae	'Monarch'
Festuca rubra ssp.commutata	'Jamestown'
Festuca rubra ssp. rubra	'Dawson'
Festuca longifolia	'Crystal'
Poa pratensis	'Baron' 'Nassau' 'Suffolk' 'Adelphi' 'Georgetown'

Inoculation of Plants

Inoculation of Seeds

Seeds were deglumed in 50% sulfuric acid, surface sterilized for 20 minutes in 50% sodium hypochlorite with Tween 20 and rinsed with sterile distilled water. They were then either a) cut in half or b)left whole. In both cases, seeds were placed overnight on sterile moist filter paper in petri dishes. A dental pick was used to scrape mycelium from the edges of fungal cultures and to inoculate the fungus into the embryo area of imbibed seeds.

Inoculated seeds were then transferred to petri plates containing water agar. Cut seeds were maintained on water agar with 20g/l sucrose, kept in the dark for 10 days and then in the light for 26 days. Specific conditions for seeds in the various whole-seed inoculation trials are noted in Table 3.2a. In general, plants were kept in plates for 10 days. All seedlings were then planted into cell packs, grown in the mist house until rooted, and then were transferred to pots and grown in a soil-mix in the greenhouse.

Inoculation of Seedlings

Seeds were deglumed, surface-sterilized, rinsed, and placed in petri dishes on moist filter paper, as above. At days 4,7,10, and 17 after germination (Table 3.2b), a dental pick was used to inoculate mycelium into the base of coleoptiles. Seedlings were then transferred to petri plates with water agar or Hoagland's solution with 8g/l agar for 10 days prior to planting in the greenhouse.

Inoculation of Callus

All callus was initiated by placing surface-sterilized seed of non-endophytic grasses in petri plates with 1/2 strength Murashige and Skoog (MS) media, 30 g/l sucrose, 5mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), and 8g/l agar at pH 5.8. Dishes were maintained in the dark at approximately 25 C. A dental pick was then used to insert fungal mycelium into callus at one of three stages: 1) Initiating callus began developing at the base of germinating shoots. At that point, callus was moved into the light until stems turned green. After inoculation they were transferred to tubes with water agar or Hoaglands solution plus agar and placed under lights until rooting occurred (Table 3.2e). 2) Mature, subcultured callus was pierced at a number of different points and then either put in tubes with growth medium (1/2 strength Murasige and Skoog basal medium, 40g/l sucrose, and 8g/l agar) or directly into tubes with hormone-free regeneration media (1/2 strength Murasige and Skoog basal medium, 10g/l sucrose, and 8g/l agar) and placed under lights (Table 3.2c). 3) Embryogenic sectors that formed spontaneously in culture were inoculated with mycelium and transferred to tubes with regeneration media and kept in the light (Table 3.2d).

Detection

Inoculated plants were maintained in the greenhouse until examined for infection. Four tillers were removed from plants containing a minimum of six tillers. The epidermis was then peeled from the inner surface of each leaf sheath and placed on a microscope slide. The tissue was stained with a standard solution of rose bengal (.5% rose bengal in 5% alcohol) and observed under the microscope at 100X for the presence of fungal mycelium [Saha et al. 1988].

Table 3.2. Combinations of grass, endophyte, and source of isolate culture used for each of the methods of inoculation.

A. Inoculation of seed.

Grass	Endophyte	Source
'Dawson'	Acremonium coenophialum	'Rebel II'*'Titan' Poa autumnalis
'Pennlawn'		'Rebel II'*
'Nassau'		'Rebel II' *
'Baron'		'Rebel II' *
'Suffolk'		'Rebel II', Poa autumnalis
'Adelphi'	A. coenophialum	'Rebel II', Poa autumnalis
	Epichloe typhina	'SR3000' hard fescue
'Crystal'	Acremonium coenophialum	'Rebel II'
'Monarch'		'Rebel II', 'Titan', Poa autumnalis

* Conditions varied for trials: 13 days in dark + 25 days in benchtop light [38 days inoculation to planting) or 25 days on benchtop or 3 weeks in growth chamber with 12 hrs.light/dark at 80 F day/ 70 F night. In other cases, and where not noted, inoculated seeds were kept under plant lights for 10 days prior to planting.

Continued next page

Table 3.2 continued

B. Inoculation of Seedlings

Grass	Endophyte	Source
'Dawson'	A. coenophialum	'Rebel II', 'Titan', Poa autumnalis
'Monarch'		'Rebel II', Poa autumnalis
'Adelphi' *	A. coenophialum	'Rebel II', Poa autumnalis
	E. typhina	'SR3000' hard fescue
'Suffolk'	A.coenophialum	'Rebel II', Poa autumnalis

Inoculations at days 4,7,10, and 17. All others only on days 4,7, and 10.

C. Inoculation of Mature Callus

Grass	Endophyte	Source
'Dawson'	A.coenophialum	'Rebel II'
'Pennlawn'		'Rebel II'
'Baron'		'Rebel II'
'Nassau'		'Rebel II'

D. Inoculation of Embryogenic Callus

Grass	Endophyte	Source
'Dawson'	A. coenophialum	'Rebel II', Poa autumnalis
'Nassau'		'Rebel II'
'Baron'		'Rebel II'

E. Inoculation of Initiating Callus

Grass	Endophyte	Source
'Dawson'	A. coenophialum	'Rebel II', Poa autumnalis
'Suffolk'		'Titan', Poa autumnalis
'Georgetown'		'Titan', Poa autumnalis
'Jamestown'	A.coenophialum	'Rebel II'
	E. typhina	'SR5000' chewings fescue
		'SR3000' hard fescue

Results

Table 3.2 lists the combinations of grass and endophyte used for each of the methods of inoculation. There was a total of 800 seeds, 1400 seedlings, and 91 mature, 21 embryogenic, and 300 initiating callus inoculated.

In a few of the seed and seedling inoculations, mycelium was visible around the base of the germinated seeds at the time of planting. Non-inoculated controls showed no signs of mycelium. There was no difference in survival for controls stuck with the dental pick compared to those that were left undamaged.

Endophyte of any type almost always grew on mature callus from any grass. Small calli were often overrun with fungus. Mycelium was observed less frequently on regenerating callus. No mycelium was visible on any of the mature callus. Only mature callus of 'Dawson' regenerated after transfer to regeneration media or when maintained on transfer media followed by regeneration media. Almost all of the embryogenic calli inoculated, including some spontaneously embryogenic bluegrasses, resulted in regenerated plants.

Most of the plants survived the treatments. Losses between inoculation and time of detection occurred largely during transplant of the seedlings, disease in the greenhouse, and lack of regeneration. However, in the over 1500 remaining cases, examination under the microscope of the stained leaf peels revealed no endophyte mycelium. Infection of non-endophytic plants was not successful for any of the grass-fungus combinations using any of the methods.

Discussion

Despite the similarity in experimental approach to that of other studies, no endophyte was detected in any of the inoculated plants. It is difficult, even in comparing the present work with studies leading to successful inoculation, to determine the reason for failure of infection. It is possible only to postulate certain methodological and/or biological factors that may have been involved.

Natural infection occurs during seed germination. At the time of flowering, the endophyte moves from the leaf sheath into the inflourescence. The hyphae then extend into the aleurone cells and into the embryo of the developing seed [Philipson and Christey 1986]. One of the goals in developing techniques of inoculation is to determine the timing that coincides with the developmental stage during which chances of infection are optimal. Latch and Christensen [1985] and Leuchtmann and Clay [1988] inoculated grasses at the seedling stage. Johnson and Siegal [1986] and Kearney et al. [1991] inserted fungal mycelium into callus and somatic embryos obtained from EF grasses. In no case has infection resulted from inoculation of mature tillers. No previous published work has reported results from the inoculation of seed. As the endophyte is normally found in the aleurone layer of seed prior to germination of the embryo, it seemed logical that artificially inoculated mycelium might also move into the developing embryo. Sterile seeds imbibed overnight proved easy to inoculate and germinated readily in almost all of the cases. In the one trial where seeds were halved, in order to allow for more direct entry of fungus into the embryo, germination was drastically reduced. In some of the earlier attempts,

fungus appeared in the water agar around the seeds. The mycelium was white and slow-growing, suggesting that it might be the endophytic fungus and not a contaminant. However, no hyphae were visible in the subsequently stained sheaths.

As mentioned previously, Latch and Christensen [1985] and Leuchtmann and Clay [1988] artificially infected EF grasses by the inoculation of seedlings. In the first, infection was highest when mycelium was inserted into split stems but also resulted when hyphae were placed at the junction of mesocotyl and coleoptile of an undamaged shoot or on the coleoptile below the first leaf. In the latter, a hypodermic syringe filled with water, mycelium, and conidia was used to inoculate fungus without damaging the meristem of seedlings. Method of inoculation, in the Latch and Christensen study [1985], had no effect on the mean percent infection of seedlings. Seemingly, use of a sterile root canal pick to insert fungal mycelium should be comparable to either method of insertion. The grooves on the pick were ideal for scraping mycelium from the edges of the cultures, and the pick could effectively pierce the coleoptile without causing mortality to the seedling.

Timing is believed to play a role in determining the likelihood of infection resulting from seedling inoculations. Latch and Christensen [1985] found that infection rates were highest when nine-day old ryegrass seedlings were inoculated with a Giocladium-like species and planted in the soil ten days later. Leuchtmann and Clay [1988b] claimed that age of the seedling was the most important factor in determining infection rate. In their trials, seedling age was also dependent upon host species: One to two centimeter high Danthonia spicata inoculated

at the time of development of the first leaf, nine days after germination, resulted in the highest infection. Cyperus virens was inoculated at the two-leaf stage, which was twenty days after germination. Inoculated seedlings were kept in petri dishes for 6-8 days prior to planting in the greenhouse. Finally, timing may also be a factor with respect to the endophyte. Viability may be affected by time in culture as well as number of times subcultured (Bacon 1990).

In this study, in an effort to determine proper timing, seedling inoculations were performed at days 4,7,10, and 17. Inoculation to planting time was 10 days. However, due to the lack of infection, no comparision can be made on the basis of the time points. In the previous studies, endophyte was detected 6-8 weeks after the inoculations. In the present research, all sheaths were examined at least 8 weeks later.

There is considerable potential for the use of callus tissue for artificial inoculation of endophytes. When placed on MS basal medium with 5 mg/L 2,4-D, callus developed readily from almost all seeds of all species, making it a readily prepared substrate. Callus was then inoculated at various stages of development. Mature, subcultured calli were inoculated at varying time points after initial development depending upon the amount of time before reaching a size of approximately 1 centimeter. Regeneration was low for mature, subcultured callus. Even upon transfer to hormone-free media, only 50% regeneration occurred for Dawson red fescue and 0% for Kentucky bluegrass.

Modification of hormone levels and incubation conditions could have yielded higher percentages of regeneration. For tall fescue, Johnson and Siegal [1986] transferred callus to a shoot regeneration media with .25 mg/L 2,4-D and to a root regeneration media with no hormone but were not successful for bluegrass or orchardgrass. Charcoal additions might also have enhanced chances of regeneration [Zaghmont and Torello, 1988]. Kearney et al. [1991] noted that tall fescue genotypes differed in auxin concentrations required to produce callus. Johnson and Siegal incubated inoculated callus in the dark for ten weeks prior to removal to hormone-free media in the light. In contrast, while some of the callus in this study was incubated in the dark to promote better fungal growth, most was transferred immediately to hormone-free media in the light in order to induce regeneration soon after inoculation. Consistant with observations by Johnson and Siegal (1986), callus of all species proved to be a suitable growth medium for all fungus. The question of fungal viability then may not explain in full the lack of infection.

Inoculation of embryogenic sectors of callus may offer the greatest potential as a technique for endophyte infection. According to Kearney et al. [1991], the developmental process of somatic embryogenesis where a cell from the callus becomes an embryo, is similar to that which occurs within a seed. While the auxin induces development of the embryo, the hormone-free media allows for germination of the embryo. The greatest number of embryos produced per tall fescue callus was in media with 30uM 2,4-D. This is in contrast with Torello et al. [1984] where additions of 20uM 2,4-D promoted best growth of red fescue callus. Kearney et al. [1991] found that succcessful infection of

regenerated tall fescue with Acremonium coenophialum was highest when somatic embryos were inoculated at the time when the callus was transferred to hormone-free medium and were thus at the point of elongation and germination. At that point, conditions are most similar to those that occur naturally in the plant. Success of regeneration in the current study was significantly higher when embryogenic sectors versus non-embryogenic callus was transferred to hormone-free media. Even when slow-growing, endophyte-looking fungus was evident on the callus or at the base of the regenerant, however, it was not visible in the sheaths of the resulting plants.

Callus initiating at the base of the shoots of germinating seeds was inoculated immediately. The seedling was then transferred to water agar in the light. Inoculation of initiating callus was tried primarily for species such as Kentucky bluegrass for which there was little success in regeneration. The callus was used merely as an easy substrate for insertion of the fungus to avoid mortality. Potential infection was thus not limited by percent regeneration as each inoculated callus produced a plant. Kearney et al. [1991] also inoculated callus at early stages (0,1, and 2 weeks after initiation] but maintained the callus on the same medium until day 28 when they were transferred to hormone-free media for regeneration. No regeneration occurred from plants inoculated at that time.

Despite the failure of infection in the present trials involving callus, there are a number of advantages to using tissue culture techniques for inoculation of fungal endophytes. Inoculation of callus enables infection of genetically identical grass with different strains

of fungus. Barring somaclonal variation, each embryo should be identical to the original plant. Thus the technique allows for a further understanding of the influence of the endophyte on the interaction. In addition, tissue culture techniques as inoculation of callus or embryogenic suspensions may be performed on a large scale enabling rapid generation of potentially infected plants.

Failure of inoculations in this study to produce infected plants may also be due to non-method related factors inherent in the host-endophyte association. The inability to infect Kentucky bluegrass may be due largely to the evolutionary relationship between grass and endophyte. The mutualistic, or type 3 relationship between grass and endophyte, where the endophyte does not reproduce sexually on the host, is thought to have evolved from a type 1 pathogenic relationship [White, 1988]. The former, however, occurs only in festucoid grasses. It is likely that ancestral varieties of the cool season festucoids established an association with the Epichloe fungus. The species coevolved due to the ability of individuals in the grass population to produce alkaloids that suppressed formation of fungal stromata. The alkaloids produced both by the grass as well as by the fungus proved to be a beneficial defense against predators [White 1988].

Recently researchers at Rutgers University have identified endophyte in two bluegrass species, Poa ampla and P. autumnalis. A. coenophialum isolated from P. autumnalis appeared more aggressive in culture than other strains of the endophyte. It was hopeful that an endophyte isolated from a species of a similar genus might be able to infect a cultivar of Kentucky bluegrass. Due to the negative results

across all trials, while this possibility may be unlikely, it should perhaps not be completely ruled out. That inoculation of EF tall fescue did not result in infection may be due to any number of factors. Further specificity of the coevolved, mutualistic relationship is evidenced by differences in compatibility of host and fungi isolated from varying sources. In all of the previous published attempts, regardless of method, successful infection resulted from inoculation of endophyte into its original host. These inoculations may serve as a check for the efficacy of the technique itself. Inoculations in the present study could have been performed on EF plants grown from fungicide-treated infected grasses. Alternately, inoculations might have been tried using genetically similar EI and EF species as 'M3' and 'M3E' or 'PE7' and 'PE7E'.

An overall summary of the results from previous inoculation trials provides significant information on the cross-compatibility of endophyte and grass. Johnson and Siegal [1986] found that 'Kentucky 31' inoculated with its own endophyte produced more shoots and a greater number of regenerated plants than did those inoculated with A. lolii from perennial rye. Infection was successful only in the host from which it was isolated. Endophytes isolated from two genotypes of tall fescue differing in alkaloid production were both able to infect somatic embryos of one of the genotypes [Kearney et al.1991]. In trials by Latch and Christensen [1985], A. coenophialum, A.loliae, Epichloe typhina, a Gliocladium-like species, and a Phialophora-like species were all able to infect tall fescue and perennial rye, giving evidence for cross-genus compatibility. Balansia cyperi isolated from either Cyperus

virens or C. rotundus produced infection when inoculated into C. virens [Leuchtmann and Clay 1988b]. However, isolates of different strains of Atkinsonella hypoxylon from Danthonia infected Danthonia but not Stipa. Strains of the same fungus isolated from different locations of Stipa populations were cross compatible with their natural host but not with Danthonia. Thus Atkinsonella did not show genus cross compatibility.

The differences in compatibility observed in the various trials may relate to what Leuchtmann and Clay [1988] refer to as 'host-specific races'. The distribution of fungal species on different hosts may be due to genetic specificity at the level of genus, species, population, or genotype, or is perhaps due to chance events. Reciprocal infection of fungal strains isolated from different plant populations and species gives information on the genetic limits of the host range and at what level the specificity of the relationship is acting. Compatibility may be explained to at least some extent by host-species biology. The native ranges of Danthonia and Stripa are non-overlapping, and cross compatibility of Atkinsonella did not occur. However, B. cyperi may have moved from the native C. virens to the sympatrically introduced C. rotundus. Morphological differences were also observed [Leuchtmann and Clay 1988] for A. hypoxylon isolated from Danthonia hosts versus the Stripa host. The same was true for Acremonium lolii isolated from different strains of perennial rye [Christensen et al. 1991]. In this study, A. coenophialum isolated from Poa autumnalis appeared to grow significantly faster than did biotypes isolated from any of the cultivars of tall fescue.

Variability in compatibility among hosts and within host populations may be better explained at the genetic level. Are fungi that infect congeneric hosts genetically different? Are endophytes isolated from a single host species or population genetically variable? Isozyme binding patterns obtained from starch gel electrophoresis have been used to determine the relationship of endophyte isolated from various sources [Leuchtmann and Clay 1989a,b, 1990]. Based on the enzymes that were selected for resolution, isozyme phenotypes were always different for hosts of different genera. In addition, there was electrophoretic variation from multiple isolates of the same host species as well as among isolates of a single host population. While there was great variation among A. coenophialum even in within-site isolates of the wild Festuca obtusa, 47 out of 52 isolates of A. coenophialum from tall fescue (Festuca arundinaceae) had the same isozyme phenotype. In general, in mutualistic versus pathogenic systems, selection should favor a broad range of compatibility [Leuchtmann and Clay 1989]. However, isozyme studies show that the host-endophyte association is specific enough that constraints must be heeded in artificially manipulating combinations of grass and fungus.

Results of inoculation studies are consistent with isozyme analysis. Where inoculation trials revealed no cross compatibility between Atkinsonella hypoxylon isolated from Danthonia and Stripa, isozyme studies revealed a large genetic distance between the taxonomically distinct genera. Isozyme phenotypes from D. spicata and D. compressa were very similar, and fungi isolated from the two species were cross-compatible in inoculation trials. Furthermore, the grass

morphology is similar, and the two species tend to hybridize in areas where they are sympatric [Leuchtmann and Clay 1989b].

Based on the isozyme evidence, it may be possible to form conjectures on which of the grass-fungus combinations in the current experiment might have been successful. With the evidence of variation in compatability, it is perhaps less surprising that infection did not occur in most cases. Despite the similarity in genus, the multilocus variation among isolates may explain the inability of Acremonium coenophialum from Poa autumnalis to infect Kentucky bluegrass. However, in accordance with trials by Leuchtmann and Clay [1991], it might be reasonable to expect infection of 'Monarch' tall fescue with some of the isolates due to less genetic variation in this cultivar.

In order to better understand the relationship of endophyte and host and to be able to produce marketable EI plants artificially, it is necessary to devise experiments to pinpoint some of the characteristics that affect the ability of the two organisms to form an association. Reciprocal inoculations of different strains and species elucidate the genetic limits of the relationship and can be backed by isozyme studies. But what are some of the factors that affect reciprocity of the relationship? Mycelium from Acremonium coenophialum and other endophytes grew well on all callus including from Kentucky bluegrass. That all callus was a suitable substrate for all types of fungus was also observed by Johnson and Siegal [1986]. Histological studies might reveal differences in growth of endophyte through callus of compatible and incompatible grasses. Ferguson and Rice [1990] examined growth of endophyte colonies growing on media supplemented with extracts from

various sources of tall fescue. While growth was affected by extract source, it was unrelated to the endophytic or non-endophytic status of the plant.

The overall goals of inoculation studies are basically twofold: 1) To provide basic research for an understanding of the biological relationship between grass and fungus and; 2) To develop reliable techniques for artificial inoculation in an effort to produce new grass-fungus combinations that capitalize on the advantages conferred by the relationship. Inoculation with strains of endophyte isolated from wildtype species will further increase the diversity of the germplasm for the breeding of enhanced endophytic cultivars suitable for use in IPM of turfgrass.

CHAPTER IV

GROWTH STUDIES

Introduction

The use of turfgrasses infected with fungal endophytes has increased in recent years [Funk et al. 1989; Saha and Johnson-Cicalese 1987]. In general, these species and cultivars have been shown to deter a variety of foliar-feeding insects effectively and to be tolerant to a high degree of environmental stresses [Arachevaleta et al. 1989; Siegel et al. 1989]. The particular agronomic characteristics and relative toxicity are, however, dependent upon the specific endophyte-grass combination. In-vitro studies of Acremonium coenophialum, Acremonium lolii, and other endophytic fungi isolated from different cultivars of tall fescue, and perennial ryegrass, respectively, have revealed differences in morphology, growth rate, and physiology [Christensen and Latch 1991; Christensen et al. 1991]. The results of these studies have yielded a greater understanding of the role of the endophyte in the mutualistic relationship. The information may also be useful in inoculation studies for producing new endophytic varieties with lower toxicities and higher beneficial qualities.

The non-choke-producing endophyte of tall fescue (Festuca arundinacea Schreber) was identified by Morgan-Jones and Gams [1982] as Acremonium coenophialum. This anamorph of Epichloe typhina was later described by White and Cole [1985] as having white, cottony mycelium growing very slowly on solid medium. The vegetative hyphae are smooth and hyaline with few solitary phialides arising from aerial hyphae. The

ability to isolate and maintain endophytic fungi in culture has enabled study of factors such as alkaloid production, and antibiosis of plant pathogens which directly affect the usefulness of endophye-infected (EI) grasses [Christensen and Latch 1991; Christensen et al. 1991; Siegel and Latch 1991].

The objectives of this preliminary study were to examine the growth characteristics of A.coenophialum isolated from Poa autumnalis compared to tall fescue isolates of A. coenophialum.

Materials and Methods

Fungal Isolation

A. coenophialum was isolated from leaf sheath or seed of P. autumnalis and from various cultivars of tall fescue. Table 4.1 (p.83) lists the source, the explant, and age of culture for each experiment. The methods of endophyte isolation were as follows.

Isolation from Leaf Sheath

Leaf sheaths were removed from plants maintained in the greenhouse. Tissue was surface-sterilized for 5 minutes in full strength sodium hypochlorite plus a few drops of Tween 20. Sheaths were then rinsed with sterile distilled water, teased apart, and placed on corn meal malt agar (CMM) [Bacon, 1988].

Isolation from Seed

Seeds were deglumed with 50% sulfuric acid for 15 minutes and rinsed with distilled water. They were then surface-sterilized with 50% sodium hypochlorite for 20 minutes, rinsed with sterile distilled water and placed on CMM agar in petri dishes.

Media

Corn Meal Malt (CMM): 17g Cornmeal agar (Difco 0386-01-3), 20g Malt extract (Sigma M-0383), 2g Yeast extract (Sigma Y-0500) in 1000 ml. distilled water (Bacon, 1990).

Potato Dextrose Agar (PDA): (Difco 0013-01-4)

M102: 30g sucrose, 20g malt extract, 2g bacto peptone (Difco), 1g yeast extract, .5g KCl, .5g $MgSo_4$, 1g KH_2PO_4 in 1000 ml. distilled water [Bacon, 1990].

Potato Dextrose Broth (PDB): (Difco 0549-01-7)

Growth of Endophyte

In all cases, a 4mm. cork borer was used to remove plugs of A. coenophialum from the actively growing outer edges of the cultures. Fungal plugs were then placed on solid or liquid media as described below.

Growth on CMM (Experiment 1)

Plugs of endophyte isolated from 'Titan', 'M3E,' 'Rebel II', 'Shenandoah', and 'Guardian' tall fescues or from P. autumnalis were placed fungus-side down on CMM in a single petri dish (Table 4.1). There were six repetitions with all isolates arranged in the same manner on the plates. Radial growth was measured in millimeters, as the average of the widest and narrowest diameter of each culture. Growth was measured at one week after initiation and at approximately 3-day intervals for the following 54 days.

Growth on CMM (Experiment 2)

Experiment 2 was the same as experiment 1 with the following exceptions (Table 4.1): 1) Several of the isolates were from different sources; 2) The age of cultures was, in some cases, older; and 3) Plugs were placed fungus-side up. Measurements were taken at 1 week and about every 3 days thereafter for 34 days.

Growth on CMM or PDA (Experiment 3)

A plug of endophyte from P. autumnalis, 'Titan', and 'Rebel II' was placed in a single petri dish containing either CMM or PDA (Table 4.1). There were five replicate dishes for each media. Radial growth was measured at 1 week and every 4 days thereafter for 19 days.

Growth in M102 (Experiment 4)

Three fungal cores from cultures isolated from either P. autumnalis or 'Titan' (Table 4.1) were placed in 250ml. ehrlenmeyer flasks containing 50ml. of M102. There were three replicates for each treatment. Cultures were maintained at room temperature on a gyratory shaker at approximately 200 rpm [Bacon 1988]. After 12 days, all media was filtered through a Buchner funnel using a 250 ml. flask with a vacuum attachment. Total mycelium was collected on filter paper and weighed. Mycelium was then dried under lights and reweighed.

Growth in M102 or PDB (Experiment 5)

Three cores of A. coenophialum from P.autumnalis or 'Titan' (Table 4.1) were placed in 250 ml. ehrlenmeyer flasks with 50 mls. of M102 or PDB and were maintained as above. Wet and dry mycelial weights were taken at day 12 as in the previous experiment.

Table 4.1. Host plant, tissue source, and age of culture for isolates of *Acremonium coenophialum*.

Experiment	Grass	Tissue	Age
1	*P. autumnalis*	sheath	6 weeks
	'Titan' tall fescue	sheath	12 weeks
	'M3E' tall fescue	sheath	7 weeks
	'Rebel II' tall fescue	seed	12 weeks
	'Shenandoah' tall fescue	seed	12 weeks
	'Guardian' tall fescue	seed	12 weeks
2	*P. autumnalis*	sheath	8 weeks
	'Titan' tall fescue	sheath	11 weeks
	'M3E' tall fescue	sheath	14 weeks
	'Shenandoah' tall fescue	seed	14 weeks
	'Guardian' tall fescue	seed	14 weeks
	'FL' tall fescue	sheath	8 weeks
	'Tribute' tall fescue	seed	14 weeks
3	*P. autumnalis*	sheath	9 weeks
	'Titan' tall fescue	seed	9 weeks
	'Rebel II' tall fescue	seed	6 months
4	*P. autumnalis*	sheath	12 weeks
	'Titan' tall fescue	seed	12 weeks
5	*P. autumnalis*	sheath	9 weeks
	'Titan' tall fescue	seed	9 weeks
	'Rebel II' tall fescue	seed	6 months

Statistical Analysis

Differences in radial growth between the isolates grown on solid media were analyzed using SAS General Linear Models Repeated Measures procedure. Duncan's multiple range test was used to compare means for the isolates. Contrasts were used to compare fungi and media in experiment 3. Due to a lack of homogeneity of variance, differences in the liquid media experiment #5 were evaluated with the Welch and with the Brown-Forsythe ANOVA on BMDP P7D.

Results

Isolation of Endophyte

From Leaf Sheaths

Aerial mycelium extended from the cut, teased ends of the pieces of leaf sheath tissue. For P. autumnalis, endophyte became visible in about 2 weeks while cultures initiated in about 5 weeks for tall fescue cultivars.

From Seed

For all sources, mycelium was not evident until about 6-8 weeks after initiation when it visibly arose from the junction between the seed and coleoptile .

Appearance

All cultures of A. coenophialum were white. In most of the cultures from tall fescue, aerial mycelium remained fairly sparse and spread in a relatively uniform, radial manner. Mycelium was not smooth along the surface of the agar but produced a hill-and-valley formation as it radiated outward. In contrast, P. autumnalis isolates had aerial mycelium which was denser, giving a more cottony appearance. Growth was less uniform resulting in an irregularly shaped colony. Mycelium at the edges of all cultures was faint and seemed to lie under the agar. A yellowish band often developed around the edges of the older tall fescue colonies.

In liquid culture, mycelium produced tightly formed, white balls. For cultures of the P. autumnalis isolate, in M102 or PDB, growth occurred by the accumulation of mycelium on the initial core producing three large fungal balls. In contrast, cultures of the 'Titan' isolate

on either media consisted of many small balls of mycelium derived from the original cores.

Growth of Endophyte

Growth on CMM (Experiment 1)

Figure 4.1 shows growth of the isolates of A. coenophialum. Overall, fungal growth was significantly different ($p=.01$) (Table A.15). The results of Duncan's multiple range test revealed that for each time point, A. coenophialum isolated from P. autumnalis was greater than for any other isolate. The overall growth rate of the isolate was .45 mm/dy. While the heirarchical order of growth of A. coenophialum from other sources varied at each time point, they were not significantly different from each other. Average growth rate of the tall fescue isolates was .33mm/dy.

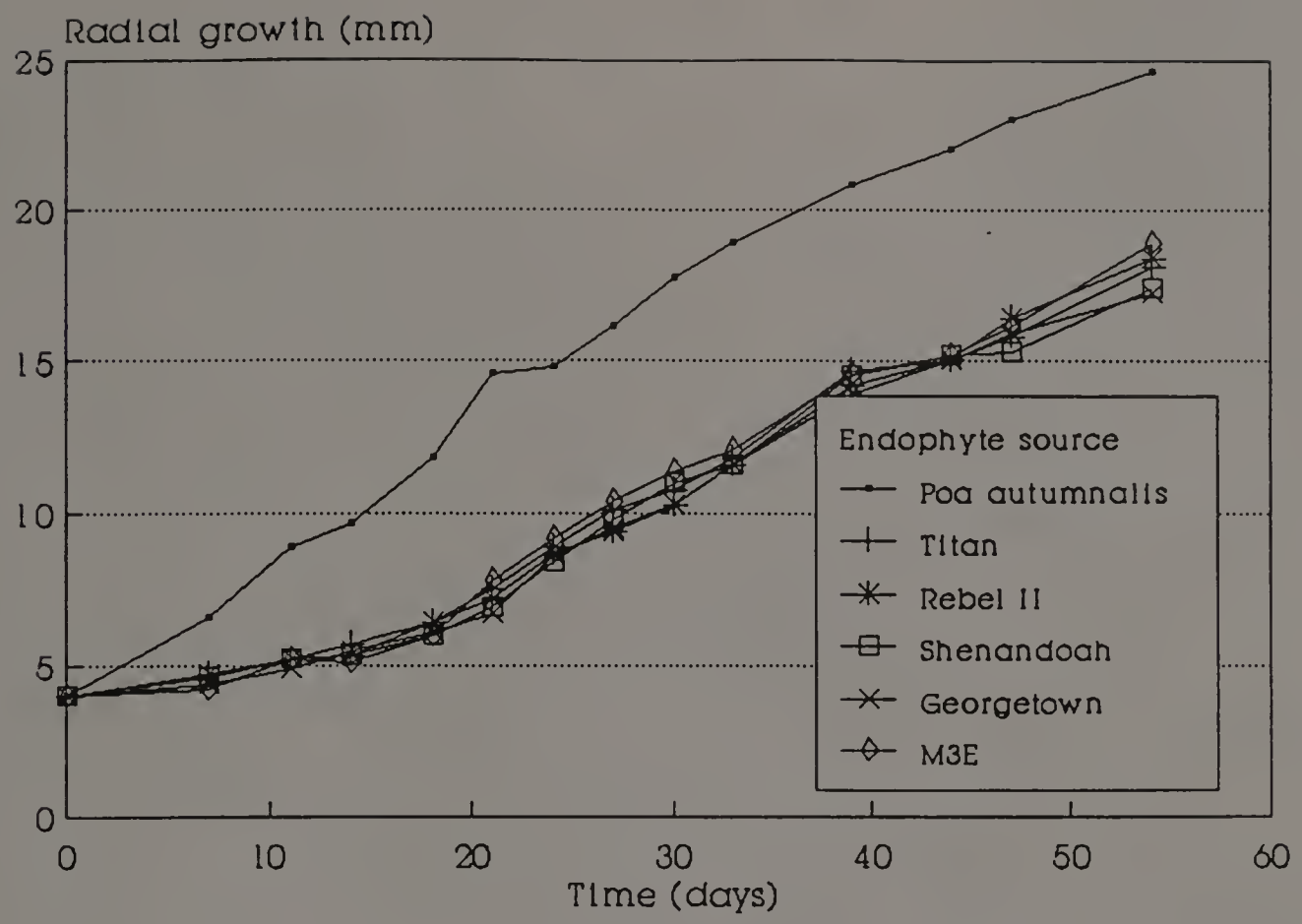


Figure 4.1. Growth of isolates of Acremonium coenophialum on CMM in experiment 1. The isolate from Poa autumnalis is significantly different from tall fescue isolates (p=.01).

Growth on CMM (Experiment 2)

Growth of A. coenophialum in experiment 2 is shown in figure 4.2. Results are similar to those in experiment 1 where a significant difference between isolates (p=.01) (Table A.16) was determined to be due to the faster growth rate of the P. autumnalis isolate (.59 mm/dy) over that of the other isolates (average .43mm/dy).

Growth on CMM or PDA (Experiment 3)

The results of the analysis yielded an interaction between fungi and media (Table A.17). As seen in figure 4.3a, mean growth of endophyte from P. autumnalis, 'Rebel II', and 'Titan' did not differ on PDA (average .62mm/day). On CMM, however, (Figure 4.3b) growth rate of the isolate from P.autumnalis (.84mm/dy) was significantly greater than that of 'Rebel II' and of 'Titan' where the average growth rate was .47mm/dy. Figures 4.4a-c show growth of each isolate on PDA or CMM. Mean growth of the isolate from P. autumnalis was greater on CMM than on PDA. Growth was significantly less on CMM than on PDA for A. coenophialum from 'Rebel II' and from 'Titan' (Table A.18).

Growth in M102 (Experiment 4)

As mentioned previously, cultures from P. autumnalis and 'Titan' appeared different in liquid culture. However, according to statistical analysis, there were no differences in rate of growth (Table A.19). Growth rate based on dry weights was .01g/dy for the P. autumnalis isolate and .007g/dy for the isolate of 'Titan.'

Growth in M102 or PDB (Experiment 5)

Figure 4.5 shows growth of the two isolates on M102 and PDB. Isolates from both sources grew better in M102. Growth rates were .01g/dy and .001g/dy for the endophyte from P. autumnalis on M102 and PDB, respectively and were .003g/dy and .001g/dy for the 'Titan' endophyte. While there appear to be differences in growth within media, due to high and unequal variances, it was not possible to reject the hypothesis that growth was the same for the isolates (Table A.20).

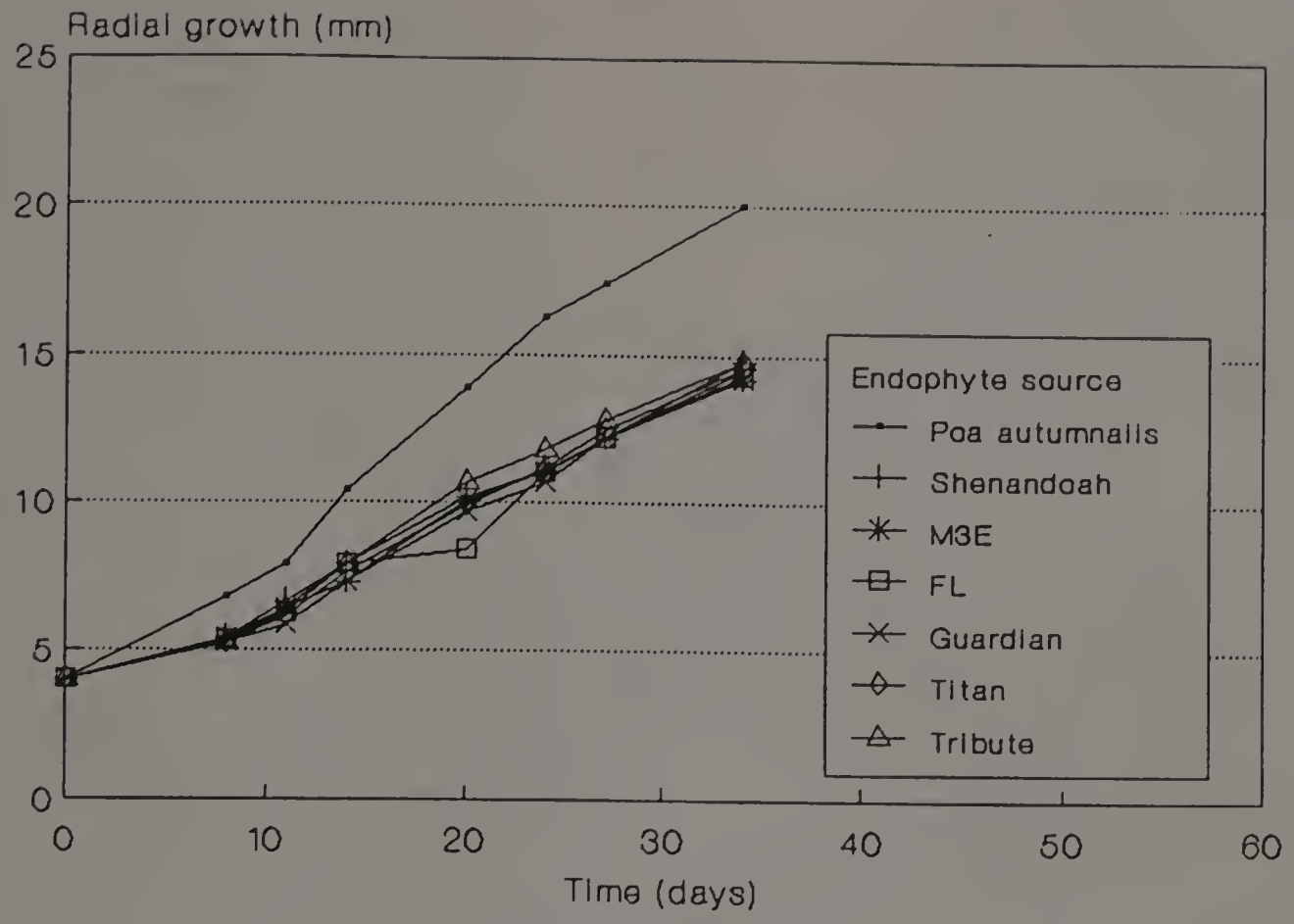


Figure 4.2. Growth of isolates of Acremonium coenophialum on CMM in experiment 2. The isolate from Poa autumnalis is significantly different from tall fescue isolates (p=.01).

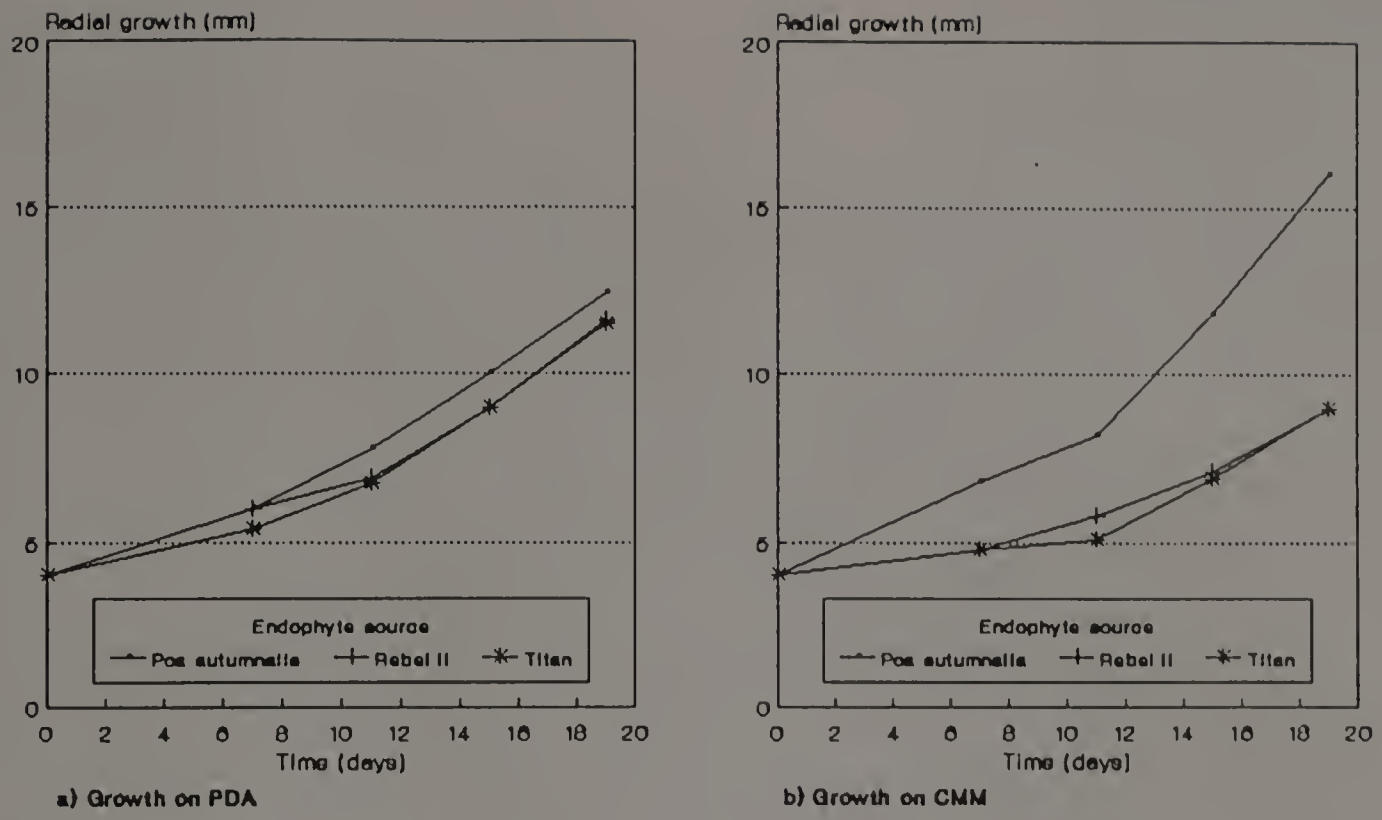


Figure 4.3. Growth of isolates of Acremonium coenophialum on PDA or CMM in experiment 3. Means are averaged from 5 replicates. a) Growth of isolates on PDA. Differences are not significant. b) Growth of isolates on CMM. Growth of the Poa autumnalis isolate differs from growth of isolates from tall fescue cultivars (p=.05).

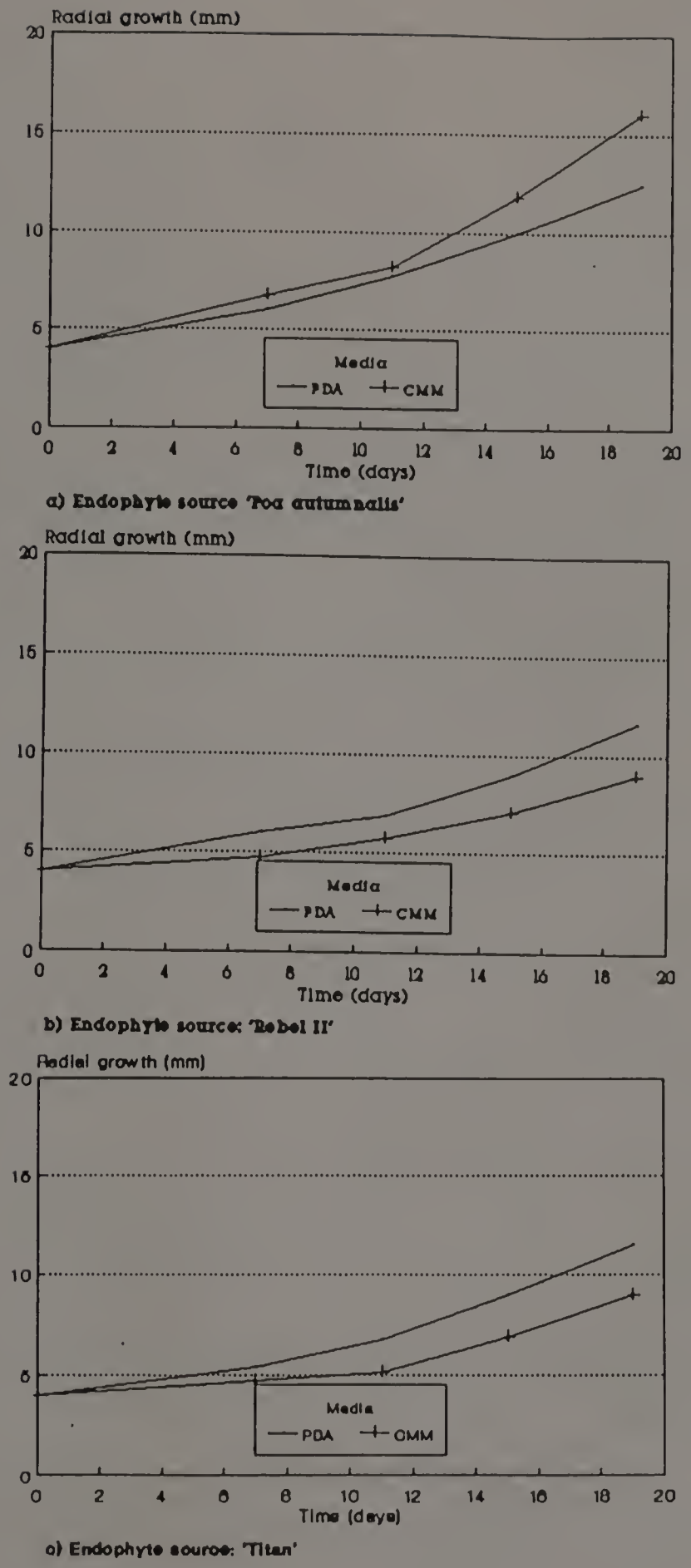


Figure 4.4. Growth of individual Acremonium coenophialum isolates on PDA or CMM in experiment 3. a) Growth of Poa autumnalis isolate on CMM or PDA. b) Growth of 'Rebel II' isolate on CMM or PDA. c) Growth of 'Titan' isolate on CMM or PDA.

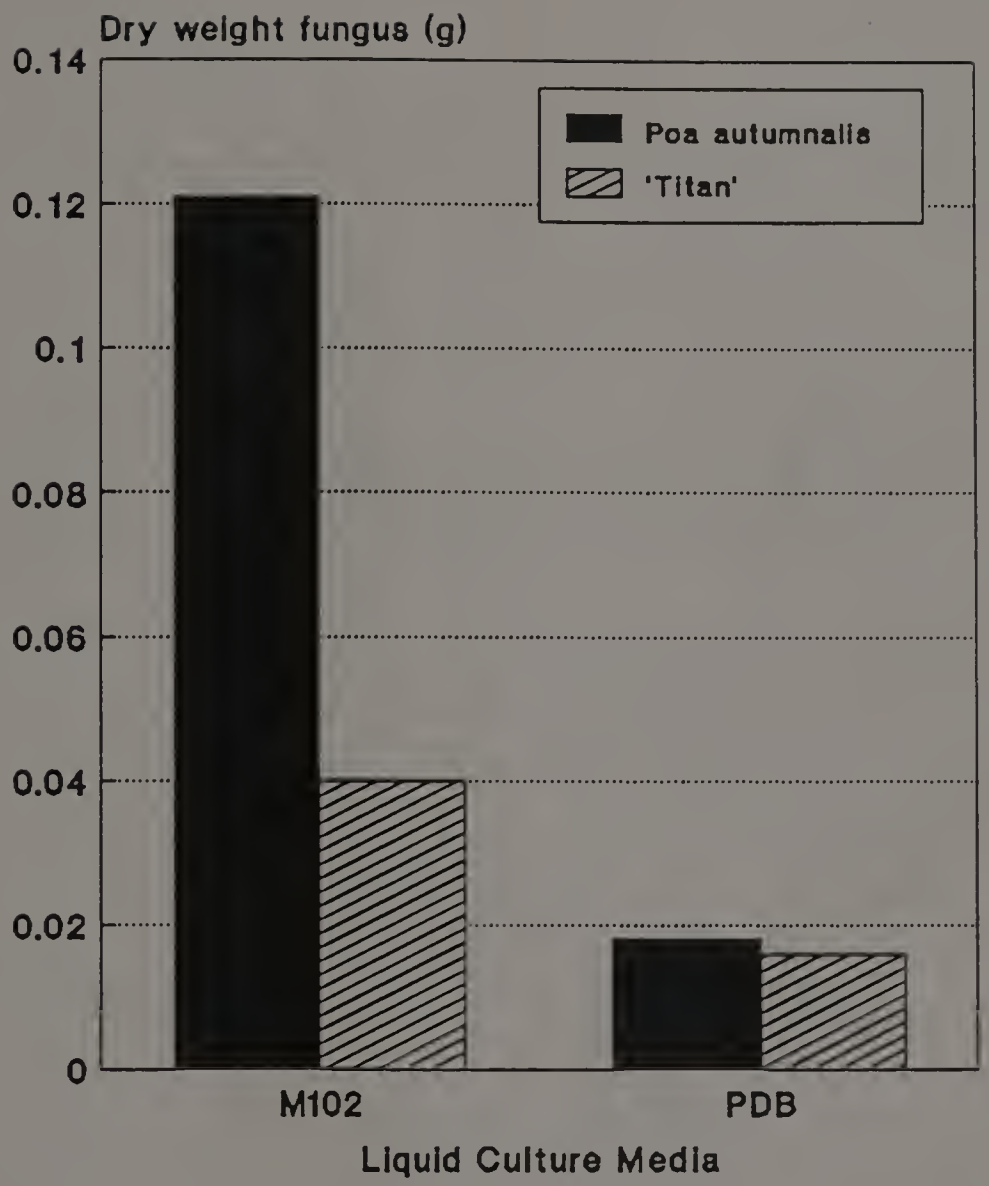


Figure 4.5. Growth of Acremonium coenophialum isolates in M102 or PDB. Growth is different between (p=.05)) but not within medias.

Discussion

Consistent with the conclusions of other researchers [Christensen and Latch 1991; Christensen et al.1991], this study shows differences in morphology and growth rates of A. coenophialum isolated from different sources. In particular, it verifies the observation that cultures initiated from the sheath of P. autumnalis grew faster than did A. coenophialum from a number of tall fescue cultivars. Further in-vitro work should focus on characterisation of the Acremonium endophyte from P. autumnalis with respect to alkaloid production and antifungal activity that would prove agronomically beneficial.

The amount of time for isolation of A. coenophialum depends on the density and location of the hyphae in the plant tissue and on the endophyte species [Bacon 1990]. Christensen and Latch [1991] reported that mycelium extended from leaf sheaths of the tall fescue cultivars within 3-7 days. Results from the present study are more consistant with those reported by Bacon [1988] where isolation time of A. coenophialum from leaf sheaths on CMM was 3-5 weeks. In his chapter on endophyte isolation and culture, Bacon [1990] states that, in general, cultures of A. coenophialum can be obtained from leaf sheath on solid media in 4-6 weeks and from seed in 3-9 weeks. Isolation is more rapid on liquid media.

The appearance of the cultures was similar to the description of the tall fescue endophyte by White and Cole [1985] as well as observations by Christensen and Latch [1991]. No attempt was made in this study to examine conidial characteristics. In their study of isolates of A. coenophialum from various tall fescue sources,

Christensen and Latch found most to produce conidia with lengths of 4.5-15um. A. coenophialum is classified as having conidial lengths of 6.5-13um [White and Morgan-Jones 1987]. Christensen and Latch thus concluded that while some of the isolates were members of the taxon A. coenophialum, those with conidial lengths of 3.5-9um may be non-choke inducing strains of A. typhinum. Those with shorter conidia tended to grow more slowly. It would be interesting to compare conidial length of A. coenophialum from P. autumnalis with length of conidia of the tall fescue isolates.

Christensen et al. [1991] classified growth rates of A.lolii on PDA according to the following criteria: Slow = 0.15mm/day, Moderate = .15-.3mm/day, Fast = .3mm/day. Growth of all isolates of A. coenophialum in the present study would thus be considered rapid. Out of 18 isolates from tall fescues collected worldwide, Christensen and Latch [1991] found that 55% fit into the category of fast growers, 28% grew moderately, and 17% were slow growing [but may be a non-choke producing strain of A. typhinum other than A. coenophialum). Thus, isolates in the present study tended to grow rapidly even as compared with other isolates of A. coenophialum. The range of growth rates overall was an average of .33mm/day in experiment 1 for the tall fescue isolates to .84mm/day for the P. autumnalis endophyte on CMM in experiment 3. In general, there was little lag time and growth seemed to increase fairly steadily (Figures 4.1-4.2). Growth had not yet levelled off even at day 54 in experiment 1 although increase was not as rapid as earlier on the curve. In accordance with the original

hypothesis, endophyte from P. autumnalis grew faster than from the tall fescues, all of which grew comparably to each other.

Comparison of results across the experiments may reflect the stability of the endophyte in culture. Replicates in the experiments were often taken from different petri plates containing cultures that had been initiated on the same date. That the replicates did not prove significantly different in the individual analyses may suggest a relative consistency in growth within each isolate, at least when initiated at a given time.

Research has shown that endophyte character may change with time in culture [Bacon 1990]. The P. autumnalis isolate used in experiment 2 was from the same original cultures as those used in experiment 1. In comparing overall growth rates for the two experiments, the older culture grew somewhat faster (.59mm/day) than the younger culture (.45mm/day). Because of the changes in rate along the growth curve, however, it is perhaps fairer to compare rates in the experiments at a given time point. At day 34 in experiment 1 and 2, growth rate of the endophyte from P. autumnalis was .57 and .59mm/day, respectively, revealing equal growth despite age differences. Contrasting all three experiments, at day 19 growth rates of isolates of P. autumnalis on CMM were .84, .34, and .49 mm/day for the 9-week old, 6-week old and 8-week old cultures. Only with a significant number of replicates varying in age might it be possible to determine if there was a true normal growth curve for an isolate and how that might be affected by age of culture. It seems unlikely, however, that it would be possible to predict growth rate at a given time even for a given age of culture.

Bacon [1990] suggests that once in culture, growth rate may increase due to nutritional selection by the fungus. There is little evidence of this occurring in the present study. The 6-month old cultures of 'Rebel II' used in experiment 3 grew comparably to the younger cultures of 'Titan' on both PDA and CMM and to the culture of 'Rebel II' in Experiments 1 and 2. Endophyte from P. autumnalis grew similarly in experiments 1 and 2. Bacon [1990] also warns of physiological and growth-related changes after subculturing. In this work none of the isolates had been subcultured prior to the start of each experiment.

Experiment 3 showed growth to be dependent upon media and fungi. There was no difference in growth of the P. autumnalis or tall fescue isolates on PDA. In contrast, endophyte from P. autumnalis grew better and tall fescue endophytes grew worse on CMM. Bacon [1990] suggests that media used for initial isolation of endophyte may need to be more complex than that used for subsequent maintenance yet recommends CMM or PDA for both isolation and culture of any of the Acremonium endophytes. He does not, however, make comparisons of culture in the two media.

In the past, A. coenophialum has been isolated and grown on a variety of medias. Welty [1986] compared growth of three tall fescue isolates and two isolates of perennial ryegrass on 10 medias. Mycelial growth was slowest on water agar and Cz-apex-Dex agar and most rapid on Bran Malt agar or PDA. Various other formulations of malt extract agar, nutrient agar, and yeast morphology agar also supported growth of the endophyte. Kulkarni and Nielson [1986] determined some of the nutritional requirements of A. coenophialum in culture: Carbon sources

included fructose, glucose, mannose, sucrose, trehalose, raffinose, sorbitol, and mannitol. Endophyte utilized ammonium more efficiently than nitrates. Nitrogen sources included amino acids such as arginine, aspargine, cysteine, glutamine, proline, and serine which influenced growth rates, pigment production, and indole and ergot alkaloid biosynthesis. Yeast extract or vitamins such as thiamine, are also considered necessary for growth. Both CMM and PDA should contain the basic nutrients to sustain adequate growth of fungi. CMM may, however, contain a greater complexity of carbon sources than PDA and also contains yeast extract. On the basis of nutrition, it would be expected that all isolates would grow better on the more enriched media.

It is also interesting to consider growth of Acremonium on different medias with respect to the nutrient relationship of endophyte and host grass. Kulkarni and Nielson [1986] note that many of the soluble carbohydrate sources as well as the amino acids present in the plant are those that are necessary to sustain growth of the endophyte. The researchers propose that the nutritional requirements of the fungi may thus be an evolutionary adaptation for their endophytic existence. In the present study, however, it is impossible to make inferences based on differences in nutrient requirements of the isolates. It seems unlikely that differences in growth of the isolates is correlated with differences in physiological and nutritional needs of A. coenophialum from varied sources. However, perhaps repeated studies measuring growth of various endophyte isolates on different medias would have some relation to the evolution of cross compatibility of specific endophyte-grass associations.

Isolation is faster and mycelial yields are greater for endophyte cultures in liquid media [Bacon 1990]. Liquid culture is thus advantageous for determination of fungal characteristics prior to changes associated with time in culture. For measurement of growth, however, the liquid cultures proved less accurate. Wet weights were highly variable. Dry weights were used by Welty [1986] and by Bacon [1985] and, as such, were the measurements reported in Figure 4.5. In contrast to results on solid media, liquid culture of all isolates grew better on the more chemically-defined M102 versus PDB. Graphically, growth of the P. autumnalis endophyte on M102 appears greater than growth of the 'Titan' endophyte (Figure 4.5). However, due to high standard deviations, differences could not be detected even in an analysis that did not assume variance to be equal. Although initial diameter of the inoculated cores was 4mm., the amount of mycelium, by weight, may not have been consistant for each replicate. In the present study, mycelium was weighed at only one time point. With measurements of a number of replicates at regular intervals, however, growth curves could be established from liquid cultures as those on solid media.

As mentioned previously, this study was a preliminary attempt to examine differences in growth of A. coenophialum isolated from different sources. In order to design an in-vitro experiment to more fully compare various physiological and morphological charactersitics of the endophyte, it is necessary to be consistent with such factors as source of plant tissue, time in culture, and number of subculture events. Isolates from seed versus sheath should be characterized individually and followed over time for changes in the initial culture and in

subcultures. Identical studies should be performed using a variety of media. In this experiment the different isolates were placed in the same dish. In order to remove chances of interaction between isolates, experiments should be conducted with a single isolate in each petri dish. Bacon [1990] suggests that most of the nutritional requirements have been determined from subcultured endophyte and there is a need for further work based on freshly isolated fungi. In this approach, the fungi is more likely to have retained the characteristics present in its endophytic status in the grass. There is also a need for more complete studies, such as those exemplified by Christensen and Latch [1991] and Christensen et al. [1991], for examination of agronomically-related traits such as alkaloid production and in vitro antibiosis of plant pathogens.

The present study as well as others [Christensen and Latch 1991] have documented, yet not explained, in vitro differences in characteristics of A. coenophialum isolated from a variety of grass sources. Christensen et al. [1991] postulate that the mechanism of variation may relate to a heterokaryotic behavior of the cells resulting in the production of different ratios of nuclear types, such as occurs in Claviceps. A number of studies have also documented isozyme variation [Leuchtmann and Clay 1990] which may affect coding for enzymes responsible for a certain characteristic. While it is essential to study fungal endophytes of grasses in vitro, it is necessary to be continuously mindful that differences may not exist in the interaction with the host. Bacon [1990] recommends choosing isolates on the basis of particular in vivo characteristics and then determining if the trait

exists and is upheld *in vitro* during culture. Variation in isolates makes it difficult to define the characteristics of the species [Christensen et al. 1991]. At the same time, variation may be advantageous for the selection of specific natural and artificial endophyte-grass combinations that will increase the success of endophytic grasses for use in IPM of turf.

APPENDIX A

ANALYSIS OF VARIANCE TABLES FOR FIELD SEASON 1990

Table A.1. Results of ANOVA for recovery of larvae from grass cores in the 'Single Density Experiment'.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Grass	12	1600.00	133.33	0.82	.6300
Error	51	8300.00	162.75		
Corrected total	63	9900.00		R-square=	.162

Table A.2. Results of ANOVA for recovery of larvae from grass cores in the 'Mixtures Experiment'.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Grass	7	969.38	138.48		.4491
Error	32	4430.00	138.44		
Corrected total	39	5399.38		R-square=	.179

Table A.3. Results of Welch and Brown-Forsythe Analysis of Variance for number of larvae recovered from field plots 8 weeks after implantation.

Source	df	F Value	Pr > F
Welch	29, 40	8.88	0.000
Brown-Forsythe			
Grass	4, 55	1.44	.2346
Density	5, 55	20.19	.0000
Grass*Density	20, 55	0.40	.9862

Table A.4. Results of Welch and Brown-Forsythe Analysis of Variance for number of third instar larvae recovered from field plots 8 weeks after implantation.

Source	df	F Value	Pr > F
Welch	29, 40	4.07	0.000
Brown-Forsythe			
Grass	4, 54	0.86	.4956
Density	5, 5	14.25	.0000
Grass*Density	20, 54	0.67	.8393

Table A.5. Results of Welch and Brown-Forsythe Analysis of Variance for weight of third instar larvae recovered from field plots 8 weeks after implantation.

Source	df	F Value	Pr > F
Welch	29, 26	1.77	0.0720
Brown-Forsythe			
Grass	4, 35	0.01	.9999
Density	5, 33	0.01	1.000
Grass*Density	20, 33	0.00	1.000

Table A.6. Results of Welch and Brown-Forsythe Analysis of Variance for root weight of cores of grasses from field plots, 8 weeks after implantation of larvae.

Source	df	F Value	Pr > F
Welch	29, 40	2.74	.0017
Brown-Forsythe			
Grass	4, 5	0.43	.7824
Density	5, 5	1.60	.3099
Grass*Density	20, 5	0.78	.6908

Table A.7. Results of Welch and Brown-Forsythe Analysis of Variance for shoot weight of cores of grasses from field plots, 8 weeks after implantation of larvae.

Source	df	F Value	Pr > F
Welch	29, 40	3.97	0.000
Brown-Forsythe			
Grass	4, 65	14.44	.0000
Density	5, 66	0.99	.4301
Grass*Density	20, 65	0.61	.8918

APPENDIX B

B. ANALYSIS OF VARIANCE TABLES FOR FIELD SEASON 1991

Table A.8. Results of Analysis of Variance for number of larvae recovered 7 weeks after implantation into potted grasses in the field.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Den	2	292.98	146.49	36.38	0.0001
Grass	2	124.27	62.14	15.43	0.0001
W [a]	1	19.55	19.55	4.86	0.0289
Den*Grass	4	34.60	8.65	2.15	0.0772
Den*W	2	.448	.224	0.06	0.9459
Grass*W	2	1.49	.744	0.18	0.8315
Den*Grass*W	4	19.01	4.75	1.18	0.3215
Error	162	652.24	4.03		
Corrected Total	179	1144.59		R-Square	0.4301

[a] 'W' represents the moisture treatment.

Table A.9. Results of Analysis of Variance for weight of larvae recovered 7 weeks after implantation into potted grasses in the field.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Den	2	.0047	.0024	3.23	0.0421
Grass	2	.0156	.0078	10.59	0.0001
W [a]	1	.0007	.0007	0.97	0.3264
Den*Grass	4	.0034	.0009	1.16	0.3287
Den*W	2	.0000	.0000	0.06	0.9427
Grass*W	2	.0028	.0014	1.92	0.1500
Den*Grass*W	4	.0002	.0000	0.09	0.9862
Error	154	.1139	.0007		
Corrected Total	171	.14179		R-Square	0.1961

[a] 'W' represents the moisture treatment

Table A.10. Results of Analysis of Variance for cumulative clipping yields from potted grasses in the field for weeks a) 1-4 and b) 5-10, prior to and after implantation of larvae.

a) Weeks 1-4.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Grass	2	18.22	9.11	80.71	0.0001
W [a]	1	.0281	.0281	.25	0.6181
Grass*W	2	.2235	.1118	0.99	0.3731
Error	230	25.96	.1129		
Corrected Total	235	44.44		R-Square	0.4160

[a] 'W' represents the moisture treatment

b) Weeks 5-10.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Den	3	8.25	2.75	5.11	0.0020
Grass	2	128.53	69.26	128.61	0.0001
W [a]	1	.0546	.0546	.10	0.7504
Den*Grass	6	1.82	.303	0.56	0.7599
Den*W	3	.4580	.153	0.28	0.8373
Grass*W	2	2.32	1.16	2.16	0.1180
Den*Grass*W	4	1.67	.279	0.52	0.7943
Error	213	114.71	.538		
Corrected Total	236	267.82		R-Square	0.5717

[a] 'W' represents the moisture treatment

Table A.11. Results of Analysis of Variance for root weights from grasses at the end of the field season, 7 weeks after implantation of larvae.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Den	3	560.50	186.83	5.47	0.0012
Grass	2	7699.58	3849.79	112.73	0.0001
W [a]	1	1.53	1.53	0.04	0.8325
Den*Grass	6	154.54	25.76	0.75	0.6068
Den*W	3	103.10	34.36	1.01	0.3910
Grass*W	2	51.07	25.53	0.75	0.4748
Den*Grass*W	6	178.74	29.79	0.87	0.5161
Error	202	6898.45	34.15		
Corrected Total	225	15647.53		R-Square	0.5591

[a] 'W' represents the moisture treatment

Table A.12. Results of Analysis of Variance for shoot weights from grasses at the end of the field season, 7 weeks after implantation of larvae.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Den	3	424.10	141.37	4.49	0.0044
Grass	2	30.12	15.09	0.48	0.6203
W [a]	1	88.82	88.82	2.82	0.0944
Den*Grass	6	193.01	32.17	1.02	0.4116
Den*W	3	81.61	27.20	0.86	0.4603
Grass*W	2	95.48	47.74	1.52	0.2217
Den*Grass*W	6	260.53	43.42	1.38	0.2239
Error	209	6575.13	31.46		
Corrected Total	232	7748.81		R-Square	0.1514

[a] 'W' represents the moisture treatment

Table A.13. Results of Analysis of Variance for number of larvae recovered from 1' X 3' sections of grasses in the natural infestation experiment.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Grass	14	668.63	47.76	1.57	0.1186
Rep	4	1270.77	317.69	10.46	0.0001
Error	53	1610.38	30.38		
Corrected total	71	3549.78		R-Square	.188

Table A.14. Repeated Measures Analysis of Variance for clipping yields from potted grasses in the field for weeks a) 1-4 and b) 5-10, prior to and after implantation of larvae.

a) Weeks 1-4.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Between Subjects Effects					
Block	9	.7086	.0787	3.01	.0021
Grass	2	4.538	2.269	86.73	.0001
W [a]	1	.0078	.0078	0.30	.5868
Grass*W	2	.0552	.0276	1.06	.3496
Error	221	5.782	.0262		
Within Subjects Effects					
Time	3	1.496	.4987	66.57	.0001
Time*Rep	27	.9729	.0362	4.84	.0001
Time*Grass	6	2.149	.3581	47.80	.0001
Time*W	3	.0129	.0043	0.58	.6312
Time*Grass*W	6	.0091	.0015	0.20	.9755
Error (Time)	663	4.468	.0074		

[a] 'W' represents the moisture treatment

Continued next page

Table A.14 continued

b) Weeks 5-10.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Between Subjects Effects					
Block	9	1.322	.1470	1.68	.0944
Den	3	1.383	.4611	5.29	.0016
Grass	2	22.85	11.43	130.99	.0001
W [a]	1	.0113	.0113	0.13	.7190
Grass*Den	6	.297	.0495	0.57	.7557
W*Den	3	.0789	.0263	0.30	.8244
Grass*W	2	.4102	.2051	2.35	.0978
Grass*W*Den	6	.278	.0463	0.53	.7840
Error	204	17.80	0.087		
Within Subjects Effects					
Time	5	7.077	1.415	193.27	.0001
Time*Rep	45	1.076	.0239	3.26	.0001
Time*Den	15	.3310	.0221	3.01	.0001
Time*Grass	10	.4946	.0495	6.75	.0001
Time*W	5	.0513	.0102	1.40	.2214
Time*Grass*Den	30	.2660	.0089	1.21	.2025
Time*W*Den	15	.3310	.0221	3.01	.0001
Time*Grass*W	10	.0918	.0092	1.25	.2524
Time*Grass*W*Den	30	.1790	.0060	0.81	.7525
Error (Time)	1020	7.470	.0073		

[a] 'W' represents the moisture treatment

APPENDIX C

C. ANALYSIS OF VARIANCE TABLES FOR GROWTH STUDIES

Table A.15. Repeated Measures Analysis for radial growth of fungal isolates on CMM in Experiment 1.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Between Subjects Effects					
Fungi	5	1905.61	381.12	48.61	.0001
Error	25	195.99	7.84		
Within Subject Effects					
Time	12	8098.45	674.87	1194.85	.0001
Time*Fungi	60	148.85	2.48	4.39	.0001
Error (Time)	300	169.44	.564		

Table A.16. Repeated Measures Analysis for radial growth of fungal isolates on CMM in Experiment 2.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Between Subjects Effects					
Fungi	6	343.83	57.30	28.59	.0001
Error	33	66.16	2.00		
Within Subject Effects					
Time	6	3055.36	509.23	665.47	.0001
Time*Fungi	36	80.44	2.23	2.92	.0001
Error (Time)	198	151.51	.765		

Table A.17. Repeated Measures Analysis for radial growth of fungi in over time in CMM or PDA in Experiment 3.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Between Subjects Effects					
Fungi	2	141.40	70.70	31.39	.0001
Media	1	9.26	9.26	4.11	.0562
Fungi*Media	2	65.60	32.80	14.56	.0001
Error	20	45.04	2.25		
Within Subject Effects					
Time	3	535.47	178.49	181.96	.0001
Time*Fungi	6	25.74	4.29	4.37	.0010
Time*Media	3	.731	.244	.25	.8621
Time*Fungi*Media	6	19.71	3.29	3.35	.0065
Error	60	58.85	.981		

Table A.18. Single degree of freedom contrasts for growth of fungi in PDA or CMM for days 7,11,15, and 19 in experiment 3. T='Titan', PA= Poa autumnalis, R= 'Rebel II'.

Day 7

Contrast	df	Contrast SS	Mean Square	F value	Pr > F
PDA vs CMM in PA	1	1.25	1.25	6.35	.0204
PDA vs CMM in T	1	3.47	3.47	17.64	.0004
PDA vs CMM in R	1	.781	.781	3.97	.0602

Day 11

Contrast	df	Contrast SS	Mean Square	F value	Pr > F
PDA vs CMM in PA	1	.450	.450	1.21	.2844
PDA vs CMM in T	1	2.94	2.94	7.90	.0108
PDA vs CMM in R	1	5.28	5.28	14.20	.0012

Day 15

Contrast	df	Contrast SS	Mean Square	F value	Pr > F
PDA vs CMM in PA	1	6.81	6.81	6.44	.0196
PDA vs CMM in T	1	7.81	7.81	7.40	.0132
PDA vs CMM in R	1	9.03	9.03	8.55	.0084

Day 19

Contrast	df	Contrast SS	Mean Square	F value	Pr > F
PDA vs CMM in PA	1	28.8	28.8	8.07	.0101
PDA vs CMM in T	1	15.0	15.0	4.21	.0536
PDA vs CMM in R	1	12.5	12.5	3.50	.0760

Table A.19. Results of Analysis of Variance for dry weight of mycelium from fungal isolates after growth in M102 in Experiment 4.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Fungi	1	.0846	.0846	.30	.6396
Rep	3	.0758	.0253	.09	.9595
Error	2	.0039	.0019		
Corrected total	6	.0075		R-Square	.4789

Table A.20. Results of Welch and Brown-Forsythe Analysis of Variance for dry weight of fungal isolates after growth in M102 or PDB in Experiment 5.

Source	df	F Value	Pr > F
Welch	3, 4	2.61	.1885
Brown-Forsythe			
Media	1, 4	9.14	.0391
Fungi	1, 4	3.84	.1215
Media*Fungi	1, 4	3.44	.1374

APPENDIX D

D. SOIL ANALYSIS FOR RESEARCH PLOTT

Soil Type: Hadley silt loam (coarse, silty, mixed, mesic type, Udifluvent)
Soil pH: 6.0
Buffer pH: 6.6
Cation Exchange Capacity: 10.8 Meq/ 100g
Percent Base Saturation: K=2.5 Mg=15.2 Ca=41.7
Aluminum: 30 ppm

Nutrient Levels

Phosphorous	18 ppm	(medium-high)
Potassium	100 ppm	(medium)
Calcium	853 ppm	(medium-high)
Magnesium	189 ppm	(very high)
Ammonium	6 ppm	(low)
Nitrate	30 ppm	(low)

Micronutrient	ppm	Soil Range
Boron	0.2	0.1-2.0
Manganese	4.7	3 - 20
Zinc	0.7	0.1-70
Copper	1.1	0.3-8.0
Iron	3.9	1.0-40

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