Top-down and bottom-up regulation of herbivores: 
*Spodoptera frugiperda* turns tables on endophyte-mediated plant defence and virulence of an entomopathogenic nematode

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**Abstract.**

1. The fungus *Neotyphodium lolii* forms a symbiotic relationship with its grass host *Lolium perenne* (perennial ryegrass). The fungus benefits from access to plant nutrients and photosynthate, whereas the plant benefits from acquired chemical defence against herbivory.

2. This study examined the potential for endophyte-mediated plant defences to influence interactions between fall armyworm *Spodoptera frugiperda*, and the entomopathogenic nematode *Steinernema carpocapsae* and clarified biological mechanisms underlying the observations made.

3. In laboratory and greenhouse experiments, *S. frugiperda* larvae were fed endophytic or non-endophytic *L. perenne* then exposed to *S. carpocapsae* or injected with the nematodes' symbiotic bacteria *Xenorhabdus nematophila*.

4. In all instances, *S. frugiperda* larvae fed endophyte-infected grass suffered significantly lower mortality than those fed non-endophytic plants. Although larvae fed endophyte-infected grass often had significantly lower biomass than those fed uninfected grass, these differences did not account for altered susceptibility to *S. carpocapsae*.

5. Endophyte-mediated reductions in herbivore susceptibility to the nematode pathogen represent a herbivore adaptation that effectively turns the tables on both plant and natural enemy by reducing the virulence of the nematodes' symbiotic bacteria while expanding the temporal window of herbivory.

**Key words.** Antagonism, multi-trophic interactions, *Neotyphodium* endophyte, *Spodoptera frugiperda, Steinernema carpocapsae, Xenorhabdus nematophila*.

**Introduction**

One of the central endeavours of ecology is to understand how top-down and bottom-up forces constrain populations of herbivores. Resources, which may vary in quality and quantity, may interact with natural enemies to regulate populations of phytophagous insects and, in theory, these interactions may vary from synergistic to antagonistic (Agrawal *et al.*, 2000; Van der Meijden & Klinkhammer, 2000). As such, their combined impact on populations of organisms is difficult to predict. Studies concerning multi-trophic interactions have demonstrated that the success of natural enemies may be influenced by plant quality (Barbosa & Letourneau, 1988; Barbosa *et al.*, 1991a). Close associations between plants and microbes such as rhizobia and mycorrhizae can have a substantial impact on plant quality by altering plant growth and nutrient content (Packovsky *et al.*, 1986; Bolan, 1991; Saxena *et al.*,...
spodoptera frugiperda

Lolium perenne

grass

Price & Hanlon to mediate interactions among its host S.carpocapsae nematode when feeding on endophyte-infected nemeria carpocapsae is less susceptible to the entomopathogenic nematode black cutworm (B. A. Kunkel and P. S. Grewal, unpubl. data). The frugiperda larvae feeding on endophyte-infected perennial have been detected in the haemolymph of 16.4 (Kunkel et al., 2004) and several of these same compounds have been detected in the haemolymph of Spodoptera frugiperda larvae feeding on endophyte-infected perennial ryegrass (B.A. Kunkel and P.S. Grewal, unpubl. data). The black cutworm Agrotis ipsilon Hufnagel, a related noctuid, is less susceptible to the entomopathogenic nematode Steinernema carpocapsae when feeding on endophyte-infected grass and the action of endophyte-mediated alkaloids on the nematodes’ symbiotic bacteria has been put forward as a mechanism to explain this phenomenon (Kunkel & Grewal, 2003). However, many insect host related factors may influence the success of entomopathogenic nematodes and some of these, such as biomass, feeding behaviour, and physiological condition are, or potentially could be, altered by feeding on endophyte-infected plants.

The work reported here investigated the ability of the fungal endosymbiont Neotyphodium loli Glenny, Bacon, Price & Hanlon to mediate interactions among its host grass Lolium perenne L., the lepidopteran herbivore Spodoptera frugiperda (Smith), and the entomopathogenic nematode S.carpocapsae. These experiments aimed to clarify underlying biological mechanisms by separating endophyte-mediated effects on S.frugiperda growth and development from effects on feeding behaviour or virulence of the nematode–bacterium complex. Susceptibility to the nematode–bacterium complex may be altered independently of any endophyte-related effects on insect biomass or feeding behaviour. Endophyte-mediated reductions in herbivore susceptibility to the nematode pathogen would represent a herbivore adaptation that effectively turns the tables on both plant and natural enemy by reducing nematode virulence while expanding the temporal window of herbivory. Conversely, enhanced susceptibility would indicate that fungal endophytes provide their grass mutualist with both direct and indirect defence against herbivory.

Materials and methods

Spodoptera frugiperda eggs for all experiments were purchased from Agripest Inc. (Zebulon, North Carolina). These insects were free of any pre-existing infection by entomopathogenic nematodes which would have killed the insect within a couple of days of eclosion. Insects were kept isolated from the nematodes prior to experimentation. Nematodes Steinernema carpocapsae (all strain) were taken from a laboratory culture maintained on larvae of the wax moth Galleria mellonella L. as a host. Groups of 10 G. mellonella larvae were placed in Petri dishes on moist filter paper and exposed to 100 infective juvenile nematodes. After 5 days, nematode-infected G.mellonella larvae were transferred to White’s traps (White, 1927) to collect newly emerged infective juveniles. Infective juvenile nematodes were stored in water in an incubator at 10°C and were used in experiments within 1–3 days of emergence from host cadavers.

Greenhouse feeding and nematode exposure assay

Seeds of perennial ryegrass (Var. Palmer III) (Loft’s Seeds Co., Bound Brook, New Jersey) were germinated on moist filter paper in the laboratory and planted in 9-cm pots filled with 1:1:1 (sand:soil:peat) soil mixture. The soil component of the mixture was a Wooster silt-loam (fine-loamy, mixed, mesic oxaquefragudalfs) collected locally on the grounds of the Ohio Agricultural Research and Development Center, Wooster, Ohio, U.S.A. After 6 weeks of growth in the greenhouse, two mature tillers from each plants were cut at the soil surface, placed into labelled vials, and returned to the laboratory where the endophyte infection status of each plant was confirmed using the Phytoscreen Immunoblot Kit (Agrinostics, Ltd. Co., Watkinsville, Georgia). Plants were watered daily and fertilised bi-weekly using a water soluble 20:20:20 (N:P:K) fertiliser.

After 4 more weeks of growth, five third-instar S.frugiperda larvae were confined to each pot using ladies hosiery. Larvae were allowed to feed for 5 days before 1600 infective

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juvenile *S. carpocapsae* were added to each pot using a 1-l plastic, hand-operated spray bottle. Nematodes were dispensed in 4 ml of water. Pots were carefully examined after 36 h and every 6 h thereafter and the number of live larvae was recorded. Each treatment (endophyte infected or uninfected) was replicated 10 times and the experiment was terminated 72 h after nematodes were added.

**Laboratory feeding and nematode exposure assay**

Perennial ryegrass, *Lolium perenne* L. (var. Repel II) (Loft’s Seeds Co.) was established in the greenhouse in window box flats (12 × 30 × 15 cm) containing Pro Mix™ potting soil (Premier Horticulure Ltd, Dorval, Quebec, Canada). After 1 month of growth, half of these flats were treated with the fungicide propiconazole at six times the label rate to eliminate the fungal endophyte. After 3 months, all plants were examined microscopically for the presence of endophyte using a staining technique modified from Saha *et al.* (1988). Two mature tillers from each plant were examined and endophyte infection was not detected in any of the plants receiving fungicide. Confirmed endophyte-infected and -uninfected plants were replanted separately in fresh potting soil to ensure pure cultures of infected and uninfected plants. Plants were watered and cut (20 cm) as needed and fertilised monthly using a water soluble 20:20:20 (N:P:K) fertiliser. These cultures were maintained in the greenhouse for 1 year prior to being used in experiments.

Neonate *S. frugiperda* larvae were transferred, in groups of 10, to 29.6 ml plastic snap-cap containers and provided with leaf clippings taken from endophyte-infected or -uninfected plants. Insect larvae were allowed to feed for 14 (trial 1) or 10 (trial 2) days prior to being exposed to nematodes and fresh plant material was provided daily. After 14 or 10 days of feeding on grass clippings, individual *S. frugiperda* larvae were weighed and placed in 4 cm Petri dishes lined with moist filter paper treated with a concentration of 0 to, 8, or 16 infective juvenile nematodes. Four Petri dishes containing the larvae and nematodes were kept at 25 °C (LD 14:10 h). Observations were made every 6 h to determine the status of each individual larva (live or dead) and experiments were terminated after 78 h. All larvae dying following exposure to nematodes were kept at 22 °C for 1 week and dissected under a microscope to confirm nematode infection as the cause of death. Two independent experimental trials were conducted using 96 larvae in each trial.

The greenhouse feeding assay was analysed using repeated-measures ANOVA employing the model 

\[ Y = E + T + (ET), \]

where \( Y \) is larval survival, \( E \) is endophyte infection status of the food plant, and \( T \) is time in hours. Univariate outputs were examined to clarify the interaction between \( E \) and \( T \).

Laboratory feeding assays were analysed in two steps. First, factorial ANOVA was performed to determine the influence of experimental trial and grass type (endophyte-infected or -uninfected) on larval biomass using the model 

\[ Y = X + E + X E, \]

where \( Y \) is mean larval biomass, \( X \) is experimental trial, and \( E \) is endophyte infection status of the food plant. Next, repeated-measures ANOVA was used to test the influence of grass type and nematode concentration on larval survival over time. Mean biomass of the four larvae grouped into each experimental unit was included as a covariate in the analysis using the model 

\[ Y = B + E + N + (EN) + T + (ET) + (NT) + (ENT) \]

where \( B \) is larval biomass, \( E \) is endophyte infection status of larval food, \( N \) is nematode concentration, and \( T \) is time in hours. Because the two experimental trials were conducted...
independently and larval age and biomass differed between them, each trial was analysed separately. Univariate outputs were examined to clarify interactions between factors (effects of endophyte and nematode concentration at each time interval) and linear contrasts were generated to test specific hypotheses.

Data from the Xenorhabdus nematophila injections experiment were analysed using repeated-measures ANOVA to test the influence of the various treatments on larval mortality using the model \( Y = X + T + (ET) \), where \( Y \) = % larval survival, \( X \) = experimental treatment, and \( T \) = time in hours. Univariate outputs were examined to clarify the interaction between \( E \) and \( T \) and linear contrasts were generated to test specific hypotheses.

In all analyses, main effects were tested at \( \alpha = 0.05 \) whereas interactions were tested at \( \alpha = 0.1 \) to decrease the likelihood of type II error. To avoid violating the assumption of compound symmetry associated with the repeated-measures analyses, Huynh–Feldt adjustments were used to determine the significance of repeated-measures factors. Percentage data were arcsin square-root transformed prior to analysis. All statistical analyses were performed using Statistica 6.1 (Statsoft Inc., 2002).

**Results**

**Greenhouse feeding and nematode exposure assay**

Thirty-six hours after exposure to nematodes, *S. frugiperda* larvae feeding on live endophyte-infected plants suffered less mortality than those feeding on live uninfected plants and although not significant thereafter, this trend was consistent up to 60 h after exposure (Table 1, Fig. 1). Larval mortality 72 h after exposure to nematodes reached approximately 40% regardless of the endophyte infection status of larval food plants.

**Laboratory feeding and nematode exposure assay**

Larvae in the first trial were 4 days older than those in the second trial and they had significantly greater biomass (b) \( (F_{1,44} = 12.0, \ P = 0.001) \) (trial 1, \( b = 0.17 \pm 0.02 \) g; trial 2, \( b = 0.11 \pm 0.02 \) g). In both trials, larvae feeding on endophyte-infected grass (E+) had lower biomass than those feeding on uninfected grass (E-) although this difference was only significant in the second trial (E+ = 0.15 ± 0.01 g, E- = 0.18 ± 0.02 g, \( F_{1,22} = 2.7, \ P = 0.1 \) and E+ = 0.08 ± 0.01 g, E- = 0.15 ± 0.02 g, \( F_{1,22} = 17.8, \ P = 0.0004 \) respectively). Larval biomass was also a significant covariate describing 11% of the total variation in cumulative larval mortality in the second trial \( (F_{1,17} = 13.9, \ P = 0.002) \) with cumulative larval mortality increasing as larval biomass decreased.

In the first trial, the influence of nematode concentration \( (F_{9,6,81.7} = 5.8, \ P < 0.001) \) and endophyte infection \( (F_{4,8,81.7} = 3.3, \ P = 0.01) \) varied over time (Table 2, Fig. 2). Larvae exposed to nematodes suffered significantly higher average mortality than unexposed larvae \( (F_{1,17} = 5.6, \ P = 0.03) \), but average mortality was similar for larvae exposed to moderate (eight) or high (16) nematode concentrations \( (F_{1,17} = 0.3, \ P = 0.58) \). Total cumulative mortality \( (78 h \text{ post-exposure}) \) increased linearly with increasing nematode concentration \( (F_{1,17} = 18.2, \ P < 0.001) \). After 36 h, larvae fed endophyte-infected grass had significantly lower mortality than those fed uninfected grass and this trend was significant for the remainder of the experiment. Although there was no significant Time × Endophyte × Nematode interaction \( (F_{9,8,81.7} = 1.3, \ P = 0.24) \), total

**Table 1.** Univariate F-values and degrees of freedom (d.f.) for repeated-measures ANOVA model describing the influence of the entomopathogenic nematode *Steinernema carpocapsae* on survival of *Spodoptera frugiperda* larvae feeding on intact plants of endophyte-infected or -uninfected perennial ryegrass in the greenhouse at 36, 42, 48, 54, 60, 66, and 72 h after exposure to nematodes.

<table>
<thead>
<tr>
<th>Factor</th>
<th>d.f.</th>
<th>36 h</th>
<th>42 h</th>
<th>48 h</th>
<th>54 h</th>
<th>60 h</th>
<th>66 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>336.0*</td>
<td>79.5*</td>
<td>79.5*</td>
<td>80.7*</td>
<td>80.7*</td>
<td>46.1*</td>
<td>46.1*</td>
</tr>
<tr>
<td>Endophyte</td>
<td>1</td>
<td>29.5*</td>
<td>1.1</td>
<td>1.1</td>
<td>0.6</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*P < 0.0001.
cumulative mortality was significantly lower for larvae fed endophyte-infected plants when these larvae were exposed to nematodes ($F_{1,17} = 14.7$, $P = 0.001$). Endophyte infection had no significant influence on total cumulative mortality for unexposed larvae ($F_{1,17} = 1.0$, $P = 0.322$).

In the second trial, larval mortality varied simultaneously over time with nematode concentration, and endophyte infection ($F_{9.7, 82.1} = 2.3$, $P = 0.004$) (Table 2, Fig. 2). Larvae suffered significantly lower average mortality when fed endophyte-infected grass regardless of exposure to nematodes ($F_{1,17} = 8.1$, $P \leq 0.01$); however, nematodes caused lower total cumulative mortality in larvae fed endophyte-infected grass compared to those fed uninfected grass ($F_{1,17} = 6.5$, $P = 0.02$). Endophyte infection had no significant influence on total cumulative mortality of unexposed larvae ($F_{1,17} = 3.0$, $P = 0.10$).

**Injection of X. nematophila**

Cumulative larval mortality varied significantly among treatments ($F_{11,2,38.1} = 3.1$, $P = 0.005$) and larval biomass had no significant effect in this regard ($F_{1,17} = 0.8$, $P = 0.77$) (Table 3, Fig. 3). Average mortality was similar for uninjected and saline injected larvae ($F_{1,17} = 1.03$, $P = 0.32$) whereas bacteria-injected larvae suffered

![Graph](image)

**Fig. 2.** Influence of *Steinernema carpocapsae* (SC) concentration (zero, eight, or 16 per Petri dish) on mortality of *Spodoptera frugiperda* larvae fed endophyte-infected (E+) or -uninfected (E−) perennial ryegrass in two independent experimental trials: (a) trial 1 (b) trial 2. Error bars = SEM.

significantly higher average mortality than saline injected or uninjected larvae ($F_{1,17} > 51.0$, $P < 0.0001$). Endophyte infection had no significant influence on average mortality of uninjected or saline injected larvae ($F_{1,17} < 0.9$, $P > 0.37$). However, endophyte infection significantly reduced average mortality of bacteria injected larvae ($F_{1,17} = 4.6$, $P = 0.04$) and although not always significant, this trend was consistent for 52 h post-injection.

**Discussion**

Although the idea that bottom-up and top-down forces act in concert to regulate populations of herbivorous insects is not new, the interaction between these forces has not received much attention. Studies have demonstrated that microbial associations with plant roots can impact plant quality directly. For example, vesicular-arbuscular mycorrhizae often assist plants in the acquisition of nutrients (particularly phosphorous) (Bolan, 1991) and may therefore alter the mineral and lipid concentration of plant tissues (Packovsky et al., 1986; Packovsky & Fuller, 1988). These data suggest that intimate associations between plants and microbes can affect the nutritional value of foliage (Benz et al., 1995) and influence plant susceptibility to herbivores (Gange & West, 1994; Borowicz, 1997); however, only recently has the possible impact of plant–microbe associations on the natural enemies of herbivores been the subject of empirical study (Barker & Addison, 1997; Bultman et al., 1997; Omacini et al., 2001). Although Neotyphodium fungal endophytes can have strong effects on plant defences that are not typically seen with mycorrhizae, current observations emphasise the important influence that microorganisms may exert on multi-trophic interactions. Neotyphodium fungal endophytes have important negative effects on herbivorous insects but, in turn, may reduce the susceptibility of herbivorous insects to their natural enemies.

Insect larvae exposed to toxic plant material may experience enhanced resistance to natural enemies through a combination of physiological and behavioural mechanisms. One physiologically based mechanism through which insect herbivores may benefit from host plant defensive compounds lies in the effect that these noxious compounds may have on the herbivores’ nutritional value for natural enemies. Barbercheck (1993) suggests that aside from secondary chemistry, plant characteristics such as protein, lipid, or carbohydrate concentrations may influence the quality of herbivorous insects as a resource for natural enemies. Some endophyte-mediated alkaloids are strong feeding deterrents (Prestidge et al., 1982; Latch, 1993) that could influence the suitability of insects feeding on endophyte-infected plants as a resource for natural enemies. Barker and Addison (1997) found that Microctonus hyperodae, a parasitoid of the Argentine weevil, displayed developmental difficulties on hosts reared on endophyte-infected grass. Likewise, Bultman et al. (1997) observed reduced survival of the parasitoid Euplectus comstockii in *S. frugiperda* larvae reared on diet containing endophyte-mediated alkaloids. More recently, Omacini et al. (2001) found that endophytes can have community-wide impacts on resource–consumer interactions. In an aphid–parasitoid system, endophyte-free grasses supported a greater abundance of insects and higher rates of parasitism than did endophyte-infected grasses. Omacini et al. (2001) concluded that observed differences were due to limited energy transfer to consumers as a result of low plant quality. Conversely, Grewal et al. (1995) demonstrated that scarab larvae feeding on the roots of endophyte-infected grasses are significantly more susceptible to entomopathogenic nematodes. However, below-ground feeding insects would not be exposed to the same high levels of endophyte-mediated alkaloids that foliage feeding insects would be exposed to. In the present study, differences in feeding behaviour were accounted for by incorporating the laboratory trials in which no food was present during larval exposure to the nematode. Although larvae fed endophyte-infected

**Table 3.** Univariate $F$-values and degrees of freedom (d.f.) for repeated-measures ANOVA model describing mortality of *Spodoptera frugiperda* fed endophyte-infected or -uninfected perennial ryegrass then injected with saline solution, or the bacteria *Xenorhabdus nematophila*, or uninjected.  

<table>
<thead>
<tr>
<th>Factor</th>
<th>d.f.</th>
<th>24h</th>
<th>31h</th>
<th>35h</th>
<th>39h</th>
<th>52h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>0.27</td>
<td>0.03</td>
<td>0.06</td>
<td>0.01</td>
<td>0.61</td>
</tr>
<tr>
<td>Biomass</td>
<td>1</td>
<td>0.07</td>
<td>0.39</td>
<td>0.31</td>
<td>0.50</td>
<td>0.02</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>0.61</td>
<td>9.36**</td>
<td>10.16**</td>
<td>8.01**</td>
<td>4.18*</td>
</tr>
<tr>
<td>Error</td>
<td>17</td>
<td></td>
<td></td>
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</table>

* $P < 0.01$, ** $P < 0.001$
grass often had lower biomass than those feeding on uninfected grass, this difference did not explain observed differences in mortality due to nematode infection.

Another physiological mechanisms that may influence susceptibility to natural enemies is the alteration and/or storage of plant-derived compounds for the purpose of defence against natural enemies. Empirical evidence for the recycling of noxious plant compounds by herbivorous insects is widespread both in terms of the kinds of plant compounds utilised and the groups of insects involved (Bowers, 1992; Nishida, 1995; Dobler, 2001). For example, several Chrysomelid beetles can sequester plant derived alkaloids and glycosides which are used to provide defence against predators (Soetens et al., 1998; Dobler, 2001). Likewise, parasitoids are sometimes affected negatively by insect hosts that sequester plant derived alkaloids (Gunasena et al., 1990; Barbosa et al., 1991a). In a study similar to that reported here, Kunkel et al. (2004) reported that endophyte-mediated alkaloids can reduce the growth and pathogenicity of X. nematophila in vitro, and these compounds can be found in the haemolymph of S. frugiperda larvae feeding on endophyte-infected grass (B. A. Kunkel and P. S. Grewal, unpubl. data). When the nematodes’ symbiotic bacteria was injected directly into the haemocoel of S. frugiperda, larvae fed endophyte-infected grass suffered lower mortality than larvae fed uninfected grass. Taken together, these findings imply that endophyte-mediated alkaloids may reduce the efficacy of entomopathogenic nematodes by acting on the symbiotic bacteria inside the insect haemocoel.

Host size was an important consideration in the study reported here and has been linked to nematode pathogenicity (Barbercheck, 1993). Several studies have demonstrated decreases in susceptibility to entomopathogenic nematodes as insect age increases (Fuxa et al., 1988; Glazer, 1992; Shapiro et al., 1999). In the present study, larval biomass was a significant covariate only in the first experimental trial using older, larger larvae. Smaller, younger larvae were more susceptible to the nematodes and this fact was reflected in the results. In the second trial using younger larvae, the highest dosage of nematodes apparently overwhelmed S. frugiperda larvae such that the influence of food source was not as strong. Furthermore, higher mortality was observed in the second trial regardless of nematode concentration.

The findings of this study emphasise the potential importance of microbes in determining plant quality and demonstrate a linkage between plant-symbiotic microbes, insect herbivores, and the natural enemies of herbivorous insects. Specifically, endophyte-infected plants reduce the virulence of entomopathogenic nematodes not so much by altering insect feeding behaviour or physiological condition, but rather by reducing the pathogenicity of the nematodes symbiotic bacteria inside the insect haemocoel. In this way, the insect was able not only to circumvent the plants’ acquired chemical defences, but turned these endophyte-mediated defences against the nematode–bacteria complex. Work is currently underway to determine which endophyte-mediated alkaloids are primarily responsible for protecting S. frugiperda from the harmful effects of the nematodes’ symbiotic bacteria.

Acknowledgements

This work was supported by the United States Department of Agriculture NRI grant number 00-35316-9249 and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center. We are also grateful for the suggestions of two anonymous reviewers.

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