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A fungal endosymbiont affects host plant recruitment through seed- and litter-mediated mechanisms

Marina Omacini*[,1], Enrique J. Chaneton[1], Lowell Bush[2] and Claudio M. Ghersa[1]

[1]*IFEVA-CONICET and Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina; and* [2]*Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky 40546 0091, USA*

Summary

1. Many grass species are associated with maternally transmitted fungal endophytes. Increasing evidence shows that endophytes enhance host plant success under varied conditions, yet studies have rarely considered alternative mechanisms whereby these mutualistic symbionts may affect regeneration from seed.

2. We performed a microcosm experiment to evaluate whether infection with *Neotyphodium occultans* affects recruitment in the annual grass *Lolium multiflorum* either directly, by infecting the seeds, or indirectly, by altering the suitability of recruitment microsites through the litter shed by host plants. Endophyte effects on establishment were tested for different litter depths and watering regimes under natural herbivory by leaf-cutting ants.

3. Seed infection increased seedling emergence through the litter as well as final recruitment, irrespective of microsite conditions. However, litter produced by infected plants delayed emergence and decreased density of both infected and non-infected grass populations.

4. Individual plant biomass did not change with seed infection but was increased under deep litter from endophyte-infected plants. Although seed infection did not protect establishing plants from leaf-cutting ants, herbivory was reduced in the presence of deep litter shed by infected plants.

5. We conclude that fungal endophytes may affect host plant recruitment across subsequent generations not only by infecting the seeds but also through the host's dead remains. While the former effect entailed an advantage to infected plants, litter-mediated effects did not discriminate by infection status, and generally promoted the establishment of fewer and larger plants. Thus hidden foliar symbionts may play an underappreciated role in maintaining host species dominance through the litter produced by prior patch occupants.

Key-words: after-life effects, endophyte, herbivory, litter, *Lolium multiflorum,* seedling emergence, symbiotic interactions

Introduction

Plant-inhabiting micro-organisms play important, but often overlooked, roles in terrestrial communities (Clay & Schardl 2002; van der Heijden 2004; Omacini *et al.* 2005). There has recently been a renewed interest for determining the ecological impacts of microbial symbionts, including fungal endophytes that live concealed within the host plant without causing apparent symptoms (Clay & Schardl 2002; Omacini *et al.* 2005; Saikkonen *et al.* 2006; Rudgers & Clay 2008). The symbiosis between cool-season grasses and endophytic fungi of the genus *Neotyphodium* (Ascomycetes: Clavicipitaceae) is widespread in both natural and agricultural ecosystems (Roberts *et al.* 2005). The fungus grows systemically in above-ground tissues and is transmitted exclusively through the host seeds. Asexual endophytes and their host grasses usually establish mutualistic relations; infection may increase plant fitness, contributing to the long-term maintenance of the symbiosis (Clay & Schardl 2002; Gundel *et al.* 2008). Nevertheless, uninfected plants do persist in natural populations. Infected plants normally produce non-infected as well as infected seeds (i.e. vertical transmission is imperfect). In addition, environmental conditions can modify the outcome of the symbiosis, diluting potential advantages of harbouring endophytic fungi (Faeth 2002; Saikkonen *et al.* 2006; Krauss *et al.* 2007; Rudgers & Swafford 2009).

*Correspondence author. E-mail: omacini@agro.uba.ar

Endophyte-induced benefits have been generally associated with increased host tolerance to multiple biotic and abiotic stresses (Malinowski & Belesky 2000; Clay & Schardl 2002). The symbiosis produces different types of alkaloids, which can protect the plant against vertebrate or invertebrate herbivores (Bush *et al*. 1997; Wilkinson *et al*. 2000). Grasses infected with fungal endophytes may also become stronger competitors than non-infected conspecifics and co-occurring plant species in the absence of herbivores (Malinowski *et al*. 1999; Clay *et al*. 2005; Omacini *et al*. 2006). Many studies have focused on how endophytes modulate the host's ability to grow in different environments. Evidence shows that the magnitude and direction of plant responses to endophyte infection vary depending on resource availability (Malinowski *et al*. 1998; Cheplick 2007; Marks & Clay 2007; Kannadan & Rudgers 2008). Much less attention has been given to patterns of seedling establishment of endophyte-infected and non-infected conspecifics under different microsite conditions (Faeth & Hamilton 2006). Differential responses to environmental factors during plant regeneration may help to understand the persistence of infected and non-infected plants within a local population. Endophyte-mediated effects on recruitment would be most critical for maintaining infection frequencies in annual grass species (Gundel *et al*. 2008).

Endophyte infection may affect host seedling recruitment in the next generation through direct and indirect mechanisms. Direct mechanisms comprise the effects of endophyte presence on seed physiology and seedling performance (Clay 1987; Faeth & Hamilton 2006; Gundel *et al*. 2006). Existing results on seed behaviour and establishment of endophyte-infected grasses are surprisingly scarce and variable. Endophyte-free and infected seeds may show no differences in response to varying soil water potentials or temperatures (Neil *et al*. 2003; Faeth *et al*. 2004). Yet, some studies found that seeds from infected plants have higher germination rates than seeds from uninfected conspecifics (Clay 1987; Novas *et al*. 2003). In contrast, others reported reduced germination and seedling success for infected seeds (Hamilton & Faeth 2005), although such negative endophyte effects may be overridden by enhanced seedling survival in later life stages (Vila Aiub *et al*. 2005). Therefore, it is far from clear whether hereditary endophytic fungi generally increase or decrease the performance of host grass species at the seed/seedling stages.

Fungal endophytes may also affect regeneration in an indirect way, by altering microsite conditions for recruitment through the host's dead remains. While the role of litter in seedling establishment has been studied in many systems (Xiong & Nilsson 1999; Olson & Wallander 2002; Hovstad & Ohlson 2008), its ecological impacts have not been related to the presence of foliar endophytes in the donor plant. Litter may alter physical (light, temperature, humidity) and chemical conditions for seeds, modifying both the timing of germination and the rates of seedling emergence (Facelli & Pickett 1991; Olson & Wallander 2002; Hovstad & Ohlson 2008). In addition, microenvironmental changes generated by litter deposition may indirectly suppress or favour certain plant species by altering the outcome of competition, or the susceptibility of seeds and seedlings to various pathogens or herbivores (Facelli 1994; Moles & Westoby 2004; Finkes *et al*. 2006). In a recent work, Antunes *et al*. (2008) showed that litter from endophyte-infected plants reduced mycorrhizal colonization of a non-endophytic species, presumably by leaching of endophyte-derived allelochemicals. Hence, there is ample potential for endophytes to drive transgenerational, litter-mediated effects involving changes in litter quality (QL), microhabitat conditions and consumer pressure.

In this study, we examine how endophyte infection affects plant establishment through seed-mediated (direct) and litter-mediated (indirect) mechanisms. We tested the hypothesis that endophyte presence in the seed enhances host recruitment, especially under stressful microsite conditions created by deep litter layers and low soil moisture levels. Moreover, we hypothesized that the litter shed by the previous generation of infected plants exerts a negative effect on current seedling recruitment. We expected litter-mediated effects of prior patch occupants to become more intense with higher litter quantities and be most negative for endophyte-free plants. To examine these hypotheses, we conducted a factorial, microcosm/glasshouse experiment, in which seeds of the annual grass *Lolium multiflorum* with contrasting levels of endophyte infection were sown into different microsites created by the amount and origin of litter (whether from infected or uninfected plants) and watering regime (WA). We allowed for an additional (biotic) stress by exposing the microcosms to natural herbivory by leaf-cutting ants. In this way, we were able to examine endophyte effects on seedling establishment and herbivory rates over a wide range of microhabitats. We expected that benefits conferred by endophytes would be partly associated with changes in host plant chemistry derived from increased alkaloid contents in the shoots (Bush *et al*. 1997).

Materials and methods

EXPERIMENTAL DESIGN

Lolium multiflorum Lam. (Italian ryegrass) is a cool-season annual species originary from the Mediterranean zone, which has become widely naturalized throughout the world (Beddows 1973; Roberts *et al*. 2005). Seeds of ryegrass populations naturally infected with the endophyte *Neotyphodium occultans* (SI+) were collected from old fields in the Inland Pampa, Argentina, where the host species is a major component of plant communities undergoing post-agricultural succession (Omacini *et al*. 1995; Fig. 1). A subset of the seeds was treated with the fungicide triadimenol (5 mg active ingredient per g seed) to obtain endophyte-free seeds (SI−). Plant monocultures from both seed types were separately grown in outdoor plots for two consecutive annual cycles (April–December). After two generations, we collected seeds and harvested all the above-ground dead material (litter) produced by endophyte-infected and non-infected monocultures. Infection levels were 98% and 9% for SI+ and SI− seeds respectively (after microscopic examination of 50 seeds for each type using aniline blue stain). The litter was harvested at the end of the summer (March),

Fig. 1. *Lolium multiflorum* seedlings emerging through the litter deposited by previous generations of this annual grass in pampean old fields (Photograph by M. Omacini).

air-dried for 21 days and stored until the start of the experiment (~40 days later).

On 31 May 2002, we established a glasshouse experiment comprising 30 plots (hereafter, 'microcosms') arranged in five blocks (Fig. 2). The microcosms were made of wooden boxes (50 cm × 50 cm, 15 cm deep) filled with soil from a native grassland dominated by a mix of tussock grasses (*L. multiflorum* was not present) (for details, see Omacini *et al.* 2004). Coarse plant debris were manually removed but the original soil fauna was left intact. Each microcosm was randomly assigned to one of three levels of litter quantity (QT) and to one of two watering treatments (WA). In each microcosm we marked two 16 cm × 34 cm subplots, which were randomly designated to receive litter from either endophyte-infected (QL+) or –non-infected (QL–) *L. multiflorum* plants (QL treatment). Litter was added to each microcosm at one of three quantities

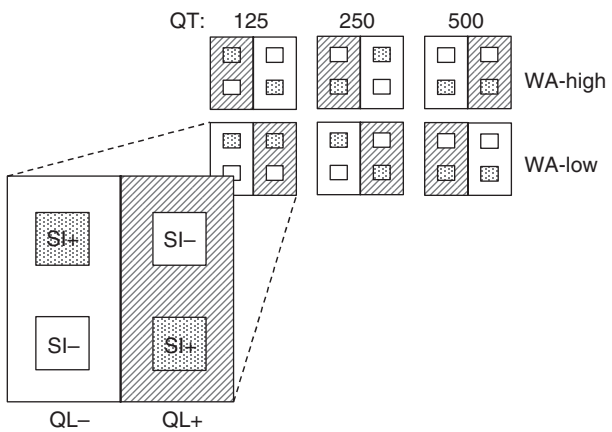

Fig. 2. Schematic of the experimental design showing all the treatments for one block. Each block included six microcosms (main plots) receiving different watering regimes (WA, -high: watered once a week, or -low: every third week) and litter quantities (QT, 125, 250 or 500 g m^{-2}). Each microcosm was split into two subplots covered with litter produced by endophyte-infected (QL+) or non-infected (QL–) plants. Each subplot was sown with endophyte-infected (SI+) and non-infected (SI–) *Lolium multiflorum* seeds in two separate microsites (100 cm^2). The full experiment comprised five complete blocks.

(QT): 125, 250 or 500 g m^{-2}, representing the range of *L. multiflorum* litter found in pampean old fields after 2–10 years of succession (Chaneton *et al.* 2001). These litter quantities reduced photosynthetically active radiation at the soil level to 25%, 8% and 3% of ambient light respectively.

The two moisture treatments were applied by regularly watering the microcosms through the litter layer to field capacity (~3000 mL) and allowing them to drain freely until the volumetric soil water content reached 70% (WA high: watered once a week) or 40% of field capacity (WA low: watered every third week). Soil moisture was measured to 6 cm depth every third day using a ThetaProbe sensor (Delta-T Devices, Cambridge, UK). During the experiment, soil water content was significantly affected by the WA (Fig. 3), but no QT or quality effects were detected. In this experiment, watering and QT had no significant effects on litter mass loss (14–20%, see Omacini *et al.* 2004).

On 16 June, 100 SI– and 100 SI+ seeds were separately sown in two 10 cm × 10 cm microsites delimited within each subplot of the microcosms (Fig. 2). Seeds were put in contact with the soil beneath the litter layer. As a result, the full experiment comprised four main factors arranged in a split-plot blocked design, with litter QT and WA treatments crossed at the main plot level, and litter QL and SI treatments crossed at the subplot level. Prior to the experiment, the germination potential of SI+ and SI– seeds was tested by incubating 100 seeds (n = 4) at 20–30 °C, with 9 h of light (ISTA 1996). No significant difference in germination was detected between SI+ (97 %) and SI– (98%) seed batches (t = 0·67, P = 0·52, d.f. = 6).

PLANT MEASUREMENTS

Every 2–3 days, the number of *L. multiflorum* plants emerging through the litter was counted in each microcosm. To avoid removing the litter, we recorded dead seedlings only when they passed through the litter layer; seedlings that germinated and died underneath the litter were not counted. Probit analysis was used to model seedling emergence dynamics for each sown microsite (Finney 1971). This procedure allows one to calculate the slope and x-intercept of the emergence curve; the slope equals the rate of emergence while the x-intercept measures the time elapsed to the start of emergence. Based on these parameters, we calculated the time to 50% emergence (E_{50}). On 20 August (12 weeks after sowing), the shoots of all established

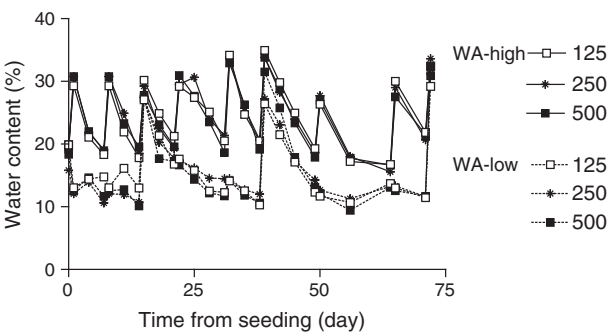

Fig. 3. Soil volumetric water content in the experimental microcosms under different watering (WA) and litter quantity (QT) treatments. WA-high: watered once a week (solid lines), WA-low: watered every third week (dotted lines). Litter was added at 125, 250 and 500 g m^{-2}. Each point shows the mean of 10 values.

plants were individually harvested and oven-dried at 80 °C for 48 h to determine final shoot dry mass per plant.

Leaf-cutting ants (*Acromirmex* sp., Formicidae: Attini) were allowed to colonize the experimental microcosms to determine whether endophyte infection could modify plant–herbivore interactions through its influence on host plant attributes (direct effect) and/or microhabitat conditions associated with QL (indirect effect). Ant damage on seedlings became apparent early in the experiment and occurred in all the microcosms. In August, just before ending the experiment, we assessed the frequency of herbivory in each subplot by counting the number of damaged seedlings (i.e. plants having at least one leaf cut cross-sectionally).

CHEMICAL ANALYSES

Nitrogen (N) and pyrrolizidine alkaloid (lolines) tissue contents were analysed at the end of the experiment. Ten plants were randomly taken from each subplot; however, as we did not have the resources to include all the treatments, subplots with intermediate litter quantities were excluded. Nitrogen content ($n = 5$, total 80) was determined using a flow injection autoanalyser (Alpkem Corporation, Wilsonville, OR). Due to logistic constraints, loline alkaloid concentrations were only evaluated for plants emerging through the QL− treatment ($n = 5$, total 40). For each subplot, 10 plants were harvested, lyophilized and ground before extraction and separation of loline alkaloids by capillary gas chromatography, using a modification of the method proposed by Yates *et al.* (1990). For each sample, 100 mg of tissue was extracted with 1 mL of CH_2Cl_2 and 50 μL of a 40% MeOH and 5% NH_4OH solution. Extracts were assayed to determine the presence of the most frequently detected pyrrolizidine alkaloids in endophyte-infected grasses, including *N*-formylloline, *N*-acetylloline, *N*-acetyl norloline, *N*-norloline and loline (TePaske *et al.* 1993; Bush *et al.* 1997).

STATISTICAL ANALYSIS

Final plant density, number of days to first emergence (*x*-intercept), E_{50}, and emergence rate (slope) were jointly analysed using a split-plot multivariate analysis of variance (MANOVA) model with blocks. WA and QT entered the model as main-plot factors, while QL and seed infection (SI) status were included as subplot factors. When MANOVA showed significant results, we used univariate ANOVAs to determine which of the response variables was most affected by the treatments (Scheiner 2001). In these analyses, four-way treatment interactions were pooled into the residual error. Cumulative mortality, final seedling biomass and frequency of plants damaged by ants were analysed using univariate split-plot ANOVA with blocks. Alkaloid concentrations were analysed using ANOVA with blocks including WA and QT as main effects (QL was not considered in these analyses). SI status was not included because alkaloids were not detected in seedlings emerged from SI− subplots. The Levene test was used to check for variance heterogeneity (test $P < 0{\cdot}05$); accordingly, final plant densities were square root-transformed before analysis. For clarity, mean values are presented in the original scale.

Results

SEEDLING EMERGENCE AND GROWTH

Between 53% and 79% of sown *L. multiflorum* seeds generated seedlings that emerged through the litter and became established in the different treatments (Fig. 4). Of the 8101 seedlings counted across all experimental microcosms, only 229 plants passed the litter layer but died thereafter (< 3%). No treatment effects on seedling mortality were detected in this experiment (split-plot ANOVA, all effects $P > 0{\cdot}10$). Hence, final plant recruitment was largely determined by early emergence patterns.

Seed infection, QL and WA all significantly affected seedling recruitment dynamics (MANOVA, Table 1). QT did not influence seedling emergence, and there were no significant treatment interactions (all $P > 0{\cdot}10$, see Table 1). Univariate ANOVA showed that SI increased final recruitment by 4–15% across microsite treatments ($F_{4,74} = 14{\cdot}08$, $P = 0{\cdot}0004$; Fig. 4), but endophyte infection did not alter the timing and rate of seedling emergence (*x*-intercept: $F_{4,74} = 0{\cdot}02$,

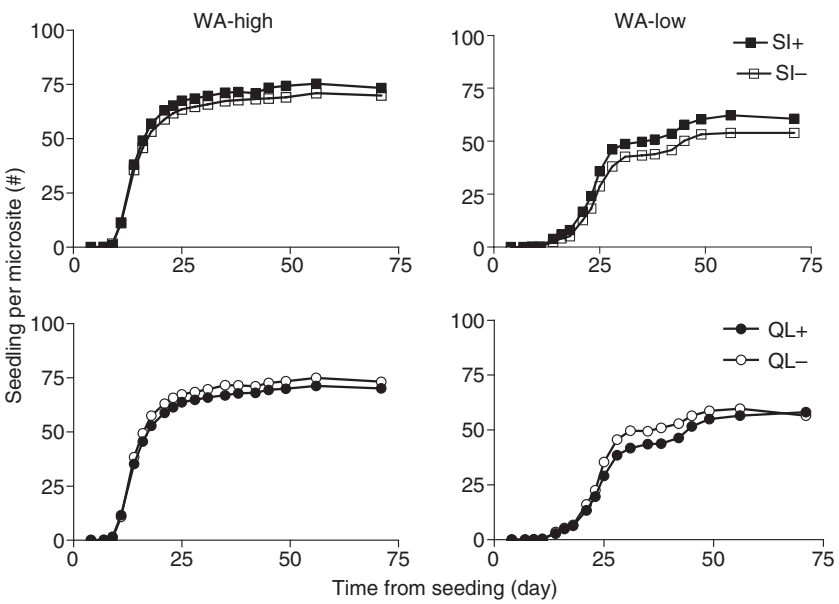

Fig. 4. Mean cumulative number of *Lolium multiflorum* seedlings emerging through the litter under different watering regimes (WA-high: watered once a week, WA-low: watered every third week). Seedling emergence differed between endophyte-infected (SI+) and non-infected (SI−) seeds irrespective of watering and litter treatment (upper panels). Emergence patterns also differed between microcosms covered with litter from infected (QL+) and non-infected (QL−) plants (lower panels). In each panel, data points represent the mean of 30 values, after pooling over nonsignificant treatments.

$P = 0{\cdot}9$; slope: $F_{4,74} = 0{\cdot}27$, $P = 0{\cdot}61$; E_{50}: $F_{4,74} = 0{\cdot}59$, $P = 0{\cdot}44$; Table 2). This endophyte effect was independent of litter and watering treatments (all interactions with SI, $P > 0{\cdot}10$).

Litter quality significantly affected both the timing of emergence and final seedling density (Table 2, Fig. 4). The presence of litter from endophyte-infected plants (QL+) delayed the onset of emergence (*x*-intercept: $F_{4,74} = 4{\cdot}17$, $P = 0{\cdot}044$) and the time elapsed to reach E_{50} ($F_{4,74} = 5{\cdot}24$, $P = 0{\cdot}025$), and also reduced the number of established plants ($F_{4,74} = 4{\cdot}22$, $P = 0{\cdot}043$). On average, we recorded 5% less seedlings in QL+ than in QL− microcosms (Table 2). Lastly, WA significantly influenced all seedling emergence parameters (Table 2) but did not modify the observed effects of SI or litter QL (interactions $P > 0{\cdot}10$). Seedlings emerged more rapidly in WA-high than in WA-low microcosms (*x*-intercept: $F_{1,20} = 60{\cdot}08$, $P = 0{\cdot}0001$; E_{50}: $F_{1,20} = 90{\cdot}91$, $P = 0{\cdot}0001$) and also attained higher final densities in WA-high microcosms ($F_{1,20} = 15{\cdot}27$, $P = 0{\cdot}001$; Fig. 4). E_{50} was reached about 12 days earlier in WA-high than in WA-low, while final recruitment was increased by 26% in WA-high plots (see Table 2). Noteworthy, the lowest numbers of established plants occurred in subplots sown with endophyte-free seeds (SI−) covered with the largest amount (500 g m^{-2}) of QL+ litter, although this three-way interaction was marginally nonsignificant (see Table 1).

Seed infection and WA did not affect final shoot mass per plant (ANOVA, SI: $F_{1,72} = 0{\cdot}13$, $P = 0{\cdot}71$, WA: $F_{1,72} = 1{\cdot}71$, $P = 0{\cdot}21$). However, seedling mass varied significantly depending on QT and quality (QT × QL: $F_{2,72} = 9{\cdot}51$, $P = 0{\cdot}0002$). Seedlings emerging through the highest QT attained greater final mass in subplots covered with QL+ than in those with QL− litter (Fig. 5).

Table 1. Results of multivariate analysis of variance (MANOVA) for the effects of watering regime (WA), litter quantity (QT), litter quality (QL) and seed infection status (SI) on four parameters describing dynamics of seedling emergence of *Lolium multiflorum* in experimental microcosms. The split-plot design included WA and QT as main plot effects, and QL and SI as subplot effects. Response variables were derived from probit analysis and included final plant recruitment (no. plants), time elapsed to the onset of emergence (*x*-intercept, days), time to half total emergence (E_{50}, days) and emergence rate (slope, day^{-1})

Effect	d.f.	Wilk's lambda	*P*-level
Block	16, 52	0·385	0·2956
Watering (WA)	4, 17	0·101	**0·0001**
Litter quantity (QT)	8, 34	0·602	0·3119
QT × WA	8, 34	0·632	0·3908
Litter quality (QL)	4, 71	0·841	**0·0142**
Seed infection (SI)	4, 71	0·816	**0·0055**
QL × SI	4, 71	0·981	0·8525
WA × QL	4, 71	0·898	0·1028
WA × SI	4, 71	0·944	0·3851
QT × QL	8, 142	0·933	0·7534
QT × SI	8, 142	0·906	0·5154
WA × QT × SI	4, 71	0·961	0·5762
WA × QT × QL	8, 142	0·864	0·2232
WA × QT × SI	8, 142	0·967	0·9655
QT × QL × SI	8, 142	0·834	0·1079

Significant effects ($P < 0{\cdot}05$) are shown in bold.

HERBIVORY BY LEAF-CUTTING ANTS

Litter quality significantly reduced the frequency of plants damaged by leaf-cutting ants at both low and high litter quantities (QL: $F_{1,72} = 6{\cdot}40$, $P = 0{\cdot}01$, QL × QT: $F_{2,72} = 4{\cdot}65$, $P = 0{\cdot}01$). On average, 11% (SE = 4·6) of the plants emerging through QL+ litter were damaged, while 30% (SE = 10·2) of the plants were damaged in QL− litter (Fig. 6). SI and WA did not affect herbivory rates ($P < 0{\cdot}60$). To explore whether the severity of ant herbivory altered biomass patterns at the subplot level, we performed analysis of covariance on final seedling mass including the frequency of damaged plants as the covariate. This analysis indicated that differences associated with litter QT and QL remained significant after controlling for the effect of herbivory (split-plot ANCOVA, QT × QL: $F_{2,71} = 6{\cdot}75$, $P = 0{\cdot}002$; covariate: $F_{1,71} = 7{\cdot}97$, $P = 0{\cdot}006$).

PLANT TISSUE CHEMISTRY

Endophyte infection did not affect plant N content (ANOVA, $P > 0{\cdot}10$ for all effects). Nitrogen concentrations ranged from 1·18% to 3·55% for SI+ plants, and from 1·04 to 3·98% for SI− plants. Regardless of endophyte infection, seedlings

Table 2. Effects of main experimental factors on four parameters describing the dynamics of seedling emergence for *Lolium multiflorum* in experimental microcosms

	Watering		Litter quantity			Litter quality		Seed infection	
Response variable	WA−	WA+	QT_I	QT_{II}	QT_{III}	QL−	QL+	SI−	SI+
R (no. plants)	**58·1** (1·7)	**73·1** (1·7)	66·0 (4·0)	68·8 (3·3)	62·0 (3·2)	**67·3** (1·9)	**63·9** (2·1)	**62·4** (2·0)	**68·8** (1·9)
x-Int (days)	**3·2** (0·02)	**2·6** (0·05)	3·0 (0·08)	2·9 (0·10)	2·9 (0·11)	**2·9** (0·06)	**3·0** (0·05)	2·9 (0·06)	2·9 (0·05)
Slope (day^{-1})	**3·1** (0·1)	**1·9** (0·1)	2·7 (0·2)	2·5 (0·3)	2·3 (0·2)	2·6 (0·1)	2·4 (0·1)	2·5 (0·1)	2·5 (0·1)
E_{50} (days)	**26·2** (0·6)	**14·4** (0·5)	21·9 (1·7)	19·6 (1·8)	19·4 (1·6)	**19·7** (1·0)	**20·9** (1·0)	20·5 (1·0)	20·1 (0·9)

Data show means (with SE in brackets) for each level of a main factor, after pooling over the rest of the treatments. Response variables were derived from probit analysis and included final recruitment (*R*), the time elapsed to the onset of emergence (*x*-intercept) and to half total emergence (E_{50}), and emergence rate (slope). For each response variable, values shown in bold under a given experimental factor were significantly different ($P < 0{\cdot}05$, univariate ANOVA).

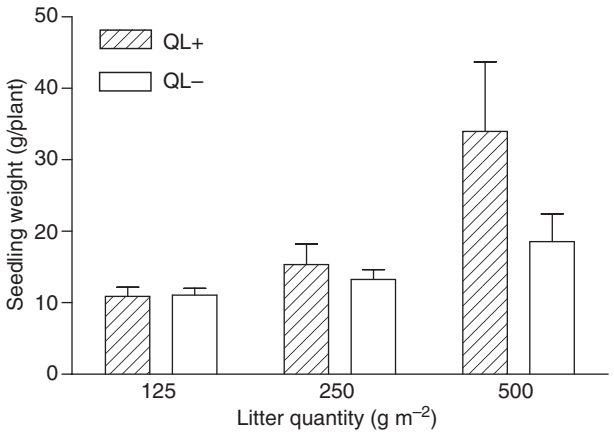

Fig. 5. Mean shoot mass (g/plant) of *Lolium multiflorum* established under different amounts of litter produced by endophyte-infected (QL+) and noninfected (QL–) plants. Values show means + SE (n = 20), with data pooled over seed infection (SI) and watering (WA) treatments.

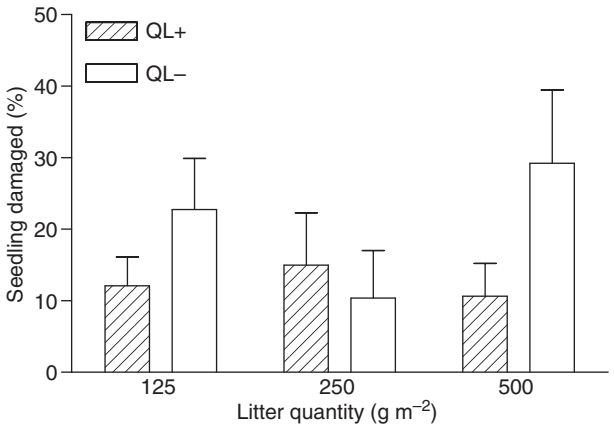

Fig. 6. Percentage of *Lolium multiflorum* plants damaged by leaf-cutting ants under different amounts of litter derived from endophyte-infected (QL+) and non-infected (QL–) populations. Values show means + SE (n = 20), with data pooled over seed infection (SI) and watering (WA) treatments.

established in WA-high microcosms had slightly lower N contents (1·54 ± 0·04, mean ± SE) than those in WA-low microcosms (1·94 ± 0·11), but this watering effect was marginally nonsignificant ($F_{1,12}$ = 3·77, P = 0·076).

After 100 days from seeding, plants grown from SI+ seeds contained 49–63 μg loline alkaloids per g dry tissue; alkaloids were not detected in SI– plants. WA did not modify loline alkaloid concentrations in SI+ plants (all P > 0·10). However, the amount of litter added significantly altered alkaloid contents, with lower overall values found in plants established under high litter cover ($F_{1,12}$ = 4·7, P = 0·05). *N*-formylloline was the most abundant pyrrolizidine alkaloid, being 26% lower in plants from QT_{III} than in those from QT_I plots ($F_{1,12}$ = 6·2, P = 0·03; Table 3). The reverse pattern was found for the less abundant *N*-acetylloline type ($F_{1,12}$ = 11·3, P = 0·006), while no differences were observed for *N*-acetyl norloline (Table 3). Two assayed alkaloids, *N*-norloline and loline, were not detected in endophyte-infected *L. multiflorum* plants.

Discussion

We found evidence for two broad and potentially conflicting mechanisms whereby vertically transmitted endophytes may affect host grass recruitment. First, SI with *Neotyphodium* – direct mechanism – enhanced *L. multiflorum* emergence and final establishment across a range of environmental conditions. Importantly, this positive endophyte effect was not limited to stressful microsites but persisted for different litter depths and soil moisture regimes. Second, the litter produced by infected plants – indirect mechanism – negatively affected recruitment, irrespective of seedling infection status. The lack of interaction between seed and litter mediated mechanisms suggests that endophyte-infected plants may enjoy a relative advantage over non-infected conspecifics and hold the ground occupied by their predecessors, even if the litter deposited by prior patch occupants creates generally poorer recruitment microsites. These results highlight the importance of mutualistic endophytes in host recruitment dynamics when the fungal symbiont is efficiently transmitted between successive generations (see Gundel *et al.* 2008).

Seed-mediated (direct) endophyte effects on host regeneration were relatively small but consistently positive. Emergence was higher for infected than for non-infected seeds across all microsite conditions. Contrary to expectation, SI effects neither depended on WA nor on the amount of surface litter. These results contradict previous evidence in that positive endophyte effects were not restricted to microsites with low water or reduced light levels (Malinowski & Belesky 2000; Kannadan & Rudgers 2008). Gundel *et al.* (2006) showed that infected seeds of *L. multliflorum* had higher base water potentials than non-infected seeds, thus requiring better soil moisture conditions for germination. Such differences in seed physiology, if they occurred at all, did not seem to determine patterns of emergence in our study, perhaps because our low WA did not create extended dry soil conditions. In our microcosms, soil drying periods were punctuated by water

Table 3. Loline alkaloid concentrations (μg g^{-1}, mean ± SE) in endophyte-infected *Lolium multiflorum* plants emerged under contrasting litter quantities (QT) and watering regimes (WA-high: once a week; WA-low: every third week)

	WA-high		WA-low	
	125 g m^{-2}	500 g m^{-2}	125 g m^{-2}	500 g m^{-2}
N-formylloline	54·8 ± 3·4^a	39·0 ± 8·0^b	55·5 ± 6·6^a	42·9 ± 9·3^b
N-acetylloline	1·3 ± 0·4^a	2·5 ± 0·2^b	1·0 ± 0·4^a	2·4 ± 0·4^b
N-acetil norloline	6·6 ± 2·3^a	7·2 ± 0·8^a	6·1 ± 0·6^a	7·4 ± 1·6^a
N-norloline	ND	ND	ND	ND
Loline	ND	ND	ND	ND
Total	62·8 ± 8·9^a	48·6 ± 17·0^b	62·6 ± 5·0^a	52·7 ± 22·9^b

Different superscript letters indicate significant differences within rows. ND, not detected.

pulses (Fig. 3), which appeared to be similarly perceived by infected and non-infected seeds. In addition, any endophyte-induced effects on germination may have been counteracted by enhanced survival of infected seedlings as they passed through the litter layer. The consistent manifestation of a mutualistic association with fungal endophytes at the seedling stage may be particularly important for annual host grasses (Vila Aiub *et al.* 2005; cf. Faeth & Hamilton 2006), the persistence of which in natural communities is strongly determined by recruitment from seed. Even small differences in recruitment may drive non-infected genotypes locally extinct, provided endophyte vertical transmission is sufficiently high and there is no immigration of endophyte-free seeds (Gundel *et al.* 2008).

Litter-mediated (indirect) endophyte effects on recruitment were negative for both infected and non-infected ryegrass. While our design did not allow specific mechanisms to be recognized, other studies have suggested that allelopathic effects could be involved in the observed dominance of some grass–endophyte associations (Orr *et al.* 2005; Antunes *et al.* 2008). Toxic compounds produced by symbiosis might persist in the host's dead remains and be leachated to the soil (Sutherland *et al.* 1999; Fletcher 2005; Antunes *et al.* 2008). This may explain why the litter of infected plants decomposed more slowly than that of endophyte-free plants (Omacini *et al.* 2004). Also, the experimental addition of loline alkaloids, or their natural release from decaying litter, has been shown to decrease germination and seedling growth of non-host species (Petroski *et al.* 1990; Antunes *et al.* 2008). Nevertheless, we cannot discount other mechanisms of suppression involving mediation by soil organisms (Matthews & Clay 2001; Omacini *et al.* 2005). For instance, litter of endophyte-infected grasses might elicit chemical signals altering growth of mycorrhizal fungi (Antunes *et al.* 2008). Unfortunately, at present, we do not have data on alkaloid concentrations in the litter of *L. multiflorum* to back this line of reasoning. Regardless of the intermediary factor, endophyte-induced changes in host QL could have lasting consequences on the regeneration and dominance of endophyte–grass associations (Clay *et al.* 2005; Omacini *et al.* 2005).

Whereas litter shed by endophyte-infected plants decreased overall recruitment irrespective of QT, it had a significant positive effect on the shoot mass of plants emerging under deep litter layers. It seems possible that this effect reflected a change in seedling root : shoot ratios, with more carbon allocated to above-ground organs in the more shaded microsites (Facelli & Pickett 1991). Yet this may not fully explain the litter QT × QL interaction (Fig. 5). Instead, growth of seedlings that managed to emerge through deep litter of infected plants likely benefited from reduced plant densities (and hence least intense competition), as well as from a concomitant decrease in herbivory rates from leaf-cutting ants in those microsites (Fig. 6). Long-term studies in successional old fields in the Inland Pampas have shown that dominance by endophyte-associated Italian ryegrass leads to accumulation of large amounts of litter (Fig. 1), which strongly inhibits recruitment of the host grass and of many ruderal species (Omacini *et al.* 1995; Chaneton *et al.* 2001). In such conditions, establishment of large *L. multiflorum* plants may be crucial for allowing host species persistence in later successional stages. More generally, our experiment revealed that endophyte infections can exert opposing direct and indirect effects on different life-history processes affecting the dynamics of host grass populations.

Endophyte infection induced the accumulation of three major loline-type alkaloids in *L. multiflorum* shoots, which corresponded to the same compounds detected in seed materials of this species (TePaske *et al.* 1993). Intriguingly, alkaloid levels decreased for infected plants grown in microsites with large litter quantities. Given that alkaloid contents are often positively related to endophyte biomass (Rasmussen *et al.* 2007), this pattern may reflect a cost in harbouring the endophyte and producing alkaloids in deep-litter patches with reduced light availability. Grasses infected with systemic endophytes can experience reduced attack from insect herbivores, an effect attributed to the presence of various alkaloids (Bush *et al.* 1997; Wilkinson *et al.* 2000). In our study, changes in host chemistry did not account for the increased recruitment of endophyte-infected plants. Seedlings in SI+ and SI− microsites were equally damaged by leaf-cutting ants. We did find, however, that the presence of litter from infected plants protected seedlings from ant herbivory. This finding suggests that endophytic grass litter may alter the environment for certain insect herbivores (Finkes *et al.* 2006; Rudgers & Clay 2008), and may thus indirectly affect host species performance in the next generation (see Figs 4 and 5). Prior work on Italian ryegrass showed that ants attending grass aphids (*Sipha maydis*) were sensitive to the presence of *N. occultans* in the host plant (Chaneton & Omacini 2007). However, it is as yet unclear how QL might influence ant activities (see also Tibbets & Faeth 1999; White *et al.* 2001). Considering that total recruitment decreased under QL+ litter, it is also possible that leaf-cutting ants preferred the higher-density seedling patches covered by non-infected (QL−) grass litter.

Although endophyte infection influenced establishment through both seed- and litter-mediated mechanisms, such effects were largely additive. We found no significant interaction between SI and litter origin (QL) that would suggest a positive feedback, whereby the litter deposited by endophyte-infected plants from a previous generation might have a stronger suppressive effect on non-infected plants than on infected conspecifics (see Table 1, Figs 5 and 6). Such a feedback mechanism would be consistent with the persistent dominance of endophyte-associated *L. multiflorum* in early successional fields (Chaneton *et al.* 2001; Vila Aiub *et al.* 2005). Given that this is the first experiment testing for an indirect, litter-mediated influence of systemic endophytes on recruitment, we believe it is premature to reject the notion that endophytic grasses may create a recruitment environment conferring an advantage to their own progeny over non-infected conspecifics and other co-occurring species (see Matthews & Clay 2001). Alternatively, if microsites occupied by infected grass litter were more disadvantageous for

infected grass seedlings (negative feedback), this might help explain the replacement of endophytic grasses by late successional species (Kardol *et al*. 2006). Clearly, more experimental work is needed on the potential feedback mechanisms involving endophyte–grass systems.

Our experimental manipulations comprised two contrasting WA with the expectation that soil moisture conditions would affect the magnitude and direction of endophyte effects on establishment (e.g. Cheplick *et al*. 2000; Gundel *et al*. 2006; Rudgers & Swafford 2009). It has been suggested that there might be a net cost in harbouring systemic endophytes in stressful environments (Cheplick 2007). By contrast, evidence indicates that endophyte symbioses may allow host grasses to cope with various abiotic stresses (Malinowski & Belesky 2000; Clay & Schardl 2002). Here we found that WA did not interact with either SI status or QL treatment in determining *L. multiflorum* recruitment and growth. Increasing the water supply strongly enhanced seedling emergence from endophyte-infected and non-infected seed pools to a similar extent. Furthermore, frequent watering did not amplify or counteract the negative impact of endophytic grass litter on recruitment. Thus the results show that water supply had an overriding influence on recruitment dynamics, which nevertheless did not offset the direct and indirect effects of fungal endophytes.

In conclusion, our study adds to the notion that microbial endosymbionts may act as support systems in promoting host plant establishment (van der Heijden 2004). We have shown that endophyte removal, or its natural loss from seeds, will have immediate effects on host plant regeneration. Moreover, fungal endophytes may have lasting effects on plant establishment patterns across successive generations by altering the quality of recruitment microsites through the accumulation of host plant litter. This observation opens a fruitful avenue for future research regarding the balance of positive and negative indirect effects of fungal endophytes on seedling performance. We expect the sign of such indirect effects to depend on the presence of other organisms, including seedling mutualists and antagonists (e.g. Antunes *et al*. 2008). Thus, hidden foliar symbionts may play an underappreciated role in maintaining host grass dominance through 'after-life' interactions modulated by the litter of prior conspecific patch occupants.

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References

Antunes, P., Miller, J., Carvalho, L., Klironomos, J.N. & Newman, J. (2008) Even after death the endophytic fungus of *Schedonorus phoenix* reduces the arbuscular mycorrhizas of other plants. *Functional Ecology*, **22**, 912–918.

Beddows, A.R. (1973) *Lolium multiflorum* Lam. *Journal of Ecology*, **61**, 587–600.

Bush, L.P., Wilkinson, H.H. & Schardl, C.L. (1997) Bioprotective alkaloids of grass–fungal endophyte symbiosis. *Plant Physiology*, **114**, 1–7.

Chaneton, E.J. & Omacini, M. (2007) Bottom-up cascades induced by fungal endophytes in multitrophic systems. *Ecological Communities: Plant Mediation in Indirect Interaction Webs* (eds T. Ohgushi, T. P. Craig & P. W. Price), pp 164–187. Cambridge University Press, Cambridge.

Chaneton, E.J., Omacini, M., Trebino, H.J. & León, R.J.C. (2001) Disturbios, dominancia y diversidad de especies nativas y exóticas en pastizales pampeanos húmedos. *Anales de la Academia Nacional de Ciencias Exactas, Físicas y Naturales*, **53**, 121–140.

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

Cheplick, G.P., Perera, A. & Koulouris, K. (2000) Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. *Functional Ecology*, **14**, 657–667.

Clay, K. (1987) Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. *Oecologia*, **73**, 358–362.

Clay, K. & Schardl, C.L. (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Midland Naturalist*, **160**, 99–127.

Clay, K., Holah, J. & Rudgers, J.A. (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 12465–12470.

Facelli, J.M. (1994) Multiple indirect effects of plant litter affect the establishment of woody seedlings in old fields. *Ecology*, **75**, 1727–1735.

Facelli, J.M. & Pickett, S.T.A. (1991) Plant litter: light interception and effects on an old field plant community. *Ecology*, **72**, 1024–1031.

Faeth, S.H. (2002) Are endophytic fungi defensive plant mutualists? *Oikos*, **98**, 25–36.

Faeth, S.H. & Hamilton, C.E. (2006) Does an asexual endophyte symbiont alter life stage and long-term survival in a perennial host. *Microbial Ecology*, **52**, 748–755.

Faeth, S.H., Helander, M.L. & Saikkonen, K.T. (2004) Asexual *Neotyphodium* endophytes in a native grass reduce competitive abilities. *Ecology Letters*, **7**, 304–313.

Finkes, L.K., Cady, A.B., Mulroy, J.C., Rudgers, J.A. & Clay, K. (2006) Plant-fungus mutualism affects spider composition in successional habitats. *Ecology Letters*, **9**, 347–356.

Finney, D. (1971) *Probit Analysis*. Cambridge University Press, New York.

Fletcher, L. (2005) Managing ryegrass-endophyte toxicoses. *Neotyphodium in Cool-Season Grasses* (eds C. Roberts, C. P. West & D. Spiers), pp. 229–242. Blackwell Publishing, OH, Ames, USA.

Gundel, P., Maseda, P., Vila Aiub, M.M., Ghersa, C.M. & Benech Arnold, R.L. (2006) Effects of *Neotyphodium* fungi on *Lolium multiflorum* seed germination in relation to water availability. *Annals of Botany*, **97**, 571–577.

Gundel, P., Batista, W.B., Texeira, M., Martínez-Ghersa, M., Omacini, M. & Ghersa, C.M. (2008) *Neotyphodium* endophyte infection frequency in annual grass populations: relative importance of mutualism and transmission efficiency. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **275**, 897–905.

Hamilton, C.E. & Faeth, S.H. (2005) Asexual, systemic endophytes in grasses: a test of the seed germination and pathogen resistance hypothesis. *Symbiosis*, **38**, *69–86*.

van der Heijden, M.G.A. (2004) Arbuscular mycorrhizal fungi as support systems for seedling establishment in grasslands. *Ecology Letters*, **7**, 293–303.

Hovstad, K. & Ohlson, M. (2008) Physical and chemical effects of litter on plant establishment in semi-natural grasslands. *Plant Ecology*, **196**, 251–260.

International Seed Testing Association (1996) International rules for seed testing. *Seed Science and Technology*, **24**, supplement.

Kannadan, S. & Rudgers, J. (2008) Endophyte symbiosis benefits a rare grass under low water availability. *Functional Ecology*, **22**, 706–713.

Kardol, P., Bezemer, T.M. & van der Putten, W.H. (2006) Temporal variation in plant-soil feedback controls succession. *Ecology Letters*, **9**, 1080–1088.

Krauss, J., Härri, S., Bush, L., Husi, R., Bigler, L., Power, S. & Müller, C.B. (2007) Effects of fertiliser, fungal endophytes and plant cultivar on the performance of insect herbivores and their natural enemies. *Functional Ecology*, **21**, 107–111.

Malinowski, D.P. & Belesky, D.P. (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Science*, **40**, 923–940.

Malinowski, D.A., Alloush, G.A. & Belesky, D.P. (1998) Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte *Neotyphodium coenophialum*. *Plant and Soil*, **205**, 1–12.

Malinowski, D.A., Belesky, D.P. & Fedders, J.M. (1999) Endophyte infection may affect the competitive ability of tall fescue growth with red clover. *Journal of Agronomy and Crop Science*, **183**, 91–102.

Marks, S. & Clay, K. (2007) Low resource availability differentially affects the growth of host grasses infected by fungal endophytes. *International Journal of Plant Science*, **168**, 1266–1277.

Matthews, J.W. & Clay, K. (2001) Influence of fungal endophyte infection on plant-soil feedback and community interactions. *Ecology*, **82**, 500–509.

Moles, A. & Westoby, M. (2004) What do seedlings die from and what are the implications for evolution of seed size. *Oikos*, **106**, 193–199.

Neil, K., Tiller, R. & Faeth, S.H. (2003) Big sacaton and endophyte-infected Arizona fescue germination under water stress. *Journal of Range Management*, **56**, 616–622.

Novas, M.V., Gentile, A. & Cabral, D. (2003) Comparative study of growth parameters on diaspores and seedlings between populations of *Bromus setifolius* from Patagonia, differing in *Neotyphodium* endophyte infection. *Flora*, **198**, 1–6.

Olson, B. & Wallander, R. (2002) Effects of invasive forb litter on seed germination, seedling growth and survival. *Basic and Applied Ecology*, **3**, 309–317.

Omacini, M., Chaneton, E.J., Leon, R.J.C. & Batista, W.B. (1995) Old-field successional dynamics on the Inland Pampa, Argentina. *Journal of Vegetation Science*, **6**, 309–316.

Omacini, M., Chaneton, E.J., Ghersa, C.M. & Otero, P. (2004) Do foliar endophytes affect grass litter decomposition? A microcosm approach using *Lolium multiflorum*. *Oikos*, **104**, 581–590.

Omacini, M., Chaneton, E.J. & Ghersa, C.M. (2005) A hierarchical framework for understanding the ecosystem consequences of endophyte grass symbioses. *Neotyphodium in Cool-Season Grasses* (eds C. Roberts, C. P. West & D. Spiers), pp. 141–161. Blackwell Publishing, OH, Ames, USA.

Omacini, M., Eggers, T., Bonkowski, M., Gange, A.C. & Jones, T. (2006) Leaf endophytes afffect mycorrhizal status and growth of co-infected and neighbouring plants. *Functional Ecology*, **20**, 226–232.

Orr, S., Rudgers, J. & Clay, K. (2005) Invasive plants can inhibit native tree seedlings: tesiting potential allelopathic mechanisms. *Plant Ecology*, **181**, 153–165.

Petroski, R.J., Dornbos, D.L. & Powell, R.G. (1990) Germination and growth inhibition of annual ryegrass (*Lolium multiflorum*) and alfalfa (*Medicago sativa*) by loline alkaloids and synthetic *N* acetylloline derivates. *Journal of Agricultural Food Chemistry*, **38**, 1716–1718.

Rasmussen, S., Parsons, A., Bassett, S., Hume, D.E., Johnson, L., Johnson, R., Simpson, W., Stacke, C., Voisey, C., Xue, H., & Newman, J. (2007) High nitrogen supply and carbon content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. *New Phytologist*, **173**, 787–797.

Roberts, C., West, C.P. & Spiers, D.E. (2005) *Neotyphodium in Cool-Season Grasses*. Blackwell Publishing, IA, Ames, USA, 379 pp.

Rudgers, J.A. & Clay, K. (2008) An invasive plant–fungal mutualism reduces arthropod diversity. *Ecology Letters*, **11**, 831–840.

Rudgers, J.A. & Swafford, A.L. (2009) Benefits of a fungal endophyte in *Elymus virginicus* decline under drought stress. *Basic and Applied Ecology*, **10**, 43–51.

Saikkonen, K., Lehtonen, P., Helander, M., Koricheva, J. & Faeth, S. (2006) Model systems in ecology: dissecting the endophyte-grass literature. *Trends in Plant Science*, **11**, 428–433.

Scheiner, S. (2001) MANOVA: multiple response variables and multispecies interactions. *Design and Analysis of Ecological Experiments* (eds S. M. Scheiner & J. Gurevitch), pp. 99–115. Oxford University Press, New York.

Sutherland, B.L., Hume, D.E. & Tapper, B.A. (1999) Allelopathic effects of endophyte-infected perennial ryegrass extracts on white clover seedlings. *New Zealand Journal of Agricultural Research*, **42**, 19–26.

TePaske, M., Powell, R. & Clement, S. (1993) Analyses of selected endophyte-infected grasses for the presence of Loline-type and ergot-type alkaloids. *Journal of Agricultural and Food Chemistry*, **41**, 2299–2303.

Tibbets, T. & Faeth, S. (1999) *Neotyphodium* endophytes in grasses: deterrents or promoters of herbivory by leaf-cutting ants? *Oecologia*, **118**, 297–305.

Vila Aiub, M.M., Gundel, P. & Ghersa, C.M. (2005) Fungal endophyte infection changes growth attributes in *Lolium multiflorum* Lam. *Austral Ecology*, **30**, 49–57.

White, B.S., Sullivan, R., Balady, G., Gianfagna, T., Yue, Q., Meyer, W. & Cabral, D. (2001) A fungal endophymbiont of the grass *Bromus setifolius*: distribution in some Andean populations, identification and examination of beneficial properties. *Symbiosis*, **31**, 241–257.

Wilkinson, H.H., Siegel, D.I., Blankenship, J., Mallory, A., Bush, J.P. & Schardl, C.L. (2000) Contribution of fungal loline alkaloids to protection from aphids in a grass–endophyte mutualism. *Molecular Plant–Microbe Interactions*, **13**, 1027–1033.

Xiong, S. & Nilsson, C. (1999) The effects of plant litter on vegetation: a meta-analysis. *Journal of Ecology*, **87**, 984–994.

Yates, S., Petroski, R.J. & Powell, R.G. (1990) Analysis of loline alkaloids in endophyte-infected tall fescue by capillary gas chromatography. *Journal of Agricultural Food Chemistry*, **37**, 182–185.

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