High *Neotyphodium* infection frequencies in tillers and seed of infected wild tall fescue plants

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Abstract

This research quantified frequencies of *Neotyphodium* infected (E+) tillers and mature seed from field-grown E+ plants of two wild tall fescue accessions from Morocco and Sardinia, Italy. Tiller infection rates were 100% (n = 50 from 10 E+ plants/accession) for each accession and over 99% of the seed (n = 2394) from E+ plants of both accessions harboured viable *Neotyphodium* endophyte. Germination rates for E+ seed were 93.8% (Morocco accession) and 97.8% (Sardinia). These results indicate that E+ wild tall fescue plants are capable of near perfect vertical transmission of viable endophyte into seed. They also suggest that viable endophyte is retained with current seed-regeneration protocols at the USDA-ARS Western Regional Plant Introduction Station, Pullman, Washington USA.

Keywords: wild tall fescue, *Neotyphodium* infection frequencies, vertical transmission

Introduction

An outgrowth of research on *Neotyphodium*–grass associations and the anti-mammalian and anti-insect properties of infection is the 'defensive mutualism hypothesis,' which views mycotoxins produced by endophytes as defense chemicals providing adaptive advantage to the infected host grass (Clay 1988). Under this hypothesis, the fitness enhancing and anti-herbivore properties of infection favour the perpetuation of infected plants and, therefore, very high *Neotyphodium* frequencies in grass populations (Faeth 2002). And because these systemic endophytes are transmitted vertically to seed progeny of maternal host grasses, this mode of transmission should facilitate high infection frequencies within grass species, especially if infection enhances host fitness compared to uninfected plants (Saikkonen *et al.* 2002 and references therein). Logically, then, infected plants are favoured by natural selection, with vertical transmission ensuring that only very high infection rates are maintained in field populations (Leuchtmann & Clay 1997; Schardl & Clay 1997). Indeed, studies have documented long-term increases in *Neotyphodium* infection rates of field populations of tall fescue (*Festuca arundinaceae*) and perennial ryegrass (*L. perenne*), indicating a mutualistic relationship between these grass–endophyte associations (e.g. Cunningham *et al*. 1993; Clay 1996).

However, the existence of highly variable endophyte frequencies in wild grass populations (e.g. Lewis *et al.* 1997; Saikkonen *et al.* 2000), have led some ecologists to question the generality of the defensive mutualism hypothesis (see Faeth 2002; Faeth & Sullivan 2003). Indeed, the existence of low infection frequencies among endophyte-infected grass populations contradicts the notion of endophyte mutualism with their host grasses (Ravel *et al.* 1997; Faeth 2002; Saikkonen *et al.* 2002; Faeth & Sullivan 2003; Hamilton & Faeth 2005). One possible mechanism to account for low to intermediate infection rates, which is widely cited in the literature, is imperfect vertical transmission of the fungus into seeds on host plants (Ravel *et al.* 1997). Mathematical modeling was used by Ravel *et al*. (1997) to develop this concept (see also Saikkonen *et al.* 2002). Apart from this hypothesis, other processes and mechanisms are thought to affect infection frequencies of <100% (e.g. Clay *et al*. 2005).

What direct evidence is available to support the generality of this concept? Specifically, how extensive is the database on tiller infection rates on infected plants and seed infection rates on infected tillers? A review of the literature indicates few databases, although the available information offers support for the concept of imperfect transmission. Wilson and Easton (1997) have shown that most seeds produced by infected tall fescue (Kentucky 31–novel *Neotyphodium* association) were infected, but the transmission rate was usually less than 100%. Hinton and Bacon (1985) indicated that endophyte-free tillers occur on infected tall fescue, similarly observed by Ju *et al.* (2006) for field-grown tall fescue plants and Hahn *et al.* (2003) for infected field plants of *Festuca* spp. Welty and Azevedo (1993) reported that environmental conditions affect vertical transmission of endophytes into seed of tall fescue.

Our research objectives were twofold: (1) to quantify frequencies of *Neotyphodium*-infected (E+) tillers and mature seed on field-grown E+ plants of wild tall fescue accessions, thereby significantly expanding our understanding of the imperfect/perfect nature of vertical transmission of endophytes in wild grasses, and (2) to use the data from objective one to determine if viable *Neotyphodium* endophyte is preserved in seed produced by E+ tall fescue accessions in the seed-regeneration programme at the USDA-ARS Western Regional Plant Introduction Station , Pullman, Washington, U.S.A. This facility is the U.S. repository for temperate grass collections (~ 17,000 accessions) and diverse *Neotyphodium* strains for use by public and private sector scientists (Clement 2001; Clement *et al.* 2001; Tapper & Latch 1999). Thus, objective two contributes to our goal of preserving valuable microbial germplasm at the Pullman seed bank.

Materials and Methods

Plant materials, field plot details, and seed harvest

Two plant inventory (PI) accessions of tall fescue were evaluated, seed of which was originally collected from wild plants in Morocco (PI 610910, M112) and Sardinia, Italy (PI 598932, S045) in 1994 (Cunningham *et al.* 1997) and stored in the Pullman seed bank. Seed was obtained from the seed bank and germinated in accordance with procedures in Clement *et al.* (2001). Newly germinated seeds were planted individually in 15-cm pots containing a commercial soil mix and maintained in a glasshouse (June–October 2002; 15–30°C, natural lighting). After 5 mo, plants were pruned and repotted into 20-cm pots, fertilised bi-weekly with soluble 20-20-20 fertiliser (0.6 g/L), then maintained in the glasshouse (11–25°C) under supplementary lighting to ensure a 14-h photoperiod between November 2002 and March 2003. Plants were checked daily and watered as needed. Plants were pruned and repotted twice during the 11 mo they were held in the glasshouse, and received a final pruning 2 d before they were transplanted into field plots. Pruning, apart from helping to maintain healthy glasshouse-grown plants, simulates livestock grazing. Seed of both accessions

was collected from "grazed" (Sardinia) and "very heavily grazed" (Morocco) tall fescue populations in their native environments (Cunningham 1996).

Two plots were established with transplants from the glasshouse in May 2003, then hand-weeded and watered, as needed, between May and July 2003. A plot consisted of two 15-m-long rows on 1 m centres, with each row containing 33 evenly spaced transplants for a total of 66 plants per plot. The spacing between the plots was 100 m. The plot area was fertilised with 45 kg N/ha in October 2002 and 2003.

Plot 1 contained 60 endophyte-infected (E+) and 6 endophyte-free (E-) plants of PI 610910, and plot 2 contained 28 E+ and 38 E- plants of PI 598932. The endophyte status of these plants was determined prior to field transplanting (September 2002) using isolation techniques and procedures described in the next section.

From 25 June to 7 July 2004, 10 E+ plants were randomly selected from each plot and seed on 5 tillers from each of these 20 plants was harvested by hand-clipping each tiller at its base. Harvest coincided with a majority of the seed on each tiller at physiological maturity, a procedure designed to restrict quantification of infection to mature seed (see Hill *et al*. 2005 for discussion of seed maturity–endophyte relationships in tall fescue). Each tiller was placed in a separate labeled paper bag. Bags were taken to a laboratory where 'filled and mature seed' was hand-stripped from each tiller, placed in a separate labeled bag and stored in the Pullman seed bank (4°C). Harvest continued into early August 2005, as seed reached maturity, with all tillers per plant bulked and placed in one paper bag. These bags were held in a seed drying room (17–22°C) for ~ 3 mo before mature seed was separated from tillers and cleaned for storage in the Pullman seed bank.

These procedures, from plots established with transplants to harvest and finally to seed cleaning operations and storage conditions, are substantially similar to the methods and procedures used to regenerate seed of temperate grass accessions at the Pullman seed bank.

Viable endophyte detection

Between November 2004 and June 2006 we removed, at various intervals, seed samples (25 seeds per tiller x 5 tillers x 10 E+ plants = 1250 seeds per accession) from the seed bank and germinated them as described by Clement *et al*. (2001). As well, we removed 25 seed from the bulked seed of one E- plant of each accession for germination. Germinated plants were planted individually in 10-cm pots in a glasshouse (11–30°C, natural lighting) and fertilised bi-weekly (as described above) before the endophyte status of each 8–9 wk-old plant was determined.

The endophyte status of each plant was determined by isolating *Neotyphodium* fungi on potato dextrose agar (PDA) with streptomycin sulfate and tetracycline hydrochloride (0.10 g of each per 1 L of PDA). Following procedures in Clement *et al.* (2001), basal stem sections (~ 1 cm) from 3–5 tillers per plant were disinfected and placed on PDA in sealed petri dishes and incubated in a laboratory (complete darkness; room temperature). Petri dishes were examined for mycelial growth from plant tissue at 2- to 3-d intervals for 30 d (PI 610910) and 45 d (PI 598932). A plant was scored as free of viable endophyte if *Neotyphodium* mycelia did not appear after these two periods of time. These different observation periods reflect the different growth rates of mycelia from plant samples of the two accessions, with mycelial growth markedly slower from samples of PI 598932 (Sardinia accession) (Clement and Elberson, unpublished). The fungi on PDA were identified as *Neotyphodium* on the basis of cultural and conidial characteristics (see Latch *et al.* 1984).

While this isolation method is very time-consuming, it generates highly reliable data on the incidence of 'viable *Neotyphodium*' in plant tissue. Although this method can result in the growth of contaminant fungi and bacteria, thereby making isolation

Table 1 Endophyte-infected plants (E+) of two wild tall fescue accessions: tiller and seed infection rates, and germination rates of seed from E+ tillers.

		Tillers (n)	Mature seed (n)	
Accession	Origin	% E+	% germination	% E+
PI 610910	Morocco	100 (50)	93.8 (1250)	99.0 (1172)
PI 598932	Sardinia	100 (50)	97.8 (1250)	99.3 (1222)

Table 2 Attributes of the experimental site, Pullman, Washington, and collection sites of endophyte-infected tall fescue seed in Morocco and Sardinia.

Attribute	Pullman (USA)[a]	Timahdite (Morocco)[b]	Torralba (Sardinia)[c]
Latitude	46°43'55"N	33°07'34"N	40°28'59"N
Longitude	117°09'25"W	005°02'47"W	008°46'58"E
Elevation	762 m	1903 m	360 m
Yearly average rainfall	453 mm	400 mm	780 mm
Min. winter temp.	-27°C	2.5°C	4°C
Max. summer temp.	37°C	31°C	30°C

[a] National Climate Data Center 2005.
[b] Cunningham 1996; Herzenni *et al.* 2001.
[c] Cunningham 1996; Chessa & Delitala 1997.

of *Neotyphodium* fungi difficult (Bacon & White 1994), we experienced very few problems with contaminants in this study. We attribute this to the selection of multiple stem sections from young tillers per undamaged plant, good surface disinfection of samples, the addition of antibiotics to PDA, and the removal of contaminant colonies from petri dishes as they appeared.

Data analyses

When appropriate, an analysis of variance was used to compare the means of specific data sets.

Results and Discussion

Tiller infection rates were 100% for each accession because all separately-harvested tillers produced E+ seed. Our results also show high vertical transmission of viable endophyte into seed on E+ tillers of plants, with almost 100% of the germinated seed from these tillers harbouring viable endophyte. Likewise, germination rates for E+ seed of each accession were high, ranging between 93 and 97.8% (Table 1). Germination rates for 25 seed from one E- plant of each accession were 96% (PI 598932) and 100% (PI 610910) and these 49 germinated seeds produced endophyte-free plants.

Sixty-three of 66 plants in plot 1 produced seed, with yields of mature seed averaging 21.73 ± 31.32 (SD) g for E- (n = 4 plants) and 60.46 ± 65.32 g for E+ (n = 59) plants. In plot 2, mature seed yields from the 28 E+ and 38 E- plants averaged 40.17 ± 29.75 g and 39.52 ± 23.13 g, respectively. Mean seed yields between E- and E+ plants of each accession were not significantly different ($P>0.05$) because there was considerable variability in the amount of mature seed produced by the plants.

Because environmental conditions can affect endophyte transmission in E+ tall fescue plants, with fewer E+ seed produced by E+ plants after hot/dry summers and cold winters (Bacon & Siegel 1988) and more E- tillers produced on E+ plants after dry and cool spring conditions (Ju *et al.* 2006), we document the conditions under which this study was conducted and associate them with our recorded infection frequencies. Rainfall during this study was similar to the yearly average for the Morocco collection site, but winter and summer temperatures for Pullman were much colder and warmer than recorded minimum winter and maximum summer temperatures for the two Mediterranean collection sites (Table 2). During this study, a wide range of ambient temperatures, specifically a minimum winter temperature of -27°C and a maximum summer temperature of 37°C (Table 2), did not adversely impact on endophyte transmission rates.

The very high E+ rates of mature seed recorded in this study suggest that current seed-regeneration procedures and operations at the Pullman seed bank are suitable for retaining viable *Neotyphodium* endophytes. However, with data from just two accessions, it is premature to conclude that the current seed-regeneration methods are optimal for preserving *Neotyphodium* endophytes that may infect some of the several thousand C_3 grass accessions at this germplasm repository.

Additionally, our results indicate that some E+ wild grasses are capable of near perfect vertical transmission of viable endophyte (> 99%), albeit under specific environmental conditions. This contrasts with the notion that *Neotyphodium* transmission through the host's seeds is imperfect (that is, failure of endophyte to propagate via seeds is greater than 10%), and thus the production of both E+ and E- progeny by E+ plants account, to a large extent, for low to intermediate infection rates in grass populations (e.g. Ravel *et al.* 1997; Saikkonen *et al.* 2002; Faeth & Sullivan 2003). Empirical studies like this one, and additional ones that focus on other wild grass–endophyte associations under a variety of environmental conditions, will generate the quantitative framework to understand how vertical transmission rates may or may not align with subsequent frequencies of E+ plants in natural grass populations.

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