

Novel perennial ryegrass-*Neotyphodium* endophyte associations: relationships between seed weight, seedling vigour and endophyte presence

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Abstract Two forage cultivars of perennial ryegrass (*Lolium perenne*) colonised by the mutualistic fungal endophyte *Neotyphodium lolii*, strain AR37, were used to investigate relationships between seed weight, seed vigour and endophyte presence. Seed was separated into six fractions according to weight, with each fraction divided into two further groups with the first being subject to accelerated ageing. Seed germination rates and proportions of viable and total endophyte frequencies were assessed for each fraction. Heavier fractions of seed produced a higher number of endophyte infected seedlings sooner than the lighter fractions for both cultivars. The highest proportion of viable endophyte was found in the fastest germinating perennial ryegrass seed for all weight fractions, from both cultivars, indicating a strong relationship between endophyte presence and seed germination rate. For one of the cultivars, after accelerated ageing, as seed weight increased the proportion of viable endophyte increased and the discrepancy between the proportion of endophyte in fresh and accelerated aged seed was reduced. This implies that for this cultivar heavier ryegrass seed provides a more favourable habitat to the dormant endophyte than lighter seed during

storage and/or allowed for a greater biomass of endophyte hyphae to proliferate in the seed tissues prior to seed dormancy, thus allowing the fungus to develop more propagules and greater nutritional reserves.

Keywords *Epichloë* · Germination · *Lolium perenne* · Symbiosis

1 Introduction

Endophytic fungi of the genus *Neotyphodium* (Ascomycota: Clavicipitaceae) are well known to increase competitive abilities of certain agronomic host grasses within the family Poaceae (Faeth et al. 2004). These asexual endophytes grow within the intracellular spaces of their grass hosts in a highly regulated manner (Christensen et al. 2002, 2008b). Lineages of single fungal genotypes are transmitted maternally (vertically) through seeds (Christensen et al. 2008a; Freeman 1904) following fungal colonisation of ovules and seed tissues during host flowering (McLennan 1920; Philipson and Christey 1986; Sampson 1933). With the onset of seed germination, hyphae within the embryo start to colonise leaf primordia and auxiliary buds, spreading systemically throughout the above ground parts of the plant (Philipson and Christey 1986). These fungal symbionts form permanent mutualistic associations with their cool season grass hosts, expressing no obvious host symptoms.

Many Poaceae grasses, including natural populations and commercial cultivar stands, may be colonised with epichloid endophytes but at highly variable frequencies, ranging from 1 to 100 % (Leyronas and Raynal 2001; Clement et al. 2006; Faeth et al. 2004; Lewis et al. 1997; Ravel et al. 1997; Saikkonen et al. 1998). These fungal symbionts often play a protective role for their hosts, such as through the production of alkaloids that deter certain herbivores. As these endophytes

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are not vital to the host, they can have complex patterns of transmission that are often not perfect and these patterns maybe important for mediating the grass-endophyte interaction (Gundel et al. 2011b). The mechanisms behind endophyte transmission failures are poorly understood with various abiotic and biotic factors implicated (Bacon and Siegel 1988; Ju et al. 2006; Pedersen et al. 1984; Bazely et al. 2007; Vila-Aiub et al. 2003) as well as the genetic compatibility between fungus and host (Saikkonen et al. 2010b; Gibert and Hazard 2013). Endophyte transmission failures can occur pre-zygotically, where the fungus fails to successfully colonise seedlings, tillers, spikes, spikelets and ovules or post-zygotically when the fungus dies within the seed (described by Gundel et al. 2008). Endophyte survival is firmly tied with that of the plant host and within the seed the fungus is reliant on the plant for nutrients, water, gas exchange and activation from dormancy. However, the detailed mechanism by which these fungi colonise the ovule and transmit via the embryo is still poorly understood (Hill et al. 2005; Scott 2001).

Neotyphodium endophytes have been shown to both positively and negatively affect seed viability and germination vigour in *Lolium* and *Festucae* host grasses (Clay 1987; Gundel et al. 2006b; Hamilton and Faeth 2005) with this relationship dependant on many factors including resource allocation, host plant fitness, and the plant/endophyte genotype. In order to study seed vigour, accelerated ageing techniques are generally used as a fast test (Cookson et al. 2001) and are designed to mimic the natural physiological ageing (or deterioration) process of seed. Although these techniques do not necessarily correlate to effects seen in the field, they are regarded as a good predictor of seed quality (McDonald 1998). Accelerated ageing techniques are also used in ecological investigations (Gundel et al. 2010) and extensively in applied *Neotyphodium* endophyte research to understand population dynamics within stands of natural grasses and to assess the storage characteristics of particular fungal strains, respectively. In general, endophyte viability decreases at a faster rate than does seed viability (Hill and Roach 2009; Hume et al. 2011; Rolston et al. 1986) and the consequential loss of viable endophyte reduces the value of commercial seed (Hill and Roach 2009). To our knowledge the effect of this technique on the physiology of epichloid fungi has not been investigated, although it is suggested that a similar deterioration process to that of the seed occurs (Grbavac pers. comm.).

Perennial ryegrass (*Lolium perenne* L.) is an important pasture, forage and turf plant and in New Zealand it is commonly and intentionally inoculated with beneficial endophyte strains of *Neotyphodium lolii*, such as AR1 and AR37 (Fletcher 1999; Fletcher and Easton 1997; Pennell et al. 2005). Understanding the biological relationships between specific seed populations of perennial ryegrass and their associated endophytes is beneficial to the pasture grass and livestock industries. Of particular interest are the areas of

endophyte transmission from plant to seed and endophyte survival during seed storage. The aim of this research was to investigate the relationships between seed weight, endophyte presence, seed vigour and accelerated ageing in two cultivars of perennial ryegrass and on a broader level investigates how one fungal genotype, artificially introduced into novel perennial ryegrass hosts, interacts with different host cultivars. This is crucial to understanding the stability of artificial (or synthetic) endophyte-grass associations for breeding purposes.

2 Materials and methods

Two accessions of perennial ryegrass, cultivars ‘Grasslands® Samson’ and ‘Grasslands® Commando’, colonised with the commercially available endophyte *N. lolii* strain AR37, were hand harvested in Jan-2009 (summer) from seed-production fields in Canterbury, New Zealand. Commando and Samson are diploid, mid-season flowering, forage cultivars developed by AgResearch Ltd. Commando was bred from Northland farm ecotypes while Samson was bred from a collection of plants originally collected from dry site pastures throughout the North Island, Marlborough and Canterbury regions of New Zealand (Lyons pers. comm.). The Commando seed was field dressed (FD) while Samson was machine dressed (MD) with both accessions stored at 0 °C with 30 % relative humidity within the Margot Forde Germplasm Centre, New Zealand’s national gene-bank of grassland plants. In Jan-2010 an 80 g seed sample from each of the two accessions was separated into six weight fractions using a column air seed separator which can separate seeds by way of an adjustable vertical air stream. Thousand seed weights (TSW) were then determined for each weight fraction (according to ISTA1999). Approximately half the seed from each fraction was subjected to an accelerated ageing regime (seed hereby referred to as aged seed), with seed placed in a paper packet suspended above 400 ml of tap water within a sealed glass desiccator to give a relative humidity close to 100 % and incubated at 40 °C for 36 h. This gave a deterioration effect equivalent to storing the seed for 6–8 months at ambient temperature and humidity (Rolston, pers. obs.).

2.1 Modification of endophyte infection frequencies after seed ageing

The infection layer (IL) method (Card et al. 2011) was performed on 100 seed from each weight fraction to determine the original pre-zygotic (total) endophyte infection frequencies. This technique has previously been shown to be more accurate than the seed squash method in assessing proportions of endophyte in fresh seed accessions but cannot differentiate between viable and non-viable endophytic mycelium (Card et al. 2011). An immunodetection protocol was used to

determine the post-zygotic endophytic frequency. For both accessions, approximately 200 seed from each fraction (fresh and aged) was sown into two replicate plastic trays (100 seed in each tray) containing potting mix, placed in a glasshouse (16–24 °C) and watered as required. Slugs were controlled using Blitzem™ (Yates, New Zealand) containing 18.0 g kg⁻¹ metaldehyde in the form of granular bait and insect pests controlled with Confidor® (Yates, New Zealand) containing imidacloprid 50 g kg⁻¹. After 6 weeks the viable endophyte infection frequency was determined for each fraction using the tissue-print immunoblot (TPIB) technique (Simpson et al. 2012) with 2 tillers per plant assessed. Fisher's Exact Test (Yates 1984) was used to test differences between the germination rates of fresh and aged seed using Microsoft Excel (Microsoft® 2007). Viable endophyte was calculated as:

$$\% \text{viable endophyte} = \frac{\text{Viable endophyte}}{\text{Viable endophyte} + \text{non-viable endophyte}} \times 100$$

and represents the percentage of seedlings that contained viable endophyte in each weight fraction. For TSW and endophyte presence data, binary logistic regression analysis was used with viable endophyte as the binary response variable (viable versus non-viable) and the predictors TSW and Endophyte method (viable endophyte in fresh seed, viable endophyte in aged seed, total endophyte). Analysis was conducted using GenStat®, 15th edition (VSN International Ltd).

2.2 Relationship between TSW, germination time and viable endophyte presence

For both accessions, approximately 400 seed from each seed weight fraction (from fresh and aged groups) were placed onto damp sterile filter paper within glass Petri plates (14 cm diameter and 2 cm deep) and incubated at 10 °C in the dark. This resulted in the majority of seeds germinating over a period of approximately 4 weeks. Over this period, Petri plates were checked twice a day for seed germination, defined as when all essential plant structures including radicle and coleoptile were visible (ISTA1999). Remaining seed were presumed non-viable. Those seed that had germinated were immediately planted in potting mix and placed in a glasshouse as mentioned previously. After 6 weeks, subsequent plants were assessed for endophyte presence using the TPIB technique. Seed weight fractions were collated into three groups, light, medium and heavy, as the data from each pair of closely weighted fractions followed a similar trend. Extended logistic curves in the form:

$$y = A + \frac{C}{[1 + T \times \text{Exp}(-B(x-m))] \frac{1}{T}}$$

were fitted to the cumulative percentage germination and endophyte data for Samson, while for Commando the cumulative percentage of the log germination was found to fit the data more accurately. *Y* is the cumulative percentage germination or endophyte, *x* is the time (hrs) to germination, parameter *A* is the initial value (nominally 0 %), *C* is the asymptote (nominally 100 %), *B* is the rate of increase, *M* is the lag before germination commences (hrs), and *T* is the parameter reflecting the shape of the curve. Time to germination (hrs) and Endophyte method (previously described) were used as the predictor variables. The Kolmogorov-Smirnov two-sample test was used (Sheskin 2007) for comparing the mean cumulative proportion of viable endophyte per number of germinated seed for the two cumulative curves for fresh and aged seed. Analysis was conducted using GenStat®, 15th edition.

3 Results

3.1 Relationship between TSW and seed germination rates

The TSWs differed between the two accessions with mean weight fractions ranging from 0.60 to 2.19 g for Commando and 1.83–3.20 g for Samson, with weighted mean TSW of all seed weight fractions being 1.24 g and 2.44 g respectively (Table 1). Germination rates also differed between the accessions with mean germination rates of 76 % and 93 % for Commando and Samson, respectively. Commando experienced a mean decrease in germination rate of 12 percentage units after ageing (76 down to 64 %). This occurred for all but the lightest fraction (0.60 g), with the third lowest weight fraction (1.10 g) experiencing the greatest loss (17 percentage units) (Fig. 1a). In contrast, the mean germination rate for Samson decreased from 93 to 92 %, a decrease of just 1 % after ageing, with only the lightest fraction (1.83 g) having a significant ($p=0.009$) decrease after aging (Fig. 1b).

3.2 Modification of endophyte infection frequencies after seed ageing

Logistic regression fits a linear model on the logit scale. Parameter estimates, standard errors, *T* values and significance values from binary logistic regression analysis with predictor variables TSW fractions and endophyte presence (viable vs total) for fresh and aged seed are provided for both accessions (Table 2, Supplementary material). A non-zero (significantly different to zero) intercept on the logit scale means that the proportion of viable endophyte is not 50 %. All the regression lines for both accessions had a non-zero intercept, except for Total endophyte for Samson ($p<0.001$) (Table 2, Supplementary material). Mean endophyte infection frequencies were 41 % and 57 % (Total endophyte) and 35 %

Table 1 TSWs, seed fraction weights and weighted contributions (%) for each seed weight fraction for Commando and Samson accessions investigated in this study

Seed factor	Seed weight fraction						
	1	2	3	4	5	6	Mean
Field dressed (FD) Commando							
TSW (g)	0.60	0.84	1.10	1.19	1.51	2.19	1.24
Weighted contribution (%)	3.6	6.4	15.1	16.4	24.7	33.8	-
Machine dressed (MD) Samson							
TSW (g)	1.83	1.93	2.43	2.49	2.74	3.24	2.44
Weighted contribution (%)	10.3	10.8	17.9	17.4	19.5	24.0	-

and 57 % (Viable endophyte) for these particular accessions of Commando and Samson, respectively (data not shown).

A non-zero slope on the logit scale would mean that the proportion of viable endophyte varies across the TSW range. This is true for Commando aged seed ($p=0.005$) but not for any of the other types for either cultivar. This indicates that ageing affected the lighter fraction of seed to a significantly greater degree than the heavier fraction, and that lighter seed may contain a greater proportion of endophyte than heavier seed (Fig. 2a and Table 2, Supplementary material). The equation for each line resulting from the regression analyses are found in Table 3, Supplementary material. A quadratic model, namely with predictors Endophyte method and (TSW + TSW²) did not improve the model fit.

3.3 Relationship between TSW, germination time and viable endophyte presence

There was a nonlinear relationship between seed weight and germination time in both accessions, with the cumulative germination percentage characterised by a sigmoidal curve (Fig. 3). For Commando, fresh seed from all weight fractions

started germinating after 200 h (Fig. 3a, b and c), with respective lag phases of 262, 248 and 224 h for the light, medium and heaviest fractions respectively (see Table 4, Supplementary material). The germination of seeds from the heaviest Commando group (1.51 and 2.19 g weight fractions), had a high frequency of germination over a 3-day period (176–248 h) after which few additional seeds germinated (Fig. 3c). The germination of seeds from the lightest (0.60 and 0.84 g weight fractions) and medium Commando groups (1.10 and 1.19 g weight fractions) germinated over a longer period of time at a lower frequency per day (Fig. 3a and b) compared to the heavier group of seed (Fig. 3c). The heaviest fraction recorded a 50 % germination rate after 248 h, the medium 275 h and the lightest 288 h (Fig. 3a, b and c) which is also reflected in the rate of increase (see Table 4, Supplementary material). After the ageing regime, seed from all weight fractions of Commando experienced a delay in their germination rates compared to the fresh seed, although this delay was less pronounced for the heavier fractions (Fig. 3a, b and c). For example, the heaviest fraction still recorded a 50 % germination rate after 295 h and the lightest fraction after 371 h (Fig. 3a and c).

(a) Field dressed (FD) Commando

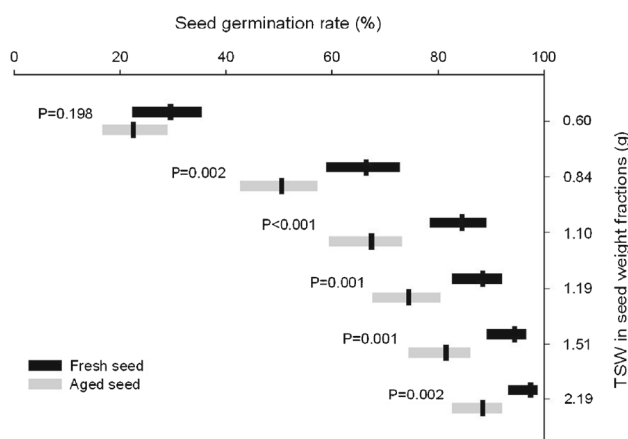
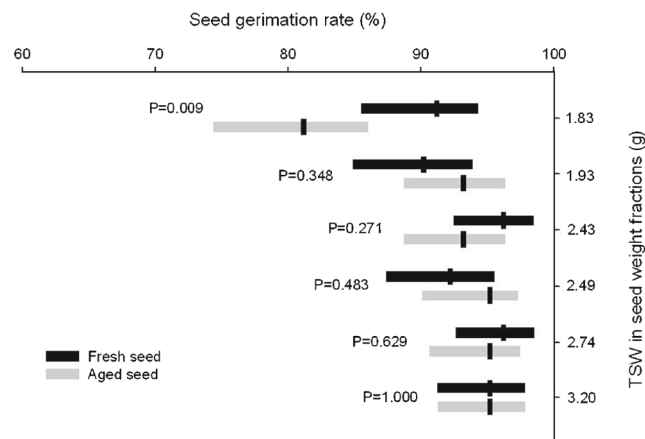


Fig. 1 Graph showing results from Fisher's exact test for germination rate (%) for field dressed perennial ryegrass Commando (a) and machine dressed Samson (b) accessions, at six TSW fractions for fresh and aged

(b) Machine dressed (MD) Samson



seed, with their respective 95 % confidence intervals and significance values. Note that the scale on both x and y axis differs among cultivars

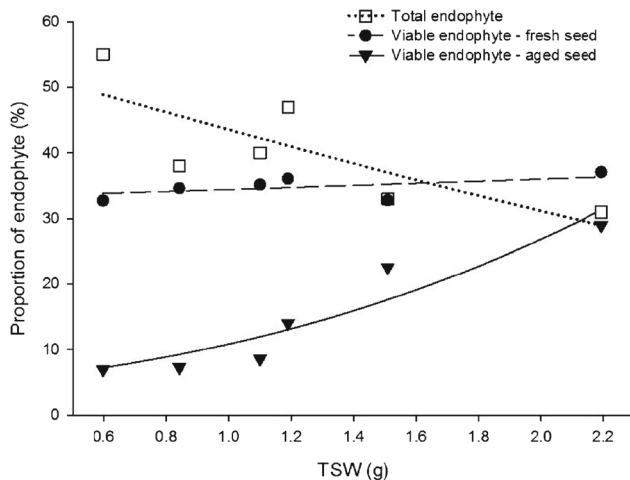
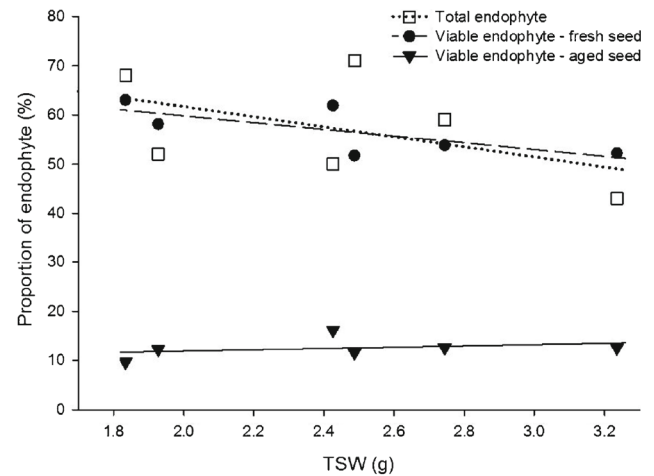
(a) Field dressed (FD) Commando

Fig. 2 Relationship between the proportion of endophyte-infected seed (viable and total) and thousand seed weight (TSW) for field dressed (FD) perennial ryegrass Commando (a) and machine dressed (MD) Samson (b) for

(b) Machine dressed (MD) Samson

fresh and aged seed. Fitted lines indicate non-linear functions fitted according to equations in Table 3 and parameter estimates in Table 2, Supplementary material. Note that the scale on both x and y axis differs among cultivar

For Samson, fresh seed from all weight fractions started germinating after 150 h, much earlier than for Commando (Fig. 3 and Table 4, Supplementary material) with respective lag phases of 160, 183 and 149 h for the light, medium and heaviest fractions respectively (see Table 4, Supplementary material). The heaviest fraction of Samson recorded a 50 % germination rate after 162 h, the medium 190 h and the lightest 175 h (Fig. 3a, b and c) which is also reflected in the rate of increase (see Table 4, Supplementary material). Interestingly the medium weight group recorded the longest time to reach this 50 % level. The germination of seeds from all the fractions followed a similar trend in that there was a high frequency of germination over a 152–224 h period, after which few additional seeds germinated (Fig. 3d, e and f). After the ageing regime, seed from all Samson weight fractions experienced a delay in their germination rates compared to the fresh seed (Fig. 3d, e and f). This is also seen in the differing values of M in the fitted equations (see Table 4, Supplementary material). For both accessions, and all three weight fractions, the Time \times Endophyte method interaction effect was significant ($p < 0.01$) with the exception of the heaviest weight fraction for Commando, for which Time and Endophyte method effects were both significant ($p < 0.001$).

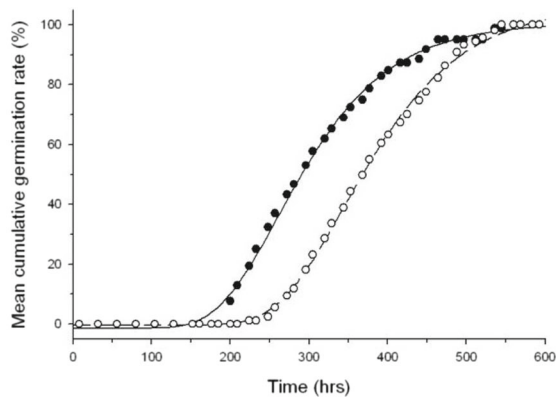
There was a nonlinear relationship for total endophyte presence/viability and germination time (hrs) for each weight fraction in both the Commando and Samson accessions. The curves followed a sigmoidal relationship similar to that of the seed germination data (Fig. 4). For both Samson and Commando the heavier groups of fresh seed produced a higher number of endophyte infected seedlings, therefore a steeper line (Fig. 4c and f) more quickly than the lighter fractions (Fig. 4a and d). This was also seen by the rate of

increase (see Table 5, Supplementary material). For aged seed this trend was reversed by Samson and followed no obvious trend for Commando (see Table 5, Supplementary material). A decrease was observed in the time taken for Commando and Samson seed that contained viable endophyte to germinate in aged seed that was also observed for fresh seed (see Table 5, Supplementary material). For both accessions, and all three weight fractions, the Time \times Endophyte method interaction was significant ($p < 0.001$). For fresh Commando seed to reach a mean cumulative proportion of 50 % endophyte infected seed took 239, 210 and 194 h respectively for light, medium and heavy weight groups (Fig. 4a, b and c) while for Samson this took 161, 184 and 158 h, respectively (Fig. 4d, e and f). Parameter estimates from fitted equations for Commando and Samson, describing the relationships between thousand seed weight (TSW) fractions and endophyte presence (viable vs total) for fresh and aged seed shown in Table 5, Supplementary material.

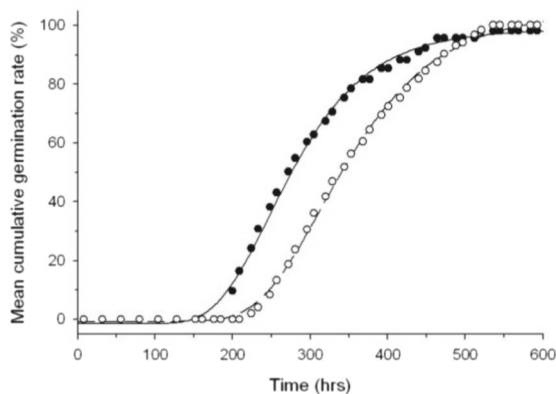
In Commando the cumulative proportion of endophyte per germinated seed for all seed fractions reached their maximum level after 200 h followed by a decline (Fig. 5). For fresh seed the maximal endophyte proportions reached were 29, 36 and 36 % for the light, medium and heaviest fractions respectively (Fig. 5a, b and c). For aged seed, there was a steep rise in endophyte proportions for all weight groups at 209 and 224 h, reaching 100 % for the lightest fraction, and then steeply decreasing for the lightest and medium fractions (Fig. 5a and b). Samson showed a similar trend for all weight groups of fresh seed, with a high frequency of endophyte infected seed observed (52 % for all weight groups) in the first 152 h of germination (Fig. 5d, e and f) that remained constant for the remainder of the germinated seeds through to the final time period of 405 h. This

Field dressed (FD) Commando

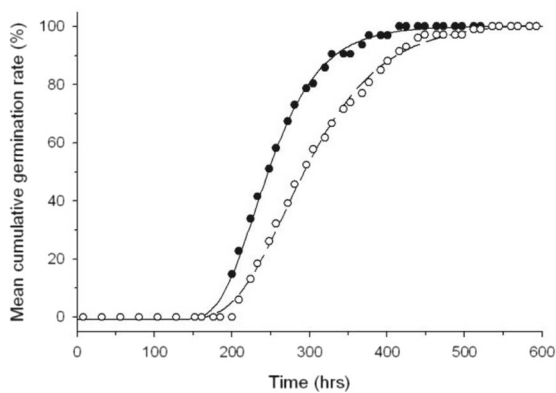
(a) Light



(b) Medium

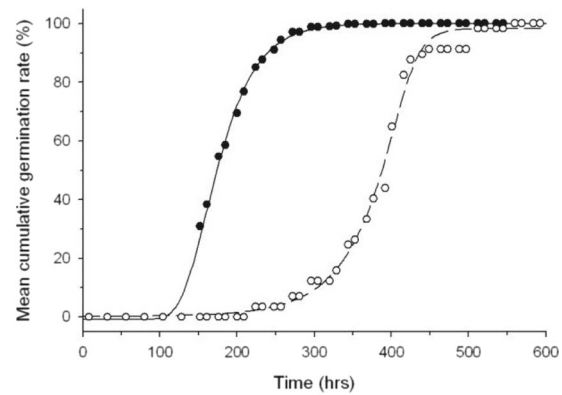


(c) Heavy

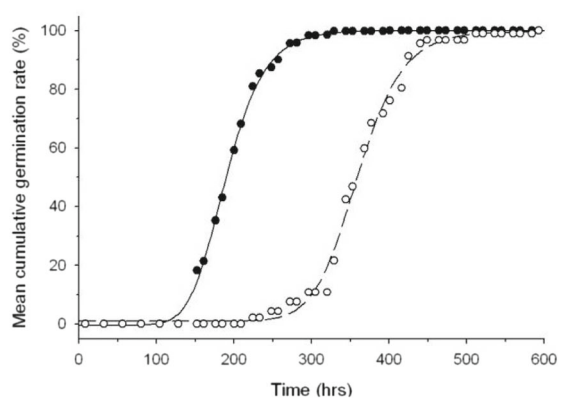


Machine dressed (MD) Samson

(d) Light



(e) Medium



(f) Heavy

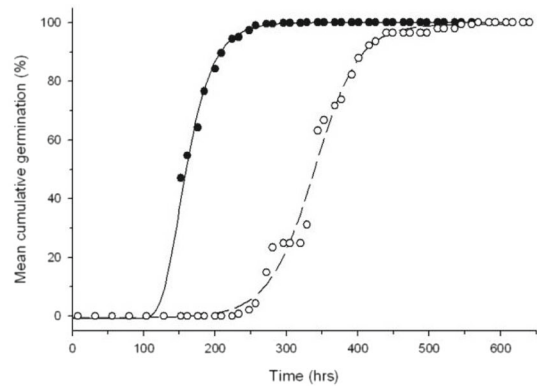


Fig. 3 Relationships between cumulative germination rates and time of germination for perennial ryegrass, Commando and Samson, at three seed weight groups, light, medium and heavy, for fresh (●) and aged (○) seed

trend was not observed for the aged Samson seed, with the lightest fraction having a high proportion of endophyte in the earlier germinating seed (33 % at 224 h), 14 % for medium seed and 0 % for heavy (Fig. 5d, e and f). All aged seed curves for both cultivars, at all seed weight groups, were highly significantly different ($p < 0.001$) to the fresh seed curves (Fig. 5) according to the Kolmogorov-Smirnov Two-sample test.

4 Discussion

Weighted seed fraction contributions, TSWs, and average germination rates differed among the accessions of Samson and Commando investigated in this study. These differences may be attributed to the growing conditions of the crop and the seed dressing methods applied to these particular seed

Field dressed (FD) Commando

Machine dressed (MD) Samson

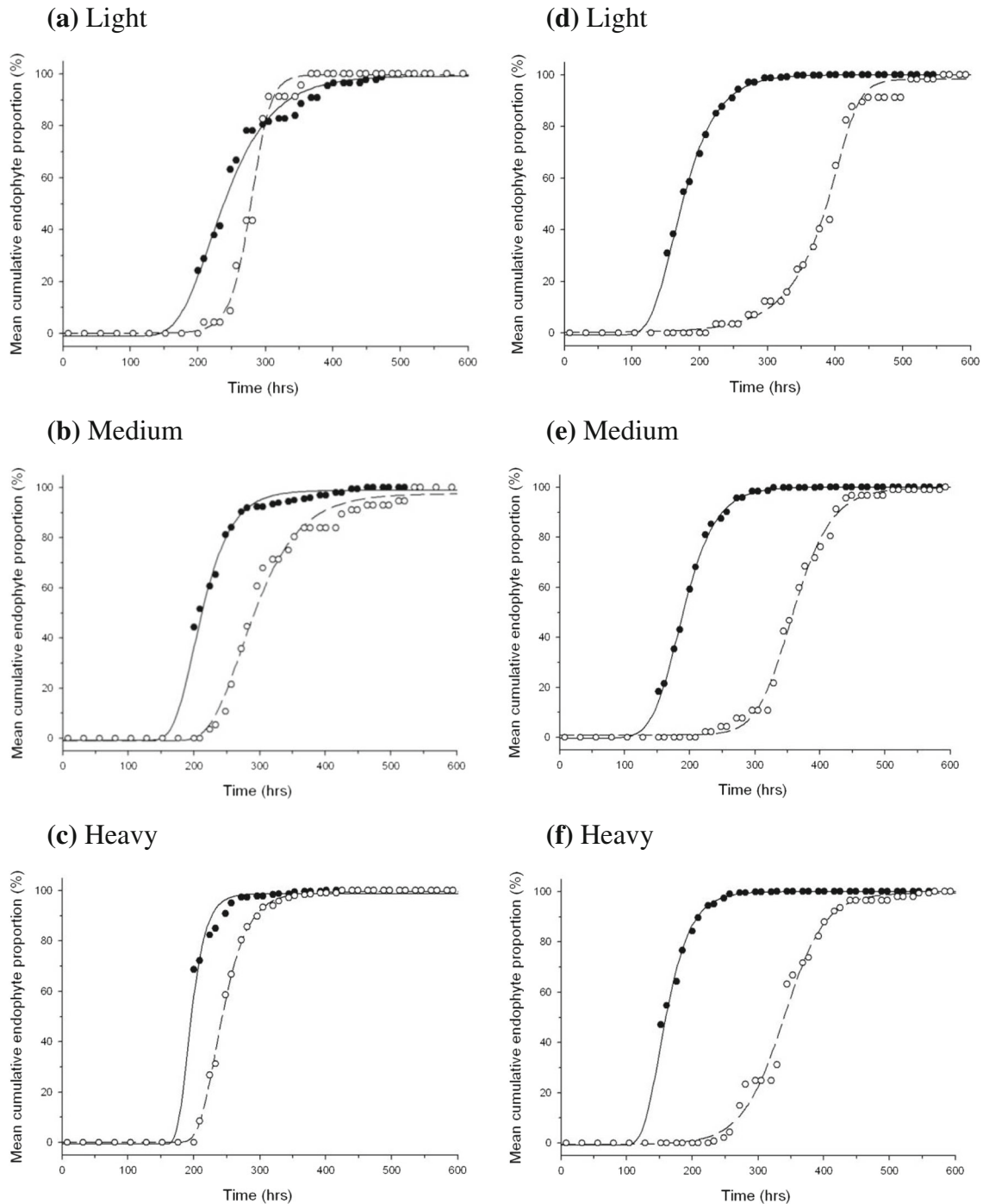


Fig. 4 Relationships between the mean cumulative number of seedlings that contained viable endophyte (*N. lolii* AR37) and time of germination for perennial ryegrass seed accessions, Commando and Samson, at three

weight fractions (a and d) light, (b and e) medium and (c and f) heavy, for fresh (●) and aged seed (○)

lines after harvest. The accession of Commando was field dressed and therefore contained many immature light seeds with a reduced germination capacity. In contrast, the Samson accession was machine dressed and contained only saleable mature grain which exhibited a higher mean TSW and

germination rate. A large number of other factors may affect TSW among seed lots within and across cultivars including; environmental factors during seed production, geographic origin, agricultural practises, seed handling, soil fertility, moisture supply and genetic factors (Larsen et al. 2004).

Field dressed (FD) Commando

Machine dressed (MD) Samson

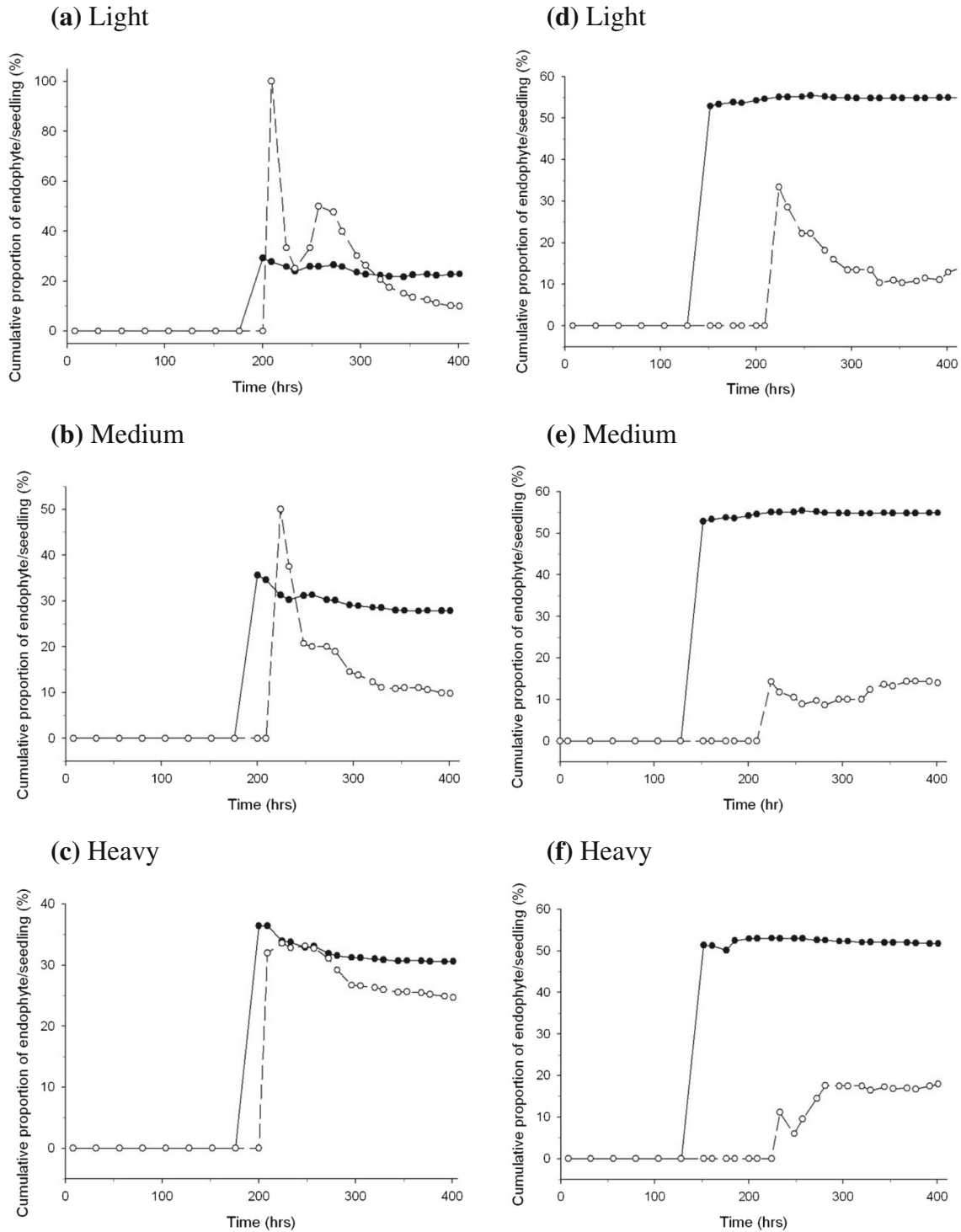


Fig. 5 Relationships between the mean cumulative proportion of viable endophyte containing seedlings per number of germinated seed and time of germination for Commando and Samson accessions, at three seed

weight groups **a** light, **b** medium and **c** heavy, for fresh (●) and aged seed (○). Note the scale on the y axis differs among cultivars and seed weight groups

Accelerated ageing reduced germination in Samson more than Commando, with mean TSW fractions of Samson losing 12 % germination and the heaviest fraction losing 9 %. In contrast,

Commando lost 1 % germination from the mean TSW fractions with no difference in germination in the heaviest seed weight fraction. Whether cultivar is a factor in response to the

ageing regime is not known (Hampton et al. 2000) and this difference is also most likely to be attributed to field history at harvest including fungicide use and/or weathering at harvest and before seed cleaning. A positive linear relationship existed between TSW and seed germination rates, with heavier seed fractions, from both cultivars, exhibiting a higher rate of germination than the lighter fractions. This relationship has also been documented in a number of other forage grass studies (Cookson et al. 2001; Larsen and Andreassen 2004; Smith et al. 2003).

Brown and Mayer (1988) explored a variety of sigmoidal-shaped curves and found the Weibull curve fitted their germination data the best. However, of the three possible sigmoidal curves offered by the statistical package GenStat[®], the extended logistic curve fitted our data the best. For the germination data for Commando, an even better fit was obtained by fitting the extended logistic to the cumulative percentage curve for log germination. The logistic and extended logistic curves differ from the other curves fitted by Brown and Mayer (1988) in that they follow the form $y \propto \frac{1}{\text{Exp}(-x)}$, whereas the other curves have the form $y \propto \text{Exp}(-x)$. Brown and Mayer (1988) also had the same issues fitting the curves, with data which ascended quickly due to the high initial proportion of seed germinating and then plateauing before the final seeds germinated.

As expected, there was a significant difference between the proportions of viable endophyte in fresh seed compared to that in aged seed, for both accessions. This indicates that accelerated ageing affected endophyte viability to a greater degree than seed viability and is a well-documented phenomenon (Hill and Roach 2009; Hume et al. 2011; Rolston et al. 1986). In Commando, it was evident that the process of accelerated ageing affected the lighter fractions of seed to a greater degree than the heavier fractions, inferring that endophyte in heavier seed are less prone to post-zygotic failure. This was supported by the positive relationship between TSW and viable endophyte proportions for aged seed. For this accession there was also a weak negative relationship between TSW and the total endophyte proportion, inferring that the lighter seed fraction contained a greater proportion of total endophyte than the heavier fraction. However, there was no relationship between TSW and viable endophyte from fresh seed possibly indicating that a higher proportion of endophyte died, or failed to colonise the seedlings as they developed, in the lighter seed fraction than the heavier seed. We speculate that heavier seed of this accession provided greater nutrient reserves and protection to the dormant endophyte than lighter seed and/or allowed for a greater biomass of endophyte hyphae to proliferate in the seed tissues prior to seed dormancy, thus allowing the fungus to develop more propagules with potentially greater nutritional reserves. This accession had a greater range of TSW than the Samson accession (due to light seed of Samson being removed at dressing) and could explain

why this relationship was not observed with the Samson accession or this relationship may be unique to this cultivar or this particular accession. Seed dressing, increases final germination percentage and also increases viable endophyte frequency and optimises the proportion of endophyte survival during seed storage. An interesting outcome from this study indicates that single fungal genotypes can exhibit specific traits when in association with different plant genotypes, even of the same host species.

Physiological ageing (or deterioration), starts before seed harvest and reduces the performance capabilities of the seed. This time dependant process causes membrane damage, loss in lipid content, changes in enzyme activity and protein synthesis and subsequent solute leakage from seeds during imbibition, whether by natural or accelerated ageing regimes (Kester et al. 1997; Walters 1998; Ouzouline et al. 2009; Powell 1988; ISTA Vigour Test Committee 1995). The outcome of this deterioration, regardless of plant species or seed weight/size, is firstly a loss in seed vigour which leads to a loss in the seeds ability to germinate, ultimately leading to seed death (ISTA Vigour Test Committee 1995). It is generally accepted that within a particular seed lot, those seed with a higher TSW will have greater carbohydrate reserves and hence greater seed vigour and subsequently a higher survival rate than those with a lower TSW. Gundel et al. (2006a) reported that after accelerated ageing, endophyte-infected seed exhibited a higher level of seed moisture content than endophyte-free seed (Gundel et al. 2006a). There is a well-known negative correlation between seed moisture content and seed longevity (Ellis and Roberts 1980), with seed moisture in equilibrium with relative humidity and we wish to investigate this further.

Evidence suggests that the relationship, between the fungus and plant, can range from mutualistic to antagonistic depending on a number of factors including the availability of nutrients in the soil, host plant fitness and the complexity of the food web (Gundel et al. 2011a; Saikkonen et al. 2006, 2010a). Both mutualistic and antagonistic (positive and negative) relationships have been observed with annual ryegrass (*L. multiflorum*) infected with the endophyte *N. occultans*. Investigations into three accessions showed that endophyte from one of these accessions negatively affected seed viability and germination rate after ageing (Gundel et al. 2006a). The authors suggest that the effect of *N. occultans* on seed quality could depend on the population genetic background and suggest that co-evolution between these vertically transmitted endophytes and their hosts could have promoted seed quality rather than quantity. In ryegrass, previous researchers showed that the rate of seedling emergence of cultivar 'Grasslands Arika' (*Lolium* × *hybridum* Hausskn) was not influenced by endophyte presence (Keogh and Lawrence 1987). However, the authors did show that there was a relationship between seed weight and endophyte presence, with one group of

endophyte-infected plants (produced from one weight category) having fewer tillers and lower shoot dry weights than the endophyte-free clonal plants. A positive correlation between the presence of endophyte and the amount of filled seed has also been observed. Clay (1987) showed that endophyte-infected tall fescue plants produced twice as many filled seed compared to endophyte-free controls. As these experiments were conducted in an herbivore free environment, free of insects and mammals, these endophytes may have enhanced the growth of their grass hosts directly.

The presence of the endophyte, *N. uncinatum*, has also been observed to increase the mean number of seeds produced by its meadow fescue (*F. pratensis*) host compared to endophyte-free plants (Saari et al. 2010). A hormonal influence has been postulated as a possible mechanism with several species of Clavicipitaceae known to produce auxin (De Battista et al. 1990; Porter et al. 1985; Tanaka 2010). In dormant seed we believe *Neotyphodium* spp. are prisoners within the plant (they are incapable of horizontal transmission that is feasible by their *Epichloë* relatives) and the hyphae start metabolizing only after the seed has broken dormancy and started the germination process (Card et al. 2013). Therefore any production of hormone, or other metabolite, must be accomplished before seed set and/or after imbibition. We were unable to determine whether endophyte affected the seed mass of Samson and Commando, as we did not include endophyte-free plant material for this comparison as this was outside the scope of this study. However, Gundel et al. (2012) observed a more complex situation with annual ryegrass whereby the trade-off between seed number and seed weight was affected by the presence of *N. occultans*. Here the endophyte induced heavier seed in the host plants under high nutrient availability but not under low nutrient availability and the authors propose that this endophyte controls the development of individual seed through a competition-like mechanism and that *N. occultans* induces heavier seeds under environmental conditions favourable to plant growth or for certain plant genotypes.

The objective of the New Zealand grass seed industry is to provide a high quality endophytic seed product that has a high level of endophyte viability (all novel endophytes developed by AgResearch Ltd are required to have an infection rate of at least 70 % at the time of sale), an alkaloid profile that is true to type and a genetically verifiable endophyte selection (Rolston and Agee 2007). Mean viable endophyte infection frequencies were 35 % and 57 % for these particular accessions of Commando and Samson, respectively. These proportions would be regarded as low for a commercial seed line. Vertical endophyte transmission is likely to be a highly regulated process (Hill et al. 2005) and understanding the factors that contribute to this regulation is crucial in the production of high quality seed products and to unravel the complexity behind this fascinating and uniquely intriguing plant-fungus interaction.

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