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Threshold modelling *Lolium multiflorum* seed germination: effects of *Neotyphodium* endophyte infection and storage environment

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Summary

Several forage species establish mutualistic associations with vertically-transmitted endophytic fungi that improve their fitness. However, the endophyte may be related to livestock toxicosis and hence ageing seeds has been used to remove the fungus since it loses viability before the seeds. The effect of endophyte on seed quality has rarely been studied. We conducted an experiment to study the relationship between the endophyte *Neotyphodium occultans* and seed germination characteristics in *Lolium multiflorum*. Infected and non-infected seeds were stored under six different conditions and seed germination rate and viability were monitored over two years. Seed water content was evaluated and the ageing-time model was used to describe the dynamics of seed germination in relation to storage period. The model provided a good description of the seed germination, and parameters appear to work better when seed viability is affected. The endophyte not only affected negatively the seed viability but also the germination rate. Although the endophyte modified the water content, this effect was not associated with the negative effect on viability. Since non-toxic endophytes are being selected to improve forage grasses, further research should incorporate the endophyte viability dynamics in the population-based model to better predict both seed quality decay and endophyte losses.

Introduction

Neotyphodium endophytic fungi are mutualistic microorganisms of many cool-season forage grasses, producing an asymptomatic and systemic infection. These endophytes grow in the green tissues of the host plants and are only transmitted by the seeds (Clay and Schardl, 2002). The association is obligate for the endophyte, and facultative for the plant (Clay and Schardl, 2002). The endophyte obtains nutrients and energy from the host, while plants benefit from the endophyte-produced alkaloids by means of which resistance to invertebrate and vertebrate herbivores is obtained (Clay and Schardl, 2002; Easton, 2007). Additionally, endophyte-infected plants are often found to be more tolerant to biotic (diseases) and abiotic (heavy metals, drought, herbicides) stresses than non-infected ones (Malinowski and Belesky, 2000; Gundel *et al*., 2006). Nonetheless, depending on the alkaloid profile, some particular grass-endophyte combinations such as *Schedonorus phoenix* (=*Festuca arundinacea*) / *N. coenophialum* and *Lolium perenne*

/ *N. lolii*, can be highly toxic to livestock. The identification of genetic variability among endophytes has enabled the selection of 'non-toxic' endophytes that have been introduced into commercial forage varieties. These new combinations maintain the benefits of plant growth and survival from endophyte infection but do not cause any harm to the health and productivity of grazing livestock (Bouton *et al*., 2002; Easton, 2007).

Although in the last few years the breeders' perception about endophytes has been changing (Bouton *et al*., 2002; Easton, 2007; Hill and Roach, 2009), *Neotyphodium* endophytes were viewed in the past as an anti-quality factor in forage crops due to their negative effects on livestock grazing. Ageing of seeds has been the most used method to eliminate toxic endophytes from commercial forage varieties, a practice that has been possible since the fungus usually presents higher rates of viability loss relative to seeds (Siegel *et al*., 1984; Rolston *et al*., 1986; Welty *et al*., 1987). It has been found that the presence of *Neotyphodium occultans* endophyte in *Lolium multiflorum* (Moon *et al*., 2000) affects seed water relations and seed viability dynamics under certain storage conditions although the underlying mechanisms are unknown (Gundel *et al*., 2006; Gundel *et al*., 2009, 2010). While the majority of works have focused on the differential rate of viability loss between endophytes and seeds, only a few have evaluated the effects of endophyte on seed ageing rate and seed quality.

A high quality seed lot is characterized by maximum values in the population parameters related to their vital functions. Thus, the highest values of germination rate and seed viability are expected to occur in non-aged seed lots, and changes in these vital functions are expected as ageing advances. For example, seed germination rate decreases before reduction in total seed germination can be detected (Dell'Aquila, 1987; Bradford *et al*., 1993). Seed quality can be affected by environmental conditions during filling, maturity state at harvest, time of after-ripening and storage conditions (Argerich *et al*., 1989; Pieta Filho and Ellis, 1991; Sinniah *et al*., 1998), and will change with population genotype (TeKrony and Hunter, 1995; Walters, 1998). In this paper, we explored the endophyte infection effects on two important aspects of seed quality: germination rate and seed viability, under several storage conditions that resulted from the combination of two constant temperatures and three relative humidity levels. We hypothesized that the negative effect of endophyte on seed viability previously observed (Gundel *et al*., 2009, 2010) translates to a higher impact on rate of germination, as the former is a more sensitive process than the latter (Dell'Aquila, 1987; Bradford *et al*., 1993).

Materials and methods

Seed materials

Lolium multiflorum Lam. seeds were hand-collected at the end of the growing season (late spring – early summer) in 2003 from an old-grassland community of the Inland Pampa region (Argentina, 34°06'S, 60°25'W). *Neotyphodium occultans* endophyte infection in this population was higher than 90%. A seed lot was treated with Triadimenol fungicide (Beta-(4-chlorophenoxy)-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol, 150 g a.i. kg^{-1}) to remove the endophyte. In 2004, both seed lots were sown on soil free of

spontaneous *L. multiflorum* seeds in 1 m^2 contiguous plots at the experimental field of the Agronomy Faculty, University of Buenos Aires (Argentina, 34°35'S, 58°35'W). Pollen interchange was allowed in order to prevent genotypic differentiation between plants. The endophyte is only seed transmitted and it is not carried in pollen (Clay and Schardl, 2002). Mature seeds from both lots were hand-harvested and stored for two month in dark glass jars at 10°C until the experiment began. For each seed lot, 100 seeds were imbibed in NaOH (2.5%) for approximately 12 h and then in Rose Bengal solution (5 ml ethanol, 0.5 g Rose Bengal stain, 95 ml distilled water). Seeds were examined using a light microscope for the presence of stained endophytic hyphae. As a result, E+ and E- biotypes showed 90 and 2% endophyte infection, respectively.

Seed storage treatments

Six seed lots (2.2 g each) of each *L. multiflorum* biotype were subjected to one of the six treatments from the combination of two constant temperatures and three levels of relative humidity (RH). Each seed lot was placed inside nylon mesh bags (0.5 mm mesh) which allowed air and humidity interchange with the surrounding atmosphere. One seed bag per biotype was placed in a plastic closed box where the RH was controlled. The three nominal values of RH inside the boxes were achieved by one of the following saturated salt solutions: $ZnCl_2$, $K_2CO_2 1.5H_2O$, and NaCl that generate 5, 43, and 75% RH, respectively. The size of the boxes was of 20 × 20 × 20 cm, with one third of the volume occupied by the saturated salt solution. Inside each box, the salt solution was placed in four open glass jars, and the bags containing the seeds (one per biotype) were located in the remaining space of the box without touching the solution. All the boxes were hermetically closed and, finally three boxes (one per RH) were placed in growth chambers set at one of the two constant experimental temperatures: 20 and 40°C. Temperatures inside chambers were controlled with a thermostat and logged throughout the experiment. The nominal values of the relative humidities were taken from Vertucci and Roos (1993) and Merritt *et al.* (2003).

Seed water content (SWC), which is at equilibrium with the surrounding atmosphere, was measured for each condition at day 12 on three samples of 30 seeds per biotype. It was determined by weighing seeds (fresh weight), drying them in an oven at 130°C for one hour, and then re-weighing (dry weight) (ISTA, 1996). Seed water content (*i.e.*, fresh weight – dry weight) was expressed as percentage (%) fresh weight. Effects of endophyte infection status, temperature, and RH on SWC were analyzed by means of standard ANOVA and differences between biotypes for each condition tested through Tukey-HSD. Analyses were performed with R (R Development Core Team, 2011).

The experiment was maintained for almost two years (729 days) and seed quality tested after 14, 39, 53, 70, 102, 132, 164, 198, 243, 313, 384, 492, 570 and 729 days. On each date three seed samples were taken per biotype from each storage condition. Each sample (20 seeds each) was sown in plastic Petri dishes (9 cm diameter) on a blotter moistened with 5 ml distilled water. Petri dishes were sealed with plastic film to prevent water loss and incubated under optimal conditions for *L. multiflorum* seed germination (15/25°C, 12/12 h) (ISTA, 1996). Seed germination as radicle appearance, was periodically recorded under laboratory fluorescence light until no further germination was observed. Germinated seeds

were removed from the Petri dishes. Non-germinated seeds which were soft and mouldy were considered dead; non-germinated firm seeds were dried at ambient room conditions and then again sown for germination under the same conditions as before, in order to identify dormant seeds. Proportion of germinated seeds (%) per Petri dish (sample) was calculated as total germinated seeds divided by the total number of sown seeds (20 seeds) and multiplied by 100. Seed viability at the beginning of the experiment was 100% for both biotypes. Viable endophyte in seeds was determined by detecting fungal hyphae in 3 week-old seedlings produced by the viable seeds (Belanger, 1996). The first 5 mm of the seedlings were incubated for about 12 h in NaHO solution (2.5%); after that, they were squashed on a slide, stained with Rose Bengal solution and flattened with a cover slip. Conspicuous hyphae of *N. occultans* were observed under light microscope. The number of seedlings depended on the number of viable seeds.

Ageing time model

We used the population-based threshold model developed by Bradford *et al.* (1993) to analyse the effects of ageing on germination rate and viability under different storage conditions. The model uses the same principles of the hydrotime model widely used to describe the relationships between seed germination and seed water relationships (Gummerson, 1986; Bradford, 1990). Similar to the hydrotime model, the ageing-time model assumes that time to germination is inversely proportional to the difference between a given quantitative factor (here ageing period), and a threshold or base level of that factor. It is assumed that there is a maximum potential lifetime (ρ_{max}) for each seed that is normally distributed. Thus, the maximum potential lifetime vary among different germination fractions or percentage ($\rho_{max}(g)$). The "ageing time constant" (θ_{age}) is defined by the equation:

$$\theta_{age} = [\rho - \rho_{max}(g)] \times t_g \qquad \text{Eq. 1}$$

where ρ is the accumulated ageing period and t_g is the time to germination of a given percentage (g).

The procedure to obtain the population parameters are well described in Bradford *et al.* (1993). Shortly, repeated probit regressions relating probit (g) against [$\rho - (\theta_{age}/t_g)$] were conducted, varying the value of θ_{age} until the best fit is obtained [*i.e.*, when the coefficient of determination (R^2) is the highest]. The procedure was conducted for each storage condition, and biotypes were compared through a test for differences between linear regressions. From these regressions, $\rho_{max}(50)$ is obtained when probit(g) = 0, and $\sigma_{\rho max}$ is derived as 1/slope (Bradford, 1990). Since maximum likelihood regression gives the data near 50% greater weight, the extreme data were removed from the regression analysis (Bradford *et al.*, 1993). Finally, using the derived parameters, seed germination time-course for each storage-time under each storage condition was obtained by running the following equation:

$$Probit(g) = [\rho - (\theta_{age}/t_g) - \rho_{max}(50)] \div \sigma_{\rho_{max}} \qquad \text{Eq. 2}$$

The adequacy of the models was checked by plotting actual versus predicted values for each time in the germination time-courses. Specifically, for each storage condition the actual germination (%) values were plotted against their respective predicted or modelled germination values.

Results

Triple-interaction between endophyte infection status, temperature and RH on seed water content (SWC) was non-significant (P = 0.893); however, infection status interacted marginally with the effect of RH (P = 0.076) and temperature (P = 0.027; table 1). SWC was higher in E+ seeds than in E- seeds in the driest atmosphere (5% RH; P = 0.046 for 20°C and P = 0.085 for 40°C), while no significant differences were observed in the wetter atmospheres (table 1).

Table 1. Seed water content (%) achieved by each *Lolium multiflorum* biotype (E- and E+) stored at different constant conditions of temperature and relatives humidity. Values are average of three samples (30 seeds each), and standard error of mean between brackets for each treatment. Tukey-HSD compares SWC between biotypes for each treatment (temperature × relative humidity).

Temperature (°C)	Relative humidity (%)	Biotype		Tukey-HSD (P)
		E-	E+	
20	5	4.3 (0.08)	8.5 (1.70)	0.046
	43	15.2 (0.19)	15.6 (0.71)	0.999
	75	13.9 (0.59)	14.5 (0.42)	0.999
40	5	4.5 (1.09)	8.4 (0.48)	0.085
	43	10.4 (1.06)	11.5 (0.81)	0.997
	75	12.8 (0.87)	13.6 (0.24)	0.999

The comparison of slopes of the linear regressions relating probit (g) against [$\rho - (\theta_{age}/t_g)$] indicated differences between E- and E+ in all the conditions except for 40°C in combination with 43% RH (table 2). Maximum difference between slopes was found at 20°C and 40°C in combination with 75% RH. R^2 values were in general higher for modelling germination dynamics when seeds were stored at 40°C, with an overall tendency to fit better for those seeds whose viability fell during the experimental period (except for 20°C and 75% RH). Conversely, lower values of R^2 were observed in those conditions in which the seeds remained viable during the whole storage period and the models were relatively bad descriptors of the seed germination dynamics (table 2; figures 1 and 2). Similarly, the parameters of the models were clearly related with RH at 40°C but not at 20°C; seed longevity was higher at lower RH and this pattern was clearly related to an increase in the θ_{age} parameter and in $P_{max}(50)$, and a decrease in σ_{pmax} (table 2).

The previous pattern of association between parameters and the effects of storage conditions on seed longevity was observed as differences between E+ and E- seeds (table 2). In general, ageing symptoms in E+ seeds were higher than in E- seeds (figure 2), and

Table 2. Parameters from the ageing-time models that describe the germination dynamics in *Lolium multiflorum* seeds non-infected (E-) and infected (E+) with the endophyte fungus *Neotyphodium occultans* incubated at 15/25°C that were stored at 20 and 40°C of constant temperature and 3 relative humidities for 729 days. The comparison of models is performed by testing for slopes difference between the linear regressions relating probit (g) against $[\rho - (\theta_{age}/t_g)]$ for E- and E+ under each storage condition.

Endophyte infection status	Seed storage treatment		Models comparison (slopes test)	Model parameters and adequacy			
	Temperature (°C)	Relative humidity (%)		θ_{age} (h·h)	$P_{max}(50)$ (h)	σ_{pmax} (h)	R^2
E-	20	5	$P = 0.016$	-41230	-883.55	454.55	0.63
E+	20	5		-52520	-1055.68	526.32	0.62
E-	20	43	$P = 0.069$	-89400	-1597.17	833.33	0.55
E+	20	43		-72440	-1457.40	666.67	0.54
E-	20	75	$P = 0.005$	-44800	-862.54	357.14	0.78
E+	20	75		-31890	-695.31	312.50	0.75
E-	40	5	$P = 0.029$	-92100	-1614.92	769.23	0.68
E+	40	5		-82000	-1373.06	625.00	0.69
E-	40	43	$P = 0.297$	-21400	-429.15	153.85	0.84
E+	40	43		-20000	-412.61	144.93	0.82
E-	40	75	$P < 0.001$	-6380	-138.59	52.08	0.77
E+	40	75		-3028	-84.60	29.94	0.86

resulted in rapid loss of germination rate and total seed viability in the worst environments (e.g. 40°C and 43 and 75% RH). Although there was not a clear pattern between E- and E+ seeds at 20°C, parameters from the model suggested a higher rate of ageing for E+ seeds relative to E- seeds (table 2).

Endophyte viability was completely lost under high temperature and humidity (table 3). At the highest RH (75%), no viable endophyte was detected after 53 and 313 days of storage at 40°C and 20°C, respectively (table 3). However, at 43% RH endophyte viability was lost after 102 days of storage at 40°C but approximately 50% of viable endophyte was maintained even until the end of the experiment at 20°C (table 3).

Discussion

Our experiment showed that the infection of cool-season grasses with *Neotyphodium* endophytes may affect the ageing rate of host seeds. These results are in accordance with our previous studies (Gundel *et al*., 2009, 2010) indicating that seed viability may be negatively affected by the endophyte infection. Nonetheless, effects may vary depending on the prevailing environmental conditions and also on the fungus and plant genotype (Hill and Roach, 2009; Kirkby *et al*., 2011).

Table 3. Proportion of endophyte viability in *Lolium multiflorum* seedlings (E+) in relation to the days of storage at 20 and 40°C of constant temperature and 3 relative humidities for 729 days. Viable endophyte was estimated on 3-week old seedlings (the number of evaluated seedlings from each treatment and for each storage time is between parentheses).

Storage time (d)	20°C			40°C		
	5% RH	43% RH	75% RH	5% RH	43% RH	75% RH
15	0.88 (54)	0.96 (58)	0.96 (50)	0.91 (58)	0.98 (53)	0.84 (58)
39	0.85 (57)	0.98 (57)	0.96 (58)	0.96 (57)	0.83 (59)	0.31 (58)
53	0.92 (55)	0.87 (55)	0.91 (56)	0.91 (56)	0.68 (57)	0.02 (45)
70	0.89 (58)	0.89 (57)	0.90 (55)	0.94 (54)	0.52 (57)	0.00 (39)
102	0.94 (55)	0.85 (55)	0.90 (55)	0.83 (54)	0.00 (52)	0.00 (6)
132	0.88 (52)	0.62 (48)	0.89 (56)	0.81 (55)	0.00 (57)	0.00 (0)
164	0.58 (51)	0.68 (57)	0.59 (52)	0.62 (56)	0.00 (56)	0.00 (0)
198	0.83 (49)	0.81 (54)	0.79 (53)	0.88 (43)	0.00 (35)	0.00 (0)
243	0.78 (50)	0.69 (56)	0.26 (52)	0.58 (53)	0.00 (34)	0.00 (0)
313	0.78 (42)	0.75 (40)	0.10 (50)	0.95 (43)	0.00 (3)	0.00 (0)
384	0.89 (49)	0.96 (52)	0.00 (49)	0.94 (52)	0.00 (0)	0.00 (0)
492	0.90 (55)	0.84 (45)	0.00 (35)	0.74 (51)	0.00 (0)	0.00 (0)
570	0.95 (41)	0.51 (52)	0.00 (12)	0.82 (36)	0.00 (0)	0.00 (0)
729	0.94 (38)	0.51 (52)	0.00 (18)	0.76 (37)	0.00 (0)	0.00 (0)

Depending on the conditions of storage, endophyte presence may change both the total seed viability and seed germination rate. In accordance with previous results, we found a negative effect of endophyte on seed viability. As we suspected, the same negative effect can be more important when analysing a more sensitive process such as seed germination rate. It is interesting to note that under highly deteriorative conditions, the negative effect of the endophyte on the germination rate of seeds was evidenced even after no viable endophyte remained. Seed ageing rate during storage depends on temperature and humidity (Bradford *et al*., 1993; Walters, 1998; Bailly, 2004), which determine seed water content. Seed deterioration is characterized by an increasing impact of free-radicals that cause oxidative damage in cell structures and seed functioning; nonetheless, seeds have their own anti-oxidant machinery to deal with the oxidative stress (Bailly, 2004; Seal *et al*., 2010). *Neotyphodium* endophytes may affect seed ageing rate by changing the seed water dynamics (Gundel *et al*., 2009, 2010) and by contributing to the anti-oxidant mechanisms in host plants (Zhang and Nan, 2007, 2010; White and Torres, 2010). In our experiment, there were no differences in SWC between E- and E+ seeds under the most deteriorative conditions (those with high RH) while a clear effect of endophyte increasing SWC was evident under drier atmospheres. A high concentration of *Neotyphodium* hyphae is found close to the embryo (Siegel *et al*., 1984) and could change seed humidity locally affecting the embryo environment and hence the ageing rate of the seed. This humid environment

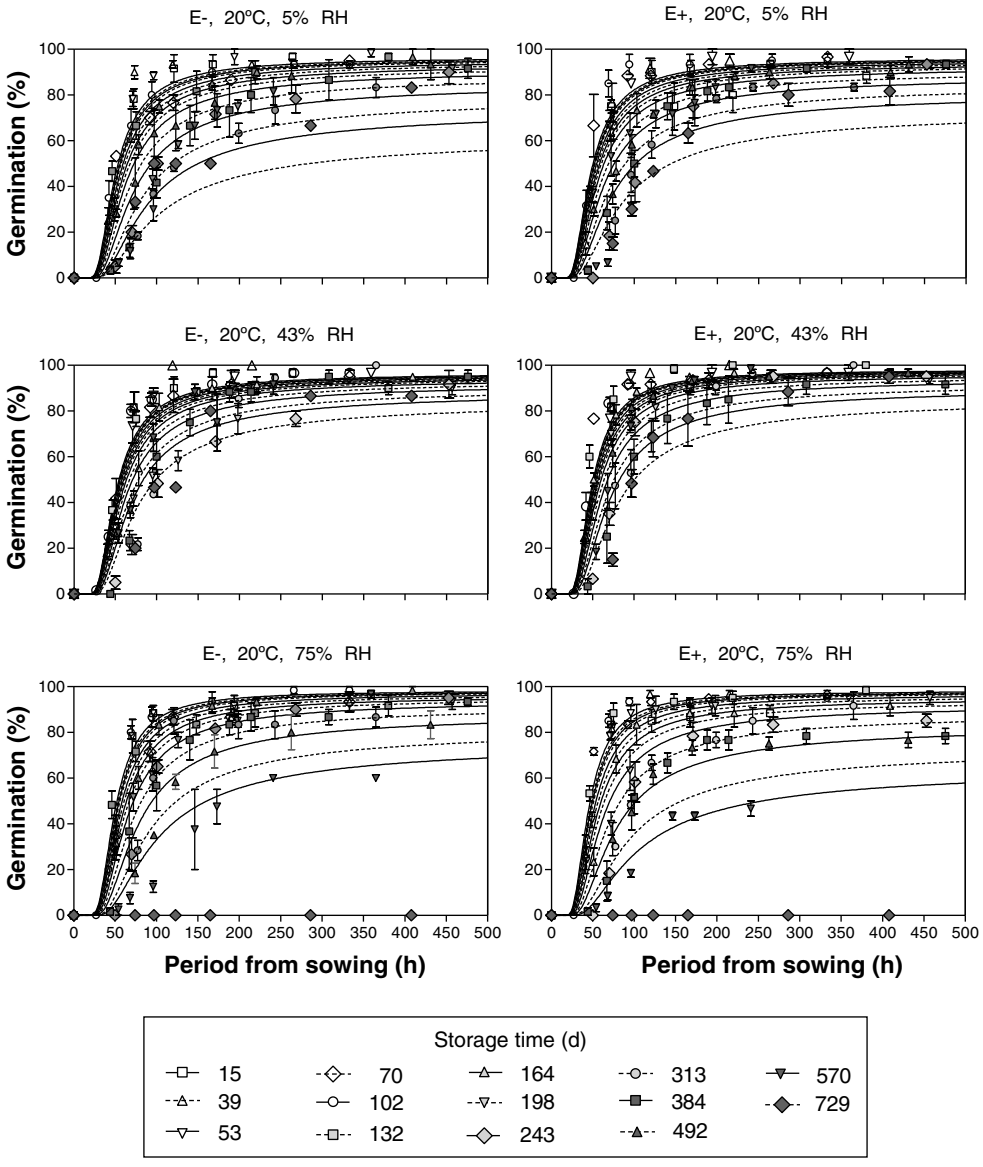

Figure 1. Germination dynamics (%) of *Lolium multiflorum* seeds infected (E+: right panel) and non-infected (E-: left panel) with the endophyte fungus *Neotyphodium occultans* incubated at 15/25°C (cycling every 12 h) that were stored at 20°C of constant temperature and 3 relative humidities (5%: high panel, 43%: medium panel, and 75%: lower panel) for 14, 39, 53, 70, 102, 132, 164, 198, 243, 313, 384, 492, 570, and 729 days (listed below). The symbols represent actual data ± SEM, and the lines correspond to the modeled dynamics of seed germination in relation to the period from sowing for the different storage times.

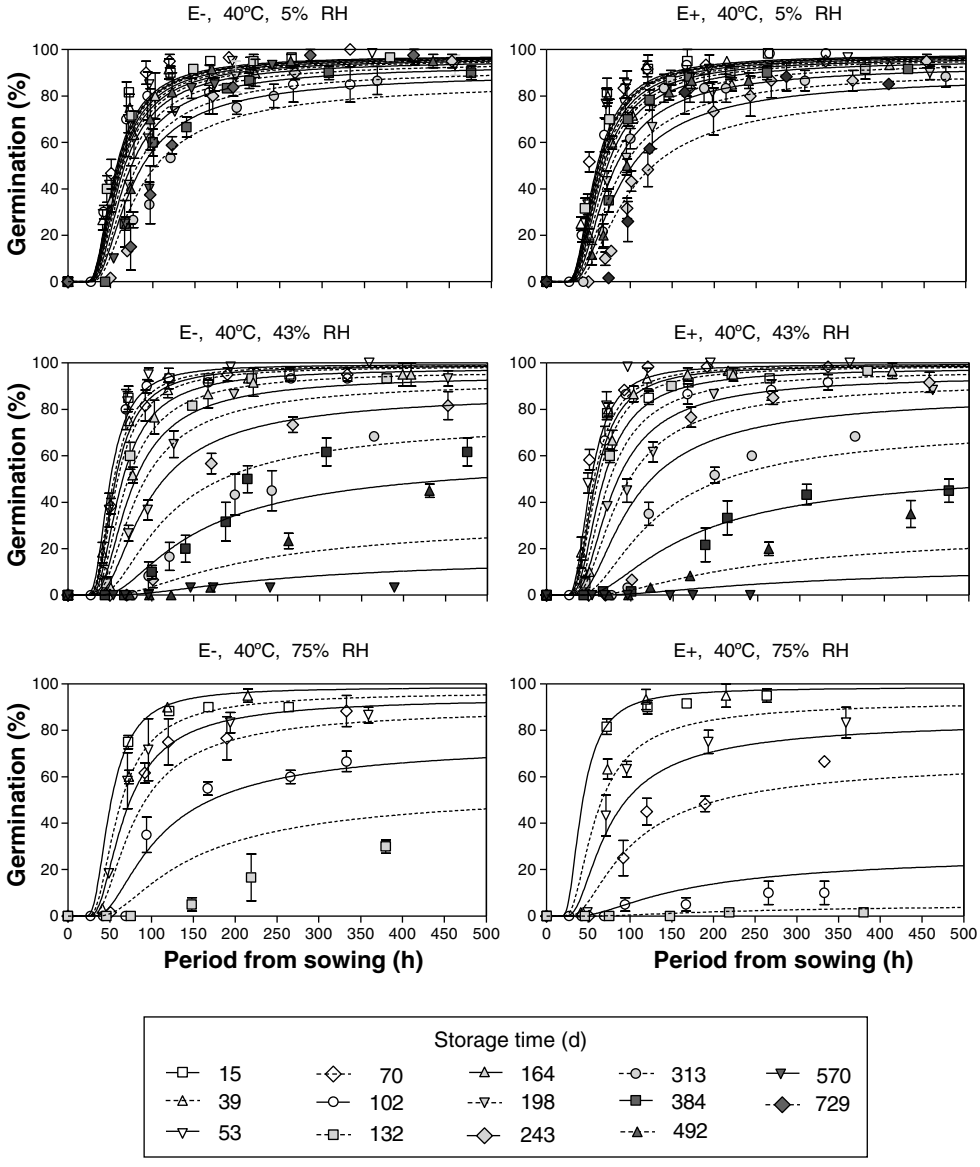

Figure 2. Germination dynamics (%) of *Lolium multiflorum* seeds infected (E+: right panel) and non-infected (E-: left panel) with the endophyte fungus *Neotyphodium occultans* incubated at 15/25°C (cycling every 12 h) that were stored at 40°C of constant temperature and 3 relative humidities (5%: high panel, 43%: medium panel, and 75%: lower panel) for 14, 39, 53, 70, 102, 132, 164, 198, 243, 313, 384, 492, 570, and 729 days (listed below). The symbols represent actual data ± SEM, and the lines correspond to the modeled dynamics of seed germination in relation to the period from sowing for the different storage times.

could increase ageing despite the endophyte enhancement of the anti-oxidant mechanisms and explain why, in our experiment, the symbiosis with *Neotyphodium* endophyte fungus did not improve seed quality in *Lolium multiflorum* under deteriorative conditions.

Endophyte infection frequency in seed lots commonly ranges between 0 and 100%, but hardly ever takes the absolute 0 or 100% (Rolston *et al*., 1986; Welty *et al*., 1987; Wheatley *et al*., 2007; Clement *et al*., 2008; Hill and Roach, 2009; Gundel *et al*., 2009, 2010; Kirkby *et al*., 2011). Changes in endophyte infection frequency can be accounted for by either the differential death rate of endophytes and host seeds, or the differential death rate of infected and non-infected seeds (Gundel *et al*., 2009, 2010). Changes in infection frequency caused by differential death between endophyte and seeds are well known (Rolston *et al*., 1986; Welty *et al*., 1987; Kirkby *et al*., 2011). These studies show that under many storage conditions, endophyte dies at higher rates than seeds of *Festuca arundinacea*, *Lolium perenne*, *L. multiflorum* and *L. rigidum*, and the difference increases with temperature and RH (Rolston *et al*., 1986; Welty *et al*., 1987; Gundel *et al*., 2009, 2010; Kirkby *et al*., 2011). Alternatively, there is almost no information about the effect of endophyte infection on seed longevity, a process that at least in *L. multiflorum* has been found to affect seed ageing and the dynamics of endophyte infection frequency in seed lots (Gundel *et al*., 2009, 2010).

The present results show that the sensitivity of ageing seed lots to the presence of the endophyte is important to predict the maintenance of seed quality under storage in forage seeds. The ageing time model and their parametrisation for specific seed commercial varieties stored under controlled environmental conditions, including endophyte viability dynamics could be useful for the seed industry. This is particularly important since non-toxic or non-ergot-producing endophytes that are being introduced into the forage commercial varieties are considered now a quality factor in the international seed industry (Bouton *et al*., 2002; Easton, 2007; Hill and Roach, 2009).

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