INTRODUCTION

Entomopathogenic fungi can infect many orders of insects, such as Coleoptera, Lepidoptera, Thysanoptera and Heteroptera (Leathers and Gupta, 1993; Schreiter et al., 1994; Sosa-Gomez et al., 1997; Tsutsumi et al., 2003; Shimazu, 2004; Yaginuma et al., 2004a, b). In particular, many studies on the virulence of these fungi have been carried out in the past as control agents of scarab grubs (Hasegawa and Koyama, 1941; Poprawski and Yule, 1991; Villani et al., 1994; Hiromori et al., 2004). Scarab grubs are major pests of agricultural land, forests and turf grasses (Shimazu et al., 1993; Hiromori and Nishigaki, 1998). These larvae are soil-inhabiting insects and are difficult to control with chemical insecticides. Beauveria brongniartii and Metarhizium anisopliae play important roles in suppressing scarab grubs (Glare, 1992; Zimmerman, 1992; Kessler et al., 2003). However, the controlling effects are often not stable because the pathogenicity of fungi may be influenced by many environmental factors (Hiromori and Nishigaki, 1998).

It is well known that entomopathogenic fungal conidia first adhere to the cuticle of host insects and then penetrate the cuticle to invade the host insects (St. Leger et al., 1989). This adhesion of conidia is considered to be one of the most important processes of infection. The conidial adhesion is inhibited by some components of the cuticle (Smith and Grula, 1981; Bidchika and Khachatourians, 1992). Therefore, the virulence of fungi is affected by the effectiveness of the conidial adhesion, which involves chemical interactions between conidia and the host cuticle.

However, the chemical dynamics between fungi and cuticle cannot sufficiently explain the mechanisms of fungal adhesion. Quintela and McCoy (1998) reported that movement of the larvae of the weevil, Diapreps abbreviatus, can facilitate the detachment of the fungal conidia from the cuticle. Scarab grubs are also soil-inhabiting insects. Yaginuma et al. (2004a) suggested that the conidia of...
the entomopathogenic fungus, *B. amorphapha*, were detached from the cuticle of the scarab grubs of *Heptophylla picea* in the searching behavior for food in the soil.

Imidacloprid is a neurotoxin that acts by binding to the nicotinic acetylcholine receptor, causing paralysis in some insects (Abbink, 1991; Boucias et al., 1996). This chemical caused paralysis in the termite, *Reticulitermes flavipes*, but did not influence fungal germination, vegetative growth and adhesion of the entomopathogenic fungus, *Beauveria bassiana* (Boucias et al., 1996). Therefore, imidacloprid was adopted in this study as an agent to suppress the insect movement.

In this study, we experimentally investigated the relationship between the movement of scarab grubs, *Anomala cuprea*, and the conidial detachment of the entomopathogenic fungus *B. amorphapha*, from the larvae.

**MATERIALS AND METHODS**

**Insect, pathogen and test soil.** Adults of *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) were collected in May from Shizuoka Pref., Japan. They were reared on artificial diets (Hatsukade et al., 1984) and allowed to lay eggs at 25 ± 1°C under a 16L : 8D photoregime. Hatched larvae were individually reared in plastic cups with leaf mold mixed with distilled water (DW) (dried leaf mold: DW = 2 : 1 w/w) at 25 ± 1°C under 16L : 8D photoregime. First stadium larvae were used in all trials.

*Beauveria amorphapha* (strain: HpBa-1) was isolated from infected larvae of the yellowish elongate chafer *Heptophylla picea* Motschulsky (Coleoptera: Scarabaeidae). This fungus showed a low virulence to *A. cuprea* larvae (Yaginuma et al., 2004b). The fungus was cultured on Sabouraud dextrose agar with 1% yeast extract at 25 ± 1°C for 10 to 14 d. Conidia were harvested in sterilized distilled water (SDW) containing 0.05% Tween 80. The suspension was filtered, and the concentration of conidia was adjusted with a hemocytometer under a phase-contrast microscope (400×) (Olympus, Tokyo, JPN).

Test soil for all trials was sterilized by heating and drying (150°C, 4 h), and then mixed with SDW (soil: SDW = 2 : 1 w/w). Thirty grams of the soil was placed in 90 mm plastic Petri dishes. In all trials, the insects were individually reared on sliced carrots in the plastic Petri dishes at 25 ± 1°C under 16L : 8D.

**Conidia labeling with fluorescein isothiocyanate (FITC).** The protocol described by Hung and Boucias (1992) was modified to label the conidia of *B. amorphapha* with FITC. Conidia were harvested in a carbonate-bicarbonate buffer containing 0.01% Tween 80 (pH 9.2) (CB buffer) and centrifuged for washing (2,000×g, 4°C, 10 min). Washed conidia were labeled with FITC (1 mg/ml) for 1 h at 20 ± 1°C. Labeled conidia were centrifuged for washing (2,000×g, 4°C, 10 min, ×5) and resuspended in SDW containing 0.05% Tween 80. Fluorescence of labeled conidia was confirmed under a fluorescent microscope prior to use (Olympus BX-FLA, 400×) (Olympus, Tokyo, JPN).

**Effect of imidacloprid concentrations on behavior of larvae.** Imidacloprid (AI: 98.5%), 1-{(6-chloro-3-pyridinyl) methyl}-N-nitro-2-imidazolidinimine, was supplied by Bayer Crop Science (Bayer Crop Science, Tsukuba, JPN). Larvae of *A. cuprea* were treated with a series of diluted imidacloprid to determine the concentration at which the larvae were not killed but were paralyzed for the duration of the test period (at least for 24 h). Imidacloprid was adjusted with acetone to 25.0, 10.0, 7.5, 5.0, 2.5, 1.0 and 0.1 ppm and applied to *A. cuprea* larvae topically (1.0 μl) behind the head capsule using a syringe equipped with a microapplicator (Burkard, Hertfordshire, UK) and a 31G needle (Hamilton Cie, Reno, USA). After application, the larvae were air-dried for 20 min. Behavior of the larvae was observed at 0, 6, 12, 18, 24 and 48 h after application. Control larvae were treated with acetone only. The effects of imidacloprid on these larvae were categorized into normal activity (similar to normal larvae), convulsions, and paralysis. Each of the trials consisted of five larvae per concentration, and was replicated twice. Larval death was checked at five and seven days after application.

**Conidial adhesion and detachment in several body regions non-paralyzed and paralyzed larvae.** Larvae were treated with diluted imidacloprid (concentration: 10.0 ppm) and air-dried for 20 min. These larvae were then dipped into a suspension of FITC-labeled conidia (concentration: 4.0 × 10^7 conidia/ml) for 30 s and air-dried for 10 min. The number of adhering conidia was observed over
time, and a decrease in conidial adhesion was considered to indicate conidial detachment. The protocol described in Altre et al. (1999) was modified for counting the number of conidia adhering to the larval cuticle. Whole bodies of dipped larvae were dissected and mounted on a slide. The number of conidia was counted on the vertex (excluding mouthparts), dorsum and abdomen (containing setae) within the field of vision of a fluorescent microscope (3 points/site/larva, Olympus BX-FLA, 400×) (Olympus, Tokyo, JPN), and the means were converted to the number of conidia per 1 mm². Non-paralyzed larvae, which were not treated with imidacloprid were assayed in the same way as control. Each trial consisted of five larvae per treatment and interval, and was replicated twice.

Conidial detachment from non-paralyzed and paralyzed larvae in the soil. Larvae were treated with diluted imidacloprid (concentration: 10.0 ppm) and air-dried for 20 min. These larvae were then dipped in a conidial suspension (concentration: 4.0×10⁷ conidia/ml) for 30 s and air-dried for 10 min. After 6, 12 and 24 h, the larvae were individually placed in a glass tubes with SDW (5 ml) containing 0.05% Tween 80 and agitated on a shaker (600 rpm, 20 min); the solution was then diluted 10, 10² or 10³ fold with SDW containing 0.05% Tween 80 (v/v). To count the colony-forming units (CFUs), samples were plated on a selective medium consisting of glucose (5 g), peptone (5 g), oxgall (7.5 g), rose Bengal (30 mg), dodeine (5 mg), cycloheximide (0.125 g), chloramphenicol (0.25 g), agar (15 g), streptomycin (60 mg/l) and penicillin G (60 mg/l) in 500 ml of SDW (Inglis et al., 1996). One milliliter of each of the diluted sample was plated on agar medium and incubated at 25±1°C in the dark for seven days. Non-paralyzed larvae, which were not treated with imidacloprid, were assayed in the same way as the control. Each trial consisted of five larvae per treatment and interval, and was replicated twice.

Soil from each of the containers (Petri dishes containing 30 g soil) used in the trials was put into a 200 ml Erlenmeyer flask with SDW (80 ml) containing 0.05% Tween 80. The containers and the sliced carrots were carefully washed with SDW (20 ml) containing 0.05% Tween 80, which was added to the same flask (total volume: 100 ml). The suspensions in the flask were agitated on a shaker (200 rpm, 20 min) and were diluted 10- or 10²-fold with SDW containing 0.05% Tween 80 (v/v). The diluted samples were plated on the selective medium to measure CFUs as described above. The number of conidia in the rearing soil was considered to reflect the number of conidia that had detached from the larvae. Rearing soil of non-paralyzed larvae was treated in the same manner as control. Each trial consisted of five containers per treatment and interval, and was replicated twice.

Movement of non-paralyzed and paralyzed larvae. Larvae were treated with diluted imidacloprid (concentration: 10.0 ppm). These larvae were individually placed at the center of a plastic container (35.0 cm×25.0 cm×4.5 cm) filled with test soil (about 1 cm in depth). Four sliced carrots were placed at the corners of the container. The movement of larvae was observed for 30 min and the distance of movement was measured by tracing the larval tracks. Non-paralyzed larvae were assayed in the same way as control. Each trial consisted of 10 larvae per treatment.

Statistical analysis. One-way ANOVA with the Scheffé’s multiple range tests and Student’s t-test were performed using Stat View ver. (SAS Institute Inc., Cary, NC).

RESULTS

Suppression of larval movement by imidacloprid treatment

The results of concentration assays are presented in Table 1. Imidacloprid concentrations of 25.0 and 10.0 ppm immediately paralyzed the larvae of A. cuprea, and the larvae did not recover for 24 h. However, the paralytic effects of at these concentrations were lost by 48 h after application. The larvae exhibited convulsions at a concentration of 7.5 ppm, but recovered by six hours after application. At concentrations of 5.0 ppm and lower, the larval activity was indistinguishable from non-paralyzed control larvae. All of these larvae were alive five and seven days after application.

Differences in conidial detachment from different body regions of non-paralyzed and paralyzed larvae

In non-paralyzed and paralyzed larvae, the numbers of conidia differed significantly between the vertex, dorsum and abdomen at 0 h after application.
tion (vertex vs. dorsal region, \( p=0.0056 \); in non-paralyzed larvae: vertex vs. dorsal region, \( p=0.0007 \); vertex vs. abdomen, \( p=0.0014 \); in paralyzed larvae) (Table 2, Fig. 1A).

Table 1. Effect of imidacloprid concentrations on behavior of Anomala cuprea larvae

<table>
<thead>
<tr>
<th>Concentration of imidacloprid (ppm)</th>
<th>Time after application (h)</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Anomala cuprea* larvae were treated with imidacloprid by topical application (1.0 μl) and air-dried for 20 min. Larval activity was checked 0, 6, 12, 18, 24 and 48 h after treatment. Imidacloprid (AI: 98.5%) was adjusted with acetone to concentrations of 25.0, 10.0, 7.5, 5.0, 2.5, 1.0 and 0.1 ppm. The larvae were treated with acetone by the same method as controls.

Activity ranking: + = normal activity, ± = convulsions, = complete paralysis.

\( N=10 \) larvae/concentration.

Table 2. Differences in conidial adhesion on the body regions of non-paralyzed and paralyzed larvae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after application (h)</th>
<th>Body region</th>
<th></th>
<th></th>
<th></th>
<th>F</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vertex</td>
<td>Dorsum</td>
<td>Abdomen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>138.1 ±24.96(^a)</td>
<td>246.2 ±23.16(^a)</td>
<td>194.7 ±15.04(^a)</td>
<td>6.327</td>
<td>2, 27</td>
<td>0.0056(^d)</td>
<td></td>
</tr>
<tr>
<td>(Non-paralyzed)</td>
<td>12</td>
<td>4.1 ±1.57(^b)</td>
<td>26.8 ±4.68(^b)</td>
<td>20.6 ±7.68(^b)</td>
<td>4.981</td>
<td>2, 27</td>
<td>0.0144</td>
<td></td>
</tr>
<tr>
<td>larvae)</td>
<td>24</td>
<td>0.4 ±0.30(^b)</td>
<td>2.4 ±1.00(^b)</td>
<td>5.1 ±1.87(^b)</td>
<td>2.517</td>
<td>2, 27</td>
<td>0.0439</td>
<td></td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>0</td>
<td>99.5 ±24.50(^a)</td>
<td>236.1 ±22.09(^a)</td>
<td>228.2 ±19.45(^a)</td>
<td>12.029</td>
<td>2, 27</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>(Paralyzed)</td>
<td>12</td>
<td>49.1 ±18.29(^a)</td>
<td>312.2 ±48.63(^a)</td>
<td>136.0 ±20.34(^a)</td>
<td>17.309</td>
<td>2, 27</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>larvae)</td>
<td>24</td>
<td>18.2 ±7.90(^a)</td>
<td>197.8 ±29.11(^a)</td>
<td>89.0 ±35.18(^a)</td>
<td>11.428</td>
<td>2, 27</td>
<td>0.0003</td>
<td></td>
</tr>
</tbody>
</table>

*Anomala cuprea* larvae were treated with imidacloprid (1.0 μl) and air-dried for 20 min. Larvae were dipped in a suspension of FITC labeled conidia (concentration: 4.0×10\(^7\) conidia/ml) for 30 s and air-dried for 10 min. After 0, 12 and 24 h, the larvae were mounted and the vertex, dorsum and abdomen observed (400×). The number of conidia on each body region was counted.

\( a \) Numbers within columns followed by the same letter are not significantly different (one-way ANOVA; \( \alpha=0.01 \); Scheffé’s multiple range test). Significance tests were conducted on non-paralyzed and paralyzed larvae. Non-paralyzed larvae: vertex \( (F=29.5; \ df=2, 27; \ p<0.0001) \), dorsum \( (F=96.7; \ df=2, 27; \ p<0.0001) \), abdomen \( (F=115.2; \ df=2, 27; \ p<0.0001) \). Treated larvae: vertex \( (F=5.1; \ df=2, 27; \ p=0.0136) \), dorsum \( (F=2.7; \ df=2, 27; \ p=0.0819) \), abdomen \( (F=7.4; \ df=2, 27; \ p=0.0027) \).

\( b \) Numbers within tables indicate the conidial adhesion on each region (conidia/mm\(^2\)). Decrease in conidial adhesion was estimated as conidial detachment from the larval cuticle.

\( c \) Numbers within columns followed by the same letter are not significantly different (one-way ANOVA; \( \alpha=0.01 \); Scheffé’s multiple range test). Significance tests were conducted on non-paralyzed and paralyzed larvae. Non-paralyzed larvae: vertex \( (F=29.5; \ df=2, 27; \ p<0.0001) \), dorsum \( (F=96.7; \ df=2, 27; \ p<0.0001) \), abdomen \( (F=115.2; \ df=2, 27; \ p<0.0001) \). Treated larvae: vertex \( (F=5.1; \ df=2, 27; \ p=0.0136) \), dorsum \( (F=2.7; \ df=2, 27; \ p=0.0819) \), abdomen \( (F=7.4; \ df=2, 27; \ p=0.0027) \).

\( d \) Numbers within rows (different conidia on observation sites) were analyzed by one-way ANOVA (\( \alpha=0.01 \)).

\( N=10 \) larvae/h/treatment.
Conidial detachment from non-paralyzed and paralyzed larvae in the soil

The number of conidia on the non-paralyzed larvae were $4.31 \times 10^4 / 1.104 \times 10^0$ at 0 h after inoculation. After 12 and 24 h, the numbers of conidia significantly decreased to $6.33 \times 10^4$ (0 h vs. 12 h, $p = 0.0279$) and $\times 10^2$ (0 h vs. 24 h, $p = 0.0145$), respectively, but the decreases were statistically not significant. In the rearing soil, the numbers of conidia were below the detectable level throughout the test period (Fig. 2).

The numbers of conidia on the paralyzed larvae were $6.46 \times 10^4$ at 0 h after inoculation. After 12 and 24 h, the numbers of conidia slightly decreased to $1.11 \times 10^4$ (0 h vs. 12 h, $p = 0.0279$) and $1.04 \times 10^4$ (0 h vs. 24 h, $p = 0.0145$), respectively, but the decreases were statistically not significant. In the rearing soil, the numbers of conidia were below the detectable level throughout the test period (Fig. 2).

**Movement of non-paralyzed and paralyzed larvae**

The non-paralyzed larvae achieved the average distance of $63.7 \pm 18.88$ cm over 30 min ($n = 10$; range, 15.9–214.4 cm). On the other hand, the paralyzed larvae were completely immobilized by imidacloprid (0.0 ± 0.0 cm; $n = 10$). The difference was, needless to say, statistically significant ($t = -3.913, p = 0.0034$).

**DISCUSSION**

We demonstrated that the movement of *A. cuprea* larvae in the soil affected the conidial detachment from the cuticle. Conidia on the non-paralyzed larvae were detached from the cuticle over time, probably because of their active movement.
Distance of the larval movement was 63.7 cm over 30 min on average, in contrast to substantially no movement (0 cm) of the paralyzed larvae. The paralyzed larvae rarely moved and merely wiggled on the soil surface in convulsions, which should be the reason why the number of conidia on the paralyzed larvae was stable over time. Furthermore, the conidial titer detected from the rearing soil was observed to be parallel with the conidial detachment from the non-paralyzed larvae. From these results, we suggest that the larval movement is associated with the detachment of fungal conidia from the cuticle, which is probably caused by the friction between the host body and the soil. Our results are in agreement with the findings of Quintela and McCoy (1997), in which immobilization of D. abbreviatus larval prevented the conidial detachment upon contact with the surface of the substrate.

On A. cuprea larvae, more conidia were found on the dorsum and the abdomen than on the vertex. The difference is probably related to the surface structure of the body regions. The surface of the vertex is smoother than those of the dorsum and abdomen. Conidia attached on the rough surface with indentations are expected to be more stable than those on a smooth surface. Hajek and Eastburn (2003) reported that conidia of Entomophaga maimaiaga were more abundantly found on the segments than on the other sites of larvae of the gypsy moth, Lymantria dispar.

We used imidacloprid to inhibit the movement of A. cuprea larvae. It has been reported that imidacloprid did not influence the fungal growth and germination of B. bassiana (Boucias et al., 1996). We confirmed that the growth of B. amorpha and another entomopathogenic fungus, Nomuraea revelyi, was not affected by imidacloprid (unpublished data). Although chemical insecticides can potentially suppress the immune system of the host insect (Hiromori and Hatsukade, 2000), low concentrations of imidacloprid did not affect several immune responses of R. flavipes, such as hemocyte reactions, humoral reactions and cuticle reactions (Boucias et al., 1996). Therefore, we suppose that side effects of imidacloprid are, if any, negligible in this study.

In this study, we demonstrated that the movement of insects is an important factor promoting the detachment of entomopathogens from the host cuticle. Our conclusion would apply not only to the scarab grubs but also to other insect living in the soil, including many notorious agricultural pests. To better control these insect pests, it may be useful to more seriously consider the interactions at the interface between the fungal conidia and the host cuticle. In future studies, in addition to the physical affecting the conidial attachment, chemical factors related to the conidial adhesion should be also investigated.

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REFERENCES


