Hybridization in Endophyte Symbionts Alters Host Response to Moisture and Nutrient Treatments

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Abstract:

When a host organism is infected by a symbiont, the resulting symbiotum has a phenotype distinct from uninfected hosts. Genotypic interactions between the partners may increase phenotypic variation of the host at the population level. *Neotyphodium* is an asexual, vertically transmitted endophytic symbiont of grasses often existing in hybrid form. Hybridization in *Neotyphodium* rapidly increases the symbiotum's genomic content and is likely to increase the phenotypic variation of the host. This phenotypic variation is predicted to enhance host performance, especially in stressful environments. We tested this hypothesis by comparing the growth, survival, and resource allocation of hybrid and nonhybrid infected host plants exposed to controlled variation in soil moisture and nutrients. Infection by a hybrid endophyte did not fit our predictions of comparatively higher root and total biomass production under low moisture/low nutrient treatments. Regardless of whether the host was infected by a hybrid or nonhybrid endophyte, both produced significantly higher root/total biomass when both nutrient and moisture were high compared to limited nutrient/moisture treatments. However, infection by hybrid *Neotyphodium* did result in significantly higher total biomass and host survival compared to nonhybrid infected hosts, regardless of treatment. Endophyte hybridization alters host strategies in response to stress by increasing survival in depauperate habitats and thus, potentially increasing the relative long-term host fitness.

Keywords: Symbiont | Neotyphodium | Endophyte | Plants

Article:

Introduction

Genetic diversity at the species level is ecologically critical at population, community, and ecosystem levels [*1–3*]. Ecological heterogeneity can act as a selective force on plant populations requiring plants to possess a high degree of genetic variance or phenotypic plasticity in order to

be successful [*2*, *4*]. Plants can respond to environmental differences such as seasonal changes in climate by shunting resources to favor successful strategies [*5–7*]. Successful phenotypic response(s) to resource availability, herbivory, pathogens, and competition result from specifically effective genotype X environment interactions [*8–11*].

Endophytic infection increases the genetic variation in the host population not only because of the genotypic interactions between host and symbiont but also as a result of the amount of genetic information present in populations containing mosaics of infected and uninfected (E-) hosts [*11–13*]. Additionally, symbionts such as *Neotyphodium* foliar endophytes and some mycorrhizal species hybridize [*14–16*], potentially increasing the genetic complexity of host populations as reviewed in Cheplick and Faeth 2009 [*17*]. As a consequence, fungal endophytes are an important but rarely explored component of genotypic variation.

Hybridization as a proximal cause of speciation as well as adaptation to and invasion of new or marginal habitats is well explored [e.g., *18–24*]. Hybridization events can lead to adaptive responses allowing for expansion into new niches and habitats [*22*, *25*]. For example, fungal hybridization has resulted in host shifts and increased the number of host species susceptible to hybrid versus nonhybrid fungal pathogens [*26–28*]. The ecological consequences of foliar endophyte hybridization have not been well explored [but see *29*] despite the abundance of hybrid endophytes in nature [*30–33*]. Initial research suggests individual populations of Arizona fescue (*Festuca arizonica*) are dominated by *Neotyphodium* infected hosts, hybrid or nonhybrid [*29*, *34*].

Two hypotheses about the quality of symbiosis between a hybrid endophyte and its grass host prevail. First, endophyte hybridization is hypothesized to increase host fitness in stressful environments by shifting the symbiotum closer to the mutualistic end of the symbiosis. Second, fungal hybridization is hypothesized to increase local adaptation to marginal and/or novel habitats thus shifting the interaction toward mutualism but only under a limited set of conditions and as a direct result of plant genotype X fungal genotype X environmental interactions [*17*, *29*, *33*]. Potential causes for these predicted outcomes are: (a) increased allelic diversity contributing to increased production of alkaloid compounds (herbivore deterrents); (b) hybridization acting as a proxy for sexual recombination thereby reducing mutation accumulation in an otherwise asexually producing fungus; and/or (c) increasing the probability of favorable genotypic interactions between host and fungal symbiont [*29*, *33*].

Endophytic fungal infection of plant hosts significantly alters host phenotype under varying ecological pressures, e.g., resistance to drought and nutrient stress [*17*, *35–39*] and increased nutrient uptake in poor soils [*40–44*]. We previously reported high hybrid endophyte infection frequencies among host plants correlated with low soil nutrients and moisture, whereas high infection frequencies by nonhybrid*Neotyphodium* were strongly correlated with higher soil nutrients and positively (albeit slightly) associated with higher soil moisture [*29*, *34*, *45*].

Despite the fact that hybrid (H) infected hosts appear to possess a lower resource requirement than nonhybrid (NH) infected hosts, NH dominate most populations with average frequencies (24 populations compared) typically greater than 55% for NH and less than 15% for H [*34*]. A possible explanation for the distribution of H versus NH infected hosts is increased genomic content associated with H endophytes that may enhance host performance via changes in host physiological or phenological response to drought and/or low soil nutrients. Such changes could be the result from increased gene expression, epistatic interactions, or some other unidentified consequence of elevated genomic content in H *Neotyphodium*.

Sullivan and Faeth [*29*] found H infected hosts produced higher volume/mass ratios in their local habitats but not in NH dominated locations, while NH hosts transplanted to H dominated locations did not produce significantly different plant architecture. They suggest that this may represent a response to light capture since H infected hosts are typically located under dense canopy, whereas, NH infected hosts are not. The relationship between drought and endophyte infection has been well explored, producing conflicting results [*12*, *38*, *40*, *46*, *47*]. Therefore, we asked if the increased genomic content present in H endophytes improves host performance in response to water and/or nutrient stress. While such ecological factors have been examined in populations of native grass systems containing multiple fungal endophyte genotypes [*48*], they have not been explored using controlled, greenhouse experiments.

Methods

Study System

Arizona fescue (*F. arizonica*) is a dominant, perennial native grass in the southwestern USA and is frequently infected by either H or NH *Neotyphodium* endophytes [*29*, *34*, *45*]. *Neotyphodium* is asexual, obligate, and vertically transmitted; hosts remain facultative. Interestingly, most infected plants in Arizona fescue populations are NH [*49*, *50*], contrary to the pattern of relatively more H *Neotyphodium* across other native grass species [*21*, *30*, *33*]. Multiple gene copies indicate *Neotyphodium* hybridization. If isozyme, DNA amplification methods of specific genes (followed by Southern blot) and sequencing produce multiple gene copies; the endophyte is considered an H, while single gene copies indicate a NH *Neotyphodium*species [*21*, *51–53*].

Plant Growth Parameters

To test the prediction that H infected Arizona fescue would perform comparatively better than NH infected hosts under limited moisture and ammonium, Arizona fescue seeds from twenty distinct maternal plants (genotypes) of known infection [*34*] status (ten H and ten NH) were planted in 12 oz cups containing a soilless mix (2:1:1 of forest mulch, vermiculite, and perlite) and watered as needed through germination. Initially, 15–20 seeds of each maternal plant were germinated in the cups [infection status determined by *49*]. Following germination, seedlings were culled from each pot leaving a single plant in the pot's center. Ten distinct maternal plants

X three replicates X two infection types X four treatments resulted in 240 plants initially. A single plant from each maternal plant was randomly selected for height measurements and total biomass. A second randomly selected plant was used to measure recovery in response to drought. The third replicate from each genotype was discarded. Seedlings were watered as needed for a month until experimental treatments began. Plants were then exposed to four treatments: (a) high moisture/high ammonium; (b) high moisture/low ammonium; (c) low moisture/high ammonium; and (d) low moisture/low ammonium. The low ammonium (nutrient) treatment consisted of a "no ammonium addition," the high ammonium (nutrient) treatment was based on a modification of Aber et al. [*54*] resulting in 0.45 g of ammonium nitrate added to each 12 oz cup during the first and fourth week of treatment (total of 0.9 g per cup). Low water treatments received 50 ml of water every 2 days and high water treatments received 100 ml of water every other day; greenhouse temperatures were kept between 18°C and 22°C.

Plant heights were measured by randomly selecting five tillers and averaging their heights to the nearest centimeter (cm) to obtain a single height measure per plant per month. After treatments were begun, measures were then taken every 21 days once seedlings had established, resulting in four measures over a total of 63 days (day 0, day 21, day 42, and day 62). Dry above- and belowground biomass was collected at the end of treatments by separating shoots from roots, carefully washing the roots of the majority of soil, and then drying for 3 days at 50°C. Plants were harvested at 80 days.

To determine if recovery from drought differed based on infection status, a replicate plant was cut 3 cm above the soil line. These plants were subsequently watered as needed for an additional 6 weeks after the 63rd day. The aboveground biomass was then harvested and treated as previously described.

Plant tissue and soil were analyzed for total carbon and nitrogen, but no significant differences were found between plants of different infection status in response to treatments or any combination of factors; results not shown.

Statistical Analysis

Root dry biomass was log-transformed to fit assumptions of normality and homogenous variances; a power analysis for several means [*55*] was run to ensure sufficient statistical power to detect treatment effects. Outliers were removed based on a 98% probability ($p \leq 0.02$) of being an outlier [*56*]. All analyses were completed with STATISTICA (StatSoft Inc. 2004).

To test the hypothesis that infection by H versus NH endophytes alters plant biomass and height measures in response to differences in resource availability (treatments), we used analysis of variance (ANOVA) with the treatments and infection status as fixed effects in a fully factorial design with a Type IV sum of squares. We chose Type IV sum of squares because not all genotypes survived resulting in an unbalanced design. The root weight fraction (RWF) was determined by calculating the ratio of the dry biomass of roots to total dry plant biomass [*5*].

Recovery biomass could not be normalized so the data was analyzed using the nonparametric Kruskal–Wallis ANOVA. This necessitated individual analysis of treatment and infection status because the test is limited to comparisons between two independent groups. We used Tukey's HSD for post hoc comparisons between means.

Host survival in response to treatments and infection status was compared using Cox proportional hazard regression. We used this model because it allows inclusion of multiple factors and because the null hypothesis assumes no difference in hazard probability based on either time or infection status [*57*]. To compare survival based solely on infection status, we used the Gehan–Wilcoxon test designed for two group comparisons of censored data [*58*].

Results

Plant Growth Parameters

Total dry weight biomass was significantly higher for H compared to NH infected hosts compared across all treatments (Table 1 and Fig. 1). There was no significant interaction between infection status and the two treatments with regard to total biomass. High moisture/high nutrient treatments produced more biomass overall, while low moisture/low nutrient treatments produced less total biomass regardless of infection status (ANOVA; $F_{(1,66)} = 8.969$, $p = 0.004$). Shoot and root biomass considered individually produced similar results with overall root and shoot biomass responding significantly to moisture and nutrient treatments and H plants producing more shoot biomass than NH plants (Table 1). However, for RWF, there was a significant interaction between treatment type (moisture/nutrient) and host infection status (Table 1 and Fig. 2). Under high nutrients, both H and NH infected hosts did not respond significantly to moisture treatments (Fig. 2). However, under the low nutrient treatment H and NH plants produced different responses to moisture treatments (ANOVA; $F_{(1,65)} = 3.764$, $p = 0.057$). These differences are not significant (Tukey's HSD; $p \geq 0.05$), but NH infected host produced a lower RWF under high versus low moisture (Tukey's HSD; $p = 0.206$) while H infected hosts produced a higher RWF under high versus low moisture (Tukey's HSD; $p = 0.573$).

Table 1. Results for dry weight biomass measures. Mixed effects ANOVA results for dry root weight, shoot, total, and root to shoot biomass measures

Effect	**Df**	***F***	***p***
Root weight fraction (root: total biomass)			
Infect	1	1.25	0.269
Moist	1	0.67	0.417
Nutrient	1	3.03	0.088

Infect × Moist[a]	1[a]	4.52[a]	0.038[a]
Infect × Nutrient[a]	1[a]	4.18[a]	0.045[a]
Moist × Nutrient	1	0.18	0.673
Infect × Moist × Nutrient[a]	1[a]	4.53[a]	0.037[a]
Error df	61		
Total biomass[a]			
Infect[a]	1[a]	6.012[a]	0.017[a]
Moist[a]	1[a]	36.086[a]	<0.0001[a]
Nutrient[a]	1[a]	8.567[a]	0.005[a]
Infect × Moist	1	0.0142	0.906
Infect × Nutrient	1	0.348	0.557
Moist × Nutrient	1	0.204	0.653
Infect × Moist × Nutrient	1	2.041	0.158
Error df	61		
Shoot biomass[a]			
Infect[a]	1[a]	8.189[a]	0.006[a]
Moist[a]	1[a]	43.234[a]	<0.001[a]
Nutrient[a]	1[a]	28.409[a]	<0.001[a]
Infect × Moist	1	1.572	0.215
Infect × Nutrient	1	2.486	0.120
Moist × Nutrient	1	2.327	0.132
Infect × Moist × Nutrient	1	0.053	0.819
Error df	61		
Root: shoot biomass[a]			
Infect	1	1.072	0.305

Moist	1	0.511	0.478
Nutrient[a]	1[a]	3.653[a]	0.061[a]
Infect × Moist[a]	1[a]	5.507[a]	0.028[a]
Infect × Nutrient[a]	1[a]	3.885[a]	0.053[a]
Moist × Nutrient	1	0.116	0.735
Infect × Moist × Nutrient[a]	1[a]	5.092[a]	0.028[a]
Error df	61		

Treatments were low and high moisture (Moist) and low and high nutrient; fully factorial with infection (Infect) status. Significant and nearly significant effects are indicated in bold

[a]Significant and nearly significant effects

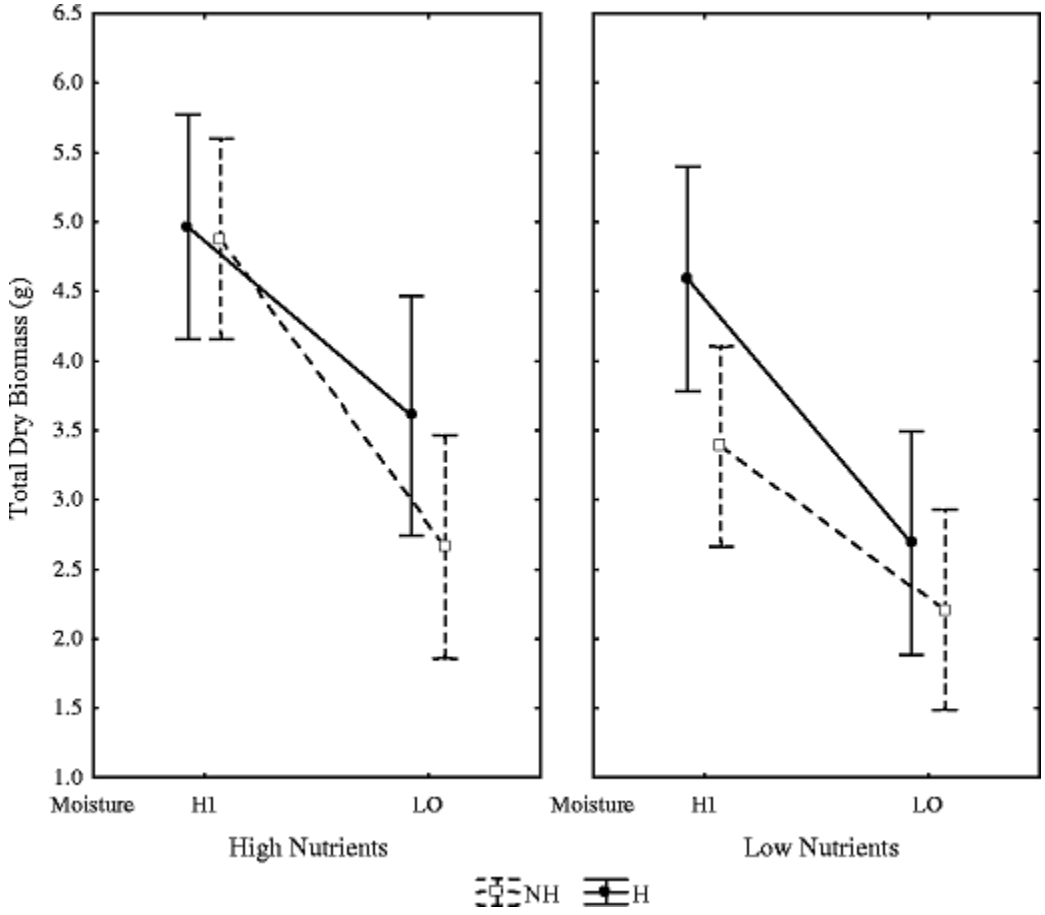

Figure 1 Average total biomass production (*dry*) of H and NH infected Arizona fescue in response to four moisture/nutrient treatments; high (*HI*) moisture/high nutrient, low (*LO*) moisture/high nutrient, high moisture/low nutrient, and low moisture/low nutrient. *Bars* indicate 95% confidence intervals. Significant differences (ANOVA, Tukey's HSD test, $p < 0.05$) include

the following pairs: (a) within H infected hosts under the high nutrient, in response to moisture treatments; (b) within H infected hosts under the low nutrient treatments, in response to moisture treatments; and (c) within NH infected hosts under high nutrient in response to moisture treatments

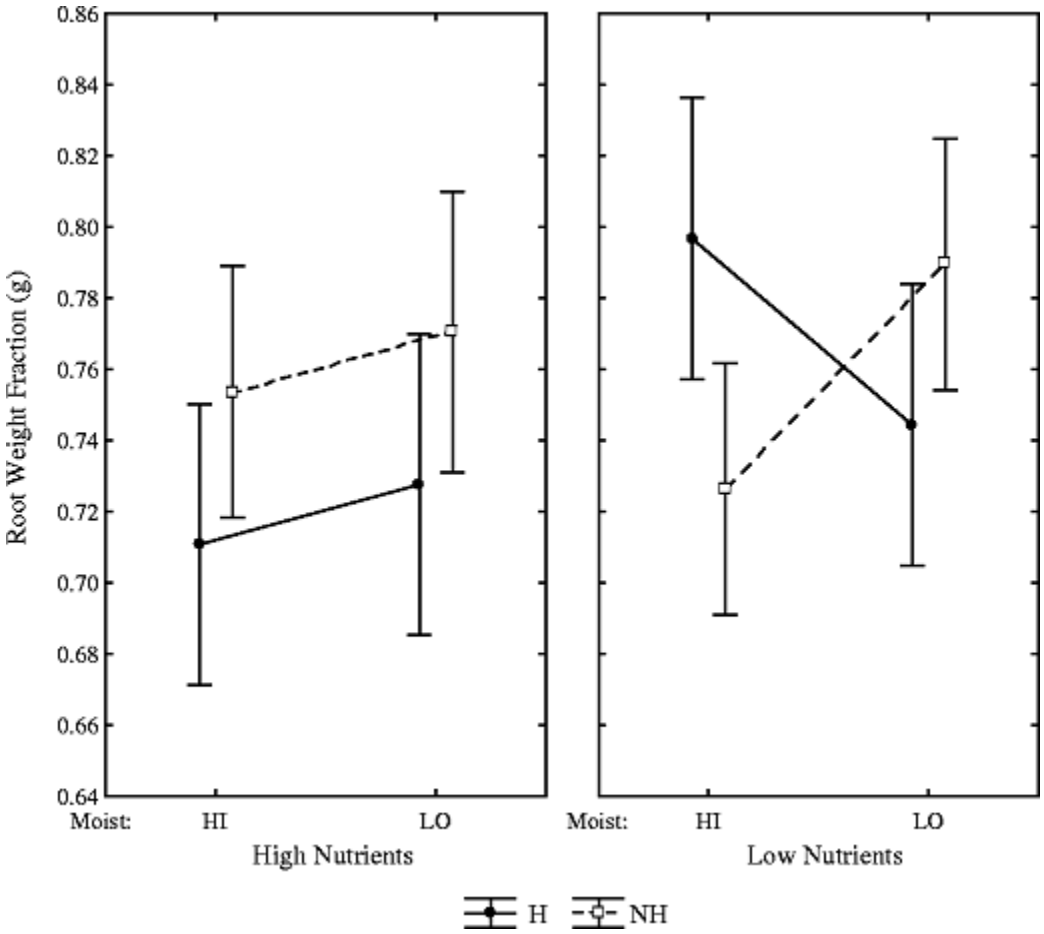

Figure 2 Average root weight fraction determined by the ratio of the root to total dry biomass weights in response to four moisture/nutrient treatments (see Fig. 1). *Bars* indicate 95% confidence intervals

Plant Recovery

The amount of aboveground dry biomass produced in recovery was not significantly different in response to either host infection status or nutrient treatments (Kruskal–Wallis test; $H = 0.005$, $p = 0.945$ and $H = 0.245$, $p = 0.620$, respectively). Exposure to previous water treatments did result in significant differences in plant recovery (Kruskal–Wallis test; $H = 5.900$, $p = 0.015$). As might be expected, previous exposure to high moisture treatments produced significantly more biomass (recovery) compared to plants previously exposed to the low moisture treatments (Kruskal–Wallis test; $H = 5.90$, $p = 0.015$; Fig. 3).

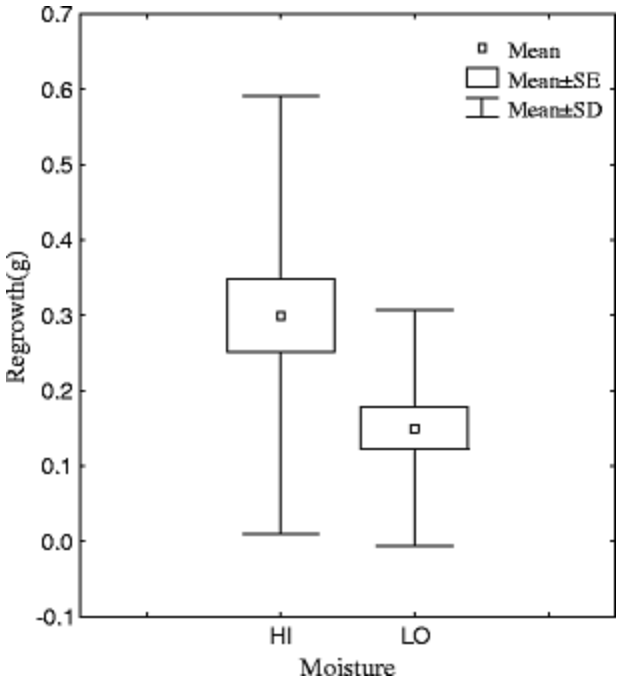

Figure 3 Average shoot biomass produced after plants were cut back to 3 cm heights (above soil surface) in response to previous moisture treatments, high (*HI*) and low (*LO*). *Large boxes* indicate standard errors and*spreads* are the standard deviation. Kruskal–Wallis test indicates a significant difference between previous moisture treatments ($H_{1,68}$ SS = 5.90, $p = 0.015$)

Survival Analysis

Survival was not significantly different between H and NH infected hosts within treatments ($X^2 = 3.132$, df = 4, $p = 0.372$). When considering infection status alone, H infected plants survived significantly better than NH (Gehan–Wilcoxon's test = 2.142, df = 56, $p = 0.032$). At the time of harvest, H had 77.9% survival compared to NH with 67.3% (Fig. 4).

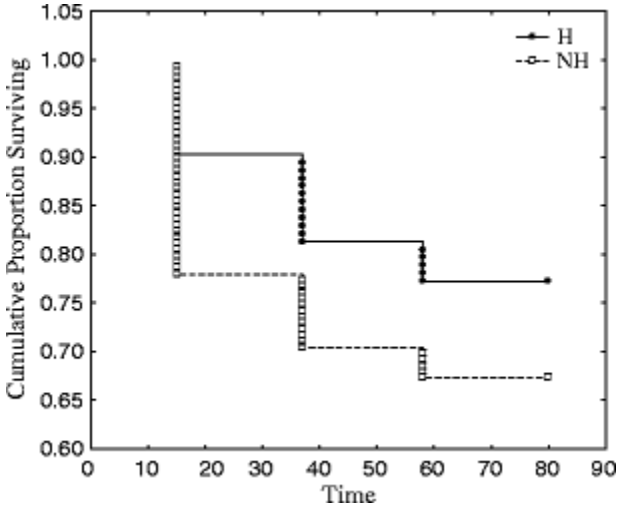

Figure 4 Cumulative survival of hybrid (*H*) and nonhybrid (*NH*) infected Arizona fescue over the course of 63 days averaged across all four treatments (see Fig. 1)

Discussion

Infection by *Neotyphodium* hybrid did not fit our predictions of increased host performance under the low moisture/low nutrient treatments. However, H *Neotyphodium* infection did result in significantly higher total biomass weights and host survival regardless of treatment. In addition, infection status altered RWF in response to moisture treatments when the soil nutrient was also limited. This suggests that infection by H versus NH endophytes alters host resource allocation to root versus shoot biomass in response to available soil moisture. Finally, host recovery was not significantly different as a result of endophyte infection status and instead, was only indicative of previous moisture treatments. Cumulatively, these data suggest that H infected hosts produce a unique response to resource stress in terms of allocation to above- versus belowground biomass and additionally increase host plant survival regardless of ecological conditions.

The greenhouse experiments reported here were designed to identify potential causes for the distribution patterns found in situ and test theoretical predictions about host response to H *Neotyphodium.* Our original predictions were that H infected hosts would outperform (higher biomass and better recovery) NH infected hosts under the most limited resource conditions. Instead, H plants tended to produce higher total biomass than NH plants and had significantly better survival when compared across all treatments (Table 1, Figs. 1, and 4). This, in combination with differences in above- versus belowground allocation strategy, may provide H infected hosts with greater relative, lifetime reproductive output.

Increased aboveground biomass production by H in the low moisture/low nutrient treatments is consistent with previous research indicating H dominated populations may be light limited [*29*]. Sullivan and Faeth [*29*] reported different plant architecture when comparing H and NH Arizona fescue. They suggested that these differences may result from the predominance of H plants under comparatively dense canopy. Under such conditions, plants are predicted to preferentially allocate carbon resources to aboveground growth as a means of increasing light interception [*5*]. The resulting increase in photosynthetic activity increases host survival and lifetime fitness [*49, 59*]. Our results support this hypothesis. Thus, NH infected hosts do not invade H dominated habitats because they are unable to successfully compete for limited light levels [*29*]. In contrast, H infected hosts are not pervasive despite their ability to inhabit marginal and presumably lower resource environments, perhaps because NH are better soil resource competitors than H infected hosts [*60*].

Significantly higher root production by NH infected hosts under low moisture/low nutrient treatments may be indicative of an adaptive response to higher light levels and competition [*5*]. High solar irradiation reduces photosynthetic efficiency and can be especially costly to plants in

arid environments where high light levels also mean lower ambient humidity and higher leaf temperatures [*7*]. Previously, we reported H dominated habitats had significantly lower understory abundances than NH dominated locations [*34*]. Increased root production by NH infected hosts could reduce light damage to leaf tissue and be advantageous when soil resources are low and plant competition is high, resulting in local adaptations [*34*, *61*].

There is also the possibility that fungal pathogens limit the distribution of H infected hosts. We found H infected hosts produced significantly more morphotypes from leaves than either uninfected or NH infected hosts (C.E. Hamilton, unpublished data). Since NH are correlated with comparatively higher soil moisture and understory abundance [*29*, *34*, *45*], microhabitats favorable to other fungi may exist in locations dominated by NH Arizona fescue. Additional indirect support comes from work by Faeth [*11*] who reported increased herbivore abundances on NH infected hosts (compared to E-). He hypothesized that herbivores might be responding to the increased water content of NH infected hosts. Increased host tissue moisture content would be attractive to both herbivores and pathogenic fungi.

To determine if our results support the above hypotheses and to differentiate between a mutualistic versus antagonistic interaction between H *Neotyphodium* requires quantification of H, NH, and E-host fitness over time spans sufficient to encapsulate the host's life span. What we have shown is that endophyte hybridization can profoundly alter host phenotypes at the population level. This is the first study to document changes in host allocation to above- versus belowground tissue production as a result of infection by H versus NH endophytes.

The genetic diversity of native grass hosts in these systems has not been tested but is assumed to be diverse due to obligate outcrossing. Testing this assumption and identifying the direct phenotypic consequences of unique host genotype X, fungal genotype interactions is warranted. Previous genotypic interactions between hosts and endophytes induce significant symbiotum phenotypic responses such as variation in alkaloid production and drought response, as reviewed in Cheplick and Faeth [*17*]. Including identification of host and fungal genetic diversity in future research will provide insights as to the quality of selection pressure experienced in response to unique ecological pressures at the population level. Determining the amount (if any) of host population isolation and the resulting degree of inbreeding will also aid in determining metapopulation dynamics influencing the symbiotic outcome [*2*, *62*].

Fungal endophytes are known to alter the host phenotype in a myriad of ways including but not limited to changes in herbivory, competitive ability, growth rate, and survival [*9*, *11*, *17*, *63*, *64*]. Previous research documents differences in host resource allocation as a result of endophyte infection [*11*, *64*]. Our results corroborate these experiments and support the hypothesis that symbiotic interactions produce uniquely adapted host-fungal complexes possibly via extended phenotypes [*11*, *17*, *65*].

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