SEASONAL VARIATION AND ENVIRONMENTAL EFFECTS ON ENDOPHYTE TRANSMISSION IN TALL FESCUE

by

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(Under the Direction of Nicholas S.Hill)

ABSTRACT

Tall fescue (*Festuca arundinacea* Schreb.) pastures are infected with a mutualistic toxin-producing endophyte (*Neotyphodium coenophialum).* Non-toxic endophyte-infected cultivars have been developed and a better understanding maternal transmission of endophyte is needed. Field, greenhouse, and growth chamber experiments were used to examine the effects of drought stress and temperature on plant and endophyte growth. Mean monthly endophyte frequency varied from 52 to 98% within field-grown plants, and mean monthly temperature was correlated with endophyte data. Growing plants at different temperature regimes demonstrated that endophyte concentration decreased within plants when temperatures decreased and increased when temperatures increased. Plant mass accumulated faster than endophyte mass at lower temperatures. Cardinal minimum temperature for endophyte growth was 10 °C but was 5 °C for the host plant. There was no water stress effect on endophyte frequency or concentration in tall fescue. Temperature appears to be the major variable affecting endophyte presence in field-grown plants.

INDEX WORDS: Tall fescue, Endophyte, Seasonal variation, Cardinal minimum temperature, Endophyte genotype, Plant genotype, Water stress, Endophyte transmission

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M.S., KonKuk University, Republic of Korea, 1997

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2003

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August 2003

ACKNOWLEDGEMENTS

Many people have contributed to my education through their guidance and support throughout my graduate school years. I would like to thank to all those who assisted me through my degree program. I especially want to express my gratitude to my major professor, Dr. Nicholas S. Hill, who accepted me into his laboratory and supported my scientific endeavors. I appreciate his vast knowledge and his assistance in writing thesis. I also thank toward my committee members, Dr. Joe H. Bouton and Dr. Ron Walcott, for the advice and time given me. To my lab-mates, Brian C. Kittle and Andrea L. Wilson Ayers, thanks for the fun and help. I would also like to thank my family for the support they provided me through my entire life and in particular, I must acknowledge my wife and best friend, Soo-Gyeong Oh, without whose love and encouragement, I would not have finished this thesis.

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CHAPTER 1

LITERATURE REVIEW

Tall fescue

The *Festuca* genus is large and diverse, and includes about 80 species that are adapted to temperate or cool zones (Terrell, 1979). It includes species that range from diploid (2n=2x=14) to decaploid (2n=10x=70) (Berg et al., 1979). The most important and widely used broad-leafed species are meadow fescue (*F. pratenisis* Hubs.) (2n=2x=14), and tall fescue (*F. arundinacea* Schreb.) (2n=6x=42) (Terrell, 1979). It has long been known that tall fescue is closely related to some *Lolium* species (Jenkin, 1933), and nucleic acid matching work using chloroplast DNA supported the claim that (Darbyshire and Warwick, 1992) and *F. pratensis* Huds. was more closely related to *L. perenne* L. than to other *Festuca* species (Xu et al., 1992; Xu and Sleper, 1994). Tall fescue is a typical allohexaploid with three closely related genomes designated as $PPG_1G_1G_2G_2$ synthesized from two progenitor species, *F. pratenisis* Hubs. and *F. arundinacea* var.*glaucescens* Boiss. (Xu et al., 1992; Humphreys and Ghesqière, 1994). The P genome is contributed by *F. pratenisis* Hubs. (diploid) and *F. arundinacea* var.*glaucescens* Boiss. is the donor of G_1G_2 genome (Sleper, 1985; Humphreys et al., 1995).

Western Europe and Northern Africa are the centers of origin of tall fescue. It was introduced from Europe into North and South America (Borrill et al., 1976). Tall fescue has a long history as a valuable forage grass in the USA, and is one of the dominant cool-season perennial grasses of economic importance. It covers over 14 million hectares in the eastern USA (Ball et al., 1996). Although early performance tests during the late 1800's showed tall fescue, to be more drought and cold tolerant, form a dense stand, competitive with weeds, and adapted to a wider range soils than other forager species, tall fescue did not attain prominence until the release of the cultivars, Alta and Kentucky 31 in 1940 and 1943, respectively (Cowan, 1956; Buckner et al., 1979). Kentucky 31 is a tall fescue ecotype that was found in 1931, evaluated at the University of Kentucky, and was released as a cultivar in 1943 (Fergus and Buckner, 1972). Alta is an ecotype selected over number of years beginning in 1918 by the Oregon Agricultural Experiment Station and the Forage and Range section of USDA (Cowan, 1956).

The response of tall fescue to warm temperatures appears to be associated mainly with soil moisture availability. When soil moisture is adequate, tall fescue remains green and continues growth, whereas stand thinning occurs if water stress becomes severe (Sleper and Buckner, 1995). Generally, tall fescue is well adapted to the humid and temperate areas of the U.S. and the world (Burns and Chamblee, 1979). This plant has adapted to a wide range of climatic conditions but grows best in the transition zone that separates the northern and southern regions of the USA (Young III, 1997). However, this cool season perennial has been widely planted in an area from the lower Midwest to eastern Oklahoma and central Georgia and from northern Florida to southern Canada (Burn and Chamblee, 1979). Tall fescue is not grown in the Northeast region of the USA because of alternative forage species with better forage quality and production (Borrill et al. 1976). Tall fescue has been grown in regions of Massachusetts (Zak, 1967). In

Wyoming, tall fescue was more productive, more winter-hardy, and persisted better through the hot summers than orchardgrass (Moyer and Seamands, 1975). Tall fescue grows best on deep, moist soils that are heavy to medium in texture and high in organic matter. It grows on soils that vary from strong acidic (pH 4.7) to alkaline (pH 9.5) (Cowan, 1956). Best growth occurs when soil pH is maintained between 6.5and 8.0 (Wilkinson and Mays, 1979). A minimum precipitation range typically is 15 to 18 inches (375 to 459 mm), although in areas of high evapotranspiration, up to 36 inches (900 mm) may be required (Hannaway et al., 1999).

In the transition zone of the USA, tall fescue is used primarily as pasture and hay for beef cattle, with lesser use by dry dairy cows, replacement heifers, sheep, and horses. Over 8.5 million beef cows and 700,000 horses are maintained on tall fescue pastures in the USA (Hoveland, 1993; Ball et al., 1996). Tall fescue is a long-lived when properly managed. Therefore, this plant is also important for industrial parks, golf courses and athletic fields, re-vegetation of roadsides and disturbed areas, and other recreation or conservation purposes throughout the northern states of the USA (Fribourg et al., 1999).

Endophyte

Various endophytes in the *Neotyphodium* spp. have been reported to infect cool-season grasses (Latch et al., 1984; Lewis and Clements, 1986; Siegel et al., 1987; Christensen et al., 1993; Cunningham et al., 1993; Ravel et al, 1995; Do Valle Ribeiro et al., 1996; Glenn et al., 1996; Oldenburg, 1997; Oliveira et al., 1997; Naffaa et al., 1998; Miles, 1998; Schulthess and Faeth, 1998; Cabral et al., 1999; Fletcher, 1999; Scott, 2001; Vinton et al., 2001; Christensen et al., 2002). The early studies on *Neotyphodium* endophytes were conducted in Europe early in the 20th century where symptomless fescues were found in seed of *Lolium* spp. (Sampson, 1935; White et al, 1993; Schardl, 2001). While Freeman (1903) failed to culture endophyte from *Lolium temulentum*, Neill (1941) successfully cultured endophytes from tall fescue and perennial ryegrass and proposed that they were strains of *Epichloë typhina*. Sampson (1935) reported characteristics of the mycelium and its distribution in the plant. *Clavicipitaceous* fungal endophytes infect many grasses and most endophyte species produce ascospores and fruiting bodies on their host (Clay, 1989). In contrast, a subset of endophyte taxa classified in the form genus *Neotyphodium* spp. do not sporulate on their host are and maternally transmitted through seeds (Clay, 1993).

The terms "fescue fungus", "endophyte", "fungal endophyte", and "fescue endophyte", have all been used to denote *Epichloë typhina*. Persoon first recorded *Epichloë typhina* in 1798 under the name *Sphaeria typhina*. It was referred later to the genus *Epichloë* by Tulasne in 1861 (Sampson, 1933), and later renamed *Acremonium coenophialum* (Morgan-Jone and Gams, 1982). Glenn, Bacon, Price and Halin again renamed it as *Neotyphodium coenophialum* in 1996 (Gleen et al, 1996). It is generally accepted that these terms refer to the same organism. Certain species of ryegrasses and fescues in the USA, New Zealand and Australia have long been recognized as hosts of fungi that reside and penetrate within their leaf sheaths and seeds (Sampson, 1933; 1935; 1937; Neill, 1941). The endophyte found in tall fescue produces no visible symptoms to indicate its presence (Morgan-Jone and Gams. 1982). An intriguing question is why hyphae of the endophyte do not elicit visible host defense responses. There is no evidence that the penetration of hyphae into cell walls is aided by the production of extra cellular pectic enzymes. Instead, it appears that penetration of hyphae occurs solely by physical pressure and growth into existing spaces, a process not likely to elicit plant defense reactions (Christensen et al., 2002). A factor that may have a role in the survival of endophyte hypae in host grasses is the presence a proteinase within the endophyte cell wall (Lindstrom and Belanger, 1994). The regulated expression of the proteinase and abundance in infected plant tissue suggest that its expression may be involved in the symbiotic interaction of the plant and the endophytes.

Two characteristics of the endophyte have great practical importance. First, the organism improves the growth or appearance of the grass. Secondly, it is seed transmitted and apparently not transmitted in any other way. The endophyte grows symbiotically within the aerial portion of the tall fescue plant. Asymptomatic seed-borne *Neotyphodium* endophytes are hereditary symbionts analogous to chloroplasts and mitochondria. The endophyte is maternally transmitted by seeds, but not by wind, rain, pollen, or physical movement. The fungus does not possess any known mechanism of contagious transmission (Clay, 1993; Siegel et al., 1984b; 1987). Welty and Azevedo (1993) found no evidence for natural transmission of endophyte from infected plants to non-infected plants and they reported that burning dormant plants did not affect endophyte content of seed harvested the following year.

Endophyte lifecycle and distribution

The hyphae of the endophyte are generally coarse, mostly unbranched, contorted, and run vertically between the host cells the intercellular spaces. Occasionally two or three hyphae are coiled in a single intercellular space (Bacon, 1983). The mycelium of

Neotyphodium coenophialum is approximately 1-2µm in diameter. In the vegetative organs of the host, it is somewhat sparsely separated and runs for long distances without branching. The mycelium of the fungus has been found in infected plants at all times of the year, residing near parenchymatous cells and xylem vessels. It can be readily demonstrated at any season in tissues of tiller buds, leaf-sheaths, creeping stems, and seeds (Sampson, 1933). This endophyte is found in seed, leaf sheath, stem, and crown but only rarely in leaf blades and roots (Siegel et al. 1984b). Hinton and Bacon (1985) reported that *Neotyphodium* was never detected in any of the tissues of the root and leaf blade. Azevedo and Welty (1995) determined the occurrence, distribution, and ultrastructure of endophyte hyphae in the roots of axenically grown tall fescue seedlings. Mycelium is usually present in abundance within the pith scraped from the primary tiller of an infected *Lolium spp.*, and is found in secondary tiller as they develop (Sampson, 1937). The endophyte has not been found outside the plant in a natural environment, and no sexual reproductive stage has been observed (Bacon and Siegel, 1988).

Neill (1941) clearly described the distinct intercellular hyphae in the aleurone layer of the seed. He found the endophyte in high concentrations in seed of tall fescue and ryegrass (Siegel et al. 1984b; Philipson and Christey, 1986). Hinton and Bacon (1985) described that in mature seed of tall fescue, the fungus was uniformly distributed between the endosperm and the epithelial layer of cell of the scutellum. Additionally, hyphae did not penetrate any part of the embryo. Philipson and Christey (1986) found hyphae of *N. lolii* within the embryo, including the plumule apex, as well as below the testa, between cells of the aleurone layer, and between the scutellum and endosperm of ryegrass seed. The same authors reported that the *N. coenophialum* of *Festuca* *arundinacea* has a similar relationship with its host and appears to be ultrastructually indistinguishable from *N. lolii* of *Lolium perenne*.

The viable endophyte grows as the seed germinates, invading the seedling shortly after germination (Bacon and Siegel, 1988). Within 2 days of germination, the fungus was observed in the first internode of an emerging shoot but it is not easy to detect. Infection of the first and subsequent leaves did not occur until sheath differentiation.

The shoot already bears hyphae of the endophyte between its apical cells and the mesocotyl upon imbibition of water by the seeds (Philipson and Christey, 1986). The hyphae invade leaf primodia as they arise on the apex. The rudimentary coleoptile is infected while in the seed and retains these hyphae as it grows as upward, ensheathing the plumule for a time during the establishment of the new plant. Active mycelium within established seedling grows into new tillers and leaf sheath (Bacon and Siegel, 1988). The fungus is located in meristematic tissue of the shoot apex and leaf sheath during dormant periods of the grass. Within the leaf sheath, hyphae are found in the intercellular spaces of the parenchyma and chlorenchyma of the leaf mesophyll. Hyphae are not found in the upper or lower epidermal cells (Hinton and Bacon, 1985). The highest concentration of the fungus occurs in the base of the leaf sheath, decreasing towards the ligule (Musgrave, 1984; Siegel et al., 1984b; Hinton and Bacon, 1985). During vegetative periods, the fungus is located in meristem tissue of shoot apices, assimilative and storage tissues of leaf sheaths, and the crown of the tall fescue (Bacon and Siegel, 1988).

During flowering, mycelia can be in the ground tissue (pith) in the center of infected fertile tillers (Sampson, 1933; 1937). When plants are dormant, the fungi are confined to the apical meristem region and when plant growth resumes in the spring, the fungi grow intercellulary, without penetrating or apparently damaging the host cell. After growth of the flowering tiller starts, the mycelium grows in the pith to the flower panicles. Mycelium may be in the flower primodia before the inflorescence develops (Siegel et al., 1985). In the spring, when the grass produces inflorescence, hyphae are located primarily in the intercellular space between sclerenchyma cells in the florescence stem. The fungus is also distributed within the intercellular spaces of chlorenchyma and parenchyma cells around the vascular bundles of the inflorescence stem and the rhizome. Hyphal density is higher in the intercellular spaces at the distal region of the inflorescent stem than proximal region (Hinton and Bacon, 1985; Bacon and Siegel, 1988). Philipson and Christey (1986) observed that the inflorescence primordia prior to stem elongation during tissue differentiation were already invaded with the endophyte. The endophyte progresses intercellularly from the vegetative apex into the inflorescence primodium and floral apices, from where it penetrates the tissues of the ovary and ovule (Philipson and Christey, 1986). The fungus invades the young floret before anthesis and could be detected in ovaries early in development (Hinton and Bacon, 1985). Although the time and site of fungal entry into the megagametophyte has not been established, fungal hyphae have been observed in antipodal cells at the stage of first division of the primary endosperm nucleus. It seems likely that endophyte invasion occurs soon after fertilization (Philipson and Christey, 1986).

Sampson (1933) reported entry of hyphae of the endophyte into the ovary of the host. From the earliest stage of embryogenesis, the endophyte is present within the embryo sac. As the embryo develops, the endophyte remains in the embryo sac. The earliest sight of the endophyte within an embryo is when the stem apex is differentiated in the developing embryo (Philipson and Christey, 1986). White et al. (1991) reported that the embryo of *Festuca* spp. was not infected until fully it became differentiated.

Endophyte transmission and survival

Musgrave (1984) found variability in seed mycelia concentration among seed lines. He thought the variability in mycelia concentration reflected variability in environmental parameters during seed production and differences between cultivars. Welty (1986) showed no effect of controlled temperatures on initial movement of endophyte from seed to seedling after incubation at 12-28 ^{o}C for 1-6 weeks. Di Menna and Waller (1986) reported that the seasonal variation of mycelium concentration of *Acremonium lolii* in leaf sheaths of perennial ryegrass (*Lolium perenne* L.) was related to variation in mean monthly temperatures. The amount of endophyte within infected plants is known to vary seasonally from a high concentration in summer to a low concentration in winter. Bacon and Siegel (1988) reported the incidence of the endophyte in seed and vegetative stem of tall fescue during 6 years of management for seed production. They found reduced endophyte frequencies in seeds from tall fescue grown in hotter and drier summers followed by cold winter, than in seeds from tall fescue grown in the other years. Seasonal variation of the distribution of the tall fescue endophyte was demonstrated in tall fescue pasture (Hill et al, 2002). Stewart (1986) reported that the treatments of nitrogen fertilizer had no effect on the percentage infection of the endophyte, but the highest N rate significantly reduced the concentration of endophyte mycelium in ryegrass seeds. Infected plants grown from seed harvested from these treatments showed that reduced concentrations of endophyte in seed carried over into seedlings, mature plants, and seed harvested from these plants (Stewart, 1986). It is speculated that reduced endophyte concentration was a result of greater growth rate of the plant than of the endophyte.

In general, endophyte frequency increases over time in pasture. However, Sampson (1937) mentioned the possibility of escapes from endophyte infection in *L perenne* populations. In the populations derived from infected parents, some of stems were recorded as non-infected. Jones et al. (1983) reported that endophyte-infected plants might result in non-infected offspring. They suggest that interaction between plant genotype and endophyte genotype may be incompatible resulting in inhibition or elimination of the endophyte in plant populations. Pedersen et al. (1984) suggested that endophyte escapes were a result of environmental factors. A computer simulation model (Clay, 1993) and a simple mathematical model (Ravel et al., 1997) suggested that lower than expected levels of infection of endophyte in wild populations may occur because fitness could vary with different environmental conditions. In other words, those reports indicate environmental factors and genetic interactions of the plant/endophyte association may affect endophyte transmission from mother plant to seed and secondary tillers. However, there are no studies investigating whether these interaction variables affected endophyte transmission in the seed.

Viability of endophyte mycelium in seed is influenced by length of storage, temperature, and moisture content. Most endophyte-infected seeds stored in warehouses for 2 years contain little or no viable endophyte. The endophyte died in 50% of infected seeds of perennial ryegrass if seeds were stored for 18 months at 10°C and 19% relative humidity (Welty et al., 1987). Rolston et al. (1986) found that less than 15% of perennial ryegrass seeds were endophyte infected after 12 months of storage in ambient temperature at 13 % relative humidity, though initially 84% of these seeds were infected. Low temperature and low humidity during storage retard loss of fungus viability. Viability of the endophyte in tall fescue seed stored for 7-11 months at 21 °C decreased to zero, whereas the viability in seed stored for 19 months at 10, 6, and –20 °C was 45, 95, and 95%, respectively (Siegel et al., 1984a). Moisture contents of tall fescue that resulted in the most rapid decrease in endophyte viability, while maintaining the highest level of germination, were 19.4, 9.6, and 8.2% at 10, 20, and 30 °C, respectively (Welty et al., 1987).

Detection of endophyte

Since toxin from endophytes can adversely affect animals, it is important to know whether pasture plants contain endophytes. For efficient breeding of tall fescue, simple detection of *Neotyphodium coenophialum* is needed. Direct methods determining endophyte infection includes histological staining and microscope examination, enzyme-linked immunosorbent assay (ELISA), and tissue immunoblot (TIB).

The light microscope method is the most common technique used to detect endophyte. Sampson (1933; 1935; and 1937), Neill (1941), and Di Menna et al. (1986) observed endophytes in ryegrass and fescue with light Microscopy by the classical histological staining method. Hinton and Bacon (1985) detected endophyte of tall fescue by electron microscopy. Philipson and Christey (1986) used electron microscopy because they felt that light microscopy had limitation and led to incorrect conclusions about the endophyte life cycle. Although light microscopy cost less than electron microscopy, it results in poor resolution. Hill et al. (1998b) reviewed immunological backgrounds for improving forage species. The immunochemical staining (immunohistology) method improved light microscopy by detecting the presence of an antigenic agent in tissue and cell *in situ* (Van Vuurde, 1983; Hiatt et al. 1997a). Unfortunately, microscopy mothods for endophyte detection are tedious and time-consuming. Therefore a rapid, inexpensive, and reproducible tissue immunoblot (TIB) technique was developed to evaluate tall fescue for endophyte (Hiatt et al., 1999). Enzyme-linked immunosorbent (ELISA) technique has been used to determine the concentration of endophyte mycelium in tall fescue and ryegrass tissues (Musgrave, 1984; Musgrave et al., 1986; Reddick and Collins, 1988; Hiatt et al., 1997b; Hiatt and Hill, 1997; Hiatt et al.1999). ELISA is a reliable quantification method for endophyte in tall fescue (Reddick and Collins, 1988). Gwinn et al. (1991) determined distribution of *Neotyphodium coenophialum* within tissues of infected tall fescue with TIB. Hiatt et al. (1999) reported that the TIB method is correlated well with histological staining, followed by microscopic analysis on tall fescue tillers, seedlings, plants, and seed. Similar results were found when ELISA and TIB tests were used to estimate rate of endophyte infection in tall fescue tillers (Gwinn et al., 1991). Therefore, ELISA and TIB methods are reliable for endophyte detection. However, when test seed using these techniques, non-viable endophytes cannot be distinguished from viable endophyte. To detect viable endophyte, seed must be germinated and the presence of mycelium determined in seedling plants that are 3-5 weeks old.

Endophyte effects on tall fescue

Grasses have evolved various structural and physiological means of adapting to water deficit stress conditions (drought), in order to ensure population stability in natural environments. Many agricultural scientists have made efforts to find such traits, when breeding for improved seed yield of crops. It has been recently discovered that the endophytic fungus, *Neotyphodium coenophialum*, enhances the drought tolerance of tall fescue (Bacon, 1993; Bouton et al., 1993; West et al., 1993; West, 1994).

Read and Camp (1986) reported low forage production and loss of stand in the pastures with low levels of the endophyte. Heavily endophyte-infected tall fescue pastures showed an average of 4 % bare ground area, whereas those with 12 % infected stands showed an average of 54 % bare ground area. Siegel et al. (1985) found no effects on seed production and plant growth due to presence of endophyte.

The effects of drought on crop plants range from relatively minor reduction in yield to the devastating destruction of crops. Underlying the ability of plants to withstand periods of drought are morphological, physiological and biochemical adaptations (Hetherington, 1998). Like other crops, water relations are important in tall fescue management. Endophyte-infected tall fescue was more productive than endophyte-free tall fescue at -0.05 MPa soil matric potential (Arachevaleta et al., 1989). At -0.5 MPa soil matric potential 75 % of endophyte-free plants died and all endophyte-infected plants survived. They also indicated that leaf rolling under drought stressed was much more common on endophyte-infected plants than endophyte-free tall fescue. Drought acclimation in tall fescue was highly dependent a particular endophyte/plant genotypic interaction (Hill et al., 1996).

Osmotic adjustment is the net increase in intracellular solute content by accumulation of ions or organic solutes. The decrease of osmotic potential resulting from water deficit is an important mean of plant adoption to water stress. Lower osmotic and water potential (more negative) increased cell turgor pressure, which is necessary for cell growth and metabolic maintenance (Turner, 1986). Elmi et al. (1989) reported that endophyte presence increased osmotic adjustment in the leaf blade and basal zone of the psuedostem of KY 31 tall fescue. White (1989) and Richardson et al. (1991) also reported that endophytes enhanced osmotic adjustment in young, emerged leaf blades by accumulating greater concentrations of fructose and glucose in infected plants than uninfected plants under drought stress. Endophyte-infected plants had enhanced osmotic adjustment, survival rate, and leaf elongation rate than endophyte-free tall fescue (Elmi et al., 1992; Elmi and West 1995). Richardson et al. (1992) detected mannitol and arabitol in leaf sheaths of E+ tall fescue after stressed for 7 days at –1.0 MPa, but not in E- plants. Since virtually no mannitol and arabitol was found in the leaf blade or leaf sheath of E- plant, these polyols are probably produced only in the presence of endophyte. Yet, the total nonstructural carbohydrate pool in drought-stressed tall fescue does appear to be affected by endophyte infection (Belesky et al., 1989), even though E+ leaf blade had greater concentrations of fructose and glucose in leaf blades and higher glucose than E- plants (Richardson et al., 1992).

There is contradictory evidence suggesting there is no endophyte-related adjustment to drought. White et al. (1992a; b) observed no endophyte-mediated drought tolerance between E+ and E- isolines of tall fescue. They suggested that endophyte-mediated drought resistance might be due to alterations in drought avoidance. Morphological and physiological adaptation can lead to drought avoidance mechanisms to maintain favorable water status. In some endophyte/tall fescue associations, endophyte infected plants had greater concentrations of total non-structural carbohydrates in tillers than non-infected plants when under non-limiting water supply (Hill et al., 1990). Extensive root systems improve water uptake from soil by a deeper and denser root. Endophyte presence increased root hair length and decreased root diameter in tall fescue (Malinowski et al., 1999). Indole acetic acid (IAA) may involve with morphological changes, allowing a faster growth rate and affecting regrowth. Drought-stressed endophyte-infected tall fescue showed an increased regrowth rate at only after drought (Belesky and Fedders, 1996). IAA was detected only in culture extracts of *Neotyphodium coenophialum* (De Battista et al., 1990), mentioning that the *in vitro* ability of endophyte to produce IAA indicates the possibility of an *in vivo* production and suggests its involvement in growth response in this symbiotic relationship.

Lewis et al. (1996) reported endophyte infection did not affect either dry matter production or N uptake. However, Richardson et al. (1999) reported that N-uptake increased in the presence of endophyte. Vázquez-de-Aldana et al. (1999) found a significant interaction between endophyte infection and nutrient element concentrations in plant tissue. Ca concentration was higher in E- than E+ tall fescue. Endophyte-infected tall fescue had higher N (both organic and inorganic) and Mg concentrations than endophyte-free tall fescue. Lyons et al. (1990) observed a greater accumulation of amino acids in leaf sheaths than leaf blades of tall fescue. They found an endophyte-related increase of NH^{+4} concentration in leaf sheath but decrease of NO^{-3} concentration in leaf sheath and leaf blade and increased glutamine synthetase. They suggests endophyte effects nitrogen metabolism in tall fescue.

There appears to be an association between lower soil nematode numbers and less acute water-deficit stress symptom (West et al., 1988; Elmi et al., 2000). Soil populations of *Pratylenchus scribneri* and *Tylenchorhynchus acutus* were substantially higher in E- than in E+ tall fescue. Thus, endophyte conferred drought tolerance to tall fescue is thought to be mediated through enhanced resistance to soil-born nematodes. The bird-cherry oat aphid *Rhopalosiphum padi* L. avoided tall fescue infected with endophyte but fed on endophyte free plants (Latch et al., 1985). Thus, it appears that tolerance of drought responses to nutrient and resistance to insects add to the competitive ability of endophyte-infected grasses (Hill et al., 1991; 1998a; Clay et al., 1993; Malinowski et al, 1997; Clay and Holah, 1999).

Endophyte-infected tall fescue competes successfully with non-infected tall fescue as well as other plant species (Clay and Holah, 1999). Endophyte-infected grasses may have direct chemical effects (allelopathy) on the competitor. Seed extracts of endophyte-infected tall fescue showed allelopathic effects such as germination inhibition, reduced root-hair length, and reduced root-hair density of *Trifolium* spp. (Stringer 1996). Hoveland et al. (1999) showed that endophyte-infected tall fescue increased competition with legumes such as red clover (*Trifolium pretense*), ladino clover (*Trifolium repens*), and alfalfa (*Medicago sativa*). Allelopathic effects of loline alkaloids which are produced in endophyte-infected grasses have been reported (Malinowski et al., 1999). Root growth of red clover was significantly depressed in competition with endophyte-infected tall fescue genotype ,EDN2, compared to its endophyte-free form. Root growth was not affected when red clover was grown in competition with EDN11 and its endophyte-free form. Plant genotype EDN2 produced twice as much loline alkaloids as EDN11. This suggests that loline alkaloids might influence the competitive ability of some endophyte-tall fescue associations through a possible allelopathic effect on companion species.

Endophyte effects on animal performance

Endophytes are responsible for producing several alkaloids which have been suspected as causal agent of the toxicosis of tall fescue (Bush et al., 1979; Bacon et al., 1986). Bacon et al. (1977) reported the presence of an endophytic fungus in tall fescue and its correlation to fescue toxicosis in cattle. Hill and Stringer (1985) found that 95% of all tall fescue pastures in South Carolina were infected with endophyte. Most tall fescue pastures are infected at levels of 60 to over 80 % (Ball et al., 1996; Hoveland et al., 1983). In Kentucky, 97% of 200 fields of tall fescue were infested with endophyte-infected plants; in Alabama, 95% of the tall fescue pastures were infested; in Missouri 91% of 193 seed samples from separate tall fescue contained 50% or more endophyte-infected seeds (Welty, 1986).

Endophyte-infected tall fescue can decrease steer weight gains by 30-100% and furnish low conception rate and poor milk production in beef cows. Beef cattle annual losses are estimated at $ 354 million in reduced calf numbers and $ 252 million in reduced weaning weights (Hoveland, 1993). Poor animal gains, intolerance to heat, excessive salivation, rough hair coats, slightly elevated body temperature, nervousness, more time spent in the shade, more time spent in water, less time spent grazing, and lowered milk production characterize fescue toxicosis (Bacon et al., 1977; Ball et al., 1996). Fescue toxicosis is common and widespread over tall fescue-growing regions, particularly during warm weather (Hoveland,et al.,1982; 1983; Ball et al., 1999; Read and Camp, 1986; Fribourg et al., 1999). Fescue toxicity causes serious reproduction problems with mares. Specific problems are abortions, prolonged gestation, dystocia (difficult birth), thick placentas, agalactia (little or no milk production) and sometimes death of mares during foaling (Cross et al., 1995; Ball et al., 1996; Cross, 1997). Fescue foot is a more acute form of toxicity and a seasonal problem usually occurring in the winter. It is expressed as a gangrenous condition of animal's extremities (Bush et al.1979; Fribourg et al., 1999). It has been proved that ergot alkaloids are the toxic components of endophyte-infected tall fescue (Hill et al., 1994).

Solutions of the fescue toxicosis

There are several approaches being employed overcome the fescue toxicosis problem. The first involves dilution which can be accomplished by maintaining complementary pasture species in addition to E+ tall fescue (Chestnut, et al., 1991). However, it is difficult to maintain pasture mixtures in E+ tall fescue. For example, loss of clover appears to be associated with competitiveness of endophyte infected tall fescue (Clay and Holah, 1999; Hoveland et al., 1999). A second strategy for overcoming fescue toxicosis is to use of a pharmacological agent (Lipham, et al., 1989) or a vaccine (Hill et al., 1994). However, there is no efficient pharmacological agent or vaccine available to date. The third strategy is avoidance. In some cases, livestock producers can reduce the impact of the endophyte by preventing certain types or classes of animals from having access to endophyte-infected pastures during the latter stages of pregnancy, thus avoiding endophyte-related foaling problems. The fourth strategy is planting endophyte-free fescue or converting infected fields to non-infected fields. However, poor persistence of

E- tall fescue does not make this a likely solution. Although animal performance is greater in the endophyte free pasture, the absence of the fungus makes the fescue vulnerable to insects, drought, or overgrazing (Joost, 1995). Converting to endophyte-free is also problematic. Remnant endophyte-infected seed may germinate and contaminate the new non-infected field and it is difficult to reduce endophyte infection levels after the pasture is established (Defelice and Henning, 1990; Vila Aiub and Ghersa, 2001). One other strategy is to use tall fescue infected with an endophyte that does not produce ergot alkaloids (Bouton et al., 1998; 1999). Fortunately, recent advances have used non-toxic endophytes that have resulted in improved plant survival in pasture without animal toxicity (Fletcher, 1999; Bouton et al., 2000; 2002).

Since non-toxin endophytes are in use, understanding endophyte transmission and maintenance is important. Although the relationship between endophyte and tall fescue has been studied, little research has been conducted to provide insight as to how plants or environmental conditions affect endophyte growth, how endophytes and plants interact, and how/when endophytes are transmitted into developing seeds.

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CHAPTER 2

SEASONAL VARIATION AND ENVIRONMENTAL EFFECTS ON ENDOPHYTE TRANSMISSION IN TALL FESCUE

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ABSTRACT

Tall fescue (*Festuca arundinacea* Schreb.) is the predominant cool season forage species and grown in the United States. Typically, tall fescue is infected with the endophyte, *Neotyphodium coenophialum,* which produces toxic alkaloids to animals, resulting in reduced animal performance. Non-toxin endophyte-infected cultivars of tall fescue have been developed and to maximize their impact on pasture ecosystems a better understanding of mechanisms affecting seed and tiller transmission is needed to maintain endophytes in seed. Little research has been conducted on endophyte transmission mechanism *in planta*. Seasonal variation of endophyte in established tall fescue pastures in Watkinsville, GA, and seed fields near Salem, Oregon was examined. Growth chamber experiments were conducted to examine temperature effects on plant and endophyte growth and to determine the cardinal minimum temperatures for each. Greenhouse experiments were conducted to examine water stress effects on endophyte transmission by imposing soil moisture deficits at different stages of panicle development. Endophyte frequency varied over months in both GA and OR. Frequency averaged 93.4% when sampled April through December but was 80.5 % when sampled January through March in GA. Frequency average 64.5% when sampled February through April but was 88.6% during other months in OR. Cardinal minimum temperature for plant growth was 5.16^{o}C (+/- 0.47) but for endophyte was 10.28 ^{o}C (+/- 0.70). There was no water stress effect on endophyte frequency or concentration in tall fescue. Endophyte genotype and plant genotype affected endophyte frequency and concentration. Temperature appears to be a major variable affecting endophyte frequency in field-grown plants.

INTRODUCTION

Tall fescue is the predominant cool season perennial grass grown in the United States. It was introduced from Europe into North and South America during the colonial period (Borrill, 1976; Buckner et al., 1979; Ball et al., 1996) and has been planted on approximately 14-20 million hectares in the USA (Bacon and Siegel, 1988; Bouton, 2000). Tall fescue is frequently infected with the endophytic fungus, *Neotyphodium coenophialum*, (Bacon and Siegel, 1988; Glenn et al., 1996). The endophyte receives nutrition and structural refuge from the host, while the host receives benefits such as enhanced competition (Hill et al., 1991; 1998; Malinowski et al., 1999). Thus, the two are in a mutualistic relationship. Tall fescue may conserve water more efficiently due to endophyte presence through increasing leaf rolling (Arachevaleta et al., 1989), decreasing stomatal conductance (Elmi and West, 1995), and lower osmotic potential increasing turgor pressure in tall fescue (Elmi et al., 1989; White, 1989; Richardson et al., 1991).

Animals grazing endophyte infected tall fescue consume endophyte-derived ergot alkaloids. This results in reduced animal performance (Read and Camp, 1986; Hoveland, 1993). Recently, non-toxic endophytes have been inserted into tall fescue to capitalize on the agronomic benefit of the endophyte, but eliminate the toxicity to grazing livestock (Bouton et al., 1998; 1999; Fletcher, 1999; Bouton, 2000; 2002). Endophytes have historically been viewed as negative components of the pasture ecosystem (Ball et al., 1996) but with emerging use of non-toxic endophytes they are viewed more positively for cultivar development (Fletcher, 1999; Bouton, 2000; 2002). Therefore, it is vital to understand endophyte growth and transmission in cool season grasses to maximize the probability of maintaining non-toxic endophytes in tall fescue.

The endophyte life-cycle is relatively simple because it has no sexual stage, produces no spores, and disseminates only in the seed though the female parent (Siegel et al., 1984). Bacon and Siegel (1988) reported the endophyte grows as the seed germinates and invades the seedling plant, shortly after germination. During vegetative periods, the fungus is located in meristematic tissue of shoot apices (Bacon and Siegel, 1988). When the flowering stem begins to elongate, the mycelium grows along with the inflorescence and infects the maternal tissues of the ovule (Hinton and Bacon, 1985; Philipson and Christey, 1986).

There appears to be variation of endophyte presence and /or transmission that is association with environmental parameters during plant growth. Di Menna and Waller (1986) found that the seasonal variation of mycelium concentration of *Neotyphodium lolii* in leaf sheaths of perennial ryegrass (*Lolium perenne* L.) was related to variation in mean monthly temperatures. Bacon and Siegel (1988) reported a decrease in endophyte level in seed after a hot and dry summer and cold winter. While these studies suggest environmental parameters affect endophyte growth and transmission, direct evidence is lacking. Therefore, the objectives of this study were to examine 1) seasonal variation of endophyte infection within tall fescue, 2) temperature effects on endophyte growth *in planta*, and 3) endophyte transmission under water stress imposed at different stages of panicle development.

MATERIALS AND METHODS

Experiment 1. Seasonal variation of endophyte in tall fescue

Fifty tillers were randomly sampled from two established pastures of 'Jesup' MaxQ while systematically walking across each. Sampling began on 1 July 2000 and continued monthly until 1 June 2002. Both pastures were replications within a larger grazing study located in Watkinsville, GA. In a separate study, two seed fields of Jesup MaxQ growing in the central Willamette valley were sampled on the first of each month from October 1999 to May 2000 and October 2000 to May 2001. Two hundred tillers were sampled while systematically waking across each field.

A 2-3 mm cross section of each tiller base was tested for endophyte frequency for each sampling date and each location by immunoblot. The Georgia samples were also analyzed for endophyte concentration in the psuedostem bases and for endophyte frequency at the base of the psuedostem base and at 3cm above from the psuedostem base. Endophyte frequency and endophyte concentration were determined by immunoblot and ELISA, respectively (Hiatt et al., 1997a; b).

Weather data were obtained from a weather station located next to the pastures in Georgia and from the Salem, OR weather station of the Oregon Climate Service.
Data from each study were analyzed by analysis of variance (SAS institution, Cary, NC) using a complete factorial of replication, year, and month as treatment variables in a randomized complete block design. Fields within each location were used as replications. For the Georgia location, sample location within the psuedostem and months were considered fixed effects while replication and years (environments) were

considered random effects. For the Oregon location, months were considered a fixed effect while replication and years (environments) were considered random effects. Treatment means were separated using a Fisher's protected least significant difference (LSD). There were no year effects or interaction with years, so data were pooled and re-analyzed for replication and month effects. A paired t test was used to determine effect of sampling location in plant on endophyte detection for the Georgia samples.

Endophyte frequency data for samples collected in Georgia and Oregon, and endophyte concentration data for samples collected Georgia were correlated with mean monthly temperature and precipitation using the PROC CORR subroutine of SAS.

Experiment 2. Temperature effects on endophyte growth

a) Growth response of endophyte and tall fescue under different temperature regimes

Seeds of Jesup Max Q were placed in water and incubated at 4 ºC for 7 days to break dormancy. Seventy two seeds were planted into each of 36 flats containing a commercial germinating mix (Fafard) (Conrad Fafard, Inc., Agawam, MA), and placed into a greenhouse until germinated. Thirty-six flats of seven-day old seedling plants randomly assigned to 12 treatment combinations consisting of a factorial of 4 temperature regimes and 3 harvest dates.

The three harvest dates were 0, 3, and 6 weeks. The four temperature regimes were 1) 12/6 ^{o}C day/night temperature for 3-weeks followed by 25/19 ^{o}C day/night temperature for 3-weeks; 2) 25/19 ^{o}C day/night temperature for 3-weeks followed by 12/6 ^{o}C day/night temperature for 3-weeks; 3) 12/6 ^{o}C day/night temperature for 6-weeks; 4) 25/19 ^{o}C at day/night temperature for 6-weeks.

Germination date was recorded when 50% of the seeds had emerged. One week from germination date was considered week 0. The flats were placed into one of two Conviron PGV36 (Conviron, Winnipeg, Canada) growth chambers one with a temperature regime of 25 / 19 ºC and the other a 12 / 6 ºC day / night temperature regime. Each maintained 14 hours day length and 512 ±16 µmol m^{-2} sec^{-1} light intensity. At week 0, 12 flats (3 from each temperature regime) were randomly and harvested. Plants were removed from the flat, their roots were washed with tap water, and the psuedostem was removed from the attached seed. The psuedostem and leaf tissue were frozen and freeze-dried using a Freezemoible 25SL Freeze drier (VirTiS Inc. Gardiner, NY), and dry weights recorded. After 3 weeks, three flats from each temperature regime were randomly selected and harvested as previously described, except a 2-mm cross-section of the psuedostem base was analyzed for endophyte presence. In addition, 3 of the 6 remaining flats from the 25/19 ^{o}C regime were randomly selected and placed into the 12/6 ^{o}C temperature regime, and 3 of the 6 remaining flats from the 12/6 ^{o}C regime were randomly selected and placed into the 25/19 ^{o}C temperature regime. Plants were grown for an additional 3 weeks and harvested, analyzed for endophyte presence and freeze-dried as previously described. Plants were fertilized weekly with Miracle Gro fertilizer (Scotts Miracle-Gro Products, INC., Port Washington, NY) at a rate of 15 g per 8 L of water applied. Plant were checked daily and watered to prevent moisture deficit.

Freeze-dried materials were analyzed for endophyte concentration using ELISA (Hiatt et al., 1997b) and the tiller cross sections analyzed for endophyte presence by immunoblot (Agrinostics Ltd. Co., Watkinsville, GA). Endophyte frequency was not determined on plants from week 0 because plants were too small for immunoblot analysis.

The experiment was replicated by randomly assigning temperature regimes to the two growth chambers and conducting the experiment exactly as previously described to serve as a replication.

The experimental design was a randomized complete block with 2 replications. Replication was considered a random effect while temperature regimes and harvest dates were considered fixed effects. Data were analyzed by analysis of variance (SAS Institution, Cary, NC). Treatment means were separated using a Fisher's protected least significant difference (LSD).

b) Determining cardinal minimum temperature for endophyte and tall fescue growth

Seeds of Jesup Max Q were placed in water and refrigerated for 7 days to break dormancy. Seventy-two seeds were planted into each of 21 flats containing a commercial germinating mix (Fafard) (Conrad Fafard Inc., Agawam, MA) and placed into a greenhouse. Germination date was recorded as day 0 when 50% of the seeds had emerged. Six flats of seven-day old seedling plants were randomly assigned to each of three Conviron PGV36 (Conviron, Winnipeg, Canada) growth chambers. The growth chambers had constant temperatures of 10, 15, or 20 ºC randomly assigned to them. Each maintained 14 hours day length, and 516 ±14 µmol m^{-2} sec^{-1} light intensity. Plants were g fertilized weekly with Miracle Gro fertilizer (Scotts Miracle-Gro Products, INC., Port Washington, NY) at a rate of 15 g per 8L of water applied. Plants were checked daily and watered to prevent moisture deficit. Each week, one flat of seedling plants was randomly selected from each growth chamber and 50 harvested. Plants were removed from the flats and roots washed with tap water. Plants were excised from the seed at the psuedostem base, a 2-mm cross-section obtained to determine endophyte presence, and the remaining excised plant freeze-dried (Virtis Freezemoble Model 25L freeze drier; Gardiner, NY). Plant dry weight was recorded. Freeze-dried materials were analyzed for endophyte concentration using ELISA (Hiatt et al., 1997b) and the tiller cross sections analyzed for endophyte presence by immunoblot (Agrinostics Ltd. Co., Watkinsville, GA). Endophyte frequency was not determined on plants from week 0 and 1 because plants were too small for immunoblot analysis.

The experiment was replicated by randomly re-assigning temperature treatments to the three growth chambers and conducting the experiment exactly as previously described. The experimental design was a randomized complete block with 2 replications. Replication was considered a random effect while temperature treatment and harvest dates were considered fixed effects. Data were analyzed by analysis of variance (SAS Institution, Cary, NC). Treatment means were separated using a Fisher's protected least significant difference (LSD). Plant dry weight and endophyte biomass data (dependent variables) were regressed with temperature (independent variable) for samples harvested during weeks 4, 5, and 6 using the PROC REG procedure of SAS. Cardinal minimum temperatures for tall fescue were determined by extrapolating the regression equation and solving for Y=0. Cardinal minimum temperature for endophyte was determined by solving for Y=0 using the regression equations from weeks 4, and 5, and by solving the regression equation from 6 week by a differentiation. Plant and endophyte mean cardinal minimum temperatures and confidence intervals were calculated for each from the X values when the regression equations were solved by Y=0 or the differentiation.

Experiment 3. Effects of water stress on endophyte transmission

Two experiments were conducted to investigate water stress effects on endophyte transmission when a) individual plant genotype was infected with different endophyte genotypes, and b) individual plant genotypes were infected by a common endophyte genotype.

a) Individual plant genotype infected by different endophyte genotypes

Propagules of a plant of a single tall fescue accession (PDN2) into which different endophyte genotypes (EDN2, EDN11 and EDN12) were previously inserted (Kearney et al., 1991; Royland et al., 1994) were used for this experiment.

Tall fescue plants were vegetatively propagated in the greenhouse during September, 1999 and 2000. Tillers from each propagule were sampled and tested for endophyte presence. Two propagules of each endophyte-infected plant were randomly assigned and planted equidistant from one another in 120-L tubs containing 30kg of fritted clay (Tidy Cat, Ralston Purina Co., St. Louis, MO) in October of 1999 and 2000. A total of nine tubs were planted for the experiment. Tubs containing the tall fescue plants were transferred outdoors on 1 December and exposed ambient growing conditions for vernalization. Tubs were irrigated with tap water as necessary to maintain soil water content. Plants were transferred back into the greenhouse on 15 February of each year.

Plants were fertilized 14 days after planted and 7 days after transferring them back into greenhouse with 1.27, 1.27, and 1.27 g tub^{-1} of N, P, and K respectively. The nine tubs were randomly assigned to one of three drought stress treatments; 1) no stress, 2) stress prior to panicle emergence from the flag leaf, or 3) stress after panicle emergence and through flowering.

Soil water content (SWC) was maintained between 0.45 and 0.35 g water g^{-1} soil for the stress treatments and the non-stressed treatments were maintained at 0.65 g water g^{-1} soil (White et al., 1992; Hill et al., 1996). To adjust SWC, the tubs were weighed every 1-2 day and water was added to re-hydrate the soils to 0.45g water g^{-1} soil. Leaf water potential and leaf osmotic potential were measured with pre-calibrated end-window thermocouple psychrometers (Model 85-12V, J.R.D. Merrill Specialty Equipment Corp., Logan, UT). A single fully expanded leaf blade of each plant was excised from the youngest fully expanded and a 3-4 cm section of the lamina from immediately above the collar placed into the thermocouple psychrometer chamber within 15 second of excision. The chambers were permitted to equilibrate in an isothermal water bath at 25 ºC for 4 h before measuring leaf water potential. Chambers were frozen at -70 ºC for 2 h, warmed to room temperature, reinserted into the water bath, and equilibrated again for 4 h before measuring osmotic potential. Leaf turgor pressure was calculated as the difference between osmotic potential and leaf potential.

At boot stage, one plant from each plant/endophyte combination was removed from the pots. Endophyte frequency in tiller and in floret was examined via immunoblot and endophyte contents were quantified in psuedostem via ELISA analysis. The remaining plants were harvested at the physiological maturity of the seed. Endophyte concentration in panicles and endophyte frequency and concentration in seeds were examined.

Data were analyzed by analyses of variance (SAS Institute, Cary, NC). Treatments were a complete factorial of endophyte genotype, year, stage of harvest, and water stress. Treatments were assigned to a randomized complete block design in which all variables were considered fixed effects except year and replication which were considered random effects. Seeds did not develop in the first year of the experiment. Therefore, seed response variables were determined for the second year of experiment only. Treatment means were separated using a Fisher's protected LSD.

b) Individual plant genotypes were infected by a common endophyte genotype

Progenies from crosses between PDN11 (female parent) and PDN2 and PDN12 (male parents) (Adcock et al., 1997; Hiatt and Hill, 1997) were used. Two progeny (full-sibs) from each cross were randomly selected from a larger population of progeny for this study. Inasmuch as tall fescue is an obligate out-crossing species, the plants used in this study were unique plant genotypes, all plants containing the same endophyte since all had the same maternal parent.

Plants used for this study were propagated, examined for endophyte, planted into 120-L tubs containing kitty litter, fertilized and vernalized, and assigned to drought stress treatments exactly as described for experiment 3a. The response variables to drought stress treatments were also as described in 3a.

Data were analyzed by analyses of variance (SAS Institute, Cary, NC). Treatments were a factorial of plant genotypes, year, stage of harvest, and water stress. The experimental design was a randomized complete block in which all variables were considered fixed effects except year and replication which were considered random effects. Treatment means were separated a Fisher's protected LSD.

Laboratory methods for endophyte analysis

a) Endophyte frequency by immunoblot

Endophyte presence in tillers was tested using the Agrinostics Ltd. Co (Watkinsville, GA) immunoblot test kit. A 7.5 x 12.5-cm cellulose sponge was placed into a plastic food storage container and saturated with approximately 200 mL of extraction buffer. A 70 x 120-mm piece of blotting paper was placed on top of the sponge, and a 60 x 100-mm piece of nitrocellulose membrane placed on top of the blotting paper. Tiller cross-sections were laid with the cut surface dawn on the nitro cellulose membrane. Samples were incubated at 4 ºC refrigerator overnight. The nitrocellulose membrane with the stem pieces was placed onto a dry piece of blotting paper and the stem pieces were gently removed with a laboratory horsehair brush. The membrane was dried at 70ºC for 15 minutes. The membrane was placed into a dry 7 x 10-cm plastic food container and 20 mL of blocking/washing solution added, and placed on an orbital shaker gyrating at 50 rpm for 30 minutes. The blocking/washing solution was removed. Anti-endophyte monoclonal antibodies were diluted into fresh blocking/washing solution and poured over the nitrocellulose membrane. The membrane was returned to the orbital shaker and incubated for 1 h. The antibody solution was removed and the membrane washed with blocking/washing solution twice, six minutes each. Anti-mouse antibody was diluted in 20 mL blocking/washing solution, added to the shaker for 1 h. The anti-mouse solution was removed and washed previously described. Protein-A with a conjugated chromophore was diluted in 20 ml blocking/washing solution and returned to the orbital shaker for 30 minutes. After a final wash, a chromogenic solution was added for 20 minutes. The membrane was washed twice with tap wash and scored for endophyte by examining for a deep pink/red where tiller interfaced the membrane.

Endophyte presence in seeds was tested using the Agrinostics Ltd. Co (Watkinsville, GA) immunoblot test kit. Seeds were soaked in 1.25 M NaOH for 1 h after which they were washed with tap water until seed no longer felt slick to the touch. Control seeds of known E+ and E- origin were also prepared. A 60 x 60-mm piece of nitrocellulose membrane was prepared as previously described. The seeds were placed on the nitrocellulose membrane along with 5 seed each from the E+ and E- controls and incubated at 45 ^{o}C overnight. The remaining steps for the immunoblot were as previously described, except the chromogen used for seed stained deep blue/black and were scored accordingly.

b) Endophyte quantification by ELISA

Freeze-dried plant tissue was ground to pass a 1-mm screen in a cyclone-type mill (Cyclotec 1093 Sample Mill, Foss Tecator Co., Sweden). Fungal proteins were extracted from 15 mg of ground lyophilized plant tissue in 1ml phosphate buffered saline (PBS; 2.70 g NaH_2PO_4, and 8.77 g NaCl / liter distilled H_2O, pH 7.3). The samples were mixed thoroughly and incubated for 1 hr at room temperature. Plant tissues and buffer were centrifuged for 15 minutes at 12,000 g to remove cellular debris and the supernatant was transferred to a new tube. Polyethylene glycol (PEG) was added to the supernatant until PEG concentration equaled 100 g/liter, and refrigerated overnight at 4ºC to precipitate protein. The solution was centrifuged for 15 minutes at 12,000g and the supernatant was discarded. The protein pellet was re-suspended in 250 µl PBS containing 0.1 % sodium dodecyl sulfate (SDS) and incubated for 5 minutes at 60 ºC. The SDS solution was centrifuged for 15 minutes at 12,000g to remove undissolved material, and the supernatant transferred to another centrifuge tube. The supernatant was diluted 1:4 (v/v) in cold acetone (-20ºC) and chilled overnight at (-20ºC) to precipitate proteins. The solution was centrifuged for 15 minutes at 12,000g, the supernatant was discarded, and the protein pellet was washed with 1 ml of cold acetone. Excess acetone was decanted and the protein pellet was dried under a stream of air. Quantification of mycelia was conducted by diluting the protein pellet (1:4) in 400 µl borate saline solution (6.19 g H_3BO_3, 9.50 g $Na_2B_4O_7 \cdot 10\ H_2O$, and 4.90 g NaCl L^{-1} deionized H_2O, pH 8.5). Fifty µl of the solution was placed into wells of an Immulon 4 microtiter plate (Dyanatech Co., Chantilly, Virginia). After permitting the protein to anneal overnight at 4ºC, the plate was washed three times with ELISA wash (1.21 g Tris, 500 µl Tween 20, and 0.20 NaN_3/ liter distilled H_2O, pH 8.0), blocked with 100 µl of bovine serum albumin blocking solution (10 g bovine serum albumin, 1.17 g Na_2HPO_4, 0.24 g NaH_2PO_4, 8.20 g NaCl, and 10 ml of 2 % NaN_3 per liter distilled H_2O), and the plate was incubated for 30 minutes at 21ºC. The plate was washed three times with ELISA wash. *Neotyphodium coenophialum*-specific monoclonal antibodies from hybridoma cell line 15D7, 4H2, and 5C7 were mixed and diluted to a final antibody dilution of 1:20 in ELISA diluent (1 liter blocking solution with 500 µl Tween 20). Fifty µl was added to each well and the antibodies allowed annealing to endophyte proteins for 2 hr at 21ºC. The plate was washed three times with ELISA wash. Fifty µl rabbit anti-mouse antibody conjugated with alkaline phosphatase (RAM-AP) (Sigma Chemical Co., St. Louis, Missouri), was diluted 1:500 in ELISA diluent. After incubating at 21ºC for 2 hr, the plate was washed three times with ELISA wash. Fifty µl of substrate solution (1 g p-nitrophenyl phosphate, 0.10 g $MgCl_2$, and 96 ml diethanolamine per liter distilled H_2O, pH 9.8) was added to each well, and incubated at 21ºC. Color development was stopped after 25 minutes by adding 50 µl of 3 M NaOH. Optical density was measured spectrally at 405 nm using a BioTek EL 311 (Bio-Tek Instruments, Winooski, Vermont) microplate reader. Fungal proteins were quantified by regressing ELISA values to those of a standard dilution of purified proteins from each fungal isolate use in this experiment.

RESULTS

Experiment 1. Seasonal variation of endophyte in field-grown tall fescue

Mean monthly temperatures showed a typical trend in both years with a progressive increase in monthly average temperatures from January to August and a progressive decrease in monthly average temperatures from September to December at both sites (Figure 1). Temperatures in Georgia had a greater fluctuation over the year than those from Oregon. Temperature in Georgia was lower in January than that in Oregon in 2001 but temperatures in all other months were higher in Georgia than in Oregon. Georgia had more precipitation during the two years with the exception of October 2000, October 2001 through February 2002, and April 2002.

Analysis of variance indicated there was a month effect but no year or month × year effect for endophyte frequency for tall fescue sampled in Georgia or Oregon. Endophyte frequency was greater when sampled from April through December in Georgia than other months (Table 1). Similarly, endophyte frequency was similar in Oregon when sampled from October through January and in May, but lower when sampled from February through April.

Analysis of variance indicated there was month × sampling height interaction for endophyte frequency at different locations within tiller psuedostems among the Georgia samples. Generally, endophyte frequency was similar at both sampling locations within tillers from April through November (Figure 2). However, the psuedostem base had significantly higher endophyte frequency than at 3 cm above the psuedostem base from December through March.

Results from the both years were similar with peak endophyte concentration of tall fescue plants in June through November. Endophyte concentrations were lower from January through May with the lowest concentration in January (Table 2). Endophyte frequency was correlated with mean monthly temperatures for Georgia and Oregon grown samples ($R = 0.44$ and $R=0.75$, respectively). However, none of the endophyte parameters (frequency or concentration) were correlated with precipitation ($R = -0.01$ and -0.18, respectively).

Experiment 2 Temperature effects on endophyte growth

a) Growth response of endophyte and tall fescue under different temperature regimes

Analysis of variance indicated there were year, temperature, and harvest date effects for plant dry weight and a temperature by harvest date interaction for endophyte concentration, endophyte biomass, and endophyte frequency. Initially, all temperature treatments had similar plant dry weight, but it increased over the time in all temperature treatments (Figure 3). At week 3, plant dry weight was similar within treatments receiving either 25/19 or 12/6 ^{o}C day /night temperature regimes, but plants grown at cooler temperature regime showed less growth than those grown at the higher temperature regime. Plants grown at the cool temperature regime for the duration of the experiment had the lowest final weight. If plants grown at 12/6 ^{o}C were switched to 25/19 ^{o}C, plant dry weight was greater than those continuously grown at the cooler regime. Plants were grown at the higher temperature regime had the greatest dry matter over the duration of experiment. However, plant dry matter was less if plants grown at 25/19 ^{o}C regime were switched to the 12/6 ^{o}C regime during the second 3-week period.

Initially, all temperature treatments had similar endophyte concentration (Figure

4) and endophyte biomass (Figure 5). After 3 weeks of growth, endophyte concentration was similar within treatments receiving either 25/19 or 12/6 ^{o}C day /night temperature, but endophyte concentration in plants receiving the 25/19 regime had approximately 12 times the concentration than those receiving the 12/6 ^{o}C regime (Figure 4). When plants were grown at the 25/19^{o}C regime, endophyte concentration increased over the duration of experiment. However, endophyte concentration decreased if plants grown at 25/19 ^{o}C were switched to the 12/6 ^{o}C regime during the second 3-week period. Plants grown at the 12/6 ^{o}C regime had similar endophyte concentrations over the duration of the experiment. However if they were switched to the 25/19 ^{o}C regime at week 3, endophyte concentration increased to over six times the concentration of those continuously grown at 12/6 ^{o}C.

Endophyte biomass increased over the duration of experiment when plants were grown at the 25/19 ^{o}C regime (Figure 5). However, endophyte biomass was less if plants grown at 25/19 ^{o}C were switched to the 12/6 ^{o}C after 3-weeks. Plants grown at 12/6 ^{o}C regime had little or no increased in endophyte biomass over the 6-week period. However, endophyte biomass increased when plants were switched from 12/6 ^{o}C to the 25/19 ^{o}C regime after 3 weeks.

Endophyte frequencies for plants grown at all temperature regimes were similar, except when plants were continuously grown at the 12/6 ^{o}C regime for the entire 6week period (Table 3).

b) Determining cardinal minimum temperature for endophyte and tall fescue growth

Germinated plants were too small to detect endophyte presence during week 0 and

1. Analysis of variance indicated there were no differences, among temperatures, weeks, and there was no interaction between temperatures and weeks for endophyte frequency. However, there was a temperature by week interaction for plant dry weight, endophyte concentration, and endophyte biomass.

Although dry weights for plants grown at different temperatures were similar for weeks 1 and 2, plant dry weight generally increased thereafter (Figure 6). Plants grown at 20 °C grew faster than those grown at 15 °C which grew faster than those grown at 10 °C. Endophyte biomass had a similar growth trend as plant dry weight when grown at 20 and 15 °C, but plants grown at 10 °C had little increase in endophyte biomass (Figure 7).

Plant dry weights (dependent variable) were regressed against temperatures (independent variable) for the week 4, 5, and 6 harvest dates. These data had best fit to a linear equation with $R^2 > 0.94$ regardless of week when plants were harvested (Figure 8). These equations were solved for $Y = 0$ to estimate the minimum cardinal temperature for plant growth. The calculated X values for all 3 equations at $Y = 0$ had a mean of 5.16 °C and confidence interval of ± 0.47 °C. Although these data were extrapolated to simulate zero plant growth, they suggest the cardinal minimum temperature for plant growth is approximately 5.0 °C.

Endophyte biomass data (dependent variable) were also regressed against temperatures (independent variable) for weeks 4, 5, and 6. Data for endophyte biomass from plants harvested at week 4 and 5 had best fit to a linear equation while that from plants grown 6 weeks had a best fit to a quadratic equation (Figure 9). The R^2 of these endophyte biomass equations were 0.86 or better. These equations were solved for $Y = 0$ to estimate the minimum cardinal temperature for endophyte growth. Minimum cardinal temperatures estimated for endophyte growth had a mean of 10.28 °C and confidence interval of ± 0.70 °C, suggesting the cardinal minimum temperature for endophyte growth is approximately 10 °C.

Experiment 3. Effects of water stress on endophyte transmission

Plants receiving water stress were grown in soils containing approximately 0.42-0.45 g water g^{-1} soil. Soil water content of those plants not receiving stress treatments were between 0.60 and 0.65 g water g^{-1} soil. When stress was imposed, it took 14 days for water content to drop from 0.65 to 0.45 g water g^{-1} soil. As soil water content decreased, leaf turgor pressures decreased from 1.1 MPa in control plants to almost 0 MPa in all endophyte genotype/plant genotype combinations (data not shown).

Water stress had a little effect on endophyte concentration or endophyte frequency within psuedostems when harvested at the boot stage. There was a statistically significant difference in endophyte frequency of psuedostem among plant regenerants of PDN2 with different endophytes, but only in vegetative tillers receiving water stress prior to inflorescence. Even though statistically significant, it is not likely this decrease was biologically significant. Endophyte concentration among those plants was not affected by drought. Endophyte EDN12 was present at a higher concentration in psuedostems than EDN2 or EDN11 (Table 4).

Endophyte frequency among florets within the panicles or mature seed was not affected by water stress. Seeds harvested from the middle of the panicle had lower endophyte frequency than seeds harvested at the base or tip of the panicle (Table 5).

Drought also had no effect on endophyte frequency or concentration in tillers of different plant genotypes containing a common endophyte when harvested at the boot stage. Statistically, there was a higher frequency of endophyte in reproductive tillers (99.7 %) than in vegetative tiller (97.8 %) but this is unlikely to have biological significance. Florets and seeds located at the base or tip of panicle had higher endophyte frequency than those in the middle of the panicle (Table 6). Endophyte concentration varied among plant genotypes when tillers were harvested at the boot stage as well as among panicles and seeds harvested at seed maturity. Panicle endophyte concentration was lower than that of seed. Plant genotype 1 had the greatest concentration of endophyte, plant genotype 2 and 3 were not different from one another and plant genotype 4 had the least amount of endophyte. Plant genotype rankings were consistent for endophyte concentration in tiller, panicle, and seeds (Table 7).

DISCUSSION

The impetus for this project was an observation that field-grown endophyte-infected plants had numerous tillers in which no endophyte was present. These plants were growing under dry and cool conditions in March of 1998. A literature search found virtually no research linking environmental conditions with endophyte transmission. Thus these experiments were conducted to perform initial investigations into environmental influences on endophyte grown *in planta*.

Di Menna and Waller (1986) noted seasonal variation in endophytes of perennial ryegrass grown under field conditions in New Zealand. They found fewer mycelia in psuedostem tissue during August, the equivalent of February in the Northern Hemisphere. Their data suggested that seasonal variability in perennial ryegrass was similar to the data for field-grown tall fescue in Georgia and Oregon in this study. Although not tested, they attributed the seasonal variation to temperature and drought stress. Bacon and Siegel (1988) noted a decrease of endophyte in seed and vegetative tissue of tall fescue after the plants had experienced hot and dry summers and cold winters. Conversely, Richardson et al. (1993) and Bruehl and Kaiser (1996) found endophyte grew best on agar when under mild water stress conditions.

The drought experiments in this study indicated there was little or no effect of drought on seed transmission of endophyte or frequency of endophyte in panicles or tillers of endophyte-infected tall fescue. Thus, we conclude drought stress is not an environmental variable affecting *in planta* growth and transmission of endophyte.

Responses of tall fescue grown under constant temperature regimes of 10, 15, or

20 °C, or when transferring plants from warm to cool or cool to warm conditions, were similar to that found by others (Roboson, 1974; Thomas and Stoddart, 1995). Plants grown at high temperatures had greater growth than those grown at cooler temperatures and plants switched from warm to cool conditions reduced growth rate while those switched from cool to warm had increased growth rates (Figure 3 and 6). Although extrapolation of regression was used to estimate the cardinal minimum temperature for plant growth weight, our estimated cardinal minimum temperatures of 5.16 ± 0.47 °C for plant growth was similar to that determined by Thomas and Stoddart (1995). The similarity of our test results with that of others provides a measure of confidence that the test conditions for experiments investigating temperature effects on plants reported herein are valid.

The calculated cardinal minimum temperature for endophyte growth was 10.28 ± 0.7 °C, approximately 5 °C higher than that for plant growth. Thus, it is not surprising to find lower endophyte frequencies in field-grown tall fescue during the winter and spring months (Table 1) when mean monthly temperatures were below the cardinal minimum temperature for endophyte growth.

It is important to note, however, that while endophyte frequency and concentration were lowest in months when temperatures were lowest, it is possible the endophyte response is related to vernalization and physiological or morphological changes occurring in the plants. It is known the endophyte resides within meristematic tissue during vegetative growth (Sampson, 1993; 1937; Bacon and Siegel, 1988) and that endophytes are in flower primordia before development of inflorescences (Siegel, et al., 1985). Hinton and Bacon (1985) suggest that an infected bud simply outgrow the endophytes when conditions are not favorable for the endophyte. Thus, it is vital for endophytes to invade developing seeds as a possible means to avoid having to maintain rapid growth rates during elongation of the culm. Endophytes are in the flower primordia before the inflorescence develops (Siegel et al., 1985) and they have been observed in the differentiating primordial tissue prior to stem elongation (Phillipson and Christy, 1986). Thus, it is likely the endophyte prioritizes invasion of the developing floret over other tissue, perhaps when vernalization conditions have been met. Since endophyte *in planta* is non-septate (Hinton and Bacon, 1985), it is possible the endophyte transports vital components back to the meristematic region to provide necessary nutrients to sustain growth and invasion of the developing panicle primodia. In doing so the endophyte may be difficult to detect in above-ground portions of the plant when the meristem is differentiating from vegetative to reproductive tissue.

From a practical standpoint, this research demonstrates that tall fescue should not be sampled for endophyte detection from January through April. This is an important finding since many producers are likely to sample pastures for endophyte frequency to determine which: a) should be replaced with non-toxic fescue, b)should be overseeded with other forage species to dilute the toxic effect, c) will need supplementation to reduce toxin intake, or d) should be avoided during periods of livestock reproduction. Pastures should be sampled from May through December to obtain valid endophyte frequency data from which to base these management decisions.

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TABLES and FIGURES

Table 1. Mean monthly endophyte frequency in psuedostems of tall fescue grown during 2000 through 2002 in Georgia and 1999 through 2001 in Oregon.

Month sampled	Georgia	Oregon
	-------------------------%-------------------------	
January	79.5	92.0
February	79.5	72.6
March	82.5	51.6
April	92.0	69.3
May	95.0	84.6
June	93.0	-[†]
July	93.5	-
August	97.5	-
September	95.0	-
October	94.5	85.3
November	87.0	91.4
December	93.0	89.5
[‡]LSD (0.05)	7.2	12.8

[†] indicates no samples harvested

[‡] Least significant difference at the 0.05 level of probability.

Table 2. Mean monthly endophyte concentration in psuedostems of tall fescue grown in Georgia during 2000 through 2002.

Month sampled	Endophyte concentration
	---------mg endophyte g^{-1} plant----------
January	0.57
February	0.77
March	0.66
April	0.93
May	1.25
Jun	1.52
July	1.93
August	1.80
September	1.87
October	2.07
November	1.61
December	1.16
[†]LSD (0.05)	0.27

[†] Least significant difference at the 0.05 level of probability.

Table 3. Effect of temperature and harvest date on endophyte frequency in psuedostems of tall fescue grown at four temperature regimes in growth chambers.

Temperature	Week 3	Week 6	[§]LSD (0.05)
	%		
12/6 °C	92.0	77.6	7.7
12/6 to 25/19 °C[†]	86.3	90.3	NS
25/19 °C	92.3	95.3	NS
25/19 to 12/6 °C[††]	89.7	94.0	NS
[§]LSD (0.05)	5.5	5.3	

[†] indicates plants grown at 12/6 °C day/night temperature during the first 3-week period were switched to the 25/19 °C day/night temperature during the second 3-week period.

[††] indicates plants grown 25/19 °C at day/night temperature during the first 3-week period were switched to the 12/6 °C day/night temperature during the second 3-week period.

[§] Least significant difference at the 0.05 level of probability.

NS = not significant.

Table 4. Endophyte concentration in greenhouse-grown psuedostems from regenerants of tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) when harvested at the boot stage.

EDN2	EDN11	EDN12	†LSD (0.05)
--- mg endophyte g^{-1} plant --------------------------------			
1.21	1.20	1.36	0.08

† Least significant difference at the 0.05 level of probability.

Table 5. Endophyte frequency in florets or seeds of regenerants of tall fescue genotype PDN2 containing three different endophyte genotypes EDN2, EDN11, or EDN12. Plants were grown in the greenhouse during 2001.

Seed stage	Panicle position			[†]LSD (0.05)
	Top	Middle	Bottom	
	---------------------------- %	----------------	----------------	----------------
Floret	62.15	55.11	55.48	4.25
Seed	69.89	59.70	65.74	4.47

[†] Least significant difference at the 0.05 level of probability.

Table 6. Mean endophyte frequency in florets or seeds of four tall fescue genotypes containing a common endophyte genotype EDN11 when grown in the greenhouse during 2000 and 2001.

Tissue type	Panicle position			†LSD(0.05)
	Top	Middle	Bottom	
	%			
Floret	61.74	54.46	57.43	4.01
Seed	77.90	73.97	76.85	2.81

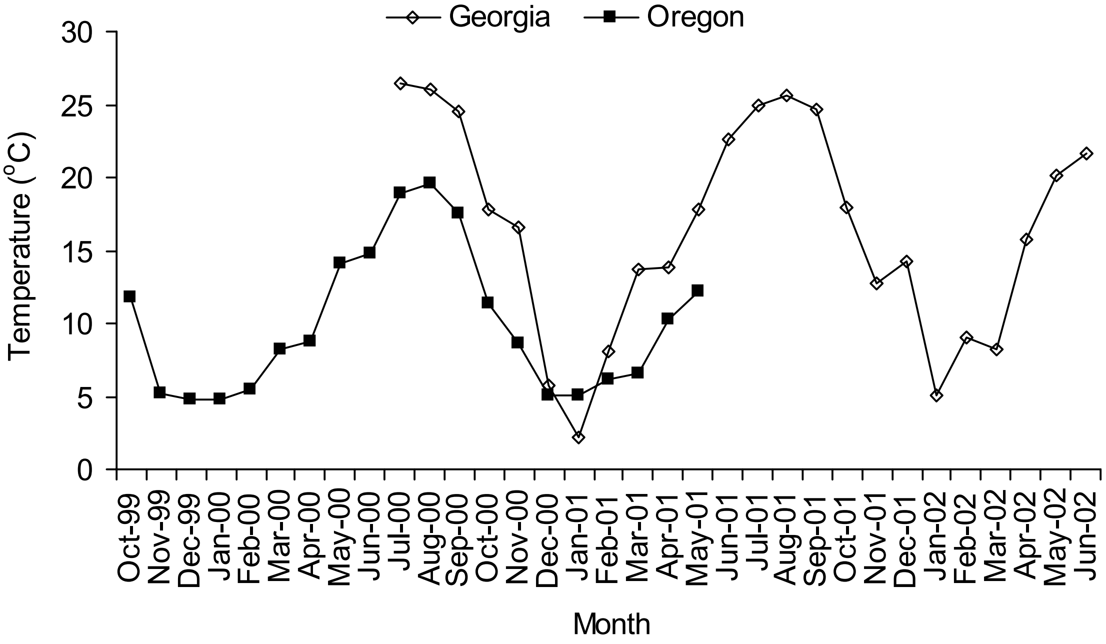
† Least significant difference at the 0.05 level of probability.

Table 7. Mean endophyte concentration in three tissues of four tall fescue genotypes containing a common endophyte genotype EDN11 when grown in the greenhouse during 2000 and 2001. Tillers were harvested in boot stage and panicles and seeds were harvested at seed maturity.

Tissue	Plant genotype				†LSD(0.05)
	1	2	3	4	
	-------------------- mg endophyte g^{-1} plant--------------------------------------				
Culm	1.35	1.00	0.97	0.69	0.09
Panicle	0.28	0.22	0.23	0.15	0.03
Seed	2.17	1.47	1.44	1.16	0.25

† indicates least significant difference at the 0.05 level of probability.

Figure 1. Weather data for Watkinsville, GA and Salem, OR for months when tillers were sampled from field-grown plants. Bar graphs and line graphs indicate precipitation and mean monthly temperature, respectively.


Georgia
Oregon
Temperature (^{o}C)
30
25
20
15
10
5
0
Oct-99
Nov-99
Dec-99
Jan-00
Feb-00
Mar-00
Apr-00
May-00
Jun-00
Jul-00
Aug-00
Sep-00
Oct-00
Nov-00
Dec-00
Jan-01
Feb-01
Mar-01
Apr-01
May-01
Jun-01
Jul-01
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Oct-01
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Jan-02
Feb-02
Mar-02
Apr-02
May-02
Jun-02
Month

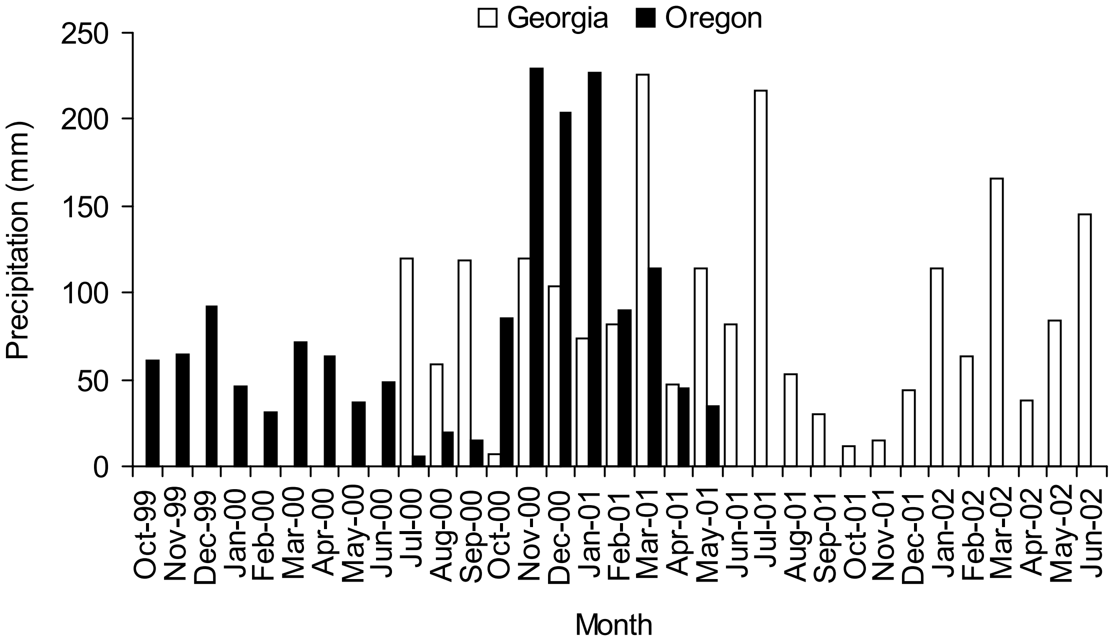

☐ Georgia
■ Oregon
Precipitation (mm)
250
200
150
100
50
0
Oct-99
Nov-99
Dec-99
Jan-00
Feb-00
Mar-00
Apr-00
May-00
Jun-00
Jul-00
Aug-00
Sep-00
Oct-00
Nov-00
Dec-00
Jan-01
Feb-01
Mar-01
Apr-01
May-01
Jun-01
Jul-01
Aug-01
Sep-01
Oct-01
Nov-01
Dec-01
Jan-02
Feb-02
Mar-02
Apr-02
May-02
Jun-02
Month

Figure 2. Mean endophyte frequency in psuedostem bases (Bottom) and 3 cm above the pseudostem base (Middle) of endophyte-infected tall fescue grown in Georgia fields during 2000 through 2002. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.1 level of probability.

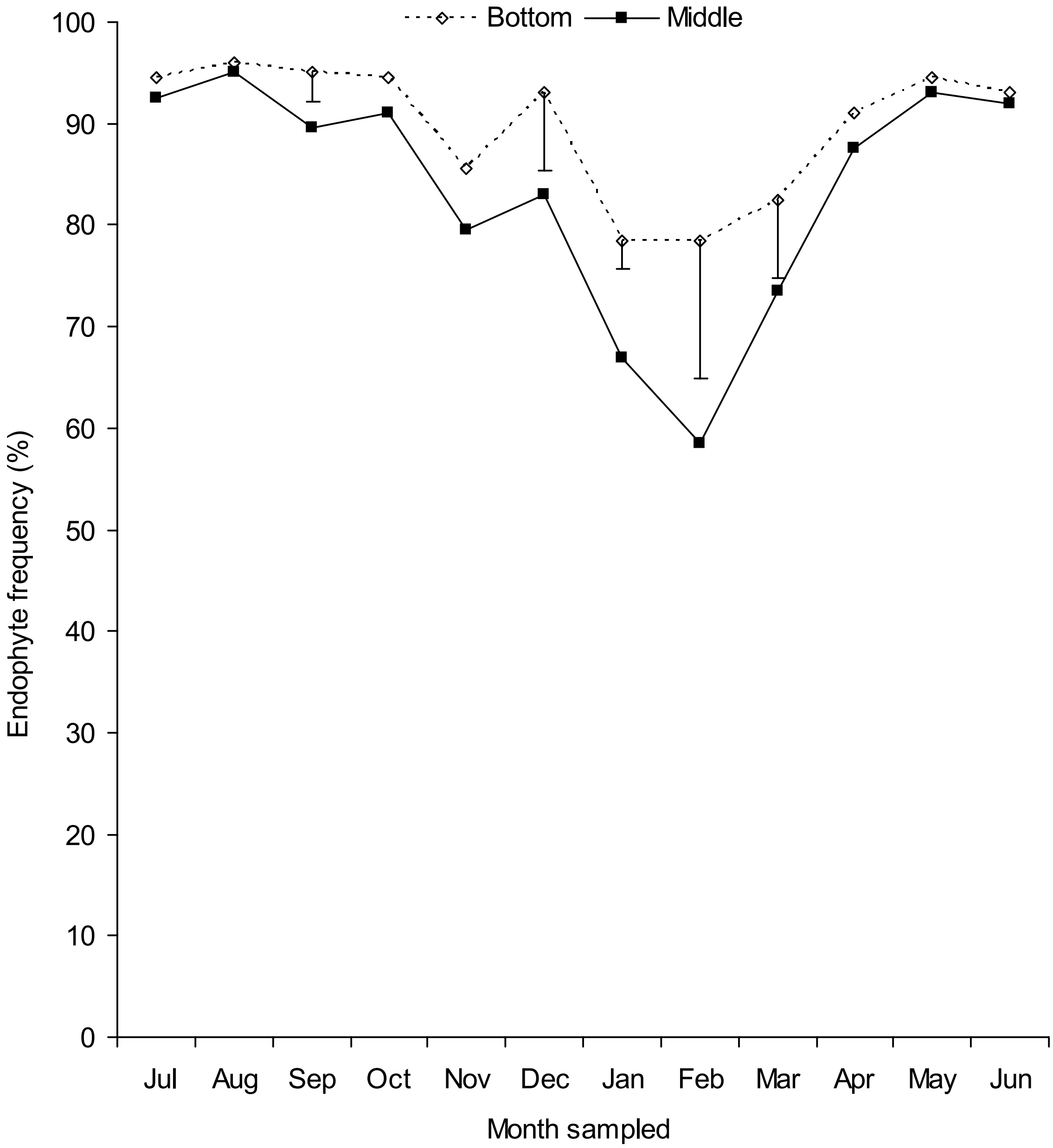
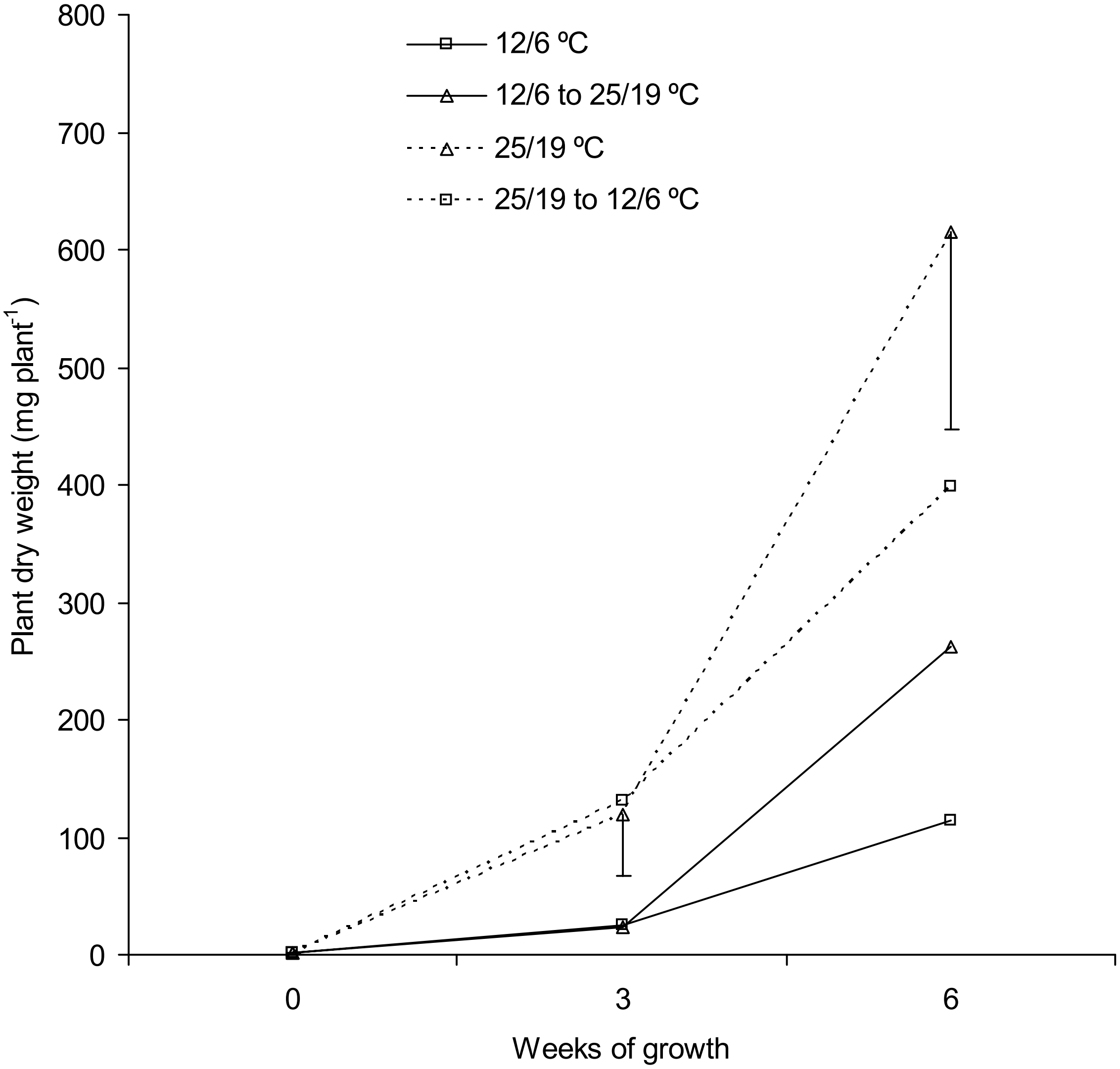

Bottom
Middle
Endophyte frequency (%)
100
90
80
70
60
50
40
30
20
10
0
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Month sampled

Figure 3. Plant dry weights of tall fescue grown in growth chambers with four different temperature regimes over a 6 week period. One half of plants were switched from one temperature regime to the other at week 3. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.05 level of probability.



800
700
600
500
400
300
200
100
0
Plant dry weight (mg plant^{-1})
0
3
6
Weeks of growth
12/6 ºC
12/6 to 25/19 ºC
25/19 ºC
25/19 to 12/6 ºC

Figure 4. Endophyte concentration in psuedostem of tall fescue plants grown in growth chambers with four different temperature regimes over a 6 week period. One half of plants were switched from one temperature regime to the other at week 3. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.05 level of probability.

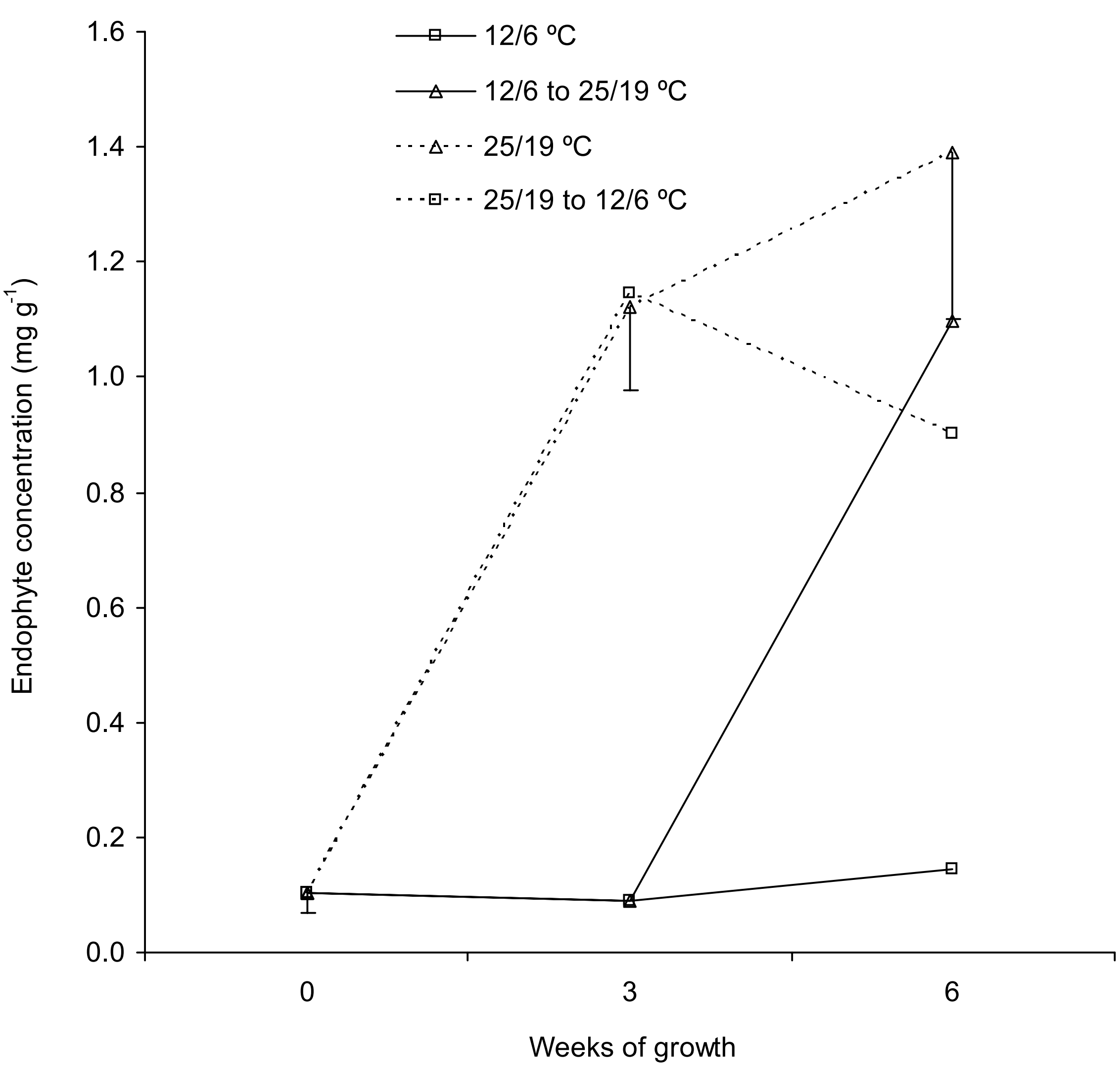

12/6 ºC
12/6 to 25/19 ºC
25/19 ºC
25/19 to 12/6 ºC
Endophyte concentration (mg g^{-1})
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
Weeks of growth

Figure 5. Endophyte biomass in tall fescue plants grown in growth chambers with four different temperature regimes over a 6 week period. One half of plants were switched from one temperature regime to the other at week 3. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.05 level of probability.

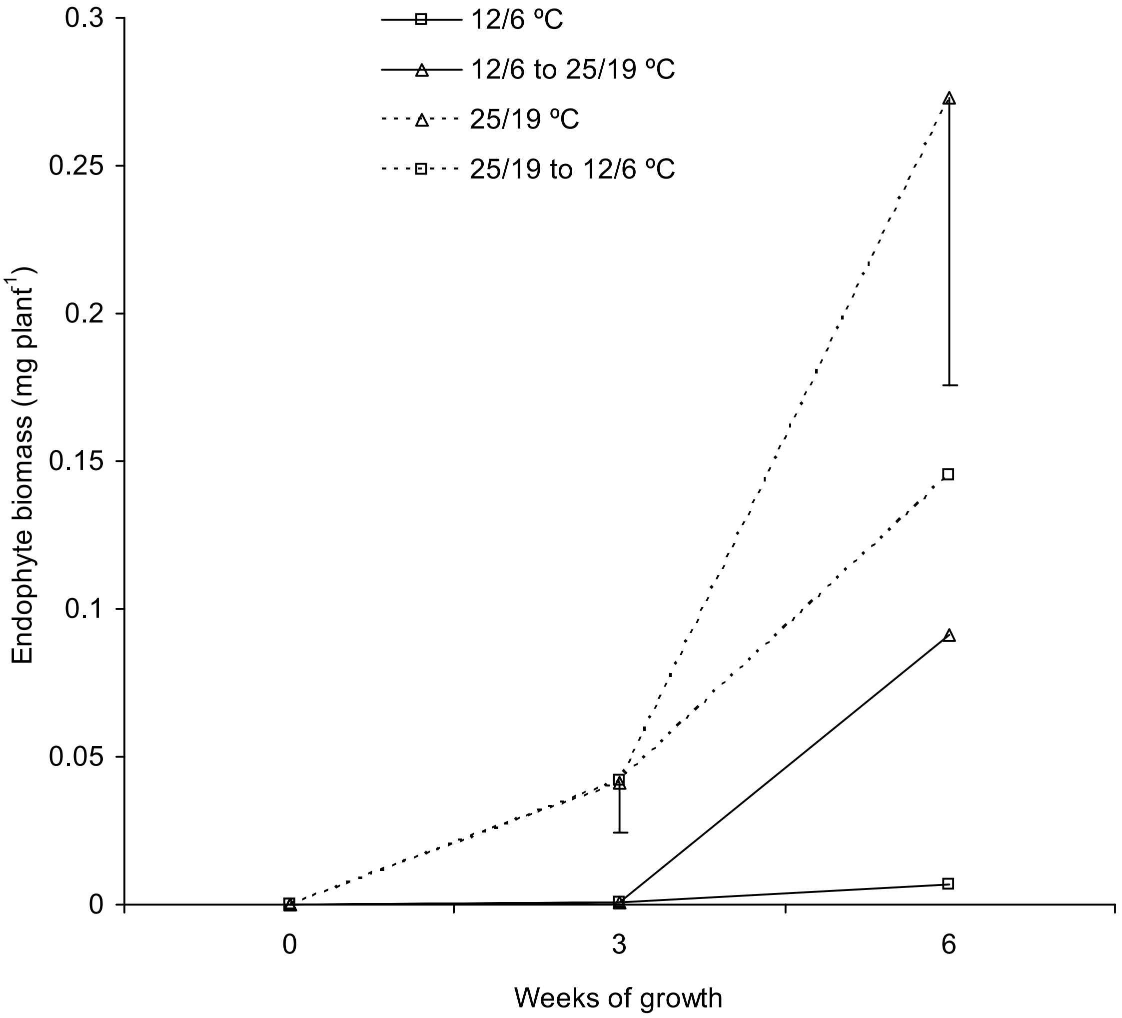

12/6 ºC
12/6 to 25/19 ºC
25/19 ºC
25/19 to 12/6 ºC
Endophyte biomass (mg plant-1)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
3
6
Weeks of growth

Figure 6. Weekly plant dry weight of tall fescue grown in growth chambers at constant temperatures of 10, 15, or 20 ^{o}C over 6 weeks. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.05 level of probability.

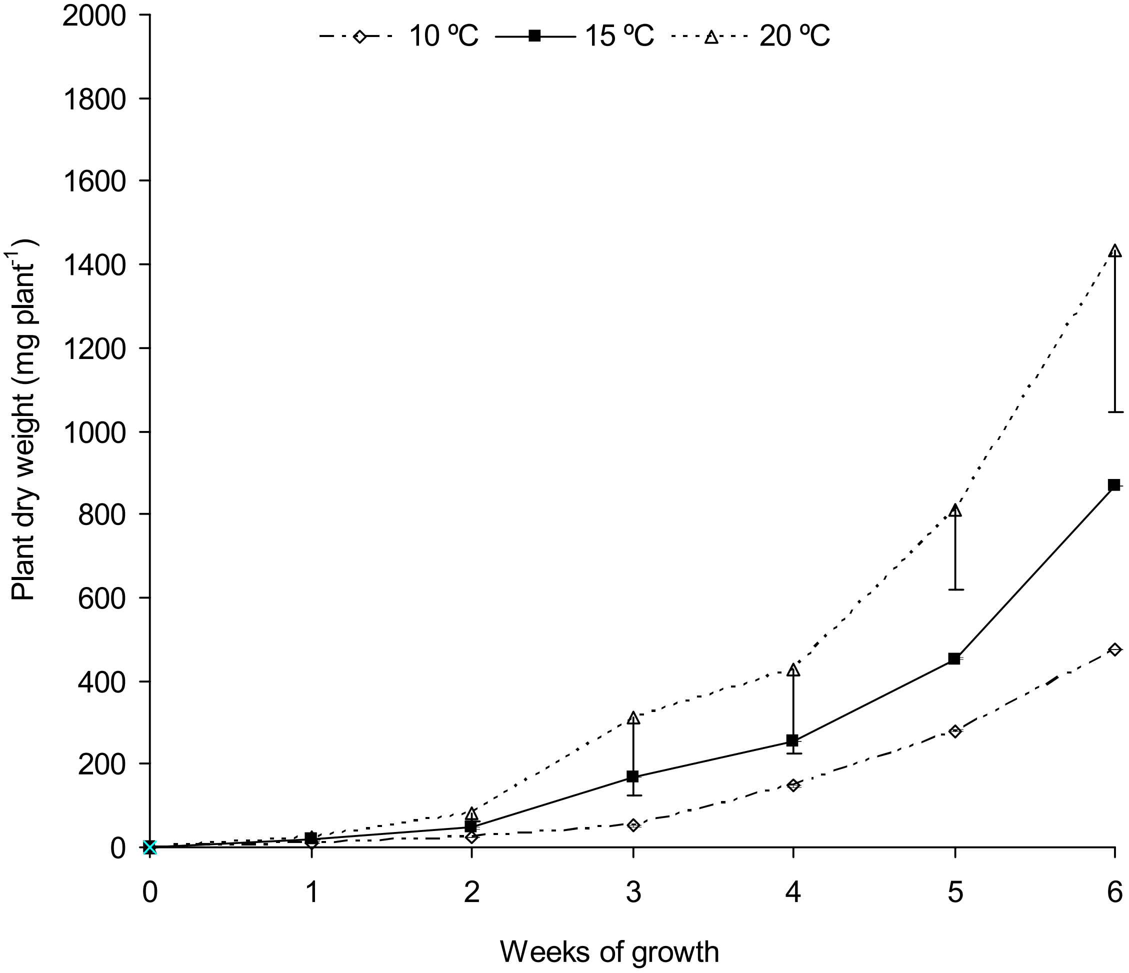

10 ºC
15 ºC
20 ºC
Plant dry weight (mg plant^{-1})
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
Weeks of growth

Figure 7. Weekly endophyte biomass in tall fescue grown in growth chambers at constant temperatures of 10, 15, or 20 ^{o}C over 6 weeks. Vertical bars on line graph indicate the value for Fisher's least significant difference at the 0.05 level of probability.

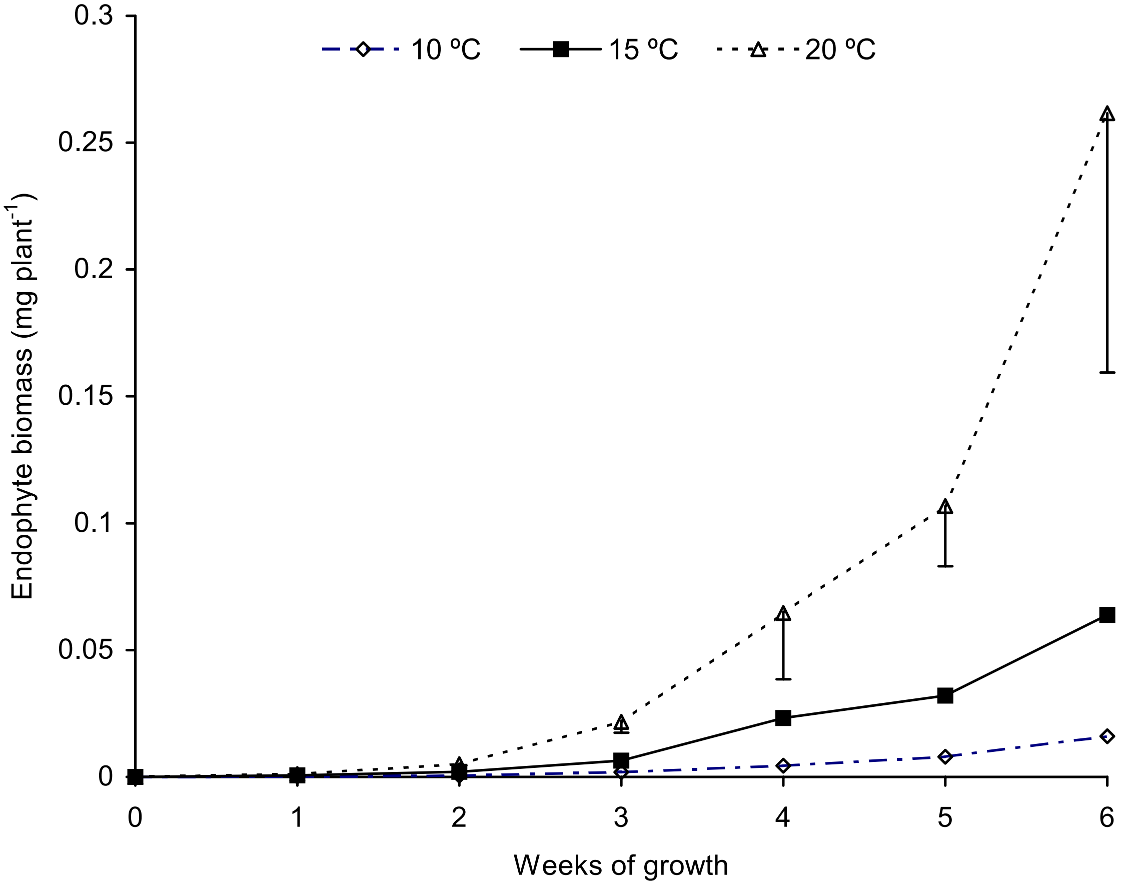

10 ºC
15 ºC
20 ºC
Endophyte biomass (mg plant^{-1})
0.3
0.25
0.2
0.15
0.1
0.05
0
Weeks of growth
0
1
2
3
4
5
6

Figure 8. Regression equations used to predict cardinal minimum temperature when solving for y=0 for tall fescue when grown at constant temperatures of 10, 15, and 20 ^{o}C. Data from weeks 4, 5, and 6 were to generate the regression equations (see text for reasons). Bold dot lines indicate an extrapolation from each regression line.

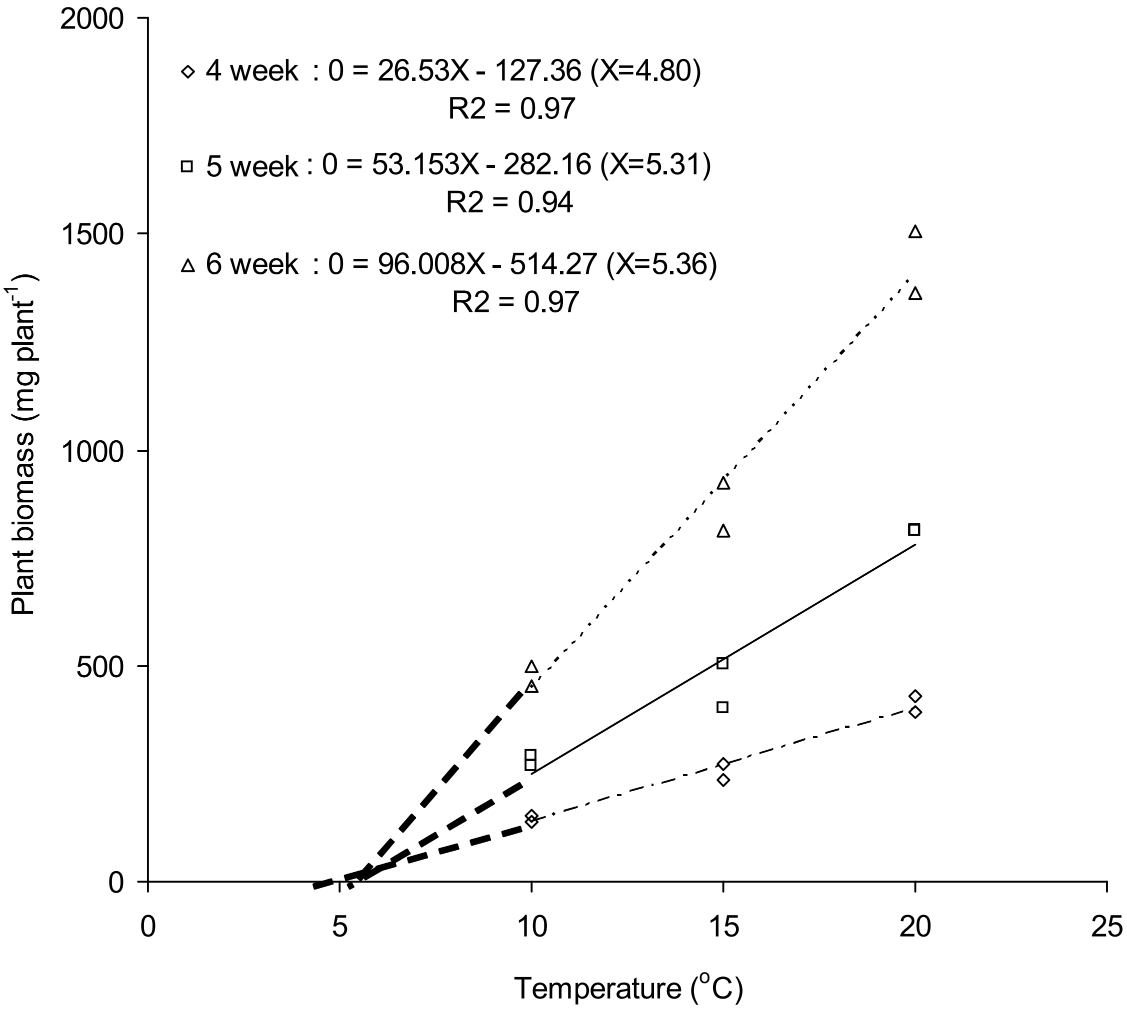
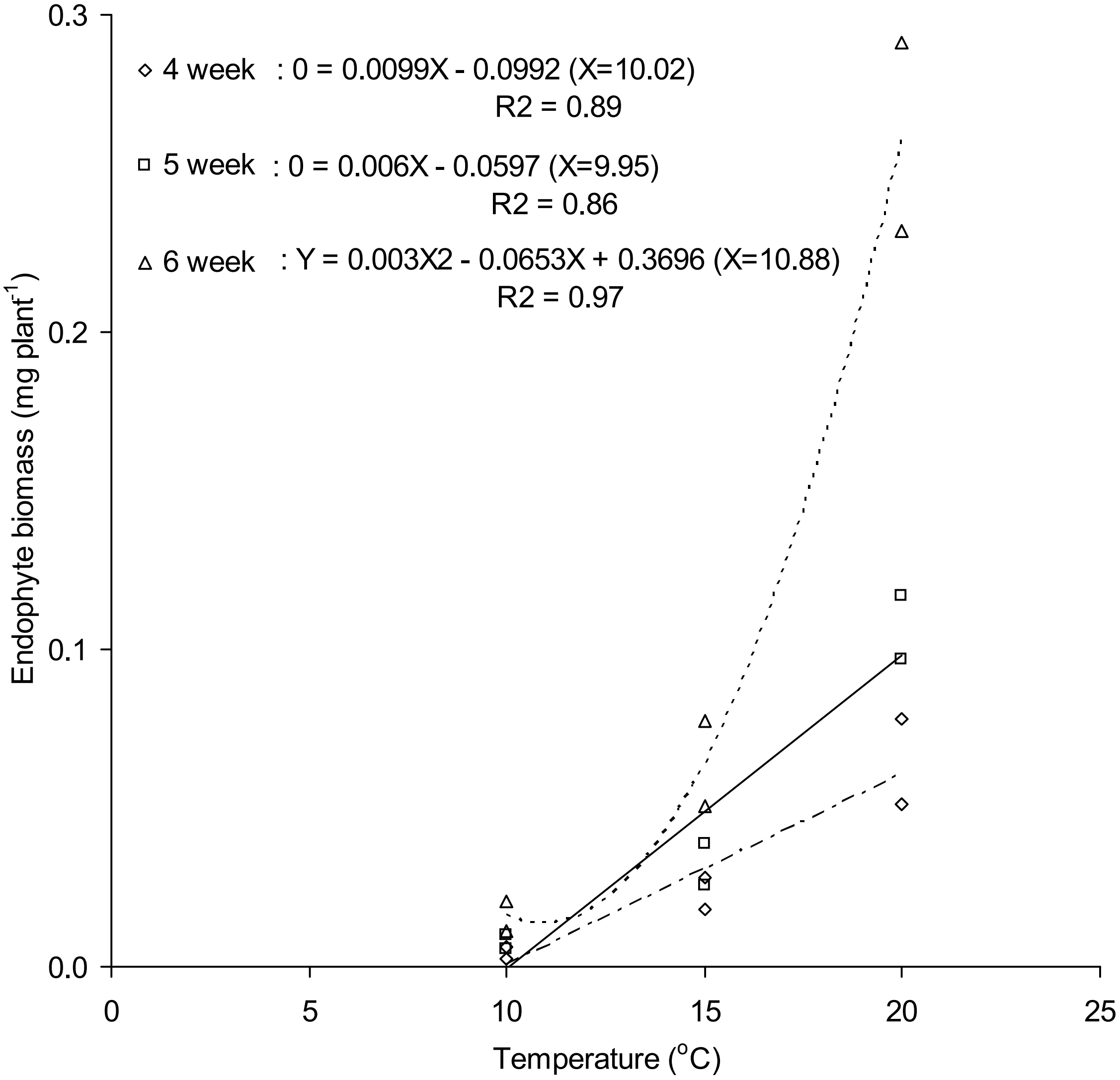

2000
1500
1000
500
0
Plant biomass (mg plant^{-1})
◇ 4 week : 0 = 26.53X - 127.36 (X=4.80)
R2 = 0.97
□ 5 week : 0 = 53.153X - 282.16 (X=5.31)
R2 = 0.94
△ 6 week : 0 = 96.008X - 514.27 (X=5.36)
R2 = 0.97
0
5
10
15
20
25
Temperature (°C)

Figure 9. Regression equations used to predict cardinal minimum temperature for endophyte in tall fescue grown at constant temperatures of 10, 15, and 20 ^{o}C. Data from weeks 4, 5, and 6 were to generate the regression equations (see text for reasons). Equations from weeks 4, and 5 were solved when Y=0 and equation from week 6 was solved by differentiation.


0.3
0.2
0.1
0.0
Endophyte biomass (mg plant^{-1})
◇ 4 week : 0 = 0.0099X - 0.0992 (X=10.02)
R2 = 0.89
□ 5 week : 0 = 0.006X - 0.0597 (X=9.95)
R2 = 0.86
△ 6 week : Y = 0.003X2 - 0.0653X + 0.3696 (X=10.88)
R2 = 0.97
0
5
10
15
20
25
Temperature (^{o}C)

APPENDICES

Appendix 1. Analysis of variance for mean monthly endophyte frequency in psuedostems of tall fescue grown in Georgia during 2000 through 2002 and Oregon during.1999 through 2001.

Source	df	Georgia	Source	df	Oregon
Replication	1	0.0199	Replication	1	0.0001
Year (Y)	1	0.3981	Year (Y)	1	0.1133
Month (M)	11	0.0001	Month (M)	7	0.0001
Y*M	11	0.1217	Y*M	7	0.0760

Appendix 2. Analysis of variance for mean monthly endophyte frequency in psuedostem base and 3 cm above the pseudostem base of endophyte-infected tall fescue grown in Georgia pasture fields during 2000 through 2002.

Source	df	Probability > F value
Replication	1	0.1697
Month (M)	11	0.0001
Sampling height (H)	1	0.0001
Year (Y)	1	0.2565
M*H	11	0.0129
M*Y	11	0.5238
H*Y	1	0.8067
M*H*Y	11	0.9811

Appendix 3. Analysis of variance for mean monthly endophyte concentration in psuedostem of tall fescue grown in Georgia during 2000 through 2002.

Source	df	Probability > F value
Replication	1	0.0013
Month (M)	11	0.0001
Year (Y)	1	0.1474
M*Y	11	0.0181

Appendix 4. Correlations (r) between endophyte frequency and concentration in psuedostem of tall fescue plants grown in pasture fields in Georgia and seed fields in Oregon and weather data collected from Georgia during 2000 through 2002and Oregon during 1999 through 2001.

	Endophyte frequency	Endophyte concentration
Endophyte concentration	0.62^{***}	-
Precipitation	-0.01^{NS}	-0.18^{NS}
Mean temperature	0.44^{***}	0.75^{***}

*** Significance at 0.001probability level.

NS = not significant.

Appendix 5. Analysis of variance for agronomic characteristics, endophyte concentration, and endophyte infection of tall fescue plants grown in growth chambers with four different temperature regimes at three sampling dates over a 6 week periods.

Source	df	Plant dry weight	Endophyte concentration	Endophyte bio-mass	df	Endophyte infection
Replication	2	0.3109	0.2415	0.6556	2	0.0346
Year (Y)	1	0.0479	0.6289	0.7187	1	0.7059
Temperature (T)	3	0.0155	0.0001	0.0001	3	0.0013
Y*T	3	0.5576	0.0679	0.1969	3	0.6031
Sampled week (W)	2	0.0001	0.0001	0.0001	1	0.6278
Y*W	2	0.3578	0.1445	0.4499	1	0.4207
T*W	6	0.3848	0.0001	0.0001	3	0.0002
Y*T*W	6	0.8241	0.0001	0.0441	3	0.6229

Appendix 6. Analysis of variance for agronomic characteristics, endophyte concentration, and endophyte infection of tall fescue plants grown in growth chambers with three different constant temperatures over a 6 week periods.

Source	df	Plant dry weight	Endophyte concentration	Endophyte bio-mass	df	Endophyte infection
Replication	1	0.5097	0.0114	0.0841	1	0.1643
Temperature (T)	2	0.0001	0.0001	0.0001	2	0.0511
Sampled week (W)	6	0.0001	0.0001	0.0001	4	0.3090
T*W	12	0.0001	0.0001	0.0001	8	0.0511

Appendix 7. Analysis of variance for endophyte frequency and endophyte concentration in psuedostem of a tall fescue genotype (DN2) affected by drought stress on different endophyte genotypes (EDN2, EDN11, or EDN12) harvested at boot stage during 2000 and 2001.

Source	df	Endophyte frequency	Endophyte concentration
Replication	2	0.3952	0.9356
Treatment (T)	2	0.0280	0.0803
Year (Y)	1	0.5812	0.0090
T*Y	2	0.9262	0.1851
Genotype (G)	2	0.8745	0.0001
T*G	4	0.7086	0.7908
Y*G	2	0.7798	0.0969
T*Y*G	4	0.4786	0.7680
Tiller Type (TT)	1	0.0299	0.0001
T*TT	2	0.0426	0.1772
Y*TT	1	0.7825	0.4602
T*Y*TT	2	0.7365	0.3004
G*TT	2	0.7798	0.0688
T*G*TT	4	0.9418	0.7369
Y*G*TT	2	0.8745	0.0704
T*Y*G*TT	4	0.9418	0.6353

Appendix 8. Interaction between water stress and tiller type on endophyte frequency in tillers of a tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) and grown in green house harvested at the boot stage during 2000 and 2001.

Water treatment	Tiller type		¶LSD (0.05)
	Reproductive tiller	Vegetative tiller	
	---------------------------- %	----------------------	
Before heading (BH)	98.89	95.00	3.16
After heading (AH)	98.89	98.89	NS
No water stress (Control)	99.17	98.61	NS
¶LSD (0.05)	N.S	2.99	

¶ indicates least significant difference at the 0.05 level of probability

NS indicates not significant.

Appendix 9. Effects of year and tiller type on endophyte concentrations in tillers of tall fescue tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress, grown in greenhouse, and harvested at the boot stage during 2000 and 2001.

	Endophyte concentration		
	--------------------------mg endophyte g^{-1} plant------------------------		
Tiller type	Reproductive tiller	Vegetative tiller	¶LSD (0.05)
	1.45	1.06	0.07
Year	2000	2001	¶LSD (0.05)
	1.30	1.21	0.07

¶ indicates least significant difference at the 0.05 level of probability

Appendix 10. Analysis of variance for endophyte frequency of tall fescue floret and seed affected by drought stress on tall fescue genotype (PDN2) containing different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress harvested at boot stage during 2000 and 2001 and at seed maturity during 2001, respectively.

Source	df	Boot stage			Seed maturity
			Year		
			1st year	2nd year	
Replication	2	0.2032	0.8236	0.0293	0.8748
Treatment (T)	2	0.2777	0.4126	0.0317	0.6025
Genotype (G)	2	0.0962	0.0002	0.6351	0.0782
T*G	4	0.5264	0.0119	0.0245	0.7790
Position (P)	2	0.0016	0.0038	0.0957	0.0001
T*P	4	0.7914	0.7146	0.8023	0.6400
G*P	4	0.4709	0.9226	0.2309	0.2813
T*G*P	8	0.9659	0.9971	0.9768	0.6422
Year (Y)	1	0.0016			
T*Y	2	0.0237			
Y*G	2	0.0073			
T*Y*G	4	0.0005			
Y*P	2	0.6627			
T*Y*P	4	0.7793			
Y*G*P	4	0.3835			
T*Y*G*P	8	0.9986			

Appendix 11. Effects of water stress on endophyte frequency among florets within the panicles of tall fescue genotype (PDN2) containing different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress harvested at boot stage in 2000 and 2001.

Year	BH	AH	Control	¶LSD (0.05)
	-------------	------------- %	-------------	-------------
2000	54.04	53.56	56.67	NS
2001	65.04	60.30	55.89	6.76

¶ indicates least significant difference at the 0.05 level of probability

NS indicates not significant.

Appendix 12. Effect of endophyte genotype on endophyte frequency of florets within the panicle of tall fescue genotype (PDN2) containing different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress harvested at boot stage in 2000 and 2001.

Year	EDN2	EDN11	EDN12	¶LSD (0.05)
	------------------------------------	------------ % ------------	------------------------------------	
2000	56.44	48.44	59.37	5.01
2001	58.78	62.00	60.44	NS

¶ indicates least significant difference at the 0.05 level of probability

NS indicates not significant.

Appendix 13. Analysis of variance for endophyte concentration in panicle (no seed) of tall fescue genotype (PDN2) containing different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress at seed maturity in 2000 and 2001and analysis of variance for endophyte concentration at different tissues (panicle or seed) on seed head of tall fescue (PDN2) containing different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought at seed maturity in 2001.

Source	df	Panicle	Source	df	Seed head		
						Tissue type	
						Panicle	Seed
Replication	2	0.0001	Replication	2	0.0001	0.0001	0.0001
Treatment (T)	2	0.4395	Treatment (T)	2	0.8015	0.1987	0.2712
Genotype (G)	1	0.0002	Genotype (G)	2	0.0500	0.0002	0.0018
T*G	2	0.4044	T*G	4	0.9685	0.6028	0.7415
Year (Y)	2	0.0008	TT (Tissue type)	1	0.0001		
T*Y	4	0.4379	T*TT	2	0.6427		
G*Y	2	0.4245	G*TT	2	0.2736		
T*G*Y	4	0.0960	T*G*TT	4	0.9817		

Appendix 14. Endophyte concentration at panicle of tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) and grown in greenhouse at seed maturity in 2000 and 2001 and endophyte concentration at seed of tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) and grown in green house at seed maturity in 2001.

Tissue	EDN2	EDN11	EDN12	¶LSD (0.05)
	---------------------------- mg endophyte g^{-1} plant			------------------------
Panicle	0.32	0.30	0.38	0.04
Seed	1.34	1.48	1.72	0.18

¶ indicates least significant difference at the 0.05 level of probability

Appendix 15. Endophyte concentration in panicle of tall fescue genotype (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress and grown in greenhouse harvested at seed maturity in 2000 and 2001.

2000	2001	¶LSD (0.05)
--	mg endophyte g^{-1} plant	--
0.36	0.30	0.03

¶ indicates least significant difference at the 0.05 level of probability

Appendix 16. Endophyte concentration (mg endophyte g^{-1} plant) at different tissues (panicle or seed) in seed head of tall fescue tall fescue (PDN2) containing three different endophyte genotypes (EDN2, EDN11, or EDN12) affected by drought stress and grown in greenhouse harvested at seed maturity in 2000-2001.

Panicle	Seed	¶LSD (0.05)
---------------------------------------mg endophyte g^{-1} plant-------------------------------------		
0.30	1.52	0.15

¶ indicates least significant difference at the 0.05 level of probability

Appendix 17. Analysis of variance for endophyte frequency and concentration in psuedostem of different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown in greenhouse harvested at boot stage during 2000 and 2001.

Source	df	Endophyte frequency	Endophyte concentration		
				Year	
				1999-2000	2000-2001
Replication	2	0.5908	0.0012	0.0215	0.0527
Treatment (T)	2	0.2923	0.2183	0.6194	0.2177
Year (Y)	1	0.8963	0.0001		
T*Y	2	0.4364	0.5724		
Genotype (G)	3	0.6170	0.0001	0.0001	0.0001
T*G	6	0.4790	0.4242	0.9412	0.1036
Y*G	3	0.7023	0.0004		
T*Y*G	6	0.8273	0.3049		
TT	1	0.0007	0.8585	0.0426	0.0251
T*TT	2	0.6321	0.1791	0.6380	0.2563
Y*TT	1	0.6960	0.0023		
T*Y*TT	2	0.3453	0.8630		
G*TT	3	0.775	0.0814	0.0169	0.7636
T*G*TT	6	0.5879	0.859	0.8765	0.9119
Y*G*TT	3	0.7342	0.1373		
T*Y*G*TT	6	0.2436	0.9230		

Appendix 18. Effect of tiller type on endophyte frequency in psuedostem of different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown greenhouse harvested at boot stage during 2000 and 2001.

Reproductive tiller	Vegetative tiller	[¶]LSD (0.05)
--%	--	
99.65	97.78	1.06

[¶] indicates least significant difference at the 0.05 level of probability

Appendix 19. Interaction between year and tiller type on endophyte concentration in psuedostem of different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown greenhouse harvested at boot stage during 2000 and 2001.

Year	Tiller type		¶LSD (0.05)
	Reproductive tiller	Vegetative tiller	
	------------------------ mg endophyte g^{-1} plant ---------------------------------		
2000	1.02	1.12	0.09
2001	0.99	0.88	0.09
¶LSD	NS	0.09	

¶ indicates least significant difference at the 0.05 level of probability

NS indicates not significant

Appendix 20. Analysis of variance for endophyte frequency in florets and seed of different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown in greenhouse harvested at seed maturity during 2000 and 2001.

Source	df	Boot stage	Seed maturity
Replication	2	0.9936	0.5402
Treatment (T)	2	0.8707	0.6925
Year (Y)	1	0.0780	0.0490
T*Y	2	0.6021	0.1803
Genotype (G)	3	0.0074	0.0020
T*G	6	0.1224	0.0913
Y*G	3	0.3086	0.9407
T*Y*G	6	0.1376	0.4682
Position (P)	2	0.0020	0.0002
T*P	4	0.8453	0.9547
Y*P	2	0.5179	0.8316
T*Y*P	4	0.7799	0.9104
G*P	6	0.9858	0.2715
T*G*P	12	0.9898	0.8160
Y*G*P	6	0.8496	0.8870
T*Y*G*P	12	0.9921	0.9601

Appendix 21. Effect of plant genotype on endophyte frequency in florets and seeds of tall fescues different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown greenhouse harvested at boot stage (for floret) and at seed maturity (for seed) during 2000 and 2001.

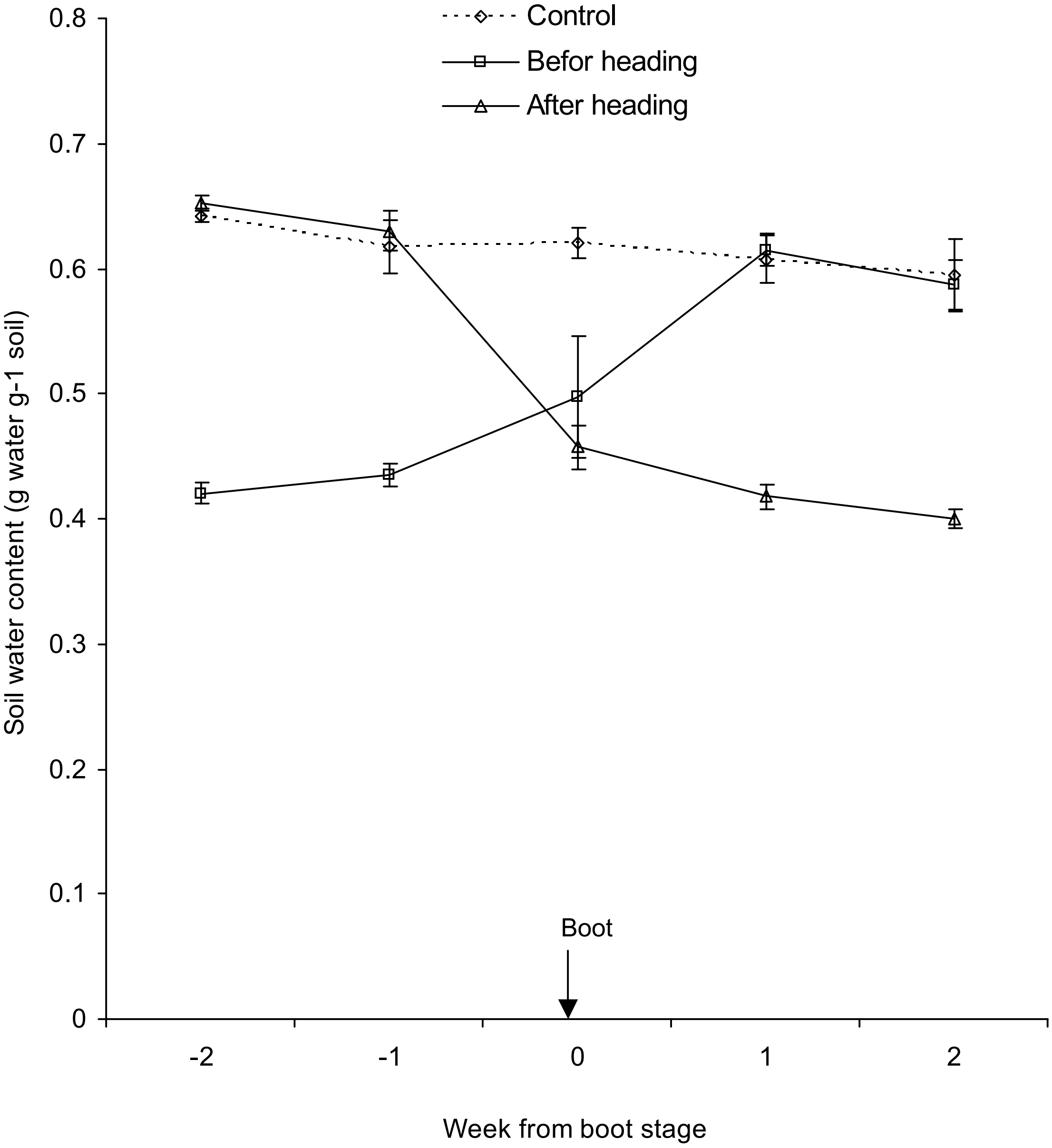
Developing stage sampled	Plant genotype 1	2	3	4	[¶]LSD (0.05)
	%				
Boot	61.63	53.42	58.19	58.28	4.63
Seed maturity	75.60	70.28	78.46	74.21	3.23

[¶] indicates least significant difference at the 0.05 level of probability

Appendix 22. Analysis of variance for endophyte concentration in florets and seed of different tall fescue genotypes infected with a common endophyte genotype (EDN11) affected by drought stress and grown in greenhouse harvested at seed maturity during 2000 and 2001.

Source	df	Combined panicle and seed	Panicle	Seed
Replication	2	0.0001	0.0001	0.0006
Treatment (T)	2	0.9478	0.0246	0.9990
Year (Y)	1	0.0009	0.4137	0.0009
T*Y	2	0.9411	0.0579	0.0932
Genotype (G)	3	0.0001	0.0001	0.0001
T*G	6	0.8459	0.0822	0.9046
Y*G	3	0.0221	0.4442	0.0221
T*Y*G	6	0.7981	0.0042	0.8113
Tissue type (TT)	1	0.0001		
T*TT	2	0.9258		
Y*TT	1	0.0071		
T*Y*TT	2	0.9188		
G*TT	3	0.0001		
T*G*TT	6	0.9604		
Y*G*TT	3	0.0379		
T*Y*G*TT	6	0.8218		

Appendix 23. Soil water content in tubs in which tall fescue are growing under three different water treatment over the time in greenhouse during 2000 and 2001. Bars on lines indicate standard error.



Control
Befor heading
After heading
Soil water content (g water g-1 soil)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Boot
-2
-1
0
1
2
Week from boot stage