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Inherited fungal symbionts enhance establishment of an invasive annual grass across successional habitats

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Abstract Plants infected with vertically transmitted fungal endophytes carry their microbial symbionts with them during dispersal into new areas. Yet, whether seed-borne endophytes enhance the host plant's ability to overcome colonisation barriers and to regenerate within invaded sites remains poorly understood. We examined how symbiosis with asexual endophytic fungi (*Neotyphodium*) affected establishment and seed loss to predators in the invasive annual grass *Lolium multiflorum* (Italian ryegrass) across contrasting successional plots. Italian ryegrass seeds with high and low endophyte incidence were sown into three communities: a 1-year-old fallow field, a 15-year-old grassland, and a 24-year-old forest, which conformed to an old-field chronosequence in the eastern Inland Pampa, Argentina. We found that endophyte infection consistently increased host population recruitment and reproductive output. Endophyte presence also enhanced aerial biomass production of ryegrass in a low recruitment year but not in a high recruitment year, suggesting that symbiotic effects on growth performance are density dependent. Endophyte presence reduced seed removal by rodents, although differential predation may not account for the increased success of infected grass populations. Overall, there was no statistical evidence for an endophyte-by-site interaction, indicating that the fungal endosymbiont benefitted host establishment regardless of large differences in biotic and abiotic environment among communities. Our results imply that hereditary endophytes may increase the chances for host grass species to pass various ecological filters associated with invasion resistance across a broad range of successional habitats.

Keywords Endophyte · Invasion · Mutualism · Neotyphodium · Recruitment

Introduction

Symbiotic microbes that live concealed inside their plant or animal hosts can exert key influences on species abundance and distribution, and on whole community organisation (Douglas 1994; Clay 2001; Stachowicz 2001; Little et al. 2004; Omacini et al. 2005; Arnold 2008). Increasing evidence shows that plant symbionts, ranging from mutualistic to parasitic, may alter the chances of a species invading a new habitat (Wolfe and Klironomos 2005; van der Putten et al. 2007). Pathogens have so far received most attention as microbial modulators of interactions between invasive and resident plant species (e.g. Parker and Gilbert 2007; Borer et al. 2007; Mangla et al. 2008). In contrast, although mutualistic plant–microbe interactions have recently become a focus of invasion research (Richardson et al. 2000; Klironomos 2002; Callaway et al. 2004; Rudgers et al. 2005; Stinson et al. 2006; Nuñez et al. 2009), their role in a variety of systems has yet to be established (Desprez-Loustau et al. 2007).

Once a plant species arrives in a new habitat, its realised invasion success depends on the ability to pass environmental filters affecting survival and performance at various life stages (Levine et al. 2004; Mitchell et al. 2006). Many species rely on mutualistic or facilitative interactions to become established (Bruno et al. 2003). Thus encountering

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suitable associates in the recipient community may constrain exotic plant invasion and spread (Richardson et al. 2000; Nuñez et al. 2009), unless partners ‘travel’ together or disperse in a coordinated fashion. This is particularly true when plant performance hinges on horizontal infection by non-maternally transmitted symbionts, like mycorrhizal fungi or nitrogen-fixing bacteria (Parker 2001; Desprez-Loustau et al. 2007). About 30% of pooid grass species are maternally infected with systemic fungal endophytes (Clay and Schardl 2002; Cheplick and Faeth 2009), and hence carry their microbial symbionts upon dispersal from the parent plant. Although several invasive grasses are known to be associated with fungal endophytes (see Rudgers et al. 2009), these cryptic leaf symbionts have been largely overlooked in the context of invasions (Desprez-Loustau et al. 2007; van der Putten et al. 2007), and only recently have been regarded as potential mediators of invasion dynamics (Rudgers et al. 2005, 2007). Most work to date focussed on the impact of endophyte–grass associations on resident communities after the host population already became widely established (Clay et al. 2005; Omacini et al. 2005; Rudgers et al. 2007). Whether seed-borne endophytes enhance the host plant’s ability to invade natural communities remains poorly understood (Cheplick and Faeth 2009).

In hereditary symbioses between grasses and asexual endophytic fungi, the endophyte grows systemically within shoot tissues and is transferred across generations only through the host seeds, i.e. there is no contagious infection (Schardl et al. 2004). Hence, seeds constitute the dispersal unit for both partners. Endophyte infection can be lost due to reduced hyphal viability in seeds or seedlings, or imperfect transmission during plant maturation (Gundel et al. 2008, 2009). These kind of intimate associations have been proposed to evolve into strong plant–microbe mutualisms (Herre et al. 1999; Clay and Schardl 2002; Saikkonen et al. 2004). Asexual endophytes often increase plant competitive ability, resistance to herbivory and tolerance to abiotic stress (Malinowski and Belesky 2000; Clay and Schardl 2002). These effects are partly related to the synthesis of alkaloids by infected plants (Bush et al. 1997). Nevertheless, positive endophyte effects are not universal. Endophyte infection sometimes provides no clear advantage and may even reduce host performance, as reported for some native grasses (e.g. Faeth et al. 2004). Further, evidence shows that endophyte effects on a given host species may shift from mutualistic to antagonistic depending on environmental context (Cheplick et al. 2000; Ahlholm et al. 2002; Cheplick and Faeth 2009).

Endophytes may create ‘niche opportunities’ for invasion by increasing the chances for a host species to overcome biotic and abiotic barriers to recruitment (Shea and Chesson 2002; Mitchell et al. 2006). In greenhouse studies, asexual *Neotyphodium* endophytes have been found to promote establishment of agronomically important grasses through enhanced seedling emergence and growth (Clay 1987; Rudgers et al. 2005; Omacini et al. 2009). In contrast, working on a native grass in field plots, Faeth and Hamilton (2006) reported lower survival of endophyte-infected relative to uninfected plants during early life stages (seeds and seedlings). Noticeably, the influence of fungal endophytes on host recruitment has not been tested for any invasive grass species under field conditions. As frequency of endophyte infection in local populations often increases over successional time (Clay and Schardl 2002), benefits of symbiosis might become more apparent as recruitment limitation (Grime 2001; Shea and Chesson 2002) and biotic interactions (Clay et al. 2005; Morris et al. 2007) intensify in late seral communities. To our knowledge, no study has yet assessed the impact of hereditary endophytes on host plant recruitment from seed across different successional habitats.

Here we examine how seed-borne endophytic fungi influenced the establishment of the invasive annual grass *Lolium multiflorum* Lam. (Italian ryegrass) in three communities—fallow field, grassland and forest—representing distinct stages of an old-field succession. We hypothesised that endophyte infection increases host invasion ability by enhancing its performance at various life stages, from seed survival, through seedling recruitment to biomass accrual and fecundity of established plants. Moreover, we expected mutualistic endophyte effects to be stronger in late successional sites, which are thought to set greater barriers to recruitment from seed (Grime 2001) and be generally more resistant to invasion than newly disturbed sites (Rejmánek 1989; Shea and Chesson 2002). Effects of endophyte symbiosis on host population recruitment, biomass production, and seed survival from predators were tested by sowing ryegrass seeds with high (E+) and low (E−) endophyte incidence in three contrasting communities along an old-field chronosequence.

Materials and methods

Study site

The study was conducted in an abandoned agricultural area located at the centre of a 290-ha crop field, near Ordoqui, Buenos Aires province, Argentina (35°55′S, 61°09′W). Mean annual rainfall for the area is ~900 mm, with temperatures varying from 7.2°C in July to 23.8°C in January. Soils are well-drained, sandy loam Typic Hapludolls. The site belongs to the eastern Inland Pampa, a region originally occupied by tussock grasslands, which was extensively transformed to row crop agriculture and livestock grazing

(Soriano 1992). Semi-natural grasslands occur in abandoned agricultural fields and grazed pastures (Omacini et al. 1995; Ghersa and León 1999). Forests did not historically occur in the mesic pampas, yet planted woody patches are common throughout the modern landscape. Old fields are often colonised by exotic tree species, which can develop dense thickets after several years of succession (Facelli and León 1986; Ghersa and León 1999).

In 2 consecutive years (2003–2004), we performed sowing experiments using Italian ryegrass E+ and E− seeds in three, 40 × 100-m plots with 1, 15 and 24 years of succession. Plots were located within 200 m distance from each other, and shared the same soil type and topographic position. The study communities comprised a newly abandoned, early successional weed assemblage (‘fallow field’), a mid-successional grassland (plot fenced in 1989), and a late-successional forest (plot fenced in 1980). The fallow field was colonised by annual/biennial forbs, including *Carduus acanthoides*, *Cirsium vulgare*, *Coniza bonariensis*, and *Hirschfeldia incana*. The grassland was dominated by perennial grasses, mainly *Bromus catharticus*, *Cynodon dactylon*, and *Deyeuxia viridiflavescens*. The forest community was dominated by the exotic tree *Ulmus pumila* (~900 stems ha^{-1}), and had a sparse herbaceous layer with *Baccharis pingraea*, *Bromus catharticus*, and *Geranium dissectum*. The study species, *L. multiflorum*, occurred naturally in all three communities. These communities were broadly representative of different seral stages commonly found on arable soils throughout the pampas (Ghersa and León 1999).

Our plots were part of a larger succession study initiated in 1978, in which plant communities established in different years have been annually surveyed to evaluate the potential for grassland restoration on set-aside farmland (Omacini et al. 1995; Tognetti et al. 2010). We are therefore confident that vegetation and environmental differences among plots mostly reflected changes brought about by old-field succession (Table 1). Unfortunately, we could not establish independent replicates of the three successional stages, which thus precludes drawing valid inferences beyond the study plots. Moreover, patterns of E+ versus E− grass performance across plots should be interpreted with caution, because of the potential influence of co-varying factors unrelated to plot age.

Study species

Lolium multiflorum is a cosmopolitan, fast-growing, C_3 annual grass widely naturalised in the Argentine Pampas. It has a ruderal-competitive life strategy, which favours its continued regeneration and persistence in successional habitats (Grime 2001). The species was introduced in the mesic pampas from Europe more than a century ago (Söyrinki 2001), and likely extended its distributional range to the whole region with the expansion of row-crop agriculture and cattle husbandry during the late 1800s (Parodi 1930). Nowadays, Italian ryegrass is a major component of semi-natural grasslands (Gundel et al. 2009) and old-field communities (Omacini et al. 1995; Tognetti et al. 2010), where it can drastically reduce the diversity of co-occurring exotic and native species due to its high competitive ability (McKell et al. 1969; Facelli et al. 1987) and production of a thick litter layer (Chaneton et al. 2001; Grime 2001). Pampean ryegrass populations bear high (>80%) levels of infection by *Neotyphodium occultans* (Moon et al. 2000; Gundel et al. 2009). In annual ryegrasses, fungal hyphae are confined to the shoot base during most of the life cycle and only colonise flowering culms and seeds as the plant enters

Table 1 Environmental and vegetation characteristics of the three old-field successional plots used in the experiments

Plot variable	*n*	Fallow	Grassland	Forest
Time since last crop (years)		1	15	24
Light penetration (%)	20	22.7 ± 5.2 a	13.1 ± 3.2 b	11.6 ± 0.1 b
Soil available N (μg g^{-1})	6	8.14 ± 1.61 a	1.48 ± 0.29 b	NA
Soil available P (μg g^{-1})	10	13.9 ± 0.8 a	4.4 ± 0.3 b	4.5 ± 0.2 b
Soil water content (%)	15	20.0 ± 1.8 c	30.4 ± 2.2 b	40.5 ± 0.6 a
Herbaceous biomass (g m^{-2})	10	377.1 ± 65.8 a	329.2 ± 65.8 a	135.0 ± 44.1 b
Total root biomass (g m^{-2})	10	44.6 ± 9.3 c	302.9 ± 50.6 b	632.1 ± 102.6 a
Forb cover (%)	10	39.5 ± 1.1 a	20.3 ± 0.8 b	13.1 ± 0.6 b
Grass cover (%)	10	12.2 ± 0.5 c	48.3 ± 0.8 a	40.2 ± 1.1 b
Lolium multiflorum (%)	10	20.4 ± 0.7 c	54.5 ± 1.3 b	64.4 ± 1.4 a

Plots occurred within 200 m from each other and shared the same soil type and topographic location, but were established in different years. Data show means ± SE. Cover values were obtained by averaging data over two growing seasons; the relative cover of *Lolium multiflorum* is shown for each plot. Different letters indicate significant differences among plots ($P < 0.05$, LSD tests after one-way ANOVA)

NA Not available

the reproductive phase. Thus endophyte presence is more readily detectable in the seeds (Moon et al. 2000). Endophyte-infected Italian ryegrass produces loline-type alkaloids, which act as feeding deterrents of herbivorous insects (Omacini et al. 2001; Sugawara et al. 2006). Seeds also may contain high alkaloid concentrations (TePaske et al. 1993). Endophyte incidence in Italian ryegrass populations has been reported to rise from 30% in recently abandoned fields to nearly 90% after several years of succession (Vila-Aiub et al. 2005). When grown in pots, infected plants sometimes perform better than endophyte-free conspecifics (Vila-Aiub et al. 2005; Omacini et al. 2006), but no comparable data exist for field populations.

Seed addition experiments

Seeds were obtained from local ryegrass populations with >90% of *Neotyphodium* infection. Seeds with low endophyte incidence (E−) were initially obtained by treating E+ seeds with the fungicide Baytan (triadimenol, 150 g kg^{-1}; Bayer CropScience, Argentina), which reduces infection rates without affecting seed viability (Omacini et al. 2001; Vila-Aiub et al. 2005). These original E+ and E− seed lines were propagated for two generations in separate garden plots maintained at the College of Agronomy, University of Buenos Aires (34°35′S, 58°35′W). Plants were allowed to cross-pollinate freely, which contributed to homogenise the plant genetic background relative to endophyte treatments. Both seed types used were produced under common environmental conditions, being harvested in late spring (December) and stored dry for 3 months before starting the field experiments. Note that we did not attempt to control for host plant genotype but worked with large seed pools drawn from wild ryegrass populations. Hence, any consistent differences between E+ and E− treatments would reflect average effects of endophyte symbiosis on host grass performance.

We tested for germination potential of E+ and E− seeds under laboratory conditions (20–30°C, 8 h light, 14 days, $n = 4$). Total germination did not differ between seed types for 2003 (E+ = 84–85% vs. E− = 71–89%) and 2004 (E+ = 88–96% vs. E− = 80–91%; both $P > 0.10$, *t*-tests, $n = 5$), in agreement with previous reports for the study species (Vila-Aiub et al. 2005; Gundel et al. 2006). Initial levels of endophyte infection were examined microscopically using aniline blue staining for 30 seeds per plant type. Infection rates for E+ and E− seed pools were 94 and 15% (2003), and 92 and 18% (2004), respectively. Therefore, we effectively compared the colonisation potential of ryegrass populations with high versus low endophyte incidence.

Sowing experiments were replicated during April–December 2003 and 2004. Total rainfall for this study season was 749 mm (2003) and 605 mm (2004), being in the range of the long-term average for the area (664 ± 159 mm). In early autumn 2003, we haphazardly selected ten 1-m^2 patches in each successional plot; patches were located at least 5 m apart. In autumn 2004, experimental patches were re-located for the second year. Ryegrass E+ and E− seeds were separately sown at the centre (100 cm^2) of two 15 × 15-cm quadrats placed at opposite corners in each patch. We sowed 2 quadrats × 10 patches × 3 plots, for a total of 60 experimental units per year. Our design assumed that sowing quadrats were independent from each other regarding the dynamics of seedling establishment. This was a valid assumption given that microsite conditions for seedlings may vary at the scale of centimetres (Fenner 1987). Seeds were added at 100 viable seeds per quadrat in mid-autumn, coinciding with the natural peak of ryegrass germination. Before sowing, the top 2 cm of soil was removed from each quadrat to reduce contamination with local seedlings (*L. multiflorum* forms annual seed banks). Overall seedling emergence in 2003 was rather low, presumably due to seed removal by granivorous rodents (personal observation). Thus, in 2004, each quadrat was covered with a 2-mm-mesh cage [20 × 15 × 5 (height) cm] to prevent rodent access during germination (seed predation was evaluated in separate trials, see below). Cages were removed soon after emergence and did not impede seedling growth. Neighbouring plants were allowed to naturally invade and overtop the sowing quadrats during the course of the experiments.

Four weeks after sowing, we counted the number of emerging ryegrass seedlings. Cumulative rainfall during this early period was higher for the second year (2003, 57 mm; 2004, 100 mm). Experiments were terminated in late spring (December) when plants reached reproductive maturity and were setting seed. We counted the final number of vegetative and reproductive tillers (flowering culms) per quadrat; the latter measure was used as a proxy for reproductive output. We noted the presence of any insect herbivores on the plants, but insect numbers and frequency of damaged leaves were too low to warrant analysis. In December, all aboveground material was harvested, sorted by individual plants to assess final recruitment, and oven-dried (60°C, 48 h) to determine biomass production on a quadrat basis. The total aerial biomass was divided by the number of recruited plants to account for changes in mean plant size (g dry mass $plant^{-1}$). In December 2004, we re-checked for endophyte infection in seeds produced by established plants. Thirty seeds were collected from each quadrat and examined under the microscope (Moon et al. 2000). Low seed production in the grassland and forest plots precluded this analysis for 2003.

Seed predation experiment

We tested whether endophyte infection affected seed loss to resident granivores. Seed removal trials were performed in May 2005, during the season of peak rodent activity for the study area. We set up a three-way factorial experiment with endophyte, seed density and successional plot as main factors. Italian ryegrass seeds with low (E− = 2%) or high (E+ = 90%) endophyte infection were presented in 9-cm-diameter dishes filled with sieved soil, at densities of 20 or 100 seeds dish^{-1}. Within each study plot, ten replicates of each endophyte-by-density treatment were randomly located at 5-m intervals forming an 8-row × 5-column grid, for a total of 40 seed dishes per plot (grand total = 120 dishes). Dishes were placed onto the soil in small natural gaps, being surrounded by herbaceous cover. This layout was assumed to allow 'instantaneous' granivory rates to be independently estimated within each successional plot. Seed removal was measured after 3 nights of field exposure during a rainless period. The proportion of intact seeds remaining reflected seed survival per dish; missing as well as damaged seeds were assumed as being lost to granivores. The presence of 'nibbled' seeds (i.e. seeds with scraped or broken glumes) was taken as evidence of predation by rodents (mainly *Akodon azarae* and *Calomys laucha*). A side experiment showed that nibbled seeds were always found in dishes open to rodents, but were absent from dishes only accessible by insects (data not shown).

Environmental measurements

To characterise differences in environmental conditions for plants establishing in contrasting successional plots, in October–December 2003 we measured several microhabitat features in the same patches selected for the sowing experiments. Within each patch, photosynthetically active radiation (PAR) was measured at ground level near the two sowing quadrats ($n = 20$ per plot) using a Li-Cor 188B radiometer (Lambda Instruments, Neb.). In the forest community, readings were taken beneath the shade cast by the tree canopy onto the sowing quadrats. Light penetration (%) was calculated as percentage PAR reaching the soil relative to that recorded under full sunlight (above the herbaceous canopy and away from trees). Soil available N was determined for 10-cm-deep soil cores taken from six patches per plot. Mineral N was extracted with a 2 mol l^{-1} KCl solution and assayed colorimetrically in an elemental analyser (Alpkem, Wilsonville, Ore.). Soil available P was measured for ten patches per plot using the Bray–Kurtz method. Lastly, volumetric soil water content was measured at 15 random points per study plot using a Theta Probe moisture sensor (Delta–T Devices, Cambridge, UK).

In addition, in December 2004, all standing herbaceous biomass was harvested from one 20 × 50-cm quadrat placed at the centre of each patch ($n = 10$). Total plant belowground biomass (including tree roots in the forest) was measured by taking a 6-cm-diameter × 10-cm-deep soil core from each harvest quadrat. All plant material was oven-dried and weighed. In October 2003 and 2004, we estimated plant species cover within each study plot using ten 1-m^2 quadrats (data were pooled over years, $n = 20$).

Statistical analyses

Data from the sowing experiments were analysed separately for each year using split-plot ANOVA, with endophyte infection (two levels) and successional plot (three levels) as main effects. Patches containing paired E+ and E− quadrats were nested within plots as a random effect, and the patch mean square was used as error term to test for differences among plots. Effects of endophyte infection and endophyte × plot interaction were then evaluated against the residual mean square (Steel and Torrie 1980). Plant biomass and number of reproductive tillers were log-transformed to meet the assumptions of ANOVA. Statistical differences in microhabitat conditions among plots were examined through one-way ANOVA. Seed removal data were analysed using generalised linear models (Proc Genmod, SAS Institute 1996). The proportion of seeds removed per dish was modelled assuming binomial errors with the logit-link function. The effect of successional plot, endophyte infection, seed density, and all corresponding interactions were tested through analysis of deviance (Crawley 1993). This analysis was repeated using only the dishes with nibbled seeds, which allowed us to focus on seed loss to rodents.

Results

Site conditions

The three study plots constituted a composite environmental gradient. Initial conditions after cessation of agriculture implied that the fallow field had greater overall resource availability than the grassland and forest plots (Table 1). Light penetration to soil level ($F_{2,57} = 8.65$, $P < 0.001$), soil available N ($F_{1,9} = 40.32$, $P < 0.0002$) and soil available P ($F_{2,27} = 128.4$, $P < 0.0001$) were all significantly higher in the fallow field than in the grassland and forest plots. Topsoil water content, however, increased from the fallow field to the forest plot ($F_{2,42} = 38.13$, $P < 0.0001$).

Herbaceous plant biomass underneath the forest canopy was less than half the aboveground biomass of the grassland and fallow field communities, which did not differ from each other ($F_{2,27} = 6.46$, $P < 0.005$). In contrast, total root biomass increased from the fallow field to the forest plot ($F_{2,27} = 18.07$, $P < 0.0001$; Table 1). Forb species cover decreased with plot age ($F_{2,54} = 19.06$, $P < 0.0001$), whereas grass species cover peaked in the grassland plot ($F_{2,54} = 48.03$, $P < 0.0001$; Table 1). Cover of Italian ryegrass increased from the fallow field, through the grassland to the forest community ($F_{2,54} = 41.39$, $P < 0.0001$), being the dominant herbaceous species in the forest understorey.

Host plant establishment

Seedling emergence in 2003 was low and represented 10–35% of sown seeds, depending on the study plot. Emergence rates in 2004 increased over 60% in all three plots (Fig. 1a, b). In 2003, both the number of emerged seedlings and final density of established plants were highest in the fallow field, intermediate in the grassland, and lowest in the forest (Table 2; Fig. 1a, c). In 2004, seedling emergence and establishment remained lower in the forest but did not differ between fallow field and grassland (Fig. 1b, d).

Endophyte effects on host plant demography depended on the life stage. Endophyte infection did not affect ryegrass seedling emergence (Table 2; Fig. 1a, b). However, endophyte presence significantly increased final ryegrass recruitment in both years, irrespective of study plot (Fig. 1c, d). Although the mean relative effect of endophyte infection on recruitment varied widely among plots (2003, 20–400%; 2004, 14–40%), there was no significant endophyte × plot interaction (Table 2).

The growth performance of establishing ryegrass plants differed strongly among communities (Table 2). Total tiller

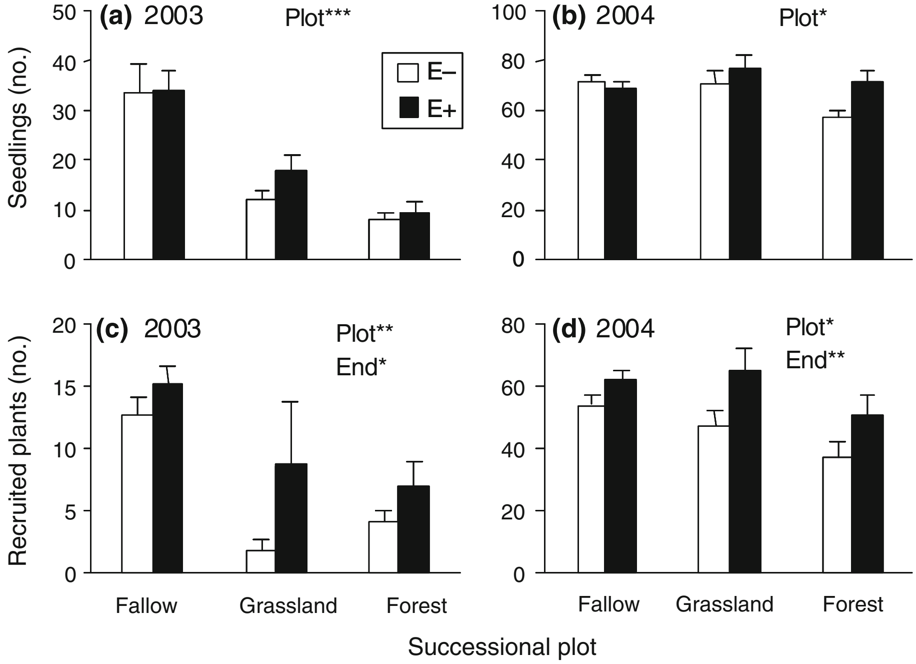


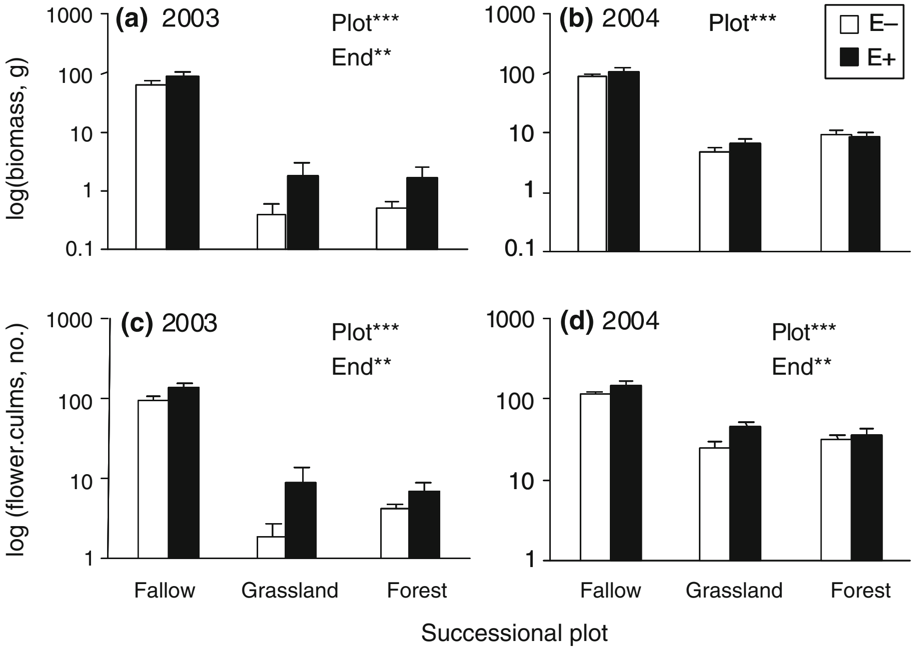
Fig. 1 Effect of fungal endophyte infection on **a**, **b** seedling emergence and **c**, **d** final recruitment of *Lolium multiflorum* in three successional plots, for two growing seasons. *Bars* show means + SE ($n = 10$) for 100-cm^2 quadrats with low (*E*−) or high (*E*+) infection by seed-borne endophytes (*End*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ [significant plot and End effects]

Table 2 Summary of ANOVA results for the effects of endophyte infection on *L. multiflorum* population performance across three contrasting successional plots, for two growing seasons (2003–2004)

	df	Emergence		Recruitment		Biomass		Flower culms	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Experiment 2003									
Plot	2, 27	22.46	**0.0001**	7.13	**0.0033**	173.32	**0.0001**	67.18	**0.0001**
Endophyte	1, 27	1.32	0.2607	5.42	**0.0277**	11.07	**0.0025**	12.14	**0.0017**
Plot × Endophyte	2, 27	0.57	0.5705	0.62	0.5474	0.07	0.9372	2.07	0.1462
Experiment 2004									
Plot	2, 27	3.64	**0.0398**	3.56	**0.0426**	125.10	**0.0001**	43.78	**0.0001**
Endophyte	1, 27	1.44	0.2406	12.04	**0.0018**	0.80	0.3794	9.05	**0.0056**
Plot × Endophyte	2, 27	1.47	0.2482	0.65	0.5312	1.74	0.1948	1.98	0.1575

Significant *P*-values are shown in *bold*

Fig. 2 Effect of End infection on **a**, **b** above-ground biomass production and **c**, **d** density of flowering culms of *L. multiflorum* in three successional plots, for two growing seasons. *Bars* show means + SE ($n = 10$) on log scale, for 100-cm^2 quadrats with E− or E+ infection by seed-borne End. $^{**}P < 0.01$, $^{***}P < 0.001$ (significant plot and End effects). For abbreviations, see Fig. 1



density per quadrat was highest in the fallow field ($P < 0.0001$, for both years) and, as a result, aboveground biomass was an order of magnitude greater in the fallow field than in the grassland and forest plots (Fig. 2a, b). At harvest, most tillers (75–98%) had developed flowering culms and were setting seed; thus, reproductive tiller density also decreased in the older successional plots (Fig. 2c, d).

Endophyte infection increased total tiller density (2003, $F_{1,27} = 12.02$, $P < 0.0018$; 2004, $F_{1,27} = 11.85$, $P = 0.0019$; endophyte × plot $P > 0.10$) and reproductive tiller production in all three communities (Table 2; Fig. 2c, d). The mean relative increase in reproductive output due to endophyte presence ranged 43–185% in 2003 and 15–87% in 2004, but again the endophyte × plot interaction was not significant (Table 2). Tiller numbers per plant did not differ between E+ and E− quadrats ($P > 0.10$). Thus, endophyte effects on total tiller density reflected changes in recruitment success (see Fig. 1c, d) rather than tiller production per plant. On the other hand, the effect of endophyte infection on ryegrass biomass production differed between years (Table 2). In 2003, when plant density was generally low, aboveground biomass was 37–200% higher in E+ than in E− quadrats (Fig. 2a). In contrast, in 2004, endophyte presence had no significant effect on biomass production (Fig. 2b). Endophyte effects on host grass productivity did not statistically differ among successional plots (see Table 2).

Endophyte infection rates

Levels of seed infection by *Neotyphodium* endophytes remained lower for ryegrass plants established in E− than in E+ quadrats (fallow $t = 9.59$, $P < 0.0001$; grassland $t = 5.14$, $P < 0.001$; forest $t = 4.44$, $P = 0.013$). Quadrats sown with E+ seeds maintained high levels of endophyte infection in all three plots (95–98%). Infection rates of E− quadrats in the fallow field averaged 12%, while mean endophyte frequencies in E− quadrats of the grassland and forest plots increased up to 63 and 53%, respectively.

Seed predation

Granivores reduced ryegrass seed survival by 63–94%. Total seed removal resulting from vertebrate and invertebrate granivores varied markedly among study plots ($F_{2,113} = 24.36$, $P < 0.0001$), but did not depend on seed density ($F_{1,113} = 0.17$, $P = 0.68$). Seed removal was highest in the grassland, where all dishes were visited by rodents (as shown by the presence of nibbled seeds), and lowest in the fallow field. Endophyte infection did not affect total seed removal ($F_{1,113} = 0.17$, $P = 0.68$). However, predation rates in dishes containing nibbled seeds were significantly lower for E+ than for E− seeds ($F_{1,77} = 18.52$, $P < 0.0001$; Fig. 3). This pattern was independent of seed density and plot (all interactions $P > 0.10$). Overall, seed removal by rodents was lower in the fallow field compared to the grassland and forest plots ($F_{2,77} = 13.21$, $P < 0.0001$; Fig. 3).

Discussion

Invasion by exotic plant species can be influenced by the presence of mutualistic partners (Richardson et al. 2000) as much as by resident competitors and consumers (Mitchell

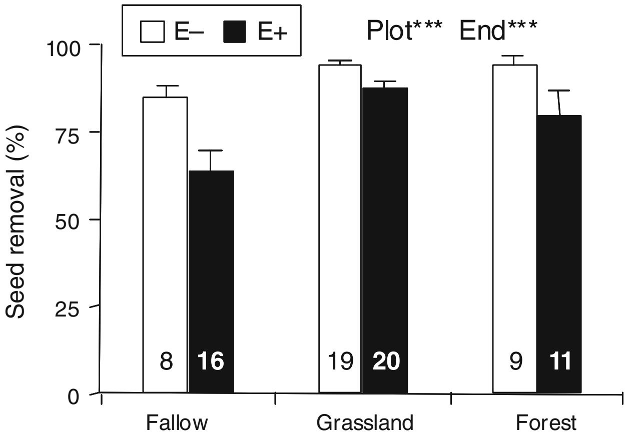


Fig. 3 Effect of End infection on *L. multiflorum* seed removal by rodents. *Bars* show means + SE for seed dishes with E− or E+ End incidence. Only dishes with rodent-nibbled seeds are shown after pooling over seed density treatments (20 or 100 seeds per dish). *Values within bars* indicate number of replicates per treatment. ***$P < 0.001$ (significant plot and End effects). For abbreviations, see Fig. 1

et al. 2006). Recently, several studies have emphasised the role of soil microbial symbionts encountered by exotic plants in the recipient community, which may either prevent or facilitate invasion (Parker 2001; Wolfe and Klironomos 2005; van der Putten et al. 2007; Nuñez et al. 2009). In this study we found that a maternally transmitted, seed-borne fungal endophyte increased the establishment of its host grass across successional communities. Our experiments showed that endophyte infection enhanced the performance of *L. multiflorum* at various life stages, increasing the chances of passing environmental filters that often prevent invasion in natural communities (see Levine et al. 2004; Mitchell et al. 2006). Furthermore, positive endophyte effects were detected in both low and high recruitment years. These findings suggest that plants carrying fungal endophytes may have an advantage over non-infected conspecifics in widely contrasting ecological conditions.

Endophyte effects on host performance shifted from neutral during the emergence phase to generally positive for establishing seedlings and reproductive plants (Figs. 1, 2). Varying germination responses to *Neotyphodium* infection had been reported under laboratory and field conditions for other grass species (e.g. Clay 1987; Faeth and Hamilton 2006) as well as for *L. multiflorum* (Vila-Aiub et al. 2005; Gundel et al. 2006). Indeed, there appears to be little evidence that fungal endophytes may consistently favour their host species through higher seed germination (Cheplick and Faeth 2009). We found, however, a positive endophyte effect on *L. multiflorum* recruitment, which likely reflected the increased survival of infected seedlings after emergence. This pattern remained significant irrespective of differences in overall recruitment between years and study plots (see Fig. 1). In a microcosm study, Omacini et al. (2009) observed that endophyte infection increased *L. multiflorum* recruitment across a range of microsite conditions created by litter deposition and soil water availability. Laboratory studies also showed enhanced establishment of *Lolium arundinaceum* (tall fescue) when infected by fungal endophytes (Clay 1987; Rudgers et al. 2005). Yet comparable evidence from field studies has been extremely rare (Eerens et al. 1998). Faeth and Hamilton (2006) reported lower establishment of E+ relative to E− populations of the perennial *Festuca arizonica*, although their experiment was conducted in a non-competitive field environment.

Putative mechanisms for improved seedling survival of endophyte-infected plants comprise increased competitive ability, resistance to herbivory, and tolerance to nutrient or drought stress (Malinowski and Belesky 2000; Clay and Schardl 2002; Cheplick and Faeth 2009). Infected plants may have greater capacity to tolerate resource shortages during the establishment phase (Rudgers et al. 2005) or to avoid damage by vertebrate or invertebrate herbivores (Clay et al. 2005). Complex interactions between biotic and abiotic factors create the template for the maintenance of intimate mutualisms in natural communities (Stachowicz 2001; Clay 2001; Morris et al. 2007). Thus, benefits from endosymbionts may become mostly apparent under varied field conditions such as those comprised by our old-field chronosequence. There is therefore a pressing need for studies examining the scope of endophyte effects on host population demography within realistic community contexts involving competitors and natural enemies (Clay et al. 1993; Morris et al. 2007).

Total tiller production increased with endophyte infection, an effect often reported for other species and interpreted as a manifestation of a grass–endophyte mutualism (Saikkonen et al. 2004; Cheplick and Faeth 2009). For instance, a more complete occupation of aboveground resource space by vegetative tillers may enhance the competitive ability of endophyte-infected plants (Clay and Schardl 2002; Omacini et al. 2006; but see Faeth et al. 2004). Nevertheless, we found that endophyte infection led to higher biomass accrual of *L. multiflorum* populations in a low recruitment year (2003), but not in a high recruitment year (2004). Study years differed in that 2003 had lower rainfall during the autumn emergence period, and the fact that in 2004 sown seeds were protected from resident granivores, although other factors could determine interannual differences in recruitment. These results suggest that endophyte infection may help to compensate for poor recruitment by increasing biomass production and reproductive output at low population densities (McKell et al. 1969). More generally, it appears that endophyte benefits on host productivity may be density dependent, and hence would be influenced by the level of recruitment limitation determined by biotic or abiotic factors (Omacini et al. 2009). Such density-dependent effects of endosymbiosis could be mostly important during early colonisation stages, when host plant densities should be generally low. Yet, how intraspecific competition affects host demography and the outcome of endophyte–grass symbioses in natural settings is still poorly known (Cheplick and Faeth 2009).

A survey performed at the end of the 2004 experiment revealed that mean endophyte infection rates increased in E− quadrats of the grassland and forest communities. Since we removed the local seed bank before sowing, this trend would have not resulted from quadrat contamination with resident (not sown) E+ seedlings. It must be noted that we initially sowed E− seed sets with ~15% endophyte infection. Given that horizontal transmission of asexual *Neotyphodium*–like endophytes from background vegetation would be unlikely (Schardl et al. 2004), it is conceivable that increased endophyte incidence in E− quadrats reflected the higher performance of E+ over E− plants at different life stages (see Figs. 1, 2, 3). Most importantly, shifts in actual infection frequencies during 2004 might have blurred differences between E+ and E− populations, which suggests that our treatments likely underestimated endophyte effects on *L. multiflorum*, especially in the older successional plots (cf. Clay et al. 2005).

Growing evidence shows that consumers may provide a mechanism of biotic resistance against invasions (Levine et al. 2004; Mitchell et al. 2006). We showed that endophyte presence protected *L. multiflorum* seeds from being preyed upon by rodents. The concentration of fungal alkaloids in grass seeds (TePaske et al. 1993) may deter granivorous consumers, yet few studies have tested this idea (Faeth and Bultman 2002). Importantly, even small differences in seed loss rates could determine that endophyte-infected plants outnumber non-infected conspecifics in the next generation (Gundel et al. 2008; Cheplick and Faeth 2009). Endophyte-induced protection at the seed stage should be most important for host grasses lacking a persistent soil seed bank, and which recruitment in newly invaded sites is seed limited (Turnbull et al. 2000; Levine et al. 2004). This is known to be the case for *L. multiflorum* in early successional communities (Chaneton et al. 2001). Thus, having better defended seeds would have a greater positive impact on host demography in recently disturbed sites, but here evidence in support of this hypothesis was weak. Although the average endophyte effect on seed predation by rodents was higher in the fallow field (Fig. 3), the endophyte-by-site interaction was not significant. Moreover, seed consumers other than rodents apparently did not discriminate between E+ and E− seeds (see also Tibbets and Faeth 1999), and total seed removal was unaffected by endophyte infection. Together with the absence of an endophyte effect on seedling emergence (Fig. 1a, b), these results suggest that differential seed predation may not account for the observed increase in recruitment of infected grass populations.

We hypothesised that positive endophyte effects would be stronger in late successional communities, because these create more restrictive conditions for plant species depending heavily on regeneration from seed (Rejmánek 1989; Grime 2001). This also seemed a reasonable expectation given that the magnitude and direction of endophyte effects are known to depend on the level of environmental stress or resource competition (Ahlholm et al. 2002; Cheplick and Faeth 2009). For instance, endophyte presence enhanced tall fescue establishment more in high than in low diversity species mixtures (Rudgers et al. 2005). In the present study, environmental conditions in the fallow field favoured *L. multiflorum* recruitment and biomass production, whereas in the grassland and forest communities, where light and soil nutrient levels were more limited (Table 1), *L. multiflorum* performed more poorly (Figs. 1, 2). Plant community structure also varied drastically among our successional plots. Nevertheless, we did not find any statistically significant endophyte-by-site interaction that would be consistent with our initial hypothesis. It thus appears that endophyte symbiosis exerted a positive influence on host establishment regardless of large environmental differences among study plots. This finding may help to explain the increase in *L. multiflorum* infection frequencies observed with successional time (Vila-Aiub et al. 2005). Yet, it also implies that the increase in *L. multiflorum* abundance along succession (Facelli et al. 1987; Omacini et al. 1995; Table 1) would not be driven by the relative effect of endosymbiosis in early versus late seral stages.

In conclusion, our study supported the view that maternally inherited endosymbionts can provide an ecological advantage to exotic grass species in invaded systems (Rudgers et al. 2005, 2007). Furthermore, we showed that the association with seed-borne endophytic fungi similarly enhanced host plant fitness across a broad range of successional habitats. Endophytes facilitated an invasive grass host whose persistence and spread in invaded old fields depend on annual regeneration from seed (Chaneton et al. 2001; Grime 2001). Mutualistic endophyte effects occurring at different stages of the host's life cycle, however subtle, may allow the maintenance of high endophyte infection frequencies in natural grass populations, provided the symbiont is efficiently transmitted across generations (Gundel et al. 2008). While novel microbial endophytes are increasingly being discovered in disparate taxa (Arnold 2008; Rudgers et al. 2009), their ecological roles are only beginning to be uncovered. We hope our findings will motivate work in other plant and animal systems to further understand how different types of endosymbionts influence population dynamics and invasion processes.

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