Neotyphodium interactions with a native grass are driven by endophyte haplotype

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Abstract

We examined the effect of endophyte infection, endophyte and host plant maternal genotype on traditional growth parameters. We also measured leaf water potential, leaf rolling, and stomatal density to provide explanations for differences in biomass production and relative growth rates. Our general findings show that *Neotyphodium* infection, *Neotyphodium* haplotype and its interaction with host maternal genotype influence Arizona fescue growth, and biomass production. Endophyte haplotype and its interaction with host maternal genotype is the most critical and consistent factor in influencing growth and physiological outcomes. Endophyte-host interactions are likely to be enormously complex because of the genetic and environmental variation that exists in natural populations. The outcome of these interactions in natural grass-endophyte systems is exceedingly difficult to predict based simply on the presence or absence of the endophyte.

Keywords: growth rate, haplotype, leaf rolling, plant biomass, water potential, stomatal density

Introduction

Increasing evidence suggests that the outcome of the interaction between *Neotyphodium* endophyte and environmental factors depends on the genotype of the host grass and the haplotype of the endophyte (systemic endophytes are haploid) (Saikkonen *et al.* 2004; Müller & Kraus 2005). This dependency is even evident in agronomic grasses, where genetic variation of both host and endophyte are limited due to bottleneck effects and selective breeding.

Far less is known about the influence of *Neotyphodium* infections on wild populations of native grasses than on agronomic grasses (e.g. Faeth 2002). *Festuca arizonica* Vasey (Arizona fescue) is a native perennial bunchgrass, that is widespread in semi-arid ponderosa pine (*Pinus ponderosa*)/grassland communities above 2000 m elevation in the southwest USA (Kearney & Peebles 1960). *Neotyphodium* infection frequencies in wild populations of Arizona fescue are usually high. Surveys show the frequency of infected plants in Arizona fescue populations range from 50-100% (Schulthess & Faeth 1998; Saikkonen *et al.* 1999). Despite high frequencies, however, the infection by asexual *Neotyphodium* does not appear to benefit the host, as predicted for *Neotyphodium* symbionts of tall fescue and ryegrass (Wilkinson & Schardl 1997).

Genetic variation in *Neotyphodium* endophytes also potentially influences interaction outcomes. Different *Neotyphodium* strains that have been intentionally manipulated or transferred to agronomic tall fescue may alter growth and physiological properties of the host (e.g. Assuero *et al.* 2000, 2002). To our knowledge, there have been no tests of how endophyte haplotype in conjunction with host genotype and environmental factors, alters host growth and biomass production, gas exchange, and water relations in native grasses infected with *Neotyphodium*.

We assessed the relative performance in terms of growth and biomass production of uninfected (E-) and *Neotyphodium* infected (E+) plants with two endophyte haplotypes within four maternal genotypes of Arizona fescue. We measured traditional growth analysis parameters, including relative growth rates, above- and below-ground biomass and below-ground:above-ground biomass ratios. We also measured water potential, leaf rolling, and stomatal density to provide explanations for differences in biomass production and relative growth rates. Our purpose here is to incorporate not just infection status but also variation in endophyte haplotypes within hosts and the environment to determine how these factors interact to influence host growth.

Methods

The host plant – *Festuca arizonica*

Festuca arizonica Vasey (Arizona fescue) is a native perennial bunchgrass that is widespread in semi-arid ponderosa pine (*Pinus ponderosa*)/grassland communities above 2000 m elevation in the southwest USA (Kearney & Peebles 1960)). *Neotyphodium* infection frequencies in wild populations of Arizona fescue are usually high.

The endophyte – *Neotyphodium*

Arizona fescue harbours at least three distinct forms of *Neotyphodium*, each likely a unique species (Sullivan & Faeth 2004). Two of the four maternal plants use in our experiments (MD 1 and MD 49) harboured one non-hybrid endophyte haplotype (termed H1) and the other two maternal plants (MD 44 and MD 46) harboured another non-hybrid endophyte haplotype (termed H2). Haplotypes of the experimental plants were confirmed by microsatellite DNA analyses of multiple loci (Sullivan & Faeth 2004).

Seed sources

To test the effect of infection, host maternal genotype and endophyte haplotype and environmental factors on host growth and physiological parameters, we used *Neotyphodium* infected (E+) and uninfected (E-) Arizona fescue seeds from four naturally-infected maternal plants (MD 1, MD 44, MD 46, and MD 49) from the same population at Merritt Draw. Merritt Draw is a drainage meadow on the Mogollon Rim (elevation 2500 m) in Arizona. Maternal plants were randomly selected in 1997 from a pool of about 50 infected plants in the population. To remove the endophyte, maternal plants were split into ramets and half were treated hydroponically with low levels of the fungicide propiconazole while the other half were treated the same except without fungicide (Faeth & Sullivan 2003). All ramets were planted individually into 16 oz cups with native soil (Brolliar stony clay loam) and continually split and re-potted as they grew for about 1 year to provide cloned replicates after hydroponic treatment. The clones were then transplanted to a plot at the Arboretum in Flagstaff in 1998. After growing for 3 years in the field, E+ and E- (endophyte experimentally removed) seeds from each of the four maternal plant genotypes used in this experiment were collected from the clones. Seeds were collected from E- and E+ plants in September 2001, cold-treated for 30 days at 5°C and then stored at room temperature.

Table 1 Summary of ANOVA for the effects of plant genotype (nested within haplotype) and infection status on biomass and growth parameters (R:S = above-ground:below-ground biomass ratio; NAR = net assimilation rate; LAR = leaf area ratio; SLM = specific leaf mass).

Source of variation	df	Total biomass	Above-ground biomass	Below-ground biomass	R:S	RGR	Total leaf area	NAR	LAR	SLM
Infection	1	0.837	0.170	0.264	**<0.001**	0.172	0.066	0.348	**<0.001**	0.386
Genotype (Haplotype)	2	0.073	0.497	**<0.001**	**<0.001**	**<0.001**	0.453	**<0.001**	0.406	**<0.001**
Infection x Genotype (Haplotype)	2	0.693	0.303	0.056	**0.001**	**0.010**	0.819	0.984	0.658	**0.002**
Error	195									

Significant (P<0.05) effects are in bold.

Table 2 Summary of ANOVA of the effects of endophyte haplotype on biomass and growth parameters. (R:S = above-ground to below-ground biomass ratio; NAR = net assimilation rate; LAR = leaf area ratio; SLM = specific leaf mass). Only infected plants were included in these analyses.

Source of variation	df	Total biomass	Above-ground biomass	Below-ground biomass	R:S	RGR	Total leaf area	NAR	LAR	SLM
Haplotype	2	**0.029**	0.418	**0.002**	**0.002**	**<0.001**	0.090	**<0.001**	0.742	0.623
Error	99									

Significant (P<0.05) effects are in bold.

Table 3 Summary of ANOVA for the between-subject effects of plant genotype (nested within haplotypes), and infection status on leaf water potential (Ψ_L), leaf rolling, and stomatal density.

Source of variation	df	Ψ_L	Leaf rolling	Stomatal density
Infection	1	**<0.001**	**0.003**	0.308
Genotype (Haplotype)	2	0.184	**<0.001**	**0.005**
Infection x Genotype (Haplotype)	2	0.467	0.562	0.119
Error	195			

Significant (P<0.05) effects are in bold.

Table 4 Summary of ANOVA for the effects of endophyte haplotype on leaf water potential (Ψ_L), leaf rolling, and stomatal density. Only infected plants were included in these analyses.

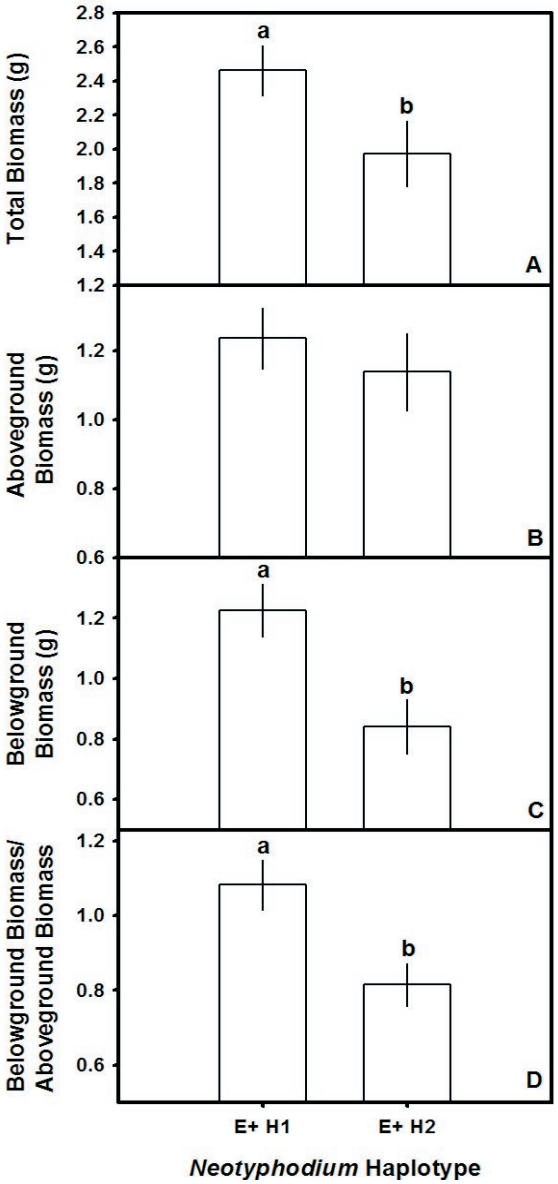
Source of variation	df	Ψ_L	Leaf rolling	Stomatal density
Haplotype	2	**<0.001**	**<0.001**	**<0.001**
Error	99			

Significant (P<0.05) effects are in bold.

The experiment

Neotyphodium infected (E+) and uninfected (E-) Arizona fescue seeds from four infected maternal plants were planted in individual pots. To remove the endophyte, maternal plants were split into ramets and half were treated hydroponically with low levels of the fungicide propiconazole while the other half were treated the same except without fungicide (Faeth & Sullivan 2003). Seeds of four genotypes of Arizona fescue, MD 1, MD 44, MD 46, MD 49 were sown in a 50:50 mix of native soil to potting soil (Super Soil, Supersoil and Rod McLellan Company, San Mateo, CA, USA). Trays were placed in a growth chamber under a 22°/15°C (day/night) temperature regime with an 18 h photoperiod, during which time they received 400 μmol/m^2/s photosynthetically active radiation (PAR) from a combination of cool white florescence tubes (F72T12/CW/VHO, Sylvania, Hart Lighting and Supply Inc., Phoenix, Arizona, USA) and incandescent bulbs (60 W XL, Sylvania, Hart Lighting and Supply Inc., Phoenix, Arizona, USA). Soils were watered to field capacity three times per week and seeds were allowed to germinate. After 21 days seedlings were transplanted from the

Figure 1 Total (A), above-ground (B), below-ground (C) biomass, and below-ground biomass:above-ground biomass ratio (D) of two endophyte haplotypes (H1 and H2) of infected (E+) Arizona fescue. Different letters indicate means are significantly different (P<0.05). Only infected plants are included.



plastic trays into individual square pots (11 x 11 x 11 cm, L x W x H) containing the above native soil/potting soil mixture and allowed to establish for 21 days after which 12 E+ and 12 E- plants were randomly sacrificed for initial above-ground and below-ground biomass.

Plant infection status was confirmed using a modified tissue print immunoblot (Schulthess & Faeth, 1998) at the start of the experiment. Twenty four E+ plants and 24 E- plants were used from each maternal plant genotype in this experiment.

Biomass production and growth

The effect of endophyte infection on biomass production and growth parameters was determined over a 49 day period which began after the 21st day of the establishment period. At the end of the period, plants were divided into roots and above-ground parts, and soil was washed from roots by hand. Specific leaf mass (SLM) was determined on a subsample of each plant (containing about 25% of the total leaves) using a leaf area meter (Decagon Devices, Pullman, WA, USA). Above-ground biomass was placed in an oven at 60°C for 24 h and below-ground biomass was placed in an oven at 105°C for 48 h. Following this drying period above- and below-ground biomass was determined by weighing samples on a balance (Mettler Toledo, Inc., OH, USA). Biomass of E+ or E- plants at the initial harvest were randomly paired with respective E+ or E- plants at the final harvest, and the relative growth rate (RGR; rate of biomass gain per biomass) and net assimilation rate (NAR; rate of biomass gain per leaf area) of each plant was estimated using the equations in Xiong *et al*. (2000), following Hunt (1990). Total leaf area was measured using a leaf area meter (Decagon Devices, Pullman, WA, USA). Leaf area ratio (LAR; leaf area per total plant biomass), leaf mass ratio (LMR; leaf biomass per total plant biomass), and below-ground:above-ground biomass ratio (R:S) were also calculated.

Water potential

Leaf water potential (Ψ_L) was measured on the 42nd day following the start of the experiment using a pressure chamber (Model 1003, PMS, Corvallis, Oregon, USA) on one leaf from each plant. The leaf was cut 6 cm from its tip. Leaf water potential was only measured once per maternal plant genotype because Ψ_L does not vary much throughout consistent water treatments and remains relatively constant (Morse *et al*. 2002).

Leaf rolling and stomatal density

Leaf rolling and stomatal density were measured on the first day of the treatments and on the day before plants were harvested for comparison (data reported only for the last day). The width of one of the longest green leaves on each plant was measured 5 cm from the tip using a caliper holding the mid-vein in the center. This measurement was termed actual leaf width. The leaf was then excised 10 cm from the tip, forcibly unrolled, and the opened leaf width was measured 5 cm from the tip using a caliper and holding the mid-vein in the center. The ratio of actual leaf width to opened leaf width was termed leaf rolling. The same leaf was then used to measure stomatal density using the method of Kubinová (1994). Stomata are only present on the adaxial side of Arizona fescue leaves.

Statistical analyses

Separate analyses of variance (ANOVA) were used to examine the effect of infection (E+ or E-), maternal plant genotype effects on biomass production and growth parameters, Ψ_L, leaf rolling, and stomatal density Plant maternal genotype was a nested factor within endophyte haplotype because each plant half-sib genotype was associated with only one of the two endophyte haplotypes. To assess the effect of endophyte haplotype on biomass production and growth parameters, $_L$, leaf rolling, and stomatal density we conducted separate analyses of variance with only infected plants, because endophyte-removed plants obviously had no endophyte haplotype associated with them.

We also tested for differences in biomass measurements and physiological parameters among maternal plant genotypes when the endophyte was removed to provide an estimation of variation

Figure 2 Relative growth rate (RGR; A), total leaf area (B), net assimilation rate (NAR; C), leaf area ratio (LAR; D), and specific leaf mass (SLM; E) of two endophyte haplotypes (H1 and H2) of infected (E+) Arizona fescue. Different letters indicate means are significantly different (P<0.05). Only infected plants are included.

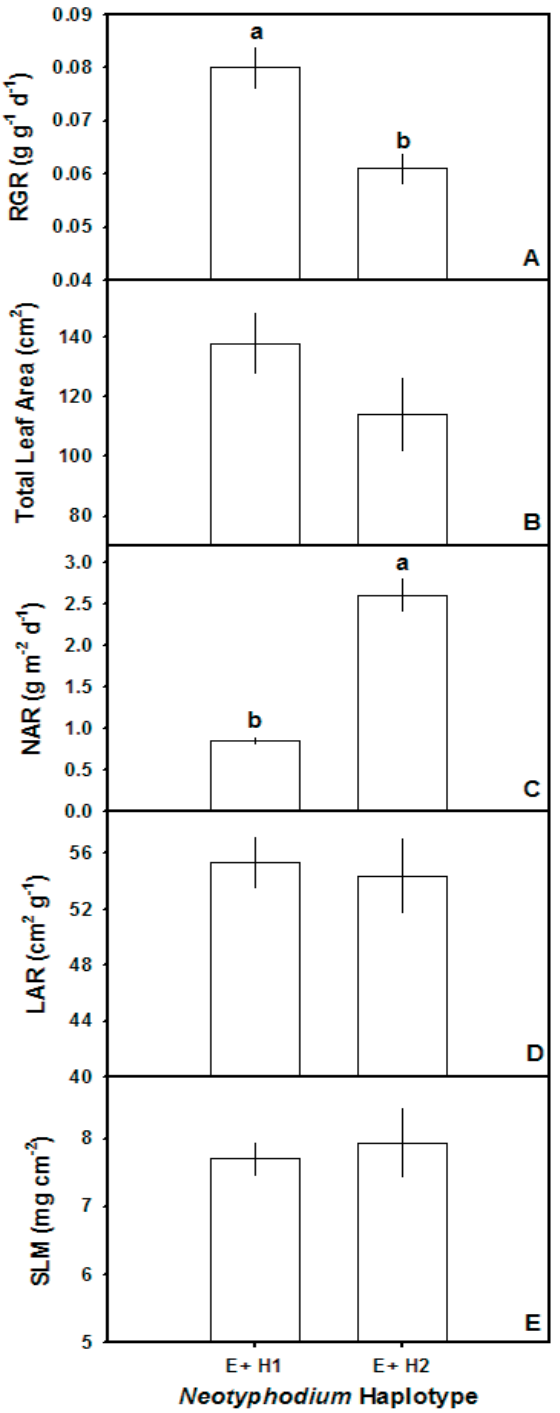
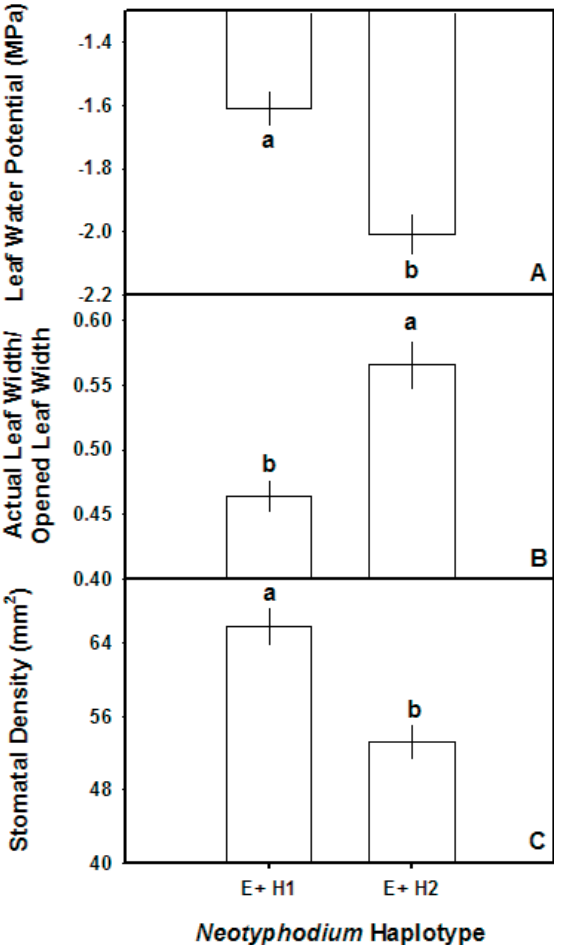


Figure 3 Leaf water potential (A), actual leaf width: opened leaf width (B), and stomatal density (C) of two endophyte haplotypes (H1 and H2) of infected (E+) Arizona fescue. Different letters indicate means are significantly different (P<0.05).



due only to maternal plant genotype without the complicating factors of infection and endophyte haplotype. All data sets satisfied the assumptions of ANOVA based on homogeneity of variances, normality of errors, and independence of errors.

Results

Biomass production and growth

Endophyte haplotype affected total, below-ground biomass and below-ground biomass:above-ground biomass (Table 1, Fig. 1). Furthermore, relative growth rates and net assimilation rate (NAR) differed significantly between the two endophyte haplotypes (Table 2, Fig. 2). In general, plants harbuoring the H1 endophyte haplotype had greater biomass (Fig. 1), net assimilation rate and relative growth rates (Fig. 2) than plants harbouring the H2 endophyte haplotype. Plants infected with the H1 haplotype had greater total leaf area than plants with the H2 haplotype, but this difference was only marginally different (P = 0.09, Fig. 2B). There was a significant endophyte haplotype effect within plant maternal genotype on below-ground biomass, below-ground biomass/above-ground biomass, NAR, and SLM.

Leaf water potential, leaf rolling, and stomatal density

The presence of *Neotyphodium* also affected leaf water potential (Ψ_L)(Table 3). *Neotyphodium* infected plants had less negative Ψ_L than plants with their endophytes removed. Leaf rolling, but not stomatal density, also varied with infection (Table 3). E+ plants showed greater leaf rolling (a lower actual leaf width:opened leaf width ratio) than E- plants.

The two *Neotyphodium* haplotypes also differed in leaf water potential. Infected plants with the H2 haplotypes had more negative Ψ_L than H1 plants (Table 4, Fig. 3A). Leaf rolling and stomatal density also varied with endophyte haplotypes (Table 4, Fig. 3 B,C). Plants harbouring the H1 endophyte rolled leaves more tightly than H2 plants (Fig. 3B), and H1 plants had higher stomatal density than H2 plants (Fig. 3C). There were no significant endophyte effects within maternal genotype (data not shown).

Discussion

In native Arizona fescue, haplotype of the endophyte is the most critical and consistent factor influencing the growth and biomass production of Arizona fescue plants, as well as physiological outcomes such as leaf water potential, leaf rolling, and stomatal density. Endophyte haplotype appears to override infection status, at least in determining growth and some physiological measures. Within infected plants, the strain of endophyte, even within the same population, greatly influences relative performance and physiological aspects of the host plant. In addition, the many complex interactions involving plant maternal genotype and endophyte haplotype, and endophyte infection indicate that the direction and magnitude of *Neotyphodium* interactions with this native host grass, even under laboratory conditions, are highly variable and complex.

Genetic background of the maternal host plants results in additional variation in below-ground biomass accumulation, and allocation to roots and shoots, as indicated by differences among maternal host plant genotypes that are without their endophytes. Host plant genetic variation, even in cultivated grasses, where host genetic variation is reduced relative to wild grasses because of cultivation and selective breeding, can also result in different growth and reproductive performance (e.g. Cheplick & Cho 2003).

Physiological and morphological variation

Consistent with previous physiological studies of *Neotyphodium* infection in Arizona fescue (Morse *et al.* 2002; Morse 2005), Ψ_L was less negative in E+ plants than E- plants and infected plants with H1 endophytes had less negative Ψ_L than plant with H2 endophytes. E+ plants also had more tightly rolled leaves than E- plants regardless of treatment. Rolling leaves more tightly may be the reason for E+ plants maintaining less negative Ψ_L than E- plants. Leaf movements, such as rolling, are common adaptive mechanisms to water stress and drought conditions in plants. These movements help in reducing incident irradiation, leaf temperature, and transpiration (Begg 1980; Ehleringer & Forseth 1980) by decreasing the exposed leaf area and leaf conductance to water vapour (g_s) (Begg 1980), although the effect of leaf rolling on transpiration is dependent on stomatal distribution and on the degree and pattern of stomatal opening in rolled leaves. Rolling prevents water loss but also restricts potential carbon gain and may place the plant at a competitive disadvantage especially if adequate soil water is available. Infected plants tended to produce less above-ground biomass than E- plants regardless of maternal genotype. Likewise, within infected plants, H1 plants rolled leaves more tightly than H2 plants, and also had less negative Ψ_L than H2 plants, further supporting the link between increased leaf rolling and less negative Ψ_L. Endophyte haplotype also affected stomatal density indicating that endophyte haplotypes create additional variation in the host plant response to environmental variation.

The effect of endophyte infection, plant maternal genotype and endophyte haplotype on stomatal density may be a result of gibberellic acid produced by the endophyte, which is the primary hormone involved in stomata formation (Saibo *et al.* 2003). Little is known about the biochemical mechanisms that alter growth and increase stress tolerance in *Neotyphodium* infected plants. De Battista *et al.* (1990) and Yue *et al.* (2001) suggested that auxin (indoleacetic acid; IAA) may play a role in plant growth alterations in infected plants. Joost *et al.* (1993) showed experimentally that endophyte infection altered the physiology of infected plants via hormonal signals, such as abscisic acid, that promote guard cell closure, although one should note that these results have not been replicated. Apparently, endophyte strain also influences levels of plant hormones, as suggested by differences in stomatal density between H1 and H2 endophyte-infected plants. Further research into the area of plant-endophyte interactions at the physiological level could help to elucidate endophyte infection and haplotype effects across different Arizona fescue genotypes.

Our general findings show that *Neotyphodium* haplotype, and to a lesser extent *Neotyphodium* infection and host plant maternal genotype, influence Arizona fescue growth and biomass production. Therefore, it is likely that these factors also influence other interactions with the host grass, such as susceptibility to herbivory and pathogens, inter- and intraspecific competition, and community structure. *Neotyphodium* infections in tall fescue can modify diversity-productivity relationships by increasing the level of competition between non-tall fescue species and tall fescue (Rudgers *et al*. 2004) and alter community and ecosystem properties in old fields (Matthews & Clay 2001; Clay & Holah 1999; Clay *et al.* 2005). Further, Omacini *et al.* (2001) showed that fungal endophyte infection in *Lolium multiflorum* (Italian ryegrass), an agronomic grass, alters food-web structure by disrupting the transfer of energy from plants to upper trophic levels. Extrapolating from agronomic grass studies to natural populations and communities may be far too simplistic, given that agronomic grasses and their endophytes typically have much less genetic diversity due to selective breeding and genetic bottlenecks (Saikkonen *et al.* 2004) and are grown in environments that are less variable than natural populations. With the greater genetic diversity in both the endophyte (e.g. Faeth & Sullivan 2003) and its grass host (e.g. Saikkonen *et al.* 2004) natural populations should exhibit much greater complexities. Based upon our results that endophyte haplotype, plant maternal genotype and environmental factors, in addition to infection, result in highly variable outcomes in terms of host performance and physiology, we conclude it will be extraordinarily difficult to predict effects at the population, community and ecosystem levels.

ACKNOWLEDGMENTS

T. Bender, C. Hayes, A. Jani, R. Overson, S. Strauss, T.J. Sullivan, S. Wittlinger NSF grants DEB 9727020 and 0128343

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