

Does Fire Maintain Symbiotic, Fungal Endophyte Infections in Native Grasses?

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Abstract

Systemic endophytic fungi in agronomic and turf grasses are well known for conferring increased resistance to herbivores and to abiotic stresses, such as drought, and increasing competitive abilities. Many native grasses also harbor high frequencies of the asexual and vertically-transmitted endophyte, *Neotyphodium*. In *Festuca arizonica* (Arizona fescue), frequency of *Neotyphodium*-infected plants is often high, but variable, within and among populations. However, *Neotyphodium* infections in native Arizona fescue, unlike infected agronomic grasses, do not increase resistance to herbivores, do not generally increase drought resistance and do not increase competitive abilities. Native grasses, however are often subjected to another important abiotic stress, fire, which is not normally associated with turf and pasture grasses. Endophyte-related resistance to frequent fires is an alternative explanation for the maintenance of high frequencies of this endophyte in native grasses. We tested the hypothesis that infection by *Neotyphodium* increases resistance to the direct and indirect effects of fire.

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We tested the fire hypothesis in several ways. First, we determined patterns of *Neotyphodium* frequency in two long-term, prescribed fire sites, each with replicated plots of varying fire intervals and intensities. Second, we conducted germination experiments with infected (E+) and uninfected (E-) seeds in the laboratory under different temperature regimes that simulated the direct effects of fire and in a field experiment during a prescribed fire. Third, we performed a field experiment comparing survival of E+ and E- Arizona fescue plants after a prescribed burn.

Our results do not support the hypothesis that *Neotyphodium* increases resistance to fire. Although infection levels were variable in long-term prescribed burn plots, endophyte infection frequency was not related to fire frequency or intensity. Contrary to our predictions, E+ seeds had lower germination rates than E- seeds in the laboratory-simulated temperature experiment. Likewise, E+ seeds in the field experiment did not have greater germination rates than E- seeds after a prescribed burn. Finally, E+ plants did not survive better than E- plants after a fire.

High levels of *Neotyphodium* infection in the native grass Arizona fescue do not appear related to fire as an abiotic stress. We propose other explanations for the maintenance of high levels of *Neotyphodium* infections in this native grass.

Keywords: Arizona fescue, endophytic fungi, *Festuca arizonica*, fire, mutualism, *Neotyphodium*, stress, symbiosis

1. Introduction

Fungal endophytes live internally, intercellularly and usually asymptotically within plants and are thought to interact mutualistically with their hosts (e.g., Clay, 1988; 1990; Schardl and Clay, 1997). Symbiotic systemic endophytes, such as *Epichloë*, and its closely-related anamorph, *Neotyphodium*, are well known for increasing fitness of their agronomic grass hosts in two basic ways. First, systemic endophytes may reduce or deter herbivory by producing alkaloids (Clay, 1988; 1990; Breen, 1994; Bush et al., 1997; Schardl and Phillips, 1997; Saikkonen et al., 1998). Second, they may also increase resistance to abiotic stresses such as drought and flooding, especially in tall fescue (e.g., West, 1994; Buck et al., 1997), as well as increasing nutrient uptake in poor soils (Belesky and Malinowski, 1999; Malinowski and Belesky, 1999). In return, the host plant provides a refuge and photosynthate to the endophyte.

The systemic endophytes in the genus *Neotyphodium* (formerly *Acremonium*, Glenn et al., 1996), which form symbioses with pooid grass species, are extreme examples of the mutualism. *Neotyphodium* endophytes, clavicipitaceous members of the Ascomycetes, are found only in the asexual stage, and transmission is always vertically, from maternal plant to seed (e.g., Wilkinson and Schardl, 1997; Schardl and Phillips, 1997). Alternatively, the systemic *Epichloë* can either be transmitted vertically via seeds or horizontally by producing stromata in grass

inflorescences that produce disease conditions (choke panicle) and both asexual and sexual spores (Bucheli and Leuchtman, 1996). As predicted by evolutionary theory (Law, 1985; Massad, 1987; Frank, 1995). *Epichloë* that are transmitted vertically are more mutualistic relative to the host grass than are horizontally transmitted ones, which are more likely to be parasitic (Bucheli and Leuchtman, 1996; Schardl et al., 1997). Since *Neotyphodium* is strictly asexual and vertically transmitted, similar to cytoplasmic organelles (Siegel and Schardl, 1992; Schardl and Tsai, 1992; but see White et al., 1996), one therefore expects strong mutualistic interactions with its host grass (Law, 1985; Massad, 1987; Frank, 1995).

Unlike *Neotyphodium* in agronomic grasses, however, systemic and seed-borne endophytes in native grasses do not always provide unequivocal benefits to their host grasses (Saikkonen et al., 1998; Faeth, 2002; Faeth and Bultman, 2002). About nine *Neotyphodium*-infected native grasses are reported as toxic to livestock (Faeth and Bultman, 2002; Faeth, 2002), at least in some populations (e.g., Jones et al., 2000), but other E+ native grass populations and species appear highly palatable to livestock and native grazers (e.g., Vinton et al., 2001; Nan and Li, 2001; Faeth, 2002). Similarly, resistance to invertebrate herbivores via systemic endophytes in native grasses is often inconsistent (Clay and Brown, 1997; Saikkonen et al., 1998) or absent (e.g., Lopez et al., 1995; Saikkonen et al., 1999; Tibbets and Faeth, 1999). Increasing evidence also suggests that *Neotyphodium* in native grasses do not always provide benefits through increased resistance to drought or enhanced competitive abilities (Ahlholm et al., 2002; Faeth and Bultman, 2002; Faeth and Fagan, 2002).

The dearth of endophyte-related benefits to the host is especially puzzling in Arizona fescue (*Festuca arizonica* Vasey). Infection frequencies are relatively high, but variable, among populations in the southwestern USA (60–100% infectivity, Schulthess and Faeth, 1998; Saikkonen et al., 1999). High infection frequencies have been used as *de facto* evidence of mutualistic interactions with grasses (Clay, 1998) because asexual *Neotyphodium* is viewed as strictly vertically transmitted. *Neotyphodium*, alternatively, may be 'lost' from plants and seeds by either imperfect transmission (*sensu* Ravel et al., 1997) or hyphal inviability (Siegel et al., 1984), but never gained. Hence, infection frequency should decline with time, unless E+ plants and seeds have some fitness advantage over E- plants (Wilkinson et al., 1997). Yet, *Neotyphodium* infections in Arizona fescue do not increase, but rather decrease, resistance to native (deer and elk) and domestic (cattle) vertebrates (Saikkonen et al., 1999), and a suite of native and pest insects (Lopez et al., 1995; Tibbets and Faeth, 1999; Saikkonen et al., 1999), and root-feeders and seed predators (Faeth and Sullivan, submitted). Moreover, infection decreases competitive abilities (Faeth, Helander and Saikkonen, submitted) and decreases growth and reproduction in most resource environments (Faeth and

Fagan, 2002; Morse et al., 2002; Faeth and Sullivan, submitted). Finally, *Neotyphodium* infections in Arizona fescue do not increase resistance to either seed or seedling pathogens, and does not increase germination success or inhibit germination of other seeds via allelopathy (Faeth et al., 2001; Hamilton and Faeth, unpublished data). Overall, *Neotyphodium* infections in Arizona fescue seemingly interact either antagonistically or neutrally, at least relative to conventional hypotheses about benefits of asexual endophytes to their hosts.

Faeth and Bultman (2002) proposed another hypothesis, the fire resistance hypothesis, to explain the high endophyte frequencies in natural grass populations, especially those inhabiting semi-arid habitats. This hypothesis proposes that the endophytes increase host resistance to fire, a prominent abiotic stress in many natural grasslands. Fire is a regular and dominant feature of most natural grasslands (e.g., Knapp et al., 1998) but is often absent or reduced in cultivated turf fields and pastures. Fire is well-known for influencing plant (e.g., D'Antonio and Vitousek, 1992; Whelan, 1995; Bond and van Wilgen, 1996) and microbial (e.g., mycorrhizae and pathogens, Dickman and Cook, 1989; Taylor, 1991; Johnson, 1995) abundances and species composition. Because *Neotyphodium* alters physiology and morphology of leaves, roots and seeds in their grass hosts (e.g., Clay, 1990; 1998; Richardson et al., 1993; Elmi and West, 1995; Belesky and Malinowski, 1999), infection may also decrease susceptibility, or conversely, increase tolerance or resistance, to the direct or indirect effects of fire (Faeth and Bultman, 2002). For example, *Neotyphodium* endophytes are well known for altering water and growth relationships of their host-grasses (e.g., Bacon, 1993; West et al., 1993; Elbersen and West, 1996), and susceptibility of plant tissues to fire may be directly related to their water content and growth patterns (Bond and van Wilgen, 1996). Protecting the host, either at the seed, seedling or adult stage, against negative effects of frequent fires, should result in concomitant increases in the fitness of the endophytic symbiont.

The fire resistance hypothesis is based upon several arguments and previous observations. First, Arizona fescue inhabits semi-arid Ponderosa pine-grassland habitats in the southwest USA that have evolved with very frequent (every 2-5 yrs.), low intensity fires (Cooper, 1960; Dieterich, 1980; Covington and Moore, 1992; 1994). Indeed, like many other grasslands, frequent fires largely maintains these Ponderosa pine-bunchgrass habitats (Covington and Moore, 1992; 1994; Sackett et al., 1995). Historically, fires occurred in June and early July, just before or during summer rains (Cooper, 1960), the period of rapid growth, flower and seed production and germination of Arizona fescue. Periodic fires thus represent a potent selective force on Arizona fescue plants through direct combustion of aboveground tissues and seeds and indirectly through heat that may kill above and below ground tissues and seeds, as well as altering light availability and soil moisture and nutrients (e.g., Tyler and D'Antonio, 1995; Bond and van Wilgen,

1996). Second, our observations indicate that *Neotyphodium starrii* in Arizona fescue resists loss of viability from heat and dry storage, suggesting that the endophyte itself may be adapted to the effects of fire. For example, *Neotyphodium* persists (remains viable and grows into seedlings) in Arizona fescue seeds after heating for 72 hrs at 50°C or when storing at room temperature for up to three years (Faeth, personal observation). This is in contrast to *Neotyphodium* in tall fescue that is naturally found, and is now cultivated, in cooler and moister habitats that are not prone to fire (Saikkonen et al., 2000); in tall fescue, the endophyte is lost after storing seed at 21°C for 7 months or heating seeds to 49°C for several days (Siegel et al., 1984). Third, preliminary sampling of prescribed fire plots at one of our study sites, Limestone Flats, showed that infection frequency was lower in four yr burn plots (79% infectivity) than in burn plots at one (93% infectivity) or two (94% infectivity) yr intervals, suggesting that more frequent fires maintain higher infection levels.

To test the fire resistance hypothesis, we first determined more extensive patterns of infection in long-term, prescribed fire sites with varying intervals of fire. We also simulated the indirect effects of fire (heat) on germination success of infected (hereafter, E+) and uninfected (hereafter, E-) Arizona fescue seeds in the laboratory. We also compared germination success of E+ and E- seeds under conditions of an actual fire in the field. Lastly, in a field experiment, we determined survival of E+ and E- Arizona fescue plants when subjected to a fire.

2. Materials and Methods

The study system

Festuca arizonica Vasey (subfamily Pooideae) is a perennial bunch grass that reproduces by seed (USDA 1988) and is native to the southwestern US. In Arizona, it is the dominant grass in Ponderosa pine-grassland habitats above 2000 m elevation (Kearney and Peebles, 1960). Arizona fescue is an important forage grass for both native elk and deer and introduced livestock. Lifespan is 5–10 years and seed production begins within two growing seasons after germination, at least under ideal conditions (Saikkonen et al., 1999). *Neotyphodium starrii* is common in Arizona fescue populations at all study sites (Schulthess and Faeth, 1998). The taxonomy of *Neotyphodium* (formerly *Acremonium*, Glenn et al., 1996) is "unsettled" and two species have been described from Arizona fescue (White et al., 1993). However, there appears to be at least three independent evolutionary origins from teleomorphic (sexual) *Epichloë* ancestors (White et al., 1993; An et al., 1992; Tsai et al., 1994; Schardl, personal communication). *Neotyphodium* in Arizona fescue from Arizona localities most closely resembles *N. starrii* (Sullivan and Faeth, 2001) and we use that convention here.

Prescribed fire plots

To determine the relationship between fire and endophyte frequencies, we randomly sampled Arizona fescue plants for *Neotyphodium* infection from replicated prescribed fire plots within two prescribed fire research sites (Chimney Spring and Limestone Flats) in central Arizona, over a two year period (1997–1999). In 1997, we began by sampling one, two, and four year plots at Limestone Flats. In subsequent years, we sampled remaining plots at both Chimney Spring and Limestone Flats. The plots were established as experimental prescribed fire areas in 1976 and 1977 (see Sackett, 1980 for details), respectively, at which time all plots were randomly assigned a fire frequency and then all but control plots were burned (Sackett, 1980). The three control (no fire) plots at each site had not been burned since the last natural fire (Limestone Flats in 1908; Chimney Spring in 1876). Both sites were free from commercial logging and grazing by domestic livestock that has been prevented for several decades (Sackett, 1980).

At each site, we removed tissue and seed samples from at least 50 plants from each of three replicate plots of varying fire frequencies [1, 2, 4, 6, 8 yrs and control (no burn)], with the exception of the 6 yr. burn plots at Chimney Spring. The total number of plants sampled was thus at least 900 at Limestone Flats (6 burn frequencies $\times$ 3 replicates $\times$ 50) and at least 750 at Chimney Spring (5 burn frequencies $\times$ 3 replicates $\times$ 50). Small samples from each plant were chilled in the field and returned to the lab for immunoblot analysis, using antibodies developed specifically for *Neotyphodium* in Arizona fescue. Details of the immunoblot assay, modified from Gwinn et al. (1991) are found in Schulthess and Faeth (1998). All immunoblot results were confirmed by staining seeds collected from each plant, and observing the presence of characteristic *Neotyphodium* hyphae between the aleurone cells of seeds (Saha et al., 1988; Schulthess and Faeth, 1998).

We used ANOVA to determine the effect of each site and burn frequency on endophyte frequency (dependent variable). Since endophyte frequencies are expressed as percentages, we arcsine square root ($\arcsin p^2$) and logistic ($\ln [p/1-p]$) transformation of percentages (Sokal and Rohlf, 1995) from each replicate plot before analyses. Since results from each transformation were similar, we report only the arcsine transformation results here. All assumptions of ANOVA were tested and met.

Laboratory seed germination experiment

To test the effect of high temperatures on germination success of E+ and E- seeds, we collected seeds from the field plants with known infection status. Infection, or lack thereof, was confirmed with seed staining (see above). Ten

seeds from ten different plant genotypes (5 E+ and 5 E- plants) were randomly selected and mixed, then subjected to four dry-heat treatment levels: 95°C, 105°C, 110°C and 115°C, plus a control group stored at room temperature (approx. 21°C) (total = 500 seeds). All treatment and control seeds had been stored at room temperature prior to the experiment for approximately 3 months. E+ and E- seeds stored in this fashion typically have a high rate (80–90%) of germination (Neil, Tiller and Faeth, unpublished data). Each group of seeds was placed on a metal tray and kept at the treatment temperature for 2 hrs in an oven.

We chose this range of temperatures and length of exposure to simulate those of actual surface and shallow soil depth temperatures and duration measured during prescribed 4 yr burns at the field study sites (Sackett and Haase, 1992; Sackett et al., 1992). After cooling, the seeds were placed on moist filter paper in petri dishes. The control seeds were also treated similarly. Seeds were observed daily for germination for one month.

We used ANOVA with germination success as the dependent variable and infection status and temperatures as factors. Because germination success is expressed as a proportion, we used an angular or arcsine ($\arcsin p^2$) and logistic ($\ln [p/1-p]$) transformation (Sokal and Rohlf, 1995) of the dependent variable. Results using these transformations are similar; therefore, we only report statistical analyses of the angular transformation. There was a significant effect of temperature and infection; therefore, we conducted *post hoc* tests to compare differences in germination of E+ and E- seeds at each temperature, using Bonferroni corrections for multiple comparisons (Sokal and Rohlf, 1995).

Field seed germination experiment

While increased temperature is one prominent feature of fire, fire also may influence seed germination in other indirect ways. For example, Keeley and Fotheringham (1998) found that simply smoke generated from fires stimulated germination of chaparral plant species, and fire is also well-known for altering microbial (Dickman and Cook, 1989; Taylor, 1991; Johnson, 1995), moisture and nutrient properties of soil (e.g., Bond and van Wilgren, 1996; Covington and Sackett, 1984; 1986; 1992). To test the effect of actual fire on germination success, we placed seeds from E+ and E- plants into native soil in fire-proof metal pans (standard muffin-tins with 12 depressions per tray) covered with a screen to prevent disturbance by rodents birds and insects. Depressions were each filled with a 1:1 mixture of sterilized native soil and potting soil. Seeds of each infection status and genotype were randomly planted (depth of 8 mm) among and within trays (10 seeds per depression). The three trays were then randomly assigned a position in a 5 × 5 m grid at the study plot. The trays were placed into the soil such that the top of the tray was flush with ground level and then covered with

the average amount of litter in a 2 yr. burn plot at Chimney Springs on 6 November 1998. We then burned the experimental plot the next day (7 November) during the regularly scheduled 2 yr. burn cycle. On 19 November, two additional trays, containing replicates of each infection status and all the genotypes, were randomly positioned within the plot. Since these trays were not burned, we could compare germination success of E+ and E- seeds with and without the effects of fire. We monitored containers monthly after the burn for germination. We used G-tests to compare germination rates and survival of E+ and E- seedlings after germination.

Field plant survival experiment

To test the effect of endophyte infection on seedling survival, we randomly placed 6 month old plants grown in the greenhouse into an experimental plot at the Limestone Flats study area. Twenty plants grown from three E+ and three E- maternal genotypes (hence, half-sibs) were randomly planted on 29 September 1999 in a 10 × 5 meter plot in a buffer zone scheduled for a yearly burn in late October 1999. Each plant was randomly placed 0.2 m from the adjacent plant in a 6 × 20 array within the plot (total of 120 plants). We monitored survival of all plants after the burn for one year, conducting a final census on 30 August 2000. One E+ plant could not be re-located, and was therefore excluded from analyses of survival frequencies. We also used a log linear analysis (SYSTAT 2000) to test for effects of endophyte status (infected and uninfected) and seed genotype on survival of plants in a multi-way table.

3. Results

Prescribed fire plots

Endophyte frequency was not related to fire frequency (Fig. 1; $F = 0.31$, $df = 4$, 20; $p = 0.87$) nor did fire frequency interact significantly with site to affect endophyte frequency (Fig. 1; $F = 1.30$, $df = 4$, 20; $p = 0.30$). However, *Neotyphodium* infections varied by site (Fig. 1; $F = 8.82$, $df = 1$, 20; $p = 0.008$) with endophyte frequencies generally lower at the Limestone Flats site (Fig. 1). Overall, mean frequency of *Neotyphodium* infected grasses was high for all fire frequencies at both sites (Fig. 1).

Laboratory seed germination experiment

Contrary to our prediction of increased relative germination rates of E+ seeds with increased temperatures, E+ seeds had lower germination rates than E- seeds

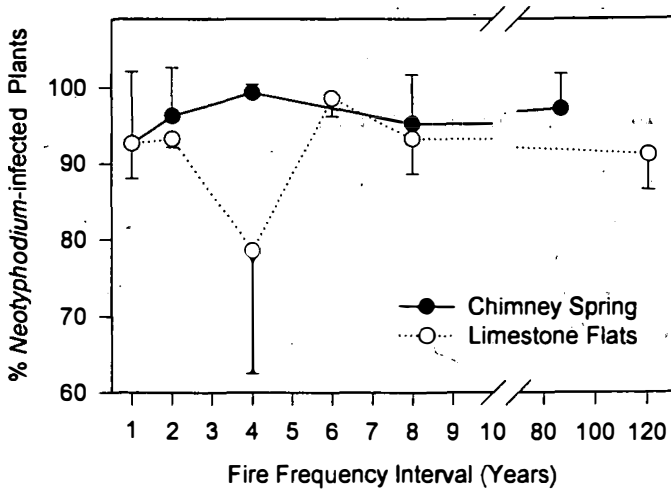


Figure 1. Mean ($\pm$ S.E.) of frequency of *Neotyphodium*-infected Arizona fescue in prescribed burn plots at Chimney Spring and Limestone Flats, Arizona. Each point is the mean of 3 replicate burn treatments within each site.

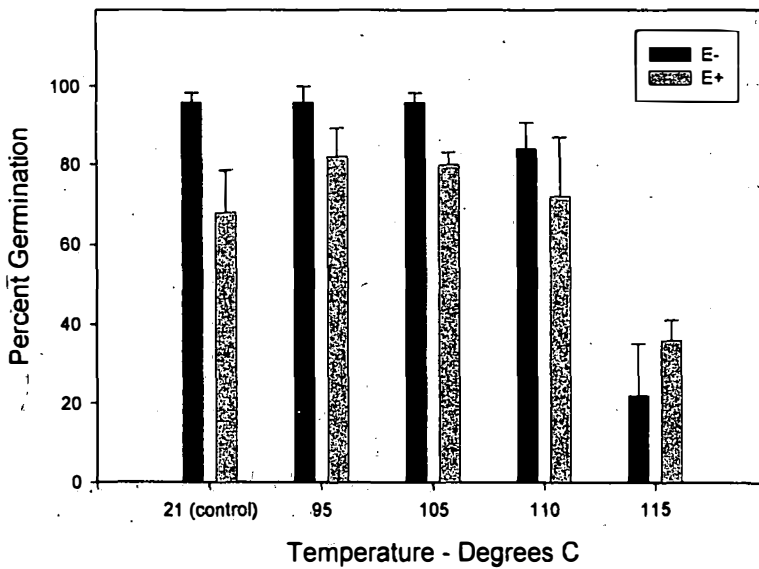


Figure 2. Mean ($\pm$ S.E.) germination of E- and E+ seeds at five different temperature treatments. Seeds were exposed to respective treatments for 2 hrs, with the exception of the controls.

(Fig. 2; infection effect, $F = 4.79$, $df = 1, 40$, $p = 0.035$). In general, germination rates of both E+ and E- seeds varied by temperature (Fig. 2, temperature effect, $F = 14.66$, $df = 4, 40$, $p < 0.001$). Germination rates of E- and E+ seeds varied in a similar fashion with temperature treatments (no infection $\times$ temperature interaction, $F = 2.33$, $df = 4, 40$, $p = 0.07$). Germination success of E+ seeds was high at all temperatures, except the extreme treatment of 115°C. Germination rates of E+ seeds were always lower than E- seeds except at this extreme treatment (Fig. 2), but the difference was not significant ($p > 0.35$). *Post hoc* tests (Tukey HSD) indicate that germination success of E- seeds was greater than that of E+ seeds at control (21°C) temperature ($p = 0.03$) and at the intermediate temperature treatment (105°C, $p = 0.001$).

Field seed germination experiment

Germination rates of E+ (21.7%) and E- (23.3%) seeds did not differ when subjected to a prescribed fire ($G = 0.034$, $df = 1$, $p > 0.50$). None of the seeds germinated until the onset on summer 'monsoon' rains the following August (1999). However, of the germinating seeds, the percent E+ seedlings (6 of 26) remaining alive after a 3 month dry period (in October 1999), was greater than E- (1 of 28) seedlings ($G = 4.94$, $df = 1$, $p < 0.05$).

Field plant survival experiment

Survival of E+ (13 of 59, 22.0%) and E- (14 of 46, 23.3%) plants did not differ after a prescribed fire ($G = 0.06$, $p > 0.80$). Neither endophyte status or genotype or their interaction contributed significantly to a log-linear model (respective p -values when terms are removed from the model: endophyte, $p = 0.67$, genotype, $p = 0.78$; endophyte $\times$ genotype, $p = 0.78$).

4. Discussion

To our knowledge, this is the first test of fire as a factor influencing systemic endophyte infections in grasses. Based upon preliminary observations from several burn frequencies, as well as observations of endophyte resistance to storage and heat, we hypothesized that endophyte-associated resistance to frequent, low intensity fires may explain high endophyte infections in Arizona fescue and perhaps other native grasses. Endophyte-related resistance to fire should be selected for since the fate of the asexual *Neotyphodium* symbiont and its grass host are closely linked. Furthermore, *Neotyphodium* occurs in aboveground tissues and seeds. Frequent, low intensity fires that characterized Arizona fescue habitats more directly affect these plant parts than below ground tissues (Sackett,

1980). After partial burning or grazing, *Neotyphodium* hyphae re-grow along with new leaf, sheath, and culm tissue from basal meristematic tissues (Azevedo and Welty, 1995).

Although frequent fire is a historical feature of the Ponderosa pine-grassland habitats occupied by Arizona fescue (Weaver, 1951; Cooper, 1960; Dietrich, 1980; Covington et al., 1997), fire does not explain variation in frequencies of *Neotyphodium* infections in this host grass. There was no relationship between fire frequency, which is inversely-related to intensity, and *Neotyphodium* infection frequency at either of the long-term prescribed fire sites (Fig. 1). Infection frequency in control plots where fire has been excluded for at least 9 decades was as high or higher than prescribed burn plots (Fig. 1). This is contrary to the prediction that infection frequencies should increase when stress is high and decrease when stress is low. *Neotyphodium* infection frequencies are known to change rapidly, even in perennial grasses, under changing selective pressures. For example, E+ tall fescue increases rapidly relative to E- fescue (within 2–4 yrs) under intense grazing pressure or abiotic stress (e.g., Clay, 1998). Like tall fescue, Arizona fescue reproduces primarily by seeds (USDA 1988); thus we also expected changes in frequencies over the span of the prescribed fire studies (~25 yrs) if fire is a selective force maintaining high infection frequencies. But clearly fire is not related to patterns of infection frequencies.

Our results from the heat-treated seed experiment also suggest that the endophyte does not increase resistance of E+ seeds to high temperatures. We chose the heat treatments to simulate temperatures typically encountered in low intensity fires that are thought to have occurred historically in these pine-grassland habitats. Germination rates of E+ seeds were generally lower than E- seeds, even at control temperatures (Fig. 2). Reduced germination is contrary to previous evidence that *Neotyphodium* infections increase germination success of agronomic tall fescue (e.g., Clay, 1987; but see Bacon, 1993). These results, however, are consistent with our other studies of seed germination of Arizona fescue; namely, the presence of the *Neotyphodium* endophyte either does not increase or sometimes decreases, germination success under varying osmotic potentials (Neil et al., submitted). Our field experiments under conditions of an actual fire corroborate the laboratory results. *Neotyphodium* infections do not increase germination success of their host seeds nor do they increase survival of their host plants.

Alternative hypotheses for high infection frequencies in natural populations

How are infection frequencies of asexual *Neotyphodium* maintained in this native grass if increased resistance to fire and other more conventional mechanisms (i.e., herbivore resistance or increased competitive abilities, see

Introduction) are not associated with endophyte infection? Wilkinson and Schardl (1997) noted that asexual, seed borne endophyte must be mutualistic or otherwise frequencies relative to E- plants would decline over time. Faeth and Bultman (2002) and Faeth (2002) provide several other possible explanations.

First, mutualistic effects of infection may be rare but nevertheless important. While *Neotyphodium* infections in Arizona fescue appear to provide few benefits and incur many costs, the benefits may occur at critical times in the lifespan of the host. For example, we found that E+ plants had increased survival in the field experiment after the fire and during the dry periods following summer rains. That infection may increase survival during periods of severe drought is also supported by recent physiological studies of infected Arizona fescue. Morse et al. (2002) found that E+ plants had higher relative growth rates and higher net assimilation rates than E- plants only under severe and prolonged water deprivation. Under more moderate water limitation, the reverse was true. However, this sporadic benefit from the endophyte may not outweigh the costs of harboring the endophyte, including lower germination rates shown here, as well as decreased resistance to herbivores and competitive abilities documented in previous studies (e.g., Saikkonen et al., 1999; Faeth and Bultman, 2002; Faeth and Fagan, 2002).

Second, White et al. (1996) and Moy et al. (2000) suggested the possibility that strictly asexual *Neotyphodium* endophytes may be occasionally transmitted horizontally because of the presence of epiphyllous nets and conidia in the leaves of some infected grasses. In culture, *Neotyphodium* also may produce asexual spores. If transmitted horizontally to adult plants, then infection frequencies could be maintained even if the interaction is neutral or parasitic. We note, however, that horizontal transmission has never been documented for *Neotyphodium* in natural populations. Furthermore, horizontal transmission rates would have to equal or exceed loss of the endophyte via hyphae inviability and imperfect transmission, which may be substantial (e.g., Ravel et al., 1997), for maintenance or increase in infection frequencies. This hypothesis, therefore, seems unlikely.

Third, asexual *Neotyphodium* may manipulate host sex allocation such that transmission probability is increased. Werren and O'Neill (1997) proposed that maternally-inherited, asexual symbionts need not act as host mutualists to persist in host populations, contrary to conventional wisdom (e.g., Law, 1985; Ewald, 1994). Instead, maternally-inherited, cytoplasmic microbes, such as *Wolbachia*, persist in many invertebrate species by reducing male function, increasing feminization and decreasing uninfected hosts. *Wolbachia* does not increase, and often decreases, host fitness. Likewise, *Neotyphodium* may promote transmission to host offspring by increasing or decreasing allocation to female and male function, respectively, or by increasing vivipary or pseudovivipary, which are

well-known in grasses (e.g., Elmqvist and Cox, 1996). Furthermore, sexual forms of endophytes drastically alter reproductive function of their hosts, such that transmission is enhanced (Clay, 1986; 1991). The hypothesis that asexual endophytes may retain the capacity to alter reproduction of their hosts has not yet been tested.

Fourth, Saikkonen et al. (1998) proposed that infected plants may persist in spatially-structured grass populations even though infection may have net negative effects on the host (e.g., Hochberg et al. 2001). In a mathematical model, Saikkonen et al. (2002) showed that asexual, vertically-transmitted endophytes need not interact mutualistically, even if transmission is imperfect, for persistence in populations, if the populations are spatially-structured (i.e., as metapopulations). For perennial bunchgrasses like Arizona fescue, seed shadows often occur near maternal plants and dispersal is limited. Thus, infections may persist in local pockets or sub-populations near maternal plants, where selective forces that drive infection frequencies may be thwarted.

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