

Effect of Tall fescue (*Schedonorus phoenix* Scop.) Genotype on Endophyte (*Neotyphodium coenophialum*) Transmission under Water stress

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ABSTRACT

It has been known that endophyte (*Neotyphodium coenophialum*) is beneficial to tall fescue (*Schedonorus phoenix* Scop.) because the mutualistic endophyte is able to confers tolerance against abiotic and biotic stresses to tall fescue. However, this fungal endophyte produces toxic alkaloid resulting in negative effects on animal performance. Recently, Non-toxic endophyte have been developed and inserted into tall fescue to avoid detrimental effect on animal but remaining positive influence on tall fescue. In order to keep this beneficial impact, it is essential to have endophyte infected tall fescue through vertical transmission from maternal plants to seeds. Little research has been carried out on endophyte transmission. To get basic information related to endophyte transmission, experiment was conducted to examine the effect of plant genotype on endophyte transmission under water stresses. Overall endophyte concentration in seeds was higher than that in panicles and endophyte concentration in seeds and panicles relied on plant. This study revealed that drought is not a critical component to control the endophyte transmission from maternal plants to seeds. Plant genotype is an important factor controlling the endophyte transmission from plant to seed.

(Key words) : Endophyte transmission, Genotype, Non-toxic endophyte, Symbiosis, Tall fescue)

I . INTRODUCTION

Tall fescue was introduced from Europe into North and South America during the colonial period. It is now predominant cool-season perennial grass grown in the United States as a popular component of pastures 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). Tall fescue provides nutrition and structural

refuge to the endophyte, while tall fescue gets many benefits including enhanced competition (Hill et al., 1991a; 1998; Malinowski et al., 1999). Thus, the two are in a mutualistic relationship. The basis for mutualism is the ability of this endophyte to enhance resistance of tall fescue to biotic and abiotic stresses including herbivory and drought (Rudgers and Clay, 2007). Tall fescue may conserve water more efficiently due to endophyte presence by increasing leaf rolling (Arachevaleta et al., 1989), decreasing stomatal conductance (Elmi and West, 1995), and lower osmotic potential increasing

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turgor pressure in tall fescue (Elmi et al., 1989; White, 1989; Richardson et al., 1991). Endophytes have historically been considered as negative components of the pasture ecosystem (Ball et al., 1996).

Animals consume endophyte-derived ergot alkaloids toxic to animal because of grazing endophyte infected tall fescue. This results in reduced animal performance (Read and Camp, 1986; Hoveland, 1993). Non-toxic endophytes that do not produce harmful alkaloids have been introduced into tall fescue to eliminate the toxicity to grazing animal, but still maintain the agronomic benefit of the endophyte to host plant, tall fescue (Bouton et al., 1998; 1999; 2002; Fletcher, 1999; Bouton, 2000).

By using these non-toxic endophytes, their negative view changes more likely positive for cultivar development (Fletcher, 1999; Bouton, 2000; Bouton et al., 2002). Therefore, endophytes in tall fescue should be considered important components for sustainable agriculture, especially in pasture ecosystems. Tall fescue toxicosis is one of serious problems for livestock industry (Bush et al., 1979; Hoveland, 1993; Read and Camp, 1986). The initial management strategy was eliminating endophyte from tall fescue pasture. Since non-toxic endophytes inserted into tall fescue, one can eliminate tall fescue toxicosis by replacing the old tall fescue pasture with cultivars that are infected to non-toxic endophytes. For the successful strategy, it is essential to understand endophyte growth and transmission in tall fescue to maximize the probability of maintaining non-toxic endophytes in pasture ecosystem.

Since this fungal endophyte has no sexual stage, does not produce spores, and disseminates only through the female parent, this endophyte

life-cycle is relatively simple (Siegel et al., 1984). The endophyte grows as the seed germinates and invades the seedling plant, shortly after germination and the fungus is located in meristematic tissue of shoot apices during vegetative periods (Bacon and Siegel, 1988). If the flowering stem starts to elongate, the mycelium of endophyte grows along with the inflorescence and infects the ovule, one of important maternal tissues (Hinton and Bacon, 1985; Philipson and Christey, 1986).

There is variation of endophyte presence and/or transmission that is associated with environmental parameters during plant growth. 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 (Di Menna and Waller, 1986) later Bacon and Siegel (1988) also reported related result that endophyte level in seed after a hot and dry summer and cold winter. Temperature seems to be the most important environmental factor for these seasonal fluctuations of endophyte concentration and frequency (Ju et al., 2006). These all indicate that the seasonal variability of the endophyte resulted from seasonal variation of temperature and water dynamics. Although it had been suggested that endophyte frequency or concentration in endophyte-infected plants may be associated to the plant genotypes (Hiatt and Hill, 1997; Hill et al., 1991b) and environmental parameters affect endophyte growth and transmission, direct evidence was lacking and there was lack of report about the virtual systemic research linking endophyte frequency or concentration in tall fescue to plant genotype and water dynamics.

The objective of this study was to examine the

effect plant genotype on endophyte transmission under water stress imposed at different stages in greenhouse.

II. MATERIALS AND METHODS

This experiment was carried out during the winter and early spring of 1999 to 2000 (year 1) and 2000 to 2001 (year 2) in a greenhouse located in the University of Georgia in Athens, Georgia.

1. Preparation different plant genotypes and water stress

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 from each cross (total 4 genotypes called 1, 2, 3, and 4) were randomly selected from a large 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 and all plants contained the same endophyte since all had the same maternal parent. Tall fescue plants were vegetatively propagated in the greenhouse and tillers infected with endophyte were randomly planted in equal distance from one another in 120-L tubs containing 30kg of fritted clay (Tidy Cat, Ralston Purina Co., St. Louis, MO). A total of nine tubs containing the tall fescue plants were kept outdoors on 1 December for vernalization were transferred into the greenhouse on 15 February. Tub were irrigated with tap water as necessary to maintain soil water content. Three water stresses were applied with three replications; 1) no stress, 2) stress before booting stage, and 3) stress after

booting stage. For the water stress treatments, 0.45 and 0.35 g water g⁻¹ soil was maintained by weighing tubs every 1~2 day and adding water to re-hydrate the soils to 0.45 g water g⁻¹ soil. The non-water stress was maintained at 0.65 g water g⁻¹ soil (White et al., 1992; Hill et al., 1996). In order to measure leaf water potential and leaf osmotic potential, pre-calibrated end-window thermocouple psychrometers (Model 85-12V, J.R.D. Merrill Specialty Equipment Corp., Logan, UT) was used. One plant from each plant genotype was removed from the tubs at boot stage. Endophyte frequency in tiller and in floret was examined via immunoblot and endophyte contents were quantified in pseudostem via ELISA. The remaining plants were harvested at the maturity of the seed to examine endophyte concentration and endophyte frequency.

2. Endophyte frequency by immunoblot

Tiller cross-sections were put on the nitro-cellulose membrane and endophyte presence in tiller was tested using the Phytoscreen Field Tiller Endophyte Detection Kit (Hiatt et al., 1997a; Agrinostics Ltd. Co, Watkinsville, GA). Seeds were soaked in 1.25 M NaOH for 1 h and were washed with tap water. Endophyte presence in seeds was tested using the Phytoscreen Seed Endophyte Detection Kit (Agrinostics Ltd. Co, Watkinsville, GA).

3. Endophyte quantification by ELISA

Endophyte concentration was measured using ELISA (Hiatt et al., 1997b). Fungal proteins were extracted from 15 mg of ground lyophilized plant tissue. Quantification of mycelia was

conducted by diluting the protein pellet (1:4) in 400 μ l borate saline solution (pH 8.5). Fifty μ l of the solution was placed into wells of an Immulon 4 microtiter plate (Dyanatech Co., Chantilly, Virginia) permitting the protein to anneal overnight at 4°C. Then, plate was washed three times with ELISA wash (1.21 g Tris, 500 μ l Tween 20, and 0.20 Na₃N/ liter distilled H₂O, pH 8.0), blocked with 100 μ l of bovine serum albumin blocking solution (10 g bovine serum albumin, 1.17 g Na₂HPO₄, 0.24 g NaH₂PO₄, 8.20 g NaCl, and 10 ml of 2% Na₃N per liter distilled H₂O), and the plate was incubated for 30 minutes at 21°C. The plate was washed three times with ELISA wash. The mixture of Neotyphodium coenophialum-specific monoclonal antibodies from hybridoma cell line 15D7, 4H2, and 5C7 were diluted to a final antibody dilution of 1:20 in ELISA diluent (1 liter blocking solution with 500 μ l Tween 20). Fifty μ l was put into each well incubated for 2 hr at 21°C. Fifty μ l rabbit anti-mouse antibody conjugated with alkaline phosphatase (RAM-AP) (Sigma Chemical Co., St. Louis, Missouri) was applied to the plate washed three times with ELISA wash and allowed incubating at for 2 hr at 21°C. Fifty μ l of substrate solution (1 g p-nitrophenyl phosphate, 0.10 g MgCl₂, and 96 ml diethanolamine per liter distilled H₂O, pH 9.8) was added to each well of the plate washed three times. After incubation at 21°C, 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.

4. Statistical analysis

Data were analyzed by analyses of variance (SAS Institute, Cary, NC). Treatments were assigned to a randomized complete block design in which all variables were considered fixed effects. Treatment means were separated using a Fisher's protected LSD.

III. RESULTS

Plants receiving water stress were grown in soils containing approximately 0.42~0.45 g water g⁻¹ (0.42 g/g) 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 to almost 0 MPa.

Table 1 presents the effect of plant genotype on endophyte frequency and endophyte concentration under water stress imposed before the

Table 1. Analysis of variance for endophyte frequency and concentration in pseudostem of different tall fescue genotypes infected with a common endophyte genotype (EDN11) harvested at boot stage

Source	df	Endophyte frequency	Endophyte concentration
Water Stress (WS)	2	ns	ns
Genotype (G)	3	ns	**
Tiller type (TT)	1	**	ns
WS \times G	6	ns	ns
WS \times TT	2	ns	ns
G \times TT	3	ns	ns
WS \times G \times TT	6	ns	ns

** Significant at p<0.01.

ns: not significant difference.

boot stage. For this analysis, although there were three water stresses, no stress (control), water stress before booting stage, and water stress after booting stage. Water stress did not show a significant effect on endophyte frequency and concentration. When tall fescue plants received water stress before heading, endophyte frequency did not depend on plant genotype. Endophyte frequency was more likely depended upon tiller type showing that endophyte frequency at different tiller types differed with higher endophyte infection ratio at the reproductive tiller compared to that of vegetative tillers (Table 2). Endophyte concentration statistically depended on plant genotype (Table 1). Endophyte concentrations within pseudostems from regenerants of tall fescue are presented at Table 3. Plant genotype 1 showed the greatest concentration of endophyte, 1.35 mg per plant.

Table 2. Effect of tiller type on endophyte frequency in pseudostem of different tall fescue genotype infected with a common endophyte genotype (EDN11) harvested at boot stage

Tiller type		LSD (0.05)
Reproductive tiller	Vegetative tiller	
99.65%	97.78%	1.06

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

Table 3. Effect of plant genotype on endophyte frequency in florets and seed harvested at boot stage

	Plant genotype				LSD (0.05)
	1	2	3	4	
Endophyte concentration (mg)*	1.35	1.00	0.97	0.69	0.09

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

* Endophyte concentration was presented as “mg endophyte /g plant”.

Based on ANOVA at Table 1, there was no interaction between factors examined in this study for endophyte frequency and concentration.

ANOVA (Table 4) exhibits that water stress did not affect endophyte frequency of florets or of seeds, whereas endophyte frequency of florets or of seeds within the panicles depended on positions of them and plant genotype. Florets harvested from the middle and base of the panicle had lower endophyte frequency compared to florets acquired from the top of the panicle. Seed acquired from the middle of the panicle contained lower endophyte frequency than seeds harvested at the base or top of the panicle (Table 5). Generally speaking, florets and seeds harvested from the top showed greater endophyte frequency (Table 5). Table 7 presents endophyte frequency of seeds from four different tall fescue genotypes showing the greatest frequency at plant genotype 3. Like endophyte frequency, there was also no drought effect on endophyte concentration, but ANOVA (Table 6) indicates that endophyte concentration in seeds and panicles relied on plant genotype on (Table 6).

Table 4. Analysis of variance for endophyte frequency in florets and seed of different tall fescue genotypes infected with a common endophyte genotype (EDN11) harvested at seed maturity

Source	df	Floret	Seed
Water Stress (WS)	2	ns	ns
Position (P)	2	**	**
Genotype (G)	3	**	**
WS × P	4	ns	ns
WS × G	6	ns	ns
P × G	6	ns	ns
WS × P × G	12	ns	ns

** Significant at p<0.01.

ns: not significant difference.

Table 5. The effect of position on endophyte frequency of florets and seeds at tall fescue genotype with common endophyte genotype EDN11 harvested at seed maturity

Tissue type	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

‡ Indicates least significant difference at the 0.05 level of probability.

Table 6. Analysis of variance for endophyte concentration in panicle and seed of different tall fescue genotypes infected with a common endophyte genotype (EDN11) harvested at seed maturity

Source	df	Panicle	Seed
Water Stress (WS)	2	ns	ns
Genotype (G)	3	**	**
WS *G	6	ns	ns

** Significant at $p < 0.01$.

ns: not significant difference.

Plants genotype 1 contained higher endophyte concentration in seed. Overall endophyte concentration in seeds was approximately 7-8 times higher than that in panicles (ranged from 0.15 to 0.28 mg per plant).

IV. DISCUSSION

The impetus for this study was an observation that field-grown endophyte infected plants had

numerous tillers in which no endophyte was present. Di Menna and Waller (1986) noted seasonal variation in endophytes of perennial ryegrass grown under field conditions in New Zealand. A literature search found virtually no direct research linking environmental conditions with endophyte transmission. It is vital to have information about endophyte vertical transmission from maternal plant to seed for better maintain non-toxic endophytes in tall fescue pasture. Knowledge of endophyte frequency and concentration in tall fescue plants will provide information to understand symbiotic relationships in pasture ecosystems. Thus this experiment was conducted to perform initial investigations into environmental influence on endophyte grown in planta.

Previous studies revealed that there were seasonal variations on endophyte biomass and frequency in perennial ryegrass (Di Menna and Waller, 1986) and tall fescue (Ju et al., 2006) grown under field conditions. They found fewer

Table 7. The effect of tall fescue genotype on endophyte frequency and concentration of seeds harvested at seed maturity

	Genotype of tall fescue				‡LSD (0.05)
	1	2	3	4	
Endophyte frequency (%)	75.60	70.28	78.46	74.21	3.23
Endophyte concentration (mg)*	2.17	1.47	1.44	1.16	0.25

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

* Endophyte concentration was presented as "mg endophyte/g plant".

mycelia or less endophyte frequency in pseudostem tissue during winters in the Southern and Northern Hemisphere. Seed and vegetative tissue of tall fescue after the plants experienced hot and dry summers and cold winters showed decreased endophyte frequency (Bacon and Siegel, 1988). Ju et al. (2006) showed that endophyte biomass in tall fescue depended on minimum cardinal temperatures playing a key role as environmental factor controlling these seasonal variations of endophyte concentration and frequency. Since water is critical environmental for the life, we expected that water affected endophyte frequency and concentration. However, in this study, the drought experiment combination with plant genotype showed little or no effect of drought on transmission of endophyte (Table 1, 4, and 6). Previous study conducted *in vitro* (Bruehl and Kaiser, 1996) partially supported these results. Endophytes grew well on agar media amended with osmoticum to obtain water potential of -3Mpa, equivalent to quite bit drought stress (Bruehl and Kaiser, 1996). In other words, water stress imposed in this experiment might not enough to affect on endophyte transmission.

Although there was statistically different endophyte frequency between vegetative tiller and reproductive tiller (Table 2), this difference was not significant from agronomical view point. Less endophyte frequency in vegetative tiller (Table 2) was more likely related limited resources. Endophytes in reproductive tillers were established before resources were reduced. Vegetative tillers, relatively younger tissues than reproductive tillers, are generally more susceptible to environment factors such as water deficiency or temperature when compared to reproductive tiller (Mostajeran and Rahimi-Eichi, 2009). It had been known that the endophyte

resides within meristematic tissue during vegetative growth (Sampson, 1933; 1937; Bacon and Siegel, 1988) and endophytes are in flower primordia before development of inflorescences (Siegel, et al., 1985). Hinton and Bacon (1985) suggested that an infected bud simply outgrow the endophytes when conditions are not favorable for the endophyte. 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 primordia. It is likely the endophyte prioritizes invasion into reproductive tissues or organs. This may explain less endophyte frequency in vegetative tiller than that in reproductive tiller.

Endophyte frequency and concentration depend on plant genotypes (Table 1, 4, and 6). Plant genotype dependency of endophyte concentration seems consistent over plant genotype by showing that plant genotype 1 showed higher endophyte concentration at booting and at seed maturity (Table 3 and 7). Table 7 indicating that higher frequency of endophyte does not correlated with concentration of that.

Table 5 presents that endophyte frequency in florets or seeds depended on the position of the panicle showing greater frequency in seed and in the floret acquired from top position of panicle. Seed maturity may relate with this phenomenon. If the endophytes invade into the seeds, the increase of endophyte in seed relies on its viability during seed development. The different harvesting time of tall fescue seed involved in various endophyte frequency (Hill et al. 2005), pointing out that late embryogenesis abundant (LEA) protein in seed reduces embryo death. This LEA may also provide endophyte

protection from death. As described earlier, constrained reserve led to failure of endophyte multiplication. Endophyte transmission from maternal tissue to seed relates with host plant fitness presenting that high seed yielding host plants had greater endophyte transmission since there is plentiful resources available for endophyte (Gundel et al., 2011). Top positioned seeds in a panicle are heavier than seeds located at other positions because they developed a longer time resulting in more stored energy. In order word, top poisoned seeds contain more resources providing more energy to endophyte when compared to seeds in other positions.

From this study, we can conclude that water stress does not have effect on endophyte vertical transmission suggesting that water stress employed in this study is not an important factor controlling the endophyte transmission from maternal plant to seed. Plant genotype and seed position in a panicle affected endophyte vertical transmission indicating that these two factors are involved in endophyte vertical transmission and may determine seed transmission of this fungal endophyte.

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