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Neotyphodium endophytes in tall fescue seed: viability after seed production and prolonged cold storage

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Summary

This research quantified frequencies of *Neotyphodium*-infected (E+) tillers and mature seed from E+ plants of two wild tall fescue (*Lolium arundinaceum* (Schreb.) S.J. Darbyshire (= *Festuca arundinacea* Schreb.) accessions from the Mediterranean basin that were stored in the seed bank at the USDA, ARS Western Regional Plant Introduction (PI) Station, Pullman, Washington USA. Tiller-infection levels were high in glasshouse (>97%) and field (100%) plants of each accession and over 99% of the seed from E+ plants of both accessions harbored viable *Neotyphodium* endophyte. Seed germination was either slightly improved (Morocco accession) or not affected (Italy) by endophyte infection. High levels of viable E+ seed were produced by E+ field plants grown under a wide range of ambient temperatures (-27° to 37°C). These collective results suggest that viable endophyte is retained with current seed-regeneration practices at the Pullman PI Station. This study also documented viable *Neotyphodium* infection frequencies (16–100%) in plants grown from seed of 20 additional Mediterranean tall fescue accessions stored for four to ten years in the Pullman seed bank. For some of these accessions, low post-storage infection frequencies, in comparison with their high initial viability levels, suggested a decline in endophyte viability during seed storage. Additional evidence for diminishing endophyte viability levels in some accessions was provided by Polymerase Chain Reaction (PCR), immunoblot, and microscopic seed assays.

Introduction

There is widespread interest in *Neotyphodium* (Clavicipitaceae) fungal endophytes because their presence in temperate grasses is linked to increased plant fitness, such as greater drought tolerance and resistance to insect and mammalian herbivores (Bacon and Siegel, 1988; Bacon and White, 1994a). These endophytes, which do not infect other plants horizontally, are transmitted vertically to seed progeny of maternal host grasses. Resistance to herbivores is the result of endophyte synthesis of secondary metabolites *in planta*. Unfortunately, some of these compounds (ergot alkaloids, indole-diterpenes) can have deleterious effects on cattle and sheep consuming infected plant material (Studemann and Hoveland, 1988; Porter, 1994). One approach to eliminate mammalian

toxicosis is to develop new grass–endophyte associations with naturally occurring strains of *Neotyphodium* that do not produce mammalian toxins, but still produce the necessary metabolites for insect resistance and other ecological benefits (Latch, 1997; Latch *et al*., 2000; Bouton *et al*., 2002). The commercialization of this approach is demonstrated by steady increases in the use and deployment of 'non-toxic' endophyte strains in cultivars of *Lolium arundinaceum* (Schreb.) S.J. Darbyshire (= *Festuca arundinacea* Schreb.; tall fescue) and *L. perenne* L. (perennial ryegrass) in Australia, New Zealand, and the USA (Easton and Tapper, 2005; Bouton and Easton, 2005; Wheatley, 2005; Ball *et al*., 2007; Bouton, 2007). Continued development of new grass–endophyte associations is contingent on the availability of a large and diverse pool of *Neotyphodium* germplasm.

The USDA, ARS Western Regional Plant Introduction (PI) Station, Pullman, Washington, is the USA seed bank for temperate grass collections (~19,300 accessions) and diverse *Neotyphodium* strains for use by public and private sector scientists (Clement, 2001; Clement *et al.,* 2001; Tapper and Latch, 1999; Cheplick, 2007). This microbial germplasm in host grasses should be conserved for research and for the possible development of new grass–endophyte combinations (Clement *et al*., 1994; Holder *et al*., 1994). Proper seed regeneration practices and cold and dry storage conditions are required to conserve viable *Neotyphodium* in grass seeds (Rolston *et al*., 1986; Do Valle Ribeiro, 1993; Holder *et al*., 1994; Clement *et al*., 1994; Clement *et al*., 2007). Additionally, commercial vendors should store *Neotyphodium*-infected (E+) grass seed under optimal conditions to market a product with high viability levels (Wheatley *et al.*, 2007).

Seed at the Pullman seed bank is stored at 4°C, 30% relative humidity and a dew point of -10°C. Although Clement *et al*. (2004) showed these conditions were optimal for preserving viable *Neotyphodium* in seeds of *L. perenne* and *Hordeum brevisubulatum* spp. *violaceum* (Boissier and Hohenacker) Tzvelev stored for eight years, their research generated ambiguous results for retention of viable endophyte in tall fescue seed stored for the same period of time.

The objectives of this research were threefold: (*i*) to assess the field seed-regeneration practices at the Pullman PI Station for production of viable *Neotyphodium* in seeds of tall fescue accessions; (*ii*) to quantify viable *Neotyphodium* in plants (percentage infection) grown from seeds of E+ tall fescue accessions stored in the Pullman seed bank; and (*iii*) to compare objective *ii* results with infection levels determined by assays that do not distinguish between viable and nonviable endophyte in seed, namely PCR (Dombrowski *et al*., 2006), microscopic (Welty *et al*., 1986) and immunoblot (Hill *et al*., 2002) assays.

Materials and methods

Seed stocks

Seed chosen for this study were from the first regenerated seed lots of E+ tall fescue accessions collected during a 1994 Australian–U.S. plant exploration trip to Mediterranean locations (Cunningham, 1996; Cunningham *et al*., 1997) and stored in the Pullman seed bank (Clement *et al*., 2001). Seed of these accessions was collected mostly from wild plants (Cunningham, 1996). We focused on this collection because Mediterranean

tall fescue is a known source of non-ergot alkaloid producing *Neotyphodium* strains. This germplasm will be useful for the development of synthetic tall fescue–endophyte combinations to mitigate animal toxicity while retaining plant persistence (Latch *et al*., 2000; Bouton *et al*., 2002).

Seed germination and maintenance of glasshouse plants (objectives i and ii)
Seeds were placed in water saturated vermiculite in covered 11- by 11- by 3.5-cm plastic boxes (25 seeds per box) and placed in a seed germinator at 16 h light/8 h dark and 25/15°C. Germination was recorded after 14 days. Germinated seeds produced seedlings with normal radicles, coleoptiles, and cotyledons (ISTA, 1999).

Newly germinated seeds were planted individually in pots (10-cm diameter) containing a commercial soil mix and maintained in a glasshouse (11–30°C, natural lighting or supplemental lighting to ensure 14 hour photoperiod). Potted plants were watered as needed and fertilized bi-weekly with a soluble 20-20-20 fertilizer (0.6g/L).

Endophyte isolation on agar (objectives i and ii)
The endophyte status of plants was determined by isolating *Neotyphodium* fungi on potato dextrose agar (PDA) supplemented with streptomycin sulfate and tetracycline hydrochloride (0.10 g of each per 1 L of PDA). Basal stem sections (~1 cm long) from three to four tillers of each four to twelve week-old glasshouse plant were surface 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 two to three day intervals for 45 days. A plant was scored 'free of viable endophyte' if *Neotyphodium* mycelia did not appear on PDA during this period of time. The fungi on PDA were identified as *Neotyphodium* on the basis of cultural and conidial characteristics (Latch *et al.,* 1984).

While this isolation method is time-consuming, it generates highly reliable data on the presence of 'viable *Neotyphodium*' in plant tissue. Indeed, "the only way to estimate viability of endophytes is to grow seed and examine seedling plants" (Hill *et al*., 2002). Although this method can result in the growth of contaminant fungi and bacteria, thereby making isolation of *Neotyphodium* fungi difficult (Bacon and White, 1994b), very few contaminant problems occurred in this study. This was attributed to the selection of multiple stem sections from young tillers from each 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.

Seed-regeneration practices (objective i)
Two Plant Inventory (PI) accessions were evaluated (Morocco, PI 610910; Italy [Sardinia], PI 598932) (Cunningham *et al.*, 1997). Seeds were germinated in June 2002 and the endophyte status (sections from four tillers per plant on PDA) of each plant was determined in September 2002. The plants were pruned and repotted twice during the 11 months they were held in the glasshouse. They received a final pruning two days before they were transplanted into field plots. Pruning, apart from helping to maintain healthy glasshouse-grown plants, simulates livestock grazing.

Two field 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 centers, with each row containing 33 evenly spaced transplants for a total of 66 plants per plot. The plots were separated by 100 m. The plot area was fertilized with 45 kg N/ha in October 2002 and 2003. No fungicides were applied in the plots. Plot 1 contained 60 E+ and 6 E- plants of PI 610910, and plot 2 contained 28 E+ and 38 E- plants of PI 598932.

On 7 July 2004, five randomly selected tillers from each of 20 E+ randomly selected plants (10 per plot) were hand-clipped at their base. This harvest was performed when the majority of the seed on each tiller was at physiological maturity, a procedure designed to restrict quantification of endophyte infection to mature seed. Hill *et al.* (2005) researched 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. Harvest continued into early August 2004, as seed reached maturity, with all remaining tillers per plant bulked and placed in one paper bag. These bags were held in a seed drying room (17–22°C) for three months before mature seed was separated from tillers and cleaned for storage in the Pullman seed bank.

These practices and procedures (plot establishment with transplants, harvest, seed cleaning, pre-storage conditions) are substantially similar to those used to regenerate seed of temperate grass accessions at the Pullman PI Station. Rainfall, ambient temperatures and other characteristics of the Pullman research site were documented and compared with those of the collection sites of the two Mediterranean accessions (table 1). This was done to provide insight into the possible influence of site-specific abiotic conditions on tiller and seed infection frequencies. Environmental conditions can affect infection levels of tall fescue tillers and seeds (Bacon and Siegel, 1988; Welty and Azevedo, 1993).

Table 1. Characteristics of the research site in Pullman, Washington, and the collection sites of tall fescue seed in Morocco and Italy (Sardinia).

Attribute	Pullman (USA)[1]	Timahdite (Morocco)[2]	Torralba (Sardinia)[3]
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

[1]National Climate Data Center, 2005; [2]Cunningham, 1996; Herzenni *et al.,* 2001; [3]Cunningham, 1996; Chessa and Delitala, 1997.

Seed samples (25 seeds per tiller × 5 tillers × 10 E+ plants = 1250 seeds per accession) were removed from the seed bank between November 2004 and June 2006 and germinated (table 2). Plants from germinated seeds were maintained in a glasshouse as described above. Additionally, 25 seeds from the bulked seed of individual E+ and E- plants (five E+, four E- plants of PI 610910; five E+, five E- plants of PI 598932) were removed (June 2007) from the seed bank and germinated to determine if endophyte infection affected germination levels. The effect of *Neotyphodium* infection on grass seed germination is unclear (Gundel *et al.*, 2006).

Table 2. *Neotyphodium*-infected plants (E+) of two wild tall fescue accessions: tiller and seed infection levels, and germination levels of seed from E+ tillers.

		% E+ tillers (n)		Mature seed (n)	
Accession	Origin	Glasshouse[1]	Field[1]	% Germination	% E+
PI 610910	Morocco	97.5 (40)	100 (50)	93.8 (1250)	99.0 (1172)
PI 598932	Sardinia	100 (40)	100 (50)	97.8 (1250)	99.3 (1222)

[1]Tillers from glasshouse plants and the same plants as field transplants.

Viable Neotyphodium in stored seed (objective ii)

Between June 2006 and January 2007, germination was quantified for regenerated seed (25 per accession) stored for four to ten years in the Pullman seed bank and representing ten, five, and five PI accessions from Morocco, Tunisia, and Italy (Sardinia), respectively (table 3). Plants from germinated seeds were maintained in a glasshouse before their endophyte status was established to reflect levels of viable *Neotyphodium* in stored seed.

PCR, immunoblot, and microscopic detection of endophyte in single seed (objective iii)

PCR detection of *Neotyphodium* fungi in single seeds was performed in accordance with protocols described in Dombrowski *et al*. (2006), with modifications and additions to template DNA extraction and isolation for PCR analysis, as follows: Seeds were soaked in 1.25 M NaOH for 60 minutes and rinsed in sterile double distilled water prior to extraction with a modified Puregene (Qiagen, Valencia, CA) DNA isolation protocol. The extraction buffer was prepared by adding 1g polyvinylpyrrolidone-40 (PVP) to 50 mL of Puregene Cell Lysis Solution (Qiagen, Valencia, CA) and incubating solution at 65°C for 10 minutes, inverting occasionally until PVP was dissolved. The solution was cooled to room temperature. Individual washed seeds (one per well) were placed in a 96 well plate cluster tube rack (Corning style rack; Sigma-Aldrich, St Louis, MO), then 250 µL of extraction buffer was added to each well. Samples were incubated overnight at room temperature. The following morning, two tungsten beads were placed in each well and samples were homogenized for three minutes at 30 Hz in a mixer mill instrument. Racks were rotated and samples were homogenized for an additional three minutes at 30 Hz. Samples were visually inspected to determine if they were completely homogenized and, if necessary, homogenized for an additional three minutes at 30 Hz. Next, they were incubated at 65°C for 60 minutes, followed by centrifuging at 4000 rpm for 25 minutes.

Table 3. Germination of seeds of tall fescue accessions after prolonged cold storage, and viable *Neotyphodium* infection status of plants from germinated seeds of each accession.

		Regenerated seed[1]		Infected plants (%)	
Country of origin	Plant Inventory (PI) no.	Years of storage	Post-storage germination (%)	Initial viability[2]	Post-storage viability
Morocco	598829	10	96	100.0	79.2
	598848	10	96	33.3	66.7
	598863	10	100	100.0	96.0
	598868	10	100	100.0	88.0
	610940	9	100	100.0	68.0
	610937	9	100	66.7	76.0
	610905	9	100	100.0	20.0
	598893	10	96	100.0	45.8
	598903	10	100	100.0	100.0
	610900	9	96	100.0	75.0
Tunisia	619480	7	72	100.0	94.4
	633833	9	100	40.0	48.0
	619476	7	100	33.3	16.0
	610956	8	96	60.0	50.0
	634229	4	96	100.0	91.7
Italy (Sardinia)	598930	10	88	66.7	18.2
	598932	10	80	60.0	40.0
	598934	10	100	60.0	96.0
	598943	10	96	66.7	41.7
	598945	9	88	100.0	27.3

[1]Seed from first regeneration cycle for each accession.
[2]Clement *et al.*,2001; Clement, unpublished.

The supernatant was transferred into a new set of cluster tubes. Samples were treated with Proteinase K for one hour at 55°C, then treated with RNase A for 30 minutes at 37°C following the manufacturer's protocols. Puregene Protein Precipitation Solution was added to cell lysate according to the manufacturer's instructions. Samples were vortexed vigorously and placed at -20°C for 15 minutes prior to centrifugation at 4000 rpm for 25 minutes at 4°C. The supernatant was removed to a new tube, vortexed vigorously, and placed at -20°C to precipitate remaining proteins and polysaccharides. Samples were spun at 4000 rpm for 20 minutes at 4°C and supernatant was collected in a new tube and precipitated overnight at -20°C with an equal volume of isopropanol. DNA was collected by centrifugation and washed with 70% ethanol. Each DNA seed extraction was dissolved in 60 µL TE buffer. PCR reactions were conducted as described (Dombrowski *et al*., 2006) using 3 µL of the TE solution per PCR reaction. PCR reactions were run a minimum of two times per seed extract and assessed for the endophyte-specific band.

The immunoblot assay for detection of endophyte in seeds was conducted using a commercial test kit (Cat. #ENDO797-1; Agrinostic Ltd. Co., Watkinsville, GA), following published procedures (Hill *et al*., 2002; Hill *et al*., 2005; Dombrowski *et al*., 2006; Trento *et al*., 2007). For microscopic analysis, seeds were stained and examined for fungal endophytes using light microscopy as described by Saha *et al*. (1988), with the modification that a 5% NaOH seed treatment was used to soften the seeds.

Calculations of *Neotyphodium*-infected seed levels were based on assays of 25–100 seeds from five accessions (table 4), which were selected on the basis of varying endophyte-infection levels from plant examinations (table 3).

Data analysis

The viable endophyte infection status of accessions was determined from the percentage of infected plants grown from seed samples of each accession. The GLM procedure (SAS Institute Inc., 1999) was used to compare mean seed yields of E+ and E- plants and mean germination percentages of seed produced by E+ and E- plants.

Table 4. *Neotyphodium* infection percentages in plants and seed of selected tall fescue accessions.

Plant Inventory (PI) number	Country of origin	Plants[1]	Seed analysis		
			PCR[2]	Immunoblot[2]	Microscope[3]
598868	Morocco	88	86	96	80
610905	Morocco	20	86	86	72
598903	Morocco	100	76	90	76
633833	Tunisia	48	54	92[4]	50
598932	Sardinia	40	56	54	48

[1]Source: table 3; [2]50 seeds tested; [3]25 seeds tested; [4]100 seeds tested.

Results and discussion

Seed-regeneration practices

Most of the tillers on E+ glasshouse plants harbored viable endophyte (97.5–100%). These same E+ plants produced only E+ tillers in the field since all separately-harvested tillers produced E+ seed. Moreover, the results show almost 100% vertical transmission of viable endophyte through tillers and into seeds on field plants of tall fescue, with 99.0% (PI 610910) and 99.3% (PI 598932) of the germinated seed from tillers producing plants with viable endophyte (table 2). Therefore, some E+ wild tall fescue populations are capable of near perfect vertical transmission of viable endophyte through tillers and into seeds. This finding contrasts with the notion that *Neotyphodium* transmission through the seeds of temperate grasses is imperfect (specifically, the failure of *Neotyphodium* hyphae to grow into tillers and into seed is greater than 10%). This concept of 'imperfect vertical transmission', first proposed by Ravel *et al.* (1997) via mathematical modeling, has been used to account for the production of both E+ and E- seed by E+ plants, and thus for low to intermediate *Neotyphodium* infection levels in fescue and ryegrass populations (Saikkonen *et al.,* 2002; Faeth and Sullivan, 2003). Additional studies that focus on a diversity of wild

grass–endophyte associations under a variety of environmental conditions would generate a stronger quantitative framework on how vertical transmission levels may or may not align with subsequent frequencies of E+ plants in natural grass populations.

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 1). The wide range of ambient temperatures during this study, albeit a minimum winter temperature of -27°C and a maximum summer temperature of 37°C (table 1), did not adversely affect the vertical transmission of *Neotyphodium* in field plants (table 2). By comparison, Bacon and Siegel (1988) reported lower E+ seed production on E+ tall fescue plants after 'hot/dry summers and cold winters' and Ju *et al.* (2006) found more E- tillers on E+ tall fescue plants after 'dry and cool spring conditions.' Other researchers reported, without reference to environmental conditions, that transmission was high on E+ tall fescue but usually less than 100% (Wilson and Easton, 1997) and that E+ fescue plants supported E- tillers (Hinton and Bacon, 1985; Hahn *et al.*, 2003).

Germination levels for E+ seed of each accession were >93.0% (table 2). In separate tests, the germination levels for seed produced by E+ and E- plants of PI 610910 averaged 98.0% and 86.4% ($P = 0.049$), respectively, whereas germination percentages (94.4%) were equal ($P > 0.05$) for both E+ and E- plants of PI 598932. These E- and E+ seed germination levels from two accessions do not enhance our understanding of whether or not endophyte infection affects the germination of tall fescue seed.

Sixty-three of 66 plants in plot 1 produced seed, with yields of mature seed averaging 25.09 ± 14.08 (SEM) g for E- (n = 4 plants) and 60.46 ± 8.50 g for E+ (n = 59) plants. In plot 2, mature seed yields averaged 39.52 ± 3.75 g for E- (n = 38) and 40.17 ± 5.62 g for E+ (n = 28) plants. Mean seed yields for E- and E+ plants of each accession were not significantly different ($P > 0.05$) because there was considerable variability (e.g., means of 0.14 to 68.21 g for per E- plant and 0.34 to 220.94 g per E+ plant in plot 1) in the amount of mature seed produced by the plants.

Viable Neotyphodium in stored seed

As revealed by seed infection levels, the 20 accessions harbored viable *Neotyphodium* endophytes (table 3). However, there was a wide range of post-storage endophyte viability levels (16–100%) for the accessions. Although initial viability percentages were based on examinations of just three to five plants per accession (only small quantities of original seed were available to establish initial levels in plants) (Clement *et al.,* 2001), their generally high levels, in comparison with some low post-storage infection levels, suggest a loss of endophyte viability in some accessions (table 3). Post-storage percentages declined for 15 accessions; however, they measured higher for four accessions, which we attribute to experimental variability associated with sample sizes. Post-storage germination levels for seed were generally good (≥88% for 18 accessions) (table 3).

PCR, immunoblot, and microscopic detection of endophyte in single seed

In general, the seed assay methods detected comparable infection levels for PI 598868, PI 598903, PI 598932, and PI 610905. However, the immunoblot assay detected higher

levels of infection than the microscopic method (table 4), which supports the findings of other investigators (Dombrowski *et al*., 2006; Trento *et al*., 2007). Seed infection levels correlated well with the plant infection levels of PI 598868, PI 598903, and PI 598932. For PI 610905, the plant infection level was much lower (20% with viable endophyte) than the seed infection levels (72–86%) measured by the three seed assays (table 4). This finding suggests the presence of high amounts of nonviable endophyte hyphae in the seed, because the seed assays cannot distinguish between viable and nonviable hyphae (Welty *et al*., 1986; Hill *et al*., 2002; Dombrowski *et al*., 2006). For PI 633833, the immunoblot estimate (92%) was much higher than the levels measured by PCR, microscopic examination, and endophyte isolation on PDA (table 4). This very high immunoblot value may be due to a cross-reaction with contaminant proteins in the seed lot (see Dombrowski *et al*., 2006).

Conclusions

Although this study recorded near perfect vertical growth of viable endophytes into tillers and seed of E+ tall fescue plants in field plots, it is important to note that the results were generated from only two accessions. Nonetheless, the results suggest that current seed-regeneration practices and operations at the Pullman PI Station are suitable for maintaining viable endophytes in seed of tall fescue accessions destined for cold storage.

This study also shows that viable *Neotyphodium* fungi has been preserved in regenerated seed of 20 accessions stored for prolonged periods of time. However, the results (tables 3 and 4) indicate that endophyte viability may have declined in the seed of some accessions. Additional research is required to identify optimal storage conditions for maintaining viable endophyte levels in large collections of diverse tall fescue–*Neotyphodium* associations.

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References

Bacon, C.W. and Siegel, M.R. (1988). Endophyte parasitism of tall fescue. *Journal of Production Agriculture,* **1**, 45–55.

Bacon, C.W. and White, J.F., Jr. (eds.) (1994a). *Biotechnology of Endophytic Fungi of Grasses*, pp. 214, CRC Press, Boca Raton.

Bacon, C.W. and White, J.F., Jr. (1994b). Stains, media, and procedures for analyzing endophytes. In: *Biotechnology of endophytic fungi of grasses*, (eds. C.W. Bacon, and J.F. White, Jr.), pp. 47–56, CRC Press, Boca Raton.

Ball, D.M., Lacefield, G.D., Agee, C.S. and Hoveland, C.S. (2007). Introduction and acceptance of novel endophyte tall fescue in USA. In: *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses,* (eds. A.J. Popay and E.R. Thom), pp. 249–251, New Zealand Grassland Association, Dunedin.

Bouton, J.H. (2007). Where do we go from here? In: *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses,* (eds. A.J. Popay and E.R. Thom), pp. 515–518, New Zealand Grassland Association, Dunedin.

Bouton, J.H. and Easton, S. (2005). Endophytes in forage cultivars. In: *Neotyphodium in Cool-Season Grasses,* (eds. C.A. Roberts, C.P. West and D.E. Spiers), pp. 327–340, Blackwell Publishing, Oxford.

Bouton, J.H., Latch, G.C.M., Hill, N.S., Hoveland, C.S., McCann, M.A., Watson, R.H., Parish, J.A., Hawkings, L.L. and Thompson, F.N. (2002). Reinfection of tall fescue cultivars with non-ergot alkaloid-producing endophytes. *Agronomy Journal,* **94**, 567–574.

Cheplick, G.P. (2007). Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. *Environmental and Experimental Botany,* **60**, 202–210.

Chessa, P.A. and Delitala, A.M.S. (1997). Objective analysis of daily extreme temperatures of Sardinia (Italy) using distance from the sea as independent variable. *International Journal of Climatology,* **17**, 1467–1485.

Clement, S.L. (2001). Overview of *Neotyphodium* incidence in seed bank collections and plants in managed and unmanaged habitats. In: *Proceedings 4th International Neotyphodium/Grass Interactions Symposium*, (eds. V.H. Paul and P.D. Dapprich), pp. 113–122, Universitat – Gesamthochschule Paderborn, Germany.

Clement, S.L., Elberson, L.R. and Kynaston, M. (2007). High *Neotyphodium* infection frequencies in tillers and seeds of infected wild tall fescue plants. In: *Proceedings 6th International Symposium on Fungal Endophytes of Grasses,* (eds. A.J. Popay and E.R. Thom), pp. 49–52, New Zealand Grassland Association, Dunedin.

Clement, S.L., Elberson, L.R., Youssef, N.N., Davitt, C.M. and Doss, R.P. (2001). Incidence and diversity of *Neotyphodium* fungal endophytes in tall fescue from Morocco, Tunisia, and Sardinia. *Crop Science,* **41**, 570–576.

Clement, S.L., Kaiser, W.J. and Eichenseer, H. (1994). *Acremonium* endophytes in germplasms of major grasses and their utilization for insect resistance. In: *Biotechnology of endophytic fungi of grasses*, (eds. C.W. Bacon and J.F. White, Jr.), pp. 185–199, CRC Press, Boca Raton.

Clement, S.L., Youssef, N., Bruehl, G.W., Kaiser, W.J., Elberson, L.R. and Bradley, V. (2004). Effects of different storage temperatures on grass seed germination and *Neotyphodium* survival. In: *Proceedings 5th International Symposium on Neotyphodium/Grass Interactions,* (eds. R. Kallenbach, C. Rosenbrans and T.R. Lock), pp. 3, University of Arkansas, Fayetteville.

Cunningham, P. (1996). Commonwealth plant introduction list (no. 129) numbers 135997-136472. In: *Australian Plant Introduction Review*, (eds. G.P. Orr, L.J. Apps and M. Burk), vol. 26, pp. 33–101, CSIRO Division of Plant Industry, Canberra.

Cunningham, P.J., Graves, W.L., Chakroun, M., Mezni, M.Y., Saidi, S., Bounejmate, M., Porqueddu, C. and Reed, K.F.M. (1997). Novel perennial forage germplasm from North Africa and Sardinia. In: *Australian Plant Introduction Review,* (eds. G.P. Orr, L.J. Apps and M. Burk), vol. 27, pp. 13–46, CSIRO Division of Plant Industry, Canberra.

Do Valle Ribeiro, M.A.M. (1993). Transmission and survival of *Acremonium* and implications for grass breeding. In: *Acremonium/Grass Interactions*, (eds. S.S. Quisenberry and R.E. Joost), pp. 195–213, Elsevier, Amsterdam.

Dombrowski, J.E., Baldwin, J.C., Azevedo, M.D. and Banowetz, G.M. (2006). A sensitive PCR-based assay to detect *Neotyphodium* fungi in seed and plant tissue of tall fescue and ryegrass species. *Crop Science,* **46**, 1064–1070.

Easton, S. and Tapper, B. (2005). *Neotyphodium* research and application in New Zealand. In: *Neotyphodium in Cool-Season Grasses,* (eds. C.A. Roberts, C.P. West and D.E. Spiers), pp. 35–42, Blackwell Publishing, Oxford.

Faeth, S.H. and Sullivan, T.J. (2003). Mutualistic asexual endophytes in a native grass are usually parasitic. *The American Naturalist,* **161**, 310–325.

Gundel, P.E., Maseda, P.H., Ghersa, C.M. and Benech-Arnold, R.B. (2006). Effects of the *Neotyphodium* endophyte fungus on dormancy and germination rate of *Lolium multiflorum* seeds. *Austral Ecology*, **31**, 767–775.

Hahn, H., Huth, W., Schöberlein, W. and Diepenbrock, W. (2003). Detection of endophytic fungi in *Festuca* spp. by means of tissue print immunoassay. *Plant Breeding,* **122**, 217–222.

Herzenni, A., Laamari, A., Boughlala, M. and Ngaido, T. (2001). Institutional options and pastoral communities in Morocco. Paper prepared for the international Conference on Policy and Institutional Options for the Management of Rangelands in dry Areas May 7–11, 2001, Hammamet, Tunisia, http//www.capri.cgiar.org/pdf/rangeland.morocco.pdf.

Hill, N.S., Bouton, J.H., Haitt III, E.E. and Kittle, B. (2005). Seed maturity, germination, and endophyte relationships in tall fescue. *Crop Science,* **45**, 859–863.

Hill, N.S., Hiatt, E.E., III, De Battista, J.P., Costa, M.C., Griffiths, C.H., Klap, J., Thorogood, D. and Reeves, J.H. (2002). *Seed Science and Technology*, **30**, 347–355.

Hinton, D.M. and Bacon, C.W. (1985). The distribution and ultrastructure of the endophyte of toxic tall fescue. *Canadian Journal of Botany,* **63**, 36–42.

Holder, T.L., West, C.P., Turner, K.E., McConnell, M.E. and Piper, E.L. (1994). Incidence and viability of *Acremonium* endophytes in tall fescue and meadow fescue plant introductions. *Crop Science,* **34,** 252–254.

ISTA (1999). International Rules for Seed Testing. *Seed Science and Technology,* **27,** Supplement, 333pp.

Ju, H.-J., Hill, N.S., Abbott, T. and Ingram, K.T. (2006). Temperature influences on endophyte growth in tall fescue. *Crop Science,* **46**, 404–412.

Latch, G.C.M. (1997). An overview of *Neotyphodium*/Grass interactions. In: *Neotyphodium/Grass Interactions*, (eds. C.W. Bacon and N.S. Hill), pp. 1–11, N.S. Plenum Press, New York.

Latch, G.C.M., Christensen, M.J. and Samuels, G.J. (1984). Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon,* **20**, 535–550.

Latch, G.C.M., Christensen, M.J., Tapper, B.A., Easton, H.S., Hume, D.E. and Fletcher, L.R. (2000). Tall fescue endophytes. U.S. Patent 6 111 170. Date issued: 29 August 2000.

National Climate Data Center, NOAA. (27 May 2005). Locate Weather Observation Station Record, www.ncdc.noaa.gov/oa/ncdc.html.

Porter, J.K. (1994). Chemical constituents of grass endophytes. In: *Biotechnology of endophytic fungi of grasses*, (eds. C.W. Bacon and J.F. White, Jr.), pp. 103–123, CRC Press, Boca Raton.

Ravel, C., Michalakis, Y. and Charmet, G. (1997). The effect of imperfect transmission on the frequency of mutualistic seed-borne endophytes in natural populations of grasses. *Oikos,* **80**, 18–24.

Rolston, M.P., Hare, M.D., Moore, K.K. and Christensen, M.J. (1986). Viability of *Lolium* endophyte fungus in seed stored at different moisture contents and temperatures. *New Zealand Journal of Experimental Agriculture*, **14**, 297–300.

SAS Institute Inc. (1999) SAS/STAT User's Guide, Version 8. SAS Institute, Cary, NC, USA.

Saha, D.C., M.A. Jackson and J.M. Johnson-Cicalese. (1988). A rapid staining method for detection of endophyte fungi in turf and forage grasses. *Phytopathology,* **78**, 237–239.

Saikkonen, K., Ion, D. and Gyllenberg, M. (2002). The persistence of vertically transmitted fungi in grass metapopulations. *Proceedings Royal Society London*, **B 269**, 1397–1403.

Stuedemann, J.A. and Hoveland, C.S. (1988). Fescue endophyte: history and impact on animal agriculture. *Journal of Production Agriculture,* **1**, 39–44.

Tapper, B.A. and Latch, G.C.M. (1999). Selection against toxin production in endophyte-infected perennial ryegrass. In: *Ryegrass Endophyte: An Essential New Zealand Symbiosis*, (eds. D.R. Woodfield and C. Matthew), pp. 107–111, Grassland Research and Practice Series No. 7, New Zealand Grassland Association, Palmerston North.

Trento, S., Elias, S., Garay, A. and Zavala, J. (2007). Comparison of endophyte detection in fescue and ryegrass seeds using an immunoblot assay and a microscopic method. *Seed Science and Technology*, **35**, 65–74.

Welty, R.E. and Azevedo, M.D. (1993). Endophyte content of seed harvested from endophyte-infected and endophyte-free tall fescue. *Journal of Applied Seed Production,* **11**, 6–12.

Welty, R.E., Milbrath, G.M., Faulkenberry, D., Azevedo, M.D., Meek, L. and Hall. K. (1986). Endophyte detection in tall fescue seed by staining and ELISA. *Seed Science and Technology*, **14**, 105–116.

Wheatley, W.M. (2005). Endophytes, quality assurance, and the seed trade in eastern Australia. In: *Neotyphodium in Cool-Season Grasses,* (eds. C.A. Roberts, C.P. West and D.E. Spiers), pp. 351–360, Blackwell Publishing, Oxford.

Wheatley, W.M., Kemp, H.W., Simpson, W.R., Hume, D.E., Nicol, H.I., Kemp, D.R. and Launders, T.E. (2007). Viability of endemic endophyte (*Neotyphodium lolii*) and perennial ryegrass (*Lolium perenne*) seed at retail and wholesale outlets in south-eastern Australia. *Seed Science and Technology*, **35**, 360–370.

Wilson, S.M. and Easton, H.S. (1997). Seed transmission of an exotic endophyte in tall fescue. In: *Neotyphodium/Grass Interactions*, (eds. C.W. Bacon and N.S. Hill), pp. 281–283, Plenum Press, New York.