SHORT COMMUNICATION

Impact of *Metarhizium brunneum* (Hypocreales: Clavicipitaceae) on pre-imaginal *Rhagoletis indifferent* (Diptera: Tephritidae) within and on the surface of orchard soil

J. Cossentinea*, S. Jaronskib, H. Thistlewooda and W. Yeea

aAgriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, BC, Canada V0H 1Z0; bUSDA-ARS, Northern Plains Agricultural Research Laboratory, Sidney, MT, USA; cUSDA-ARS, Yakima Agricultural Research Laboratory, Wapato, WA, USA

(Received 22 June 2011; final version received 16 September 2011)

When last instar laboratory-reared *Rhagoletis indifferent* were allowed to pupate within non-sterile orchard soil containing incorporated *Metarhizium brunneum* isolate F52 conidia, a dose-related proportion died from developmental abnormalities and mycosis. When larvae entered soil superficially treated with *M. brunneum*, over 80% of the pupae died of developmental abnormalities.

**Keywords:** *Metarhizium brunneum*; *Rhagoletis indifferent*; Tephritidae; cherry fruit fly; mycosis; entomopathogenic fungi

The western cherry fruit fly, *Rhagoletis indifferent* Curran (Diptera: Tephritidae) oviposits eggs into sweet, *Prunus avium* (L.) and sour, *Prunus cerasus* L. cherries (Rosaceae) in western Canada (Banham and Arrand 1978). The larvae feed in the fruit through three instars before they drop to the orchard floor and burrow into the soil to pupate for ca. 9–10 months (Madsen and Procter 1985). Tropical fruit fly species, including *Anastrepha ludens* (Loew) and several *Ceratitis* species (Diptera: Tephritidae), are susceptible in pre-imaginal stages to infection by several isolates of *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) *sensu latu* (Lezama- Gutiérrez et al. 2000; Ekesi, Maniania, and Lux 2002; Ekesi, Maniana, and Lux 2003; Destefano, Bechara, Messias, and Piedrabuena 2005; Mochi, Monteiro, DeBortoli, Dória, and Barbosa 2006; Quesada-Moraga, Ruiz-García, and Santiago-Álvarez 2006). This susceptibility suggests the possible use of the entomopathogenic fungus as a suppressive soil treatment for tephritid flies. However, studies to date have concluded that temperate Tephritidae are not susceptible to infection by *M. anisopliae* isolates when exposed in pre-adult stages (Yee and Lacey 2005; Daniel and Wyss 2009). The European fruit fly, *Rhagoletis cerasi* (Linnaeus), was found to be unsusceptible to two *M. anisopliae* isolates when dipped in suspensions as late third instars (Daniel and Wyss 2009) and *R. indifferent* larvae allowed to pupate in soil superficially treated with a *M. anisopliae* isolate were also found to be uninfected (Yee and Lacey 2005).

Bioassays of temperate *Rhagoletis* species with pathogens are difficult to evaluate due to the insects’ extensive pupal diapause. Cold storage of *Rhagoletis* pupae under...
controlled conditions can result in low adult emergence (Yee and Lacey 2005). Cossentine, Thistlewood, Goettel, and Jaronski (2010) demonstrated pre-imaginal R. indifferens susceptibility to Beauveria bassiana incorporated into soil, based on the incidence of abnormal pupal development and mycosis and the assumption that these insects would not result in viable adults. The objective of the present study was to similarly evaluate if a commercial strain of M. brunneum (formerly M. anisopliae) can infect and cause mortality in pre-imaginal R. indifferens within non-sterile orchard soil. We also tested whether a lethal Metarhizium infection would result from larvae passing through spores on the surface of the soil.

Larval R. indifferens were reared on an agar-based diet (Cossentine et al. 2010) and were judged appropriate for use in the study based on their size and behavior (i.e., exited diet to seek a pupation site, AliNiazee and Brown 1977). All larvae immediately began to descend into the soil when placed on the soil surface.

Metarhizium brunneum isolate F52 (originally obtained from the Lethbridge Research Centre, Lethbridge, AB, Canada) was plated on Sabouraud Dextrose Agar (SDA) and incubated for at least 2 weeks at 24°C. Conidia were harvested immediately prior to use, suspended in 0.05% Tween 80 in sterile dH2O, and vigorously stirred with glass beads before determining the conidia concentration with a haemocytometer. Twenty-four-hour germination rates were > 90%.

A silty-loam (60% silt, 20% sand, 20% clay) soil obtained from a non-chemically treated cherry orchard in the Okanagan Valley of British Columbia, Canada, was air-dried before use (Cossentine et al. 2010). For incorporation of M. brunneum into the soil, sufficient sterile dH2O was used with the appropriate conidial concentration to result in concentrations of 0, 105, 106, 107, and 108 conidia g−1 soil with a moisture level of 20% of the soil’s water holding capacity (WHC) (0.12 mL H2O g−1 soil). Each 100 g of treated soil was distributed between four 60-mL plastic Solo® cups. For surface treatment of soil with M. brunneum, 1 mL of a 1 × 108 conidia mL−1 suspension was sprayed using an air-brush (Paasche Airbrush Company, Harwood Heights, IL, USA) over the surface of the same soil already containing 0.12 mL H2O g−1 soil to yield 4.5 × 106 conidia cm−2. Five last-instar R. indifferens larvae were dropped onto the soil in each of the four cups. Cups were sealed and incubated at 25°C under a 16 h L:8 h D photoperiod before puparial mycosis was assessed ≥ 28 days post-treatment. Cups were contained within a sealed container with a moist paper towel to maintain high humidity. Trials were replicated four times over separate days.

All puparia were retrieved from each treatment, surface sterilized by immersion in 70% EtOH, and rinsed in dH2O, placed in 5% NaClO for 1 min and rinsed three times in sterile dH2O. The health of each puparium was visually evaluated and the developing imago (pupa) aseptically removed, examined and placed on Potato Dextrose Agar (PDA) and incubated to allow the growth of M. brunneum if present (Cossentine et al. 2010). Active Metarhizium was judged to be M. brunneum F52 solely based on its color and conidia morphology. A puparium was recorded as abnormal if the cuticle was degraded and a pupa was considered abnormal (dead) if it was absent, decomposing, mycosed, or desiccated.

Percent abnormal development, mycosis and confirmation of viable M. brunneum within the interior pupa in treatments were arcsine transformed to normalize data prior to analysis. The treatments were evaluated in two-way ANOVAs (SAS 2008) with replication and treatments examined as factors. Means were compared using...
Tukey’s student range test. The incorporated *Metarhizium* trials and the control were analyzed separately, as was the superficial treatment of $1 \times 10^8$ conidia mL$^{-1}$ and the control, before all treatments were compared in a single analysis.

Replication did not have a significant impact on any of the variables studied ($P > 0.05$). Increasing concentrations of *Metarhizium* conidia incorporated into the soil resulted in a significant dose-related impact on the number of abnormally developing puparia ($F_{4,12} = 72.2; P < 0.0001$) and pupae ($F_{4,12} = 72.0; P < 0.0001$; Table 1). Although the incorporated *M. brunneum* conidia did not result in a significant number of mycosed puparia as was observed when *B. bassiana* was incorporated into soil (Cossentine et al. 2010), it did result in a significant number of dead pupae exhibiting superficial fungal growth within the puparia ($F_{4,12} = 47.9; P < 0.0001$; Table 1). It is important to note that not all of the fungal growth was necessarily the result of F52. The soil used was intentionally not autoclaved to reflect field conditions and other fungal species may have grown on the dead insect tissue and suppressed the growth of the F52. The incidence of active *Metarhizium* on 5.7 ($\pm 3.9$) to 17.1 ($\pm 8.5$)% of the pupae removed from soil with incorporated *M. brunneum* conidia was low, although significantly higher ($F_{4,12} = 3.3; P = 0.049$) than the 0% found on control pupae. When *M. brunneum* conidia were applied to the surface of the soil, the fungus was isolated from 6.1 $\pm 2.2$% of the dissected pupae, which was not significantly greater than the 0% found among control pupae ($P > 0.05$). The low recovery of active *Metarhizium* infections may have been due to the high incidence of abnormally developed puparia, which would allow the EtOH and/or NaClO to penetrate and kill conidia on the pupae during the surface sterilization procedure. Over 80% of the puparia and pupae were found to have developed abnormally after passing through the *M. brunneum* surface treated soil (puparia: $F_{1,3} = 22.1; P = 0.02$; pupae: $F_{1,3} = 32.6; P = 0.01$; Table 1). A significant percentage of these pupae supported fungal growth ($F_{1,3} = 23.4; P = 0.02$; Table 1).

Table 1. Mean percent of abnormally developed (assumed dead) *Rhagoletis indifferens* puparia and pupae, and percent mycosed pupae, from non-sterile orchard soil treated with *M. brunneum* F52 conidia by direct incorporation or surface treatment. The fungus causing the mycosis was confirmed as *Metarhizium* for a small percentage of the pupae. N $= 20$, replicated 4 times.

<table>
<thead>
<tr>
<th></th>
<th>Incorporation method</th>
<th>% abnormal puparia</th>
<th>% abnormal pupae</th>
<th>% mycosed pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. brunneum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incorporated into soil (conidia g$^{-1}$ soil)</td>
<td>0</td>
<td>6.3 $\pm$ 4.7 a$^1$</td>
<td>8.8 $\pm$ 4.2 a</td>
<td>1.3 $\pm$ 1.3 a</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^5$</td>
<td>17.1 $\pm$ 3.8 a</td>
<td>21.0 $\pm$ 3.9 a</td>
<td>13.4 $\pm$ 5.4 a</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^6$</td>
<td>51.9 $\pm$ 5.9 ab</td>
<td>54.6 $\pm$ 6.2 ab</td>
<td>41.7 $\pm$ 5.5 ab</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^7$</td>
<td>80.7 $\pm$ 2.7 bc</td>
<td>84.5 $\pm$ 2.3 bc</td>
<td>78.3 $\pm$ 4.6 c</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^8$</td>
<td>96.9 $\pm$ 3.1 c</td>
<td>96.9 $\pm$ 3.1 c</td>
<td>92.3 $\pm$ 3.0 c</td>
</tr>
<tr>
<td>Superficially applied to soil</td>
<td>4.5 $\times 10^6$ conidia cm$^{-2}$</td>
<td>81.5 $\pm$ 9.2 bc</td>
<td>84.2 $\pm$ 9.8 c</td>
<td>73.7 $\pm$ 12.5 bc</td>
</tr>
</tbody>
</table>

$^1$Means within columns followed by the same letter are not significantly ($P > 0.05$) different as determined using Tukey’s range test (SAS 2008).
Mycosis and abnormal development were not observed when prepupal *R. cerasi* larvae were dipped in $1 \times 10^7$ *M. anisopliae* conidia mL$^{-1}$ (Daniel and Wyss 2009) or when prepupal wild *R. indifferentens* entered soil superficially sprayed with as high as $4.8 \times 10^6$ *M. anisopliae* conidia cm$^{-2}$ (Yee and Lacey 2005). It has been recognized that fungal isolates and soil moisture and temperature are important factors in efficacy trials (Ekesi et al. 2002, 2003). For these reasons we were careful to use the same conditions under which pre-imaginal *R. indifferentens* were found to be susceptible to *B. bassiana* GHA (Cossentine et al. 2010). Our data demonstrate that last instar *R. indifferentens* reared on meridic diet are susceptible to infection by *M. brunneum* when entering the soil to pupate. If this phenomenon is not restricted to laboratory reared insects, orchard floor management of *R. indifferentens* with entomopathogenic fungi may be a practical option.

**Acknowledgements**

The authors are grateful for the help of P. Randall and B. Rozema (AAFC, Summerland, BC). This research was funded by Agriculture and Agri-Food Canada.

**References**


