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RESEARCH ARTICLE

Dynamics of *Neotyphodium* endophyte infection in ageing seed pools: incidence of differential viability loss of endophyte, infected seed and non-infected seed

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Abstract

Symbiotic associations between grasses and vertically transmitted endophytic fungi are widespread in nature. Within grass populations, changes in the frequency of infected plants are driven by influence of the endophyte on the fitness of their hosts and by the efficiency of endophyte transmission from parent plants to their offspring. During the seed stage, the endophyte might influence the fitness of its host by affecting the rate of seed viability loss, whereas the efficiency of endophyte transmission is affected by losses of viability of the fungus within viable seeds. We assessed the viability losses of *Lolium multiflorum* seeds with high and low level of infection of the endophyte *Neotyphodium occultans*, as well as the loss of viability of the fungus itself, under accelerated seed ageing and under field conditions. Starting with high endophyte-infected accessions of *L. multiflorum*, we produced their low endophyte-infected counterparts by treating seeds with a fungicide, and subsequently multiplying seeds in adjacent plots allowing pollen exchange. In our accelerated ageing experiments, which included three accessions, high endophyte-infected seeds lost viability significantly faster than their low endophyte-infected counterpart, for only one accession. High endophyte-infected seeds of this particular accession absorbed more water than low endophyte-infected seeds. In contrast, the endophyte lost viability within live seeds of all three accessions, as the proportions of viable seeds producing infected seedlings decreased over time. In our field experiment, which included only one accession, high endophyte-infected seed lost viability significantly but only slightly faster than low endophyte-infected seed. In contrast, the loss of viability of the endophyte was substantial as the proportions of viable seeds producing infected seedlings decreased greatly over time. Moving the seeds from the air to the soil surface (simulating seed dispersion off the spikes) decreased substantially the rate of seed viability loss, but increased the rate of endophyte viability loss. Our experiments suggest that, in ageing seed pools, endophyte viability loss and differential seed mortality determine decreases in the proportions of endophyte-infected seeds in *L. multiflorum*. Endophyte viability loss within live seeds contributes substantially more to infection frequency changes than differential viability losses of infected and non-infected seeds.

Introduction

Many cool-season grasses establish symbiotic relationships with endophytic fungi of the genus *Neotyphodium* (Saikkonen *et al.*, 1998; Clay & Schardl, 2002). These fungi grow systemically consuming photosynthates in the aerial parts of the plants and, when their hosts flower, the endophytes infect developing ovaries and persist in the seeds (Siegel *et al.*, 1984; do Valle Ribeiro, 1993). The endophytes often induce morphological, physiological and biochemical changes in their hosts, which may affect the performance of plants or seeds under different abiotic or biotic stresses such as drought, soil salinity, high soil metal concentration, herbicides and herbivore or predator attack (Madej & Clay, 1991; Clay, 1993; Saikkonen *et al.*, 1998; Malinowski & Belesky, 2000; Popay *et al.*, 2000; Clay & Schardl, 2002; Faeth, 2002; Omacini *et al.*, 2009). Depending on the ecological context, the endophytes may increase, not affect, or decrease the fitness of their hosts (Faeth, 2002; Cheplick, 2004; Faeth & Hamilton, 2006). Transmission of the *Neotyphodium* endophytes occurs exclusively from parent plants to offspring (Siegel *et al.*, 1984; do Valle Ribeiro, 1993). Failures of transmission result from either the endophyte not growing hyphae into all ovaries of an infected plant or from the death of the endophyte within mature seeds (Rolston *et al.*, 1986; Welty *et al.*, 1987; Ravel *et al.*, 1997). In each grass population, changes in the endophyte infection frequency are driven by the relative fitness of infected and non-infected individuals and by the efficiency of endophyte transmission between parent plants and their offspring (Clay, 1993; Ravel *et al.*, 1997; Gundel *et al.*, 2008).

Similarly, during the seed stage of the grasses, the two drivers of infection frequency change are respectively determined by differential survival of infected and non-infected seeds (i.e. relative fitness) and by the rate of endophyte mortality within viable seeds (i.e. transmission efficiency) (Gundel *et al.*, 2008). Differential survival of infected seeds may result from deterrence of some seed predators by endophyte-produced alkaloids (Madej & Clay, 1991; Popay *et al.*, 2000; Uchitel *et al.*, 2006). In addition, differential seed survival might result from endophyte effects on the process of seed ageing, an influence hitherto little explored (Hume & Barker, 2005; Gundel *et al.*, 2006*a*, 2007). Because fungal mycelium present in the seeds could modulate the distribution and dynamics of their water content, rates of viability loss of infected seeds might be different from those of non-infected seeds (Roberts & Ellis, 1989; Baskin & Baskin, 1998; Gundel *et al.*, 2007). Endophyte death within viable seeds has been well documented by studies showing that, under storage conditions, the viability of endophyte mycelium usually decays faster than the viability of host seeds (Siegel *et al.*, 1984; Rolston *et al.*, 1986; Welty *et al.*, 1987; do Valle Ribeiro, 1993; Wheatley *et al.*, 2007). In contrast, for seeds buried in the soil, endophyte and seeds have been found to have similar survival rates (Hume & Barker, 2005; Canals *et al.*, 2008). However, seed and endophyte survival have not been assessed for seeds lying on the soil surface, the most usual location of grassland seeds after dispersion (Ghersa & Martínez-Ghersa, 2000).

In this article, we present results from a set of experiments aimed at assessing the effects of *Neotyphodium occultans* endophytes on the rates of viability loss of *Lolium multiflorum* seeds as well as the dynamics of viability loss of endophytes. We assessed these dynamics under laboratory (i.e. accelerated ageing) and natural field conditions. To our knowledge, this is the first study in which the effect of endophyte infection on host seed viability as a driver of endophyte infection frequency in a seed pool is compared to the well-known endophyte mortality in infected host seeds.

Materials and methods

Study model

Lolium multiflorum Lam. is an annual cool-season grass which establishes mutualistic symbiotic associations with the endophytic fungus *N. occultans* (Moon *et al.*, 2000; Christensen *et al.*, 2002). Frequencies of *N. occultans*-infected individuals in naturalised *L. multiflorum* populations from Pampean grasslands of Argentina are often higher than 0.90 (De Battista, 2005; Gundel *et al.*, 2009). Infected and non-infected seeds have been found to differ in their requirements for germination (Vila-Aiub *et al.*, 2005; Gundel *et al.*, 2006*a*,*b*), and may differ in longevity (Gundel *et al.*, 2007). Loss of endophyte viability in stored *L. multiflorum* seeds has been reported (Medvescigh *et al.*, 2004), but never examined under natural field conditions.

Plant material

We used seeds from three wild *L. multiflorum* accessions (Traditional, Lucero and Picaflor) collected from different old-field grassland communities at the Inland Pampa region (Carlos Casares, Argentina). Traditional and Lucero accessions were collected in 1998 and 2003, respectively from a plot (>2 ha) that was abandoned to agricultural activities since 1989 (35°55′22″S, 61°09′35″W, 87 m). Instead, accession Picaflor was collected from another plot (>2 ha) that was abandoned to livestock production activities for more than 20 years (35°55′12″S, 61°09′29″W, 86 m). Seeds were obtained from many mature plants that were hand-harvested, and each collection yielded approximately 500 g of seed. The three accessions presented a high

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endophyte infection level (≈90%, based on 100 evaluated seeds). For each one, a low endophyte-infected level counterpart was obtained by treating half of the seed with a systemic fungicide during 1 h [Triadimenol, β-(4-chlorophenoxy)-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol, 150 g a.i. kg^{-1}, dose rate: 5 mg g^{-1} seed]. Fungicide treatments have been useful to kill the fungus without evidence of toxic effects (Faeth & Hamilton, 2006). The resulting pools of high endophyte-infected and low endophyte-infected seeds were maintained and multiplied by annually cultivating them at the experimental field of the Faculty of Agronomy, University of Buenos Aires (34°35′27S, 58°28′49″W, 20 m), on adjacent plots free from wild *L. multiflorum* but allowing pollen exchange between them in order to prevent genetic segregation.

All seeds used for the experiments were harvested at the end of the 2004 growing season, corresponding to the first (Lucero and Picaflor) and the sixth generation after the fungicide treatment (Table 1). Although Traditional and Lucero were collected from the same site, we considered them as different accessions. Unlike to Lucero accession, Traditional accession was cultivated for six generations in the experimental field of the Faculty of Agronomy, which differed from the original site ecological conditions. Seeds were hand-harvested from dry plants in the field, cleaned, and stored in glass jars at room temperature for about 3 months. Before the experiments, the proportion of endophyte-infected seeds in each seed pool was estimated by examining 100 randomly selected seeds under a light microscope and recording the presence or absence of endophyte mycelia in each seed. For the examinations, seeds were incubated (≈12 h) in sodium hydroxide (2.5%) and subsequently stained with Rose Bengal stain (0.5% in 95% ethanol; Bacon & White, 1994).

Table 1 Endophyte infection level (%) for fungicide-untreated (labelled as high endophyte-infected) and fungicide-treated (labelled as low endophyte-infected) *L. multiflorum* seed pools of the Traditional, Lucero and Picaflor accessions[a]

Accession	Fungicide	Endophyte Infection (%)	Infection Label
Traditional	Untreated	89	High
	Treated	27	Low
Lucero	Untreated	90	High
	Treated	2	Low
Picaflor	Untreated	86	High
	Treated	4	Low

[a]The percentage of endophyte infection was calculated by inspecting 100 seeds under a light microscope.

Experimental procedures

Accelerated ageing experiments

We carried out two separate experiments, one aimed at assessing the effects of endophyte infection on seed viability and its dynamics (*seed viability experiment*), and the other one aimed at evaluating the dynamics of endophyte viability within infected seeds (*endophyte viability experiment*). For these experiments, we exposed seeds to a controlled deteriorative condition, consisting of high constant temperature and high seed water content (SWC) (i.e. accelerated ageing), and we periodically measured the proportion of viable seeds and the proportion of seeds infected with viable endophyte (cf. Roberts & Ellis, 1989; McDonald, 1998). Although the results from standard laboratory experiments may fail in predicting seed performance in the field (McDonald, 1998), an association between seed performance under accelerated ageing experiments and seed performance under field conditions has been found for *L. multiflorum* (Marshall & Naylor, 1985).

To increase the water content of seeds, we placed them in humidification devices for 6 h at 10°C. The humidification devices were transparent plastic boxes 21 cm long, 15 cm wide and 5 cm tall. Within each box, there was a 1-cm deep water layer and a plastic net (0.1 cm mesh) located 1 cm above it. Seeds were homogeneously distributed on the plastic net, and the boxes were closed at the top. After humidification of each pool, we determined SWC by weighing a sample of 0.2 g (≈100 seeds), drying it at 130°C for an hour and reweighing (ISTA, 1999). We expressed SWC in percentage (%) of fresh weight as: g H_2O 100 g^{-1} fresh weight.

For the *seed viability experiment*, we took three seed lots weighting 2.5 g each from each of our six seed pools (Traditional, Lucero and Picaflor accessions either high endophyte-infected or low endophyte-infected), we subjected them to the humidification treatment, placed each lot in a separate 250 cm^3 hermetically closed dark glass jar and incubated them in a chamber at 40°C. Temperature inside the chamber was controlled with a thermostat and logged throughout the experiment, and cycled over a maximum range of ±2°C around the nominal value. Every 5 days we measured seed viability on a sample of 30 seeds per glass jar (see section on *Seed viability measurement* below). SWC after humidification was compared between high endophyte-infected and low endophyte-infected seed of each accession by means of *t*-tests. We modelled the changes in the proportions of viable seeds over accelerated ageing time by logistic regression (Agresti, 2002). In the models, time, seed pool infection status (coded as 0 for low endophyte-infected and 1 for high endophyte-infected) and the product of

these two variables were used as predictor variables; seed viability was treated as a binary response variable (viable or not-viable), and a binomial error structure with logit link function was assumed (Agresti, 2002). The analysis was performed separately for each accession to test for differences between high endophyte-infected and low endophyte-infected seed in the patterns of change in seed viability. Calculations were performed with SAS Proc Logistic accommodating for overdispersion by the Williams's method (SAS Institute, 1990).

For the *endophyte viability experiment*, we took three seed lots of 2.5 g each from each of the high endophyte-infected seed pools of the Traditional and Lucero accessions, and two seed lots of 2.5 g each from the high endophyte-infected seed pool of the Picaflor accession, and we subjected them to accelerated ageing in glass jars as we did for *seed viability experiment*. At periods of about 10 days, we measured seed viability in each jar (see section on *Seed viability measurement* below) and assessed the proportion of viable seeds that produced endophyte-infected seedlings based on samples of 30 seeds per accession (see section on *Endophyte viability measurement* below). Differences in SWC after the humidification treatment among accessions were tested by one-way analysis of variance (ANOVA). Based on the seed and endophyte viability data, we constructed separate multinomial logit models (Agresti, 2002) to examine the changes in the partition of each seed pool in the following categories: endophyte-infected viable seed, non-infected viable seed, viable seed with unknown infection status and not-viable seed. We used SAS Proc Catmod to fit these models (SAS Institute, 1990).

Field experiment

We assessed the viability changes of high endophyte-infected and low endophyte-infected seeds and of the endophyte itself under field conditions. The experiment was installed on 1 April 2005. The experimental procedure mimicked the sequence of environmental conditions to which seeds are exposed prior to and following dispersion from the spikes to the surface of the soil. We loaded 108 nylon mesh bags (4 × 6 cm; 0.5 mm mesh) with 30 seeds of either the high endophyte-infected or the low endophyte-infected seed pools of the Lucero accession, and we hung each bag from a wire pole 15 cm above the soil surface. Periodically (≈19 days), we retrieved three bags from each of the high endophyte-infected and low endophyte-infected seed pools (air treatment) and took them to the laboratory for seed and endophyte viability assessment (see sections on *Seed viability measurement* and *Endophyte viability measurement* below). At the same time, we transferred three bags from the high endophyte-infected and three from the low endophyte-infected seeds pools from the wire to the soil surface at a 15 × 15 cm quadrat randomly selected within a grid placed on a 8 m^2 plot. We left these bags on the soil for 10 days and then took them to the laboratory for seed and endophyte viability assessment (air-soil treatment). At each time of seed retrieval from the air treatment, we measured SWC on three independent seed samples of 0.1 g (≈50 seeds each) of another *L. multiflorum* population. Air temperature (°C) and relative humidity (%) were recorded using an automatic meteorological station situated near the experiment (Campbell Scientific, Logan, UT, USA); and records of rainfall events (mm day^{-1}) were obtained from the Argentinean National Meteorological Service (Argentina).

Based on the data from this experiment, we examined the changes in the viability of seeds and of endophyte by means of two separate logistic regression analyses (Agresti, 2002). In the first analysis, seed viability was treated as a binary response variable (viable or not-viable), whereas infection status, treatment (air and air-soil) and time were treated as predictors. In addition, two dummy variables were included in the model for testing for differences in the rate of viability change between high endophyte-infected and low endophyte-infected seeds and between treatments (time × infection and time × location). In the second analysis, endophyte viability within live seeds was treated as a binary response (viable or not-viable), while treatment (air and air-soil) and time were treated as predictors. A dummy variable was included for testing for differences in the rate of change in endophyte viability between seeds retrieved directly from the air and seeds placed for 10 days on the soil surface (time × location). Only data from the infected seed pool were included in the second analysis. Because the number of live seeds on which endophyte viability could be checked decreased over time, we could only run this analysis until the day 152. For both analyses, calculations were performed with SAS Proc Logistic (SAS Institute, 1990).

Seed viability measurement. Seed viability was assessed as the proportion of seeds germinating when subjected to optimal conditions for germination of *L. multiflorum*. Seeds were sown in plastic Petri dishes (0.9 cm) on a filter paper moistened with 5 mL of distilled water. Petri dishes were incubated at 15/25°C with a light/dark cycle of 12/12 h (ISTA, 1999). Seed germination was recorded under laboratory fluorescent light until no further germination was observed. Germinated seeds were removed from the Petri dishes upon detection.

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Not-germinated seeds with soft and mouldy aspect were considered non-viable; firm not-germinated seeds were dried at atmospheric condition and re-hydrated to identify eventual dormant seeds.

Endophyte viability measurement. We assessed the viability of the endophyte within live seeds as the proportion of young seedlings originated from seeds from the infected pool that had endophyte mycelia in the leaf sheaths. After seeds were germinated in Petri dishes for seed viability measurement, seedlings were transplanted to plastic speedling trays (30 plugs of 4 cm diameter and 8 cm length) at a rate of one seedling per plug. Plugs of speedling trays were filled with a mixture of black soil, sand and peat moss (50/25/25%), and watered as needed. Speedling trays were placed in a greenhouse at a nominal temperature of 15/25°C (12/12 h) with natural photoperiod. We examined the seedlings after 3 weeks, when they had developed two to four leaves, based on preliminary tests showing that this time was sufficient to detect the endophyte infection at leaves sheath bases. We followed the method proposed by Belanger (1996) with minor modifications: Alkaline Rose Bengal stain (see Bacon & White, 1994) was directly applied on epidermal pieces peeled from leaf sheath base placed on a glass slide. After approximately 10 min, stained endophyte was searched for under a light microscope in the first 1–2 mm of the leaf sheath (see Christensen *et al.*, 2002). The endophyte infecting *L. multiflorum* (i.e. *N. occultans*) has been morphologically well described, allowing us to identify and to distinguish it from other seed fungi without growing them in plate cultures (Moon *et al.*, 2000; Christensen *et al.*, 2002).

Results

Fungicide treated (low endophyte-infected) and untreated (high endophyte-infected) seed pools of the three populations showed contrasting endophyte infection levels (Table 1). Nonetheless, the infection level of low endophyte-infected seeds (fungicide-treated) in the Traditional population was the highest after six generations, possibly as a result of endophyte-infected plant with greater seed yield relative to non-infected plants (Table 1).

Accelerated ageing experiment

For the three accessions, the initial proportions of viable seeds did not differ significantly between high endophyte-infected seeds and their low endophyte-infected counterparts (see 'infection' parameters in Table 2). Accelerated ageing resulted in significant decreases in the proportions of viable seeds of all three accessions both high endophyte-infected and low endophyte-infected (see 'time' parameters in Table 2; Fig. 1). For one of the three accessions (Lucero), high endophyte-infected seeds lost viability at a significantly faster rate than low endophyte-infected seeds. In contrast, for the two remaining accessions (Traditional and Picaflor), the rate of viability loss did not differ significantly between high endophyte-infected and low endophyte-infected seeds (see 'time × infection' parameters in Table 2; Fig. 1). Only seeds from Lucero accession with the natural high endophyte infection were able to absorb significantly more water than their low endophyte-infected counterparts ($P = 0.025$), therefore, beginning the accelerated ageing with higher water content (Table 3, seed viability experiment).

Table 2 Logistic regression analyses of the changes in the proportions of viable seeds over incubation time under accelerated ageing as affected by the infection status of the populations for three *Lolium multiflorum* accessions Traditional, Lucero and Picaflor

Parameter	Degrees of freedom (DF)	Estimate	Chi-square	*P*
Traditional				
Intercept	1	5.5496	56.5709	<0.0001
Infection	1	−0.7328	0.5656	0.4520
Time	1	−0.2595	62.6191	<0.0001
Time × infection	1	0.0178	0.1617	0.6876
Lucero				
Intercept	1	5.1939	62.5919	<0.0001
Infection	1	1.2473	1.1932	0.2747
Time	1	−0.1976	69.1449	<0.0001
Time × infection	1	−0.0967	4.1528	0.0416
Picaflor				
Intercept	1	5.4719	50.6854	<0.0001
Infection	1	−0.8712	0.7507	0.3862
Time	1	−0.2475	56.2163	<0.0001
Time × infection	1	0.0166	0.1379	0.7104

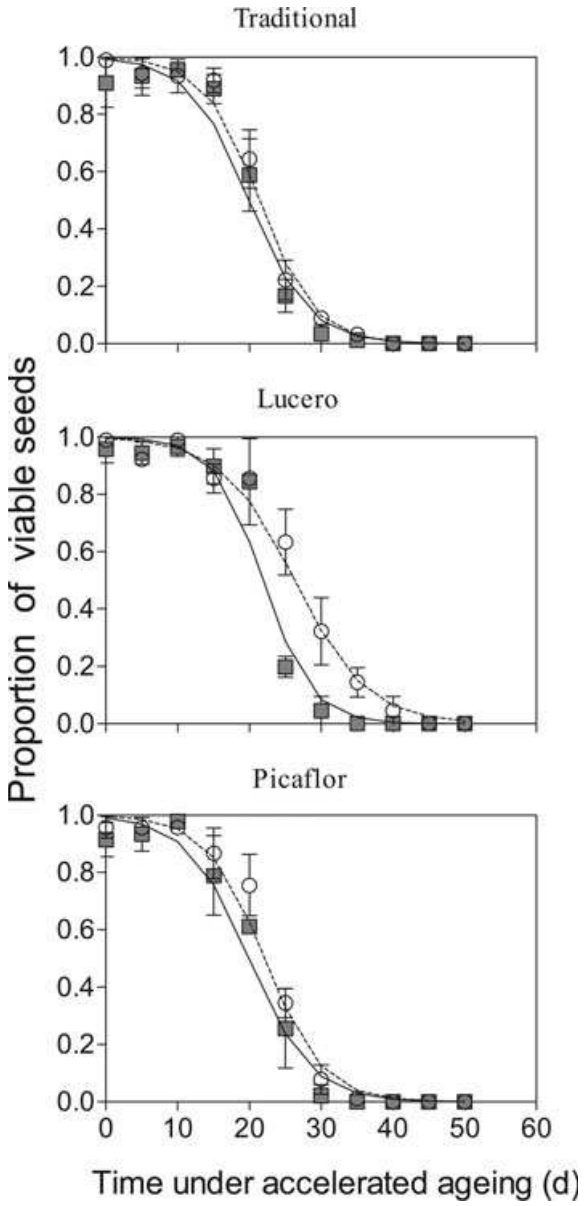

Figure 1 Viability of high endophyte-infected (■) and low endophyte-infected (○) seeds of Traditional, Lucero and Picaflor *L. multiflorum* accessions in relation to the incubation time (days) under accelerated ageing condition. Symbols are the actual data (mean ± SE, $n = 3$), and lines indicate the logistic regression model fitted for each type of seeds.

Seeds entering the accelerated ageing experiment with their natural endophyte infection frequencies exhibited significant changes in the proportions of endophyte-infected viable seed, non-infected viable seed and not-viable seed. The multinomial logit models used to estimate the patterns of change fitted reasonably well to the data (Table 4). For all three accessions, the proportions of not-viable seed increased continuously over time, the proportions of seed producing infected seedlings decreased continuously and the proportions of seed producing non-infected seedlings initially increased, peaked at about the 20 days of storage, and decreased later (Table 4; Fig. 2). Differences in SWC among accessions were not statistically significant ($P = 0.222$; see Table 3).

Table 3 Seed water content (%) measured after the humidification treatment for high endophyte-infected and low endophyte-infected *L. multiflorum* seeds of Traditional, Lucero and Picaflor accessions during *seed viability experiment*, and only for high endophyte-infected *L. multiflorum* seeds of Traditional, Lucero and Picaflor accessions during *endophyte viability experiment*[a]

Accession	Infection Label	SWC after Humidification (%)
Seed viability experiment		
Traditional	High	14.23 (1.17)
	Low	12.95 (0.33)
Lucero	High	14.13 (0.96)
	Low	12.85 (0.12)
Picaflor	High	13.35 (0.30)
	Low	13.07 (0.09)
Endophyte viability experiment		
Traditional	High	13.30 (0.51)
Lucero	High	14.36 (0.47)
Picaflor	High	14.71 (0.74)

SWC, seed water content.
[a]Values are the average and between parentheses (SEs).

Table 4 Maximum likelihood analyses for multinomial models describing the dynamics of the proportions of different seed categories (endophyte-infected viable, non-infected viable, not-viable and viable with unknown infection status) over incubation time (days) under accelerated ageing condition for the three endophyte-infected *L. multiflorum* accessions: Traditional, Lucero and Picaflor

Source	DF	Chi-square	*P*
Traditional			
Intercept	3	148.08	<0.0001
Time	3	108.49	<0.0001
Likelihood ratio	48	38.85	0.8241
Lucero			
Intercept	3	109.52	<0.0001
Time	3	140.89	<0.0001
Likelihood ratio	48	49.24	0.4234
Picaflor			
Intercept	3	104.68	<0.0001
Time	3	104.68	<0.0001
Likelihood ratio	39	46.52	0.1903

Field experiment

Under field conditions, the SWC of the Lucero accession varied over time in approximate correspondence with the changes in air humidity, reaching higher values up

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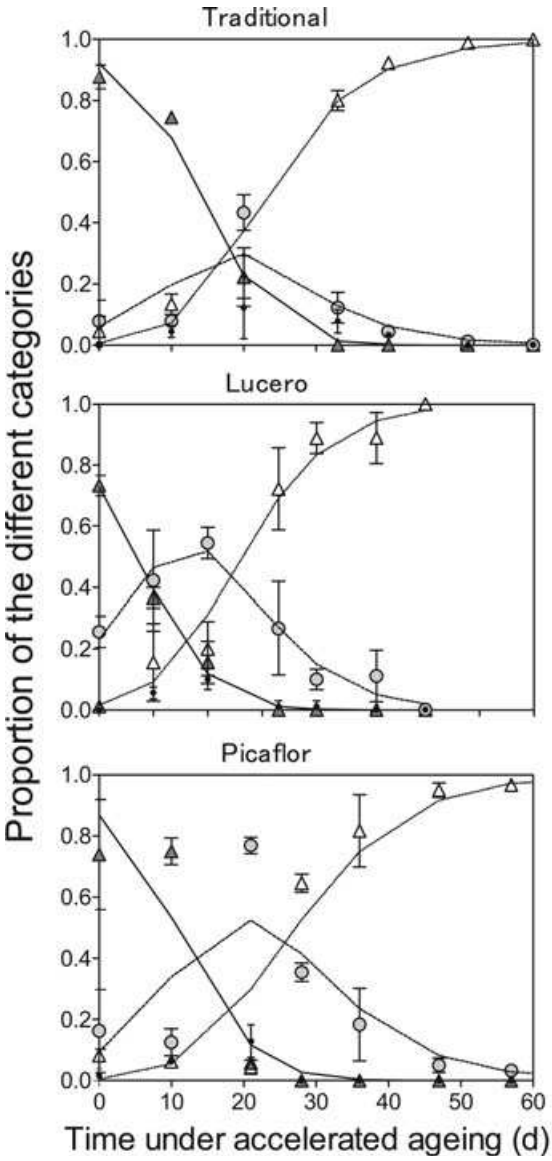

Figure 2 Proportion of different seed categories [endophyte-infected viable (▲), non-infected viable (◯), not-viable (△) and viable with unknown infection status (●)] in relation to incubation time (days) under accelerated ageing condition for the three *L. multiflorum* accessions (Traditional, Lucero and Picaflor). Symbols are the actual data (mean ± SE, $n = 3$), and lines are the modelled proportion dynamics of each category.

to approximately 50% (Fig. 3). These changes were not directly associated with rainfall events (Fig. 3). Along the experiment, the seeds were exposed to temperatures varying between 5°C and 26°C (Fig. 3).

As in the accelerated ageing experiment, the initial proportions of viable seed did not differ significantly between high endophyte-infected and low endophyte-infected seed ('infection' parameter in Table 5); and the

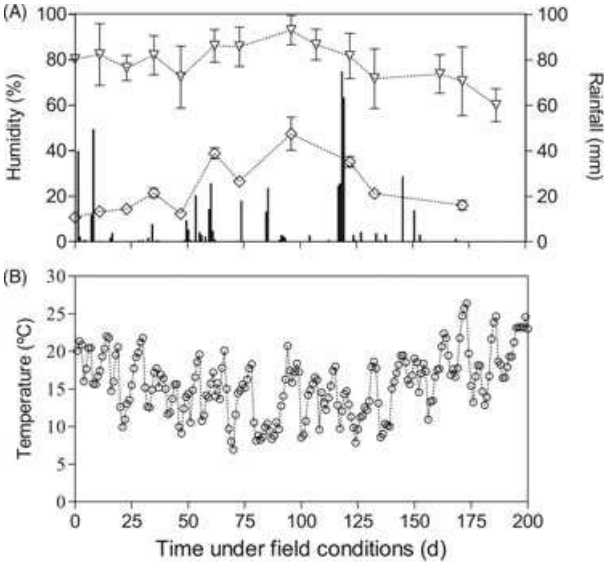

Figure 3 (A) Air relative humidity (▽), rainfall events (dark bars) and seed water content (◇) during the experimental time (days) under field conditions. (B) Dynamics of daily mean temperature (◯) during the experimental time (days) under field condition. Temperature (°C) and relative humidity (%) were recorded using an automatic meteorological station, while rainfall data (mm day^{-1}) were provided by de National Meteorological Service (Argentina). Relative humidity values are means of 10 daily measurements before each seed retrieval time; vertical bars represent temporal standard error over the same periods. Seed water content (%) was estimated on three samples of 50 seeds at each extraction time, and bars are the standard error on these three samples.

Table 5 Logistic regression analysis of the changes in the proportion of viable seeds of the Lucero *L. multiflorum* accession as affected by seed infection status and location in the field experiment

Parameter	DF	Estimate	Chi-square	*P*
Intercept	1	3.6700	401.3848	<0.0001
Infection	1	0.4279	2.4690	0.1161
Time	1	−0.0288	407.4307	<0.0001
Time × infection	1	−0.00470	5.3598	0.0206
Time × location	1	0.00474	33.9020	<0.0001

proportions of viable seed decreased significantly over the duration of the field experiment ('time' parameter in Table 5; Fig. 4, *Seeds*). The rate of viability loss varied significantly with the infection status and location of the seed. High endophyte-infected seed lost viability at a significantly faster rate than the low endophyte-infected seed ('time × infection' parameter in Table 5; Fig. 4, *Seeds*); in addition, seeds moved to the soil lost viability at a slower rate than those kept in the air ('time × location' parameter in Table 5; Fig. 4, *Seeds*).

Seeds entering the field experiment with the natural endophyte infection frequency decreased their proportion

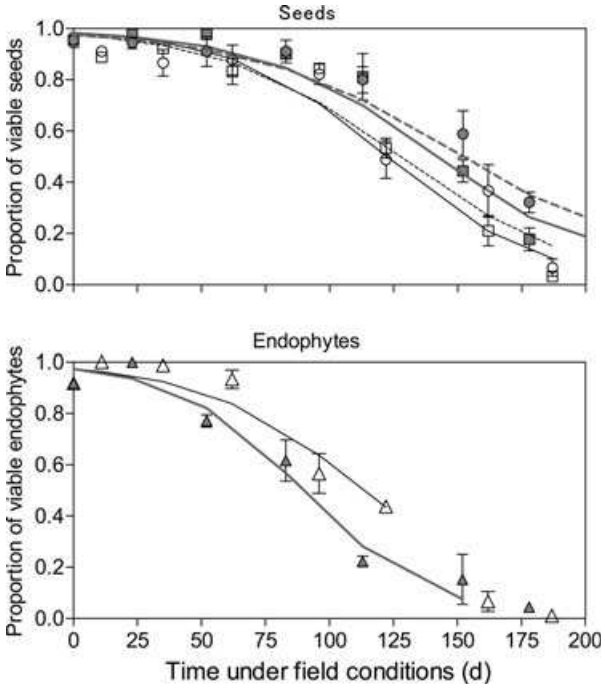

Figure 4 (Seeds) Dynamics of viability proportion of high endophyte-infected (□) and low endophyte-infected (○) seeds of Lucero *L. multiflorum* accession in relation to experimental time (days) under field conditions. Open symbols are air treatment and dark symbols are air-soil treatment. Lines indicate the regression models fitted for viability proportion of each of high endophyte-infected (continuous lines) and low endophyte-infected (dashed lines) seeds at the air (thin lines) and at the air-soil treatments (thick lines). Each symbol is the average of seed viability from three independents bags (30 seed each) and vertical bars are standard errors. (Endophytes) Dynamics of viable endophyte proportion in viable infected seeds from Lucero *L. multiflorum* accession in relation to experimental time (days) under field condition. Open (Δ) and dark triangles (▲) are air and air-soil treatments, respectively. Lines indicate the regression models fitted endophyte viability proportion at air (thin lines) and air-soil (thick lines) treatments. Each symbol is the average of endophyte viability from three independents bags and the vertical bars represent standard errors.

of infected seeds over time ('time' parameter in Table 6). The rate of loss in the proportion of infected seeds was significantly lower for the seeds kept in the air than for those moved to the soil surface ('time × location' parameter in Table 6, Fig. 4, *Endophytes*).

Discussion

Changes in the proportions of *Neotyphodium*-infected seeds in ageing seed pools are driven by differences between the survival rates of non-infected and infected seeds and by the mortality of the endophyte within live seeds. Differential survival of infected seeds may, in turn, result either from selective predation or from differences between the rates at which infected and non-infected seeds lose viability as they age (Madej & Clay, 1991; Popay *et al*., 2000; Hume & Barker, 2005; Gundel *et al*., 2006*a*). In our experiments, where seed predation was prevented and any differential survival was related to viability loss of extant seeds, the proportions of endophyte-infected seeds always decreased continuously over time. Results indicate that such decreases were accounted for in larger proportion by death of the endophyte within live seeds than by differential viability loss of infected seeds. According to our experimental results, endophyte infection does not mitigate and may occasionally increase seed viability loss (cf. Faeth & Hamilton, 2006; Gundel *et al*., 2007). Under accelerated ageing, the proportion of viable seeds dropped significantly faster in the high *N. occultans*-infected pool than in its low endophyte-infected counterpart for one of the accessions. In contrast, for the other two accessions, the proportion of viable seeds in the high endophyte-infected and low endophyte-infected pools did not differ significantly until pool exhaustion. Under field conditions, the proportion of viable seeds decreased significantly faster in high endophyte-infected than in low endophyte-infected pools, but these differences only resulted in slightly depressed proportion of viable seeds in the infected pools after 3 months in the field. Effects of endophyte infection on seed viability loss appear to be minor and conducive to only small changes in the proportion of endophyte-infected seeds.

Table 6 Logistic regression analysis of the changes in the proportion of endophyte-infected seeds of the Lucero *L. multiflorum* accession as affected by seed location in the field experiment

Parameter	DF	Estimate	Chi-square	*P*
Intercept	1	3.6628	242.1559	<0.0001
Time	1	−0.0319	118.6980	<0.0001
Time × location	1	−0.00848	14.5537	0.0001

Losses of endophyte viability within viable seeds were substantial in both accelerated ageing and field experiments. Under accelerated ageing, the proportion of all seeds entered in the experiments that produced endophyte non-infected seedlings increased for about 20 days. In addition, as the number of viable seeds decreased, the proportion of those that produced non-infected seedlings increased continuously until the seed pools became exhausted. For one accession (Lucero), this may result in part from the decreased survival of infected seeds. For the other two accessions, however, because high endophyte-infected and low endophyte-infected seeds lost viability at similar rates, the continuously increasing proportion of seeds producing non-infected seedlings must largely result from the endophyte losing

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the ability to infect the seedlings. Similar results have been shown for stored seeds of different endophyte-infected grass species (Rolston *et al.*, 1986; Welty *et al.*, 1987; Medvescigh *et al.*, 2004; Wheatley *et al.*, 2007). Under field conditions, the proportion of viable seeds producing infected seedlings decreased substantially over the duration of our experiment (about 90%). Because seed viability loss was only slightly faster for high endophyte-infected than for low endophyte-infected seeds, these observed decreases resulted from large rates of endophyte viability loss. As seed pools age, endophyte viability losses have the potential to drive large decreases in the proportion of seed that produce endophyte-infected seedlings.

Increased viability loss of endophyte-infected ageing seeds in our experiments might have been related to some extent with the amount of water absorbed by the seeds, as the higher the water content in ageing seeds, the faster they lose viability (Roberts & Ellis, 1989; Baskin & Baskin, 1998). In the accelerated ageing experiment, a negative effect of endophyte infection on seed viability was only apparent for the Lucero accession, which coincidentally was the only one exhibiting significantly higher water content in high endophyte-infected compared with low endophyte-infected seeds. The other two accessions (Traditional and Picaflor) tested in the accelerated ageing experiments exhibited no significant differences between high endophyte-infected and low endophyte-infected pools in the rates of seed viability loss and in SWCs. A tendency of highly humidified seeds to lose more viable endophyte has been shown (Rolston *et al.*, 1986; Welty *et al.*, 1987); a pattern that can be affected by both the quality of seeds and genotype of symbionts, and hence account for intra-specific variation (Hill *et al.*, 2005; Wheatley *et al.*, 2007).

The rates of viability loss of seeds and endophytes were substantially lower under field than under accelerated ageing conditions. These differences are likely to be related to the fact that seeds under accelerated ageing were exposed to continuous deterioration, whereas seeds in the field were exposed to environmental fluctuations; as cycles of hydration–dehydration have been observed to increase the chances of repair of deteriorated structures and re-establishment of seed functions (McDonald, 1998). According to the results of the field experiment, the infection dynamics of seeds placed on the soil surface differs from that reported for seeds buried in the soil. While loss of endophyte viability was not detected in live buried seeds (Hume & Barker, 2005; Canals *et al.*, 2008), in our experiment substantial numbers of endophyte-infected seeds placed on the soil surface failed to produce endophyte-infected seedlings when germinated. In addition, our field experiment indicated that moving the seeds from the air to the soil surface results in decreased rates of seed viability loss, but also in increased rates of viability loss of the endophyte. In this stage of the host life cycle, the fitness of both partners in the symbiosis appears to be dissociated, because the same environmental condition affected their viability in opposite ways.

Endophyte viability loss and differential seed mortality, respectively, determine transmission failures and fitness costs, inducing substantial decreases in the proportions of endophyte-infected seeds in ageing seed pools. In *L. multiflorum* populations, the proportion of endophyte-infected individuals is often high (De Battista, 2005; Gundel *et al.*, 2009), and the effect of endophyte on the fecundity of plants is not so large (Vila-Aiub *et al.*, 2005). Therefore, for the endophyte to persist and to reach high infection frequency, these processes must be offset by endophyte effects increasing the production of endophyte-infected seeds for the following generation (Gundel *et al.*, 2008). During the seed stage, one such endophyte effect is the deterrence of seed predators, an effect that would tend to increase the proportion of infected seeds of *L. multiflorum* (Uchitel *et al.*, 2006; Omacini *et al.*, 2009). Alternatively, seed dormancy, even when prolonging the ageing process of infected seeds, and thus increasing the chance of transmission failures (Clay & Schardl, 2002; Baskin & Baskin, 1998; Ghersa & Martínez-Ghersa, 2000), could be another way to increase the fitness of infected seeds, as chances to germinate in a safe site are enhanced by the endophyte infection (Gundel *et al.*, 2006*a*,*b*; Omacini *et al.*, 2009).

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