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Influence of genetic variation in the fungal endophyte of a grass on an herbivore and its parasitoid

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Abstract

*Neotyphodium coenophialum* (Glenn, Bacon, Price & Hanlin) (Ascomycota: Clavicipitaceae) is an endophytic fungus that lives symbiotically within grasses and produces alkaloids that can help protect its hosts from some insect pests. We used laboratory-based experiments to investigate whether fungal genotype influences an herbivore and its parasitoid. We tested whether variation in novel isolates, plus a control lacking fungal infection, affected preference by fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae: Amphipyrini), and growth and survival of *Euplectrus comstockii* Howard (Hymenoptera: Eulophidae: Euplectrini), a parasitoid of fall armyworm. Caterpillars preferred leaf blades in choice experiments from uninfected tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh., cultivar Jesup (Poaceae)] and tended to avoid blades from plants containing fungal isolates AR502, AR542, or the most common strain from pastures in Georgia, USA, in tall fescue. However, caterpillars fed as much on leaf blades from plants containing isolate AR502 as from those lacking infection. Parasitoid pupal mass was not influenced by fungal isolate, yet fungal isolate did influence parasitoid survival. Survival was higher than expected for parasitoids reared from hosts fed plants lacking fungal infection, but was lower than expected for those reared from hosts fed plants infected with the common strain or AR542 isolates. In contrast, parasitoids reared from hosts fed plants infected with isolate AR502 did not experience higher mortality than expected by chance. Our results show *N. coenophialum* can modify bottom-up trophic cascades through direct effects on herbivores, as well as indirect effects on a natural enemy of the herbivores and that the fungus may influence the tritrophic interaction in ways that counterbalance herbivore protection provided by the symbiont. Our work also shows these effects are influenced by fungal genotype. As attempts are made to produce forage cultivars with strains of fungal endophyte that lack negative influences on livestock, it will be prudent for investigators to assess the multитrophic effects of these novel associations within agroecosystems.
Introduction

*Neotyphodium coenophialum* (Glenn, Bacon, Price & Hanlin) (Ascomycota: Clavicipitaceae) is an endophytic fungus that infects stems, leaf sheaths, and seeds of its grass hosts. The fungus produces alkaloids that can have detrimental effects on herbivores (Clay, 1990; Clement et al., 1994; Faeth & Bultman, 2002; Popay & Bonos, 2005). Alkaloids include the ergots and lolitrem which primarily affect vertebrates, and lolines and peramine which mainly influence invertebrates (Siegel & Bush, 1996; Bush et al., 1997; Clay & Schardl, 2002; Schardl et al., 2007). The types of alkaloids produced appear to be primarily under the control of fungal genes, while the plant/fungal genotype interaction can modify the quantities of these alkaloids (Lane et al., 2000). The fungal microbes are important ecologically due to their widespread presence in grasses and the impacts they can have on their host, its herbivores, and potentially the entire ecosystem (Rudgers et al., 2004; Omacini et al., 2005). Furthermore, they can have substantial economic implications when they infect agronomic forage grasses, where they cause toxicoses of livestock that result in hundreds of millions of dollars lost annually in the USA alone (Ball, 1997).

Given the agronomic costs of endophytes, researchers have aimed to develop forage grasses that produce lower levels of ergot and/or lolitrem alkaloids to reduce negative effects on livestock, but retain beneficial pest protection offered by lolines and peramine. A successful strategy in this endeavor has been to collect isolates of *N. coenophialum* from tall fescue, *Lolium arundinaceum* (Schreb.) Darbysh. (Poaceae), growing in its native Eurasian range and then artificially inoculate these into high-yield grass cultivars. This process has produced several novel associations, including isolate AR542 which was introduced into tall fescue cultivars Georgia 5 and Jesup (Bouton et al., 2002) and is marketed under the trade name MaxQ™.

Development of novel fungal isolates associated with grasses has allowed investigation of the effects of fungal genotype on herbivores. Initial studies have shown that both sucking (Clement et al., 2001; Bultman et al., 2004, 2006; Hunt & Newman, 2005) and chewing insects (Ball et al., 2006) can be affected by variation in fungal genotype. In light of the negative effects endophytes can have on primary consumers, one
might expect these fungal symbionts, and the alkaloids they produce, to have influences that reach to secondary consumers and beyond.

The importance of trophic cascades in community structuring is well recognized by ecologists (Oksanen et al., 1981; Polis, 1999). Bottom-up cascades occur when resources and productivity at the base of the food web regulate productivity at higher levels, while top-down cascades occur when higher-level consumers play important roles in regulating levels below them. Endophytes may play roles in bottom-up cascades, at least in part through the alkaloids they produce, and their effects on higher trophic levels (Faeth & Bultman, 2002; Chaneton & Omacini, 2007).

Tritrophic interactions within the endophyte/grass system have been investigated in New Zealand, where perennial ryegrass (*Lolium perenne* L.) is a common pasture grass and is often infected by *Neotyphodium lolii* Latch, Christensen, & Samuels (Easton & Fletcher, 2006). Argentine stem weevil, *Listronotus bonariensis* (Kuschel), is a major pest of the grass. A parasitoid (*Microctonus hyperodae* Loan) was introduced from the weevil's native range as a biocontrol agent (Goldson et al., 1990, 1993). Investigators found that parasitoids reared from hosts fed endophyte-infected perennial ryegrass had decreased development and survival in laboratory experiments (Barker & Addison, 1996; Bultman et al., 2003, but see Urrutia et al., 2007). Furthermore, *M. hyperodae* survival and development varied when reared from weevil hosts fed different isolates of the fungus (Bultman et al., 2003). Similar results were obtained in the USA in a study of two *Euplectrus* parasitoid species reared from hosts (fall armyworm) fed infected or uninfected tall fescue (Bultman et al., 1997). The parasitoids experienced reduced growth when reared from hosts fed endophyte-infected tall fescue.

To extend this work, we investigated whether endophyte isolates within a single grass cultivar have negative effects on the second and third trophic levels. Specifically, we tested whether variation in fungal isolate affected the preference of fall armyworm and the growth and survival of one of its parasitoids.

**Materials and methods**

To assess the influence of genetic variation in endophyte we used seeds of tall fescue, *L. arundinaceum*, cultivar Jesup (Bouton et al., 1997), that were infected with the most
common strain (hereafter CS) of *N. coenophialum* from pastures in Georgia, USA, or strains AR502 and AR542 isolated from native, wild populations of tall fescue in the Mediterranean region. Strains AR502 and AR542 were obtained from AgResearch Grasslands, Palmerston North, New Zealand.

Both AR502 and AR542 isolates produce low levels of ergot alkaloids (or produce none at all), but still produce loline alkaloids (Bouton et al., 2002). In contrast, the CS isolate produces ergot alkaloids as well as lolines. With respect to the loline alkaloids, the CS and AR502 isolates produce three loline derivatives (N-formyl loline, N-acetyl loline, and N-acetyl norloline), whereas AR542 produces only N-acetyl norloline (Ball & Tapper, 1999; Pennell & Ball, 1999).

Fungal isolates were cultured from parent plants and then introduced into tall fescue plants using inoculation techniques of Christensen et al. (1997). Uninfected seeds were produced from seed of infected host plants through disinfection by heat treatment (Nott & Latch, 1993); both E- (uninfected) and E+ (infected) seeds were all from the same maternal line. Plants used in the experiment were several generations removed from the heat treatment. Microscopic inspection of 100 seeds of each type showed that all infected lines were at least 90% infected, whereas E- seeds were 0% infected. Infection in plants was confirmed with immunoblot (Hiatt et al., 1999) following completion of the experiment. Plants were grown in 15-cm-diameter pots and fertilised every other week with Peter’s liquid fertilizer (20N:20P:20K) at 300 ppm N.

**Fall armyworm preference**

*Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae: Amphipyrini) is a polyphagous herbivore that prefers grasses (Luginbill, 1928) and can be a serious pest of tall fescue in the USA (Bair et al., 1991). The lepidopteran has been used extensively in research involving grass endophytes (Clay et al., 1985; Breen, 1993; Bultman & Ganey, 1995; Boning & Bultman, 1996; Bultman & Conard, 1998; Ball et al., 2006). Larvae were obtained from French Agricultural Research (MN, USA) and were reared from eggs on artificial medium (Southland Products, AR, USA) until the beginning of the second instar.
Ten second-instar fall armyworms were introduced into Petri dishes for choice experiments. Each Petri dish contained 4-cm sections of two leaf blades. The following combinations were used: E- vs. CS, E- vs. AR542, and E- vs. AR502. Caterpillars were starved for 2 h prior to the experiment. Petri dishes were kept in an environmental chamber at 26 °C for 5 h in the dark, after which time the number of caterpillars on either blade was recorded. Feeding damage to leaf blades was also assessed. We used a subjective score in which one blade received a ‘1’ and the other a ‘0’ if the first blade clearly had more damage. If the level of damage to both blades was visually indistinguishable or nearly so, they both were scored ‘1’ unless neither was damaged, in which case they both were scored ‘0’. This approach allowed us to make comparisons between blades with each Petri dish rather than attempt to visually score damage to blades from one Petri dish relative to blades from other dishes.

**Parasitoid performance**

*Euplectrus* parasitoids are multivoltine, gregarious ectoparasitoids of lepidopteran larvae (Gerling & Limon, 1976). They inject venom into their hosts that arrests development (Coudron & Brandt, 1996). Thus, offspring of the ectoparasite are not shed with ecdysis since the host remains in the same instar until death. Females deposit from 1 to >10 eggs in a cluster on the dorsum of the host. Parasitoid larvae develop externally and move to the host's venter where they spin partial cocoons. Thus, pupae of parasitoids can easily be removed from the host and weighed. *Euplectrus comstockii* Howard (Hymenoptera: Eulophidae: Euplectrini) is a polyphagous parasitoid (Krombein et al., 1979; Coudron & Puttler, 1988) and fall armyworm is a recorded host (Coudron & Puttler, 1988). It is regularly recovered from fall armyworm within several cropping systems in Missouri, USA (Coudron & Puttler, 1988).

Fall armyworm caterpillars were allowed to feed on clippings of leaf blades from plants within a treatment group from the second to third or fourth instar. Caterpillars (n = 74 and 91 for caterpillars from which female and male parasitoids were reared, respectively) were then parasitized by *E. comstockii* parasitoids in the lab. Caterpillars continued to receive food from the appropriate treatment group and were housed separately in Petri dishes in an environmental chamber at 26 °C, 60% RH, and a L14:D10
photoperiod. We monitored parasitoid growth and survival, recording sex, pupal mass, and successful development to the adult stage.

Statistical analyses
Preferences by fall armyworm larvae were evaluated using the $\chi^2$ goodness-of-fit test with the null hypothesis of no preference between E- and endophyte-infected leaf blades contained within individual Petri dishes. Each of the combinations of E- and endophyte-infected blades (see above) was replicated 25 times. Parasitoid performance was determined by recording mass of parasitoid pupae. Differences in mass of parasitoids reared from hosts fed grass containing different endophyte isolates or lacking endophyte infection were assessed using one-way ANCOVA, with number of eggs/host and host mass as covariates. One hundred ninety-nine and 245 female and male parasitoids, respectively, were reared. However, we used the mean mass of female ($n = 71$) and male ($n = 89$) parasitoids for each host as our replicate (the host and not the individual parasitoid was the sample unit). Differences in survival of developing parasitoids among treatment groups were assessed using contingency table analysis and the $\chi^2$ test. All statistical analyses were conducted with Systat (Wilkinson, 1998).

Results
Fall armyworm preference
*Spodoptera frugiperda* caterpillars tended to select E- leaf blades over blades containing fungal isolates AR502, AR542, or CS (Figure 1). Yet, the level of feeding damage caterpillars caused to leaf blades varied some from this result; caterpillars caused more damage to blades of E- and AR502-infected plants than to blades from plants infected with AR542 or CS isolates (Figure 2). So, caterpillars were less strongly deterred to feed on blades from plants infected with isolate AR502 than those harboring AR542 or CS isolates.

Parasitoid performance
Parasitoid pupal mass was not influenced by fungal isolate for either females ($F_{3,67} = 1.21, P = 0.31$) or males ($F_{3,85} = 0.76, P = 0.52$). In contrast, fungal isolate did affect
parasitoid survival from egg to adult (Figure 3). Survival of parasitoids (n = 444) was higher than expected by chance for adult parasitoids emerging from hosts fed E- grass or grass infected with fungal isolate AR502, and lower than expected for parasitoids emerging from hosts fed grass infected with the CS or AR542 fungal isolates.

**Discussion**

Our study supports a large body of literature that shows infection of agronomic grasses by *Neotyphodium* often helps protect them from herbivores (Clay & Schardl, 2002; Faeth & Bultman, 2002). The mechanism for this protection appears to primarily be the production of toxic alkaloids by the fungus (Clay, 1988; Schardl et al., 2007). We found fall armyworm caterpillars tended to select leaf blades lacking fungal infection (Figure 1). Although avoidance of blades was similar among those infected with the common strain and novel isolates, larvae did vary in their feeding damage, showing greater feeding on plants with isolate AR502 than those with AR542 or CS isolates (Figure 2). Hence, our results suggest genetic variation in fungal endophyte can modify the defensive mutualism, as has been found in other studies (Clement et al., 2001; Bultman et al., 2004, 2006; Hunt & Newman, 2005).

Our results deviate from those reported by Ball et al. (2006), who found fall armyworm larvae failed to avoid tall fescue leaf blades infected with AR502 or AR542. Two important factors differed between our study and theirs: the source of fall armyworm larvae and the cultivar of tall fescue. The fall armyworm we used originated from maize fields in Minnesota, USA, whereas Ball et al. (2006) used caterpillars from Missouri, USA (on alfalfa) and Georgia, USA (on Bermuda grass). *Spodoptera frugiperda* contains at least two strains and these differ in many developmental and ecological/behavioral characters (Pashley, 1988). Furthermore, we used cultivar Jesup whereas Ball et al. (2006) used the Kentucky-31 cultivar. This difference is also a likely cause for variation in fall armyworm preference since plant genotypic variation often influences the effect of specific endophyte genotypes on herbivores (Timper & Bouton, 2005; Bultman et al., 2006).

Our results suggest that the presence of CS and AR542 fungal isolates can influence bottom-up trophic cascades. Changes in plant traits that accompany infection
by CS and AR542 isolates, i.e., trait-mediated indirect interactions (Wootton, 1994), reduced parasitoid performance (Figure 3). This result mirrors what has been found for other tritrophic systems involving endophyte-infected grass. For example, in a laboratory-based study, de Sassi et al. (2006) found that ladybird beetles (Coccinella septempunctata L.) preying on Rhopalosiphum padi L. aphids fed endophyte-infected perennial ryegrass showed reduced fecundity and impaired reproductive performance compared to those preying on aphids fed uninfected grass. Similarly, parasitoids reared from coleopteran hosts fed endophyte-infected perennial ryegrass had decreased development and survival in laboratory experiments (Barker & Addison, 1996; Bultman et al., 2003, but see Urrutia et al., 2007). Although we did find differences in parasitoid survival, we did not find that endophyte infection reduced parasitoid pupal mass, as it does for Euplectrus parasitoids reared from fall armyworm feeding on endophyte-infected tall fescue (cultivar Kentucky-31) (Bultman et al., 1997). The different outcomes between the previous study and that reported here may be due to differences in plant and fungal genotypes used in the two studies. In sum, most reports to date show that endophytes of agronomic grasses negatively impact third-trophic-level consumers.

We caution that our study was based in the laboratory. Extrapolation of our results to the field must be done carefully. If caterpillars were to completely avoid endophyte-infected plants in the field, effects of N. coenophialum on the third trophic level would be absent because there would be no caterpillars feeding on endophyte-infected grass for E. comstockii to parasitize. However, complete avoidance is unlikely since caterpillars in our choice experiments did select and feed on some endophyte-infected plants. The incidence of parasitism of hosts feeding on uninfected vs. endophyte-infected grasses in nature will likely be a function of many factors, like the distribution and dispersion of both grass types in the field, alkaloid production and higher-order interactions involving multiple trophic levels (Krauss et al., 2007).

Our findings follow those of Bultman et al. (2003), who found that variation in fungal isolates infecting perennial ryegrass influenced the performance of parasitoids of Argentine stem weevil. Some N. lolii isolates had little or no effect on parasitoids, while others markedly reduced performance. Similarly, we found that AR542 and CS isolates both resulted in similar reductions in E. comstockii survival, whereas effects of isolate
AR502 on parasitoid survival were similar to those associated with uninfected grass (Figure 3). Taken together these studies suggest that not only the presence of endophyte but also variation in the genotype of the fungus can influence natural enemies in grass-endophyte systems; we caution however that a general conclusion about genotype influences of endophytes on tritrophic systems cannot yet be reached without further study that includes more grass species.

The results of our study only partially follow the known patterns of alkaloid production in the host plants. The CS isolate produces ergots, lolines, and peramine (Bouton et al., 2002), which may explain the reduced survival of parasitoids. Isolate AR502 produces three derivatives of lolines, whereas isolate AR542 produces only one (N-acetyl norloline) (Ball & Tapper, 1999; Pennel & Ball, 1999). Yet, fall armyworm feeding and parasitoid survival were lower with AR542 than with AR502. Differences in loline concentrations, which were not assessed in our study, may explain the variation we found between these two isolates.

Combining the effects we found for endophyte infection on an herbivore and its parasitoid, our study suggests that *N. coenophialum* can influence the tritrophic interaction in ways that counterbalance the herbivore protection the fungus provides. Furthermore, variation in fungal isolate may affect the benefits (herbivore protection) and possible costs (reduced parasitoid fitness) to the plant. Plants infected with fungal isolates that are more toxic (CS and AR542) may provide greater benefits in terms of herbivore protection, but weaken parasitoid control of herbivores. Plants infected with less toxic isolates (AR502) should provide slightly less direct protection from herbivores, but should also allow stronger top-down control of those herbivores. So, the net effect of variation in fungal genotype on protection from herbivory may be negligible if effects on herbivores and parasitoids are counterbalanced. Determining the generality of this result will require further study.

Attempts to produce high-yield forage grass cultivars with strains of fungal endophyte that lack negative influences on livestock have begun (Bouton et al., 2002). Investigators have primarily been concerned with ergot levels in plants and have selected fungal isolates that produce low or no ergot alkaloids to reduce prevalence of vertebrate toxicoses (Fletcher & Harvey, 1981; Stuedeman & Hoveland, 1988). Little research on
the effects of these novel endophytes on insect herbivores or their natural enemies has been conducted. Our results suggest that variation in fungal isolate can influence the multitrophic interaction within the forage grass agroecosystem. The development of new endophyte/grass associations will provide a unique opportunity to explore the effects of genetic variation in a microbial symbiont on multitrophic systems and will allow for a more complete understanding of the ecological ramifications of utilizing novel endophyte-grass associations in grazing systems.

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References


Figure 1 Observed (Obs) and expected (Exp) numbers of fall armyworm larvae selecting leaf blades of Jesup tall fescue. Expected values generated from $\chi^2$ goodness-of-fit test which assumed no preference for either blade type. Larvae preferred blades lacking fungal infection over those containing (A) the common strain (CS) of the fungus ($\chi^2 = 7.0$, d.f. = 1, $P<0.05$), (B) isolate AR542 ($\chi^2 = 4.0$, d.f. = 1, $P<0.05$), or isolate AR502 ($\chi^2 = 5.4$, d.f. = 1, $P<0.05$). The experiment used 25 replicated Petri dishes. E- = blades lacking fungal endophyte throughout.
**Figure 2** Observed (Obs) and expected (Exp) feeding damage during 5 h by fall armyworm on leaf blades of Jesup tall fescue. Paired blades were assigned a ‘1’ or ‘0’ based on damage relative to one another and these scores were analyzed by a $\chi^2$ goodness-of-fit test. Larvae caused more feeding damage on blades lacking fungal infection (E-) compared to those containing (A) the common strain of the fungus ($\chi^2 = 5.0$, d.f. = 1, $P<0.05$) and (B) isolate AR542 ($\chi^2 = 6.1$, d.f. = 1, $P<0.01$). Damage to blades containing fungal isolate (C) AR502 and E- blades did not differ ($\chi^2 = 0.1$, d.f. = 1, $P>0.05$).

**Figure 3** Observed (Obs) and expected (Exp) survival of *Euplectrus comstockii* parasitoids when reared from fall armyworm hosts fed tall fescue with differing endophyte isolates or lacking infection (E-). $\chi^2 = 58.34$, d.f. = 3, $P<0.0001$. 
a) Frequency of Larvae on Blades

b) Frequency of Larvae on Blades

c) Frequency of Larvae on Blades
Frequency of Surviving Parasitoids

Treatment

E-  CS  502  542