Infectivity of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) to *Phlebotomus papatasi* (Diptera: Psychodidae) Under Laboratory Conditions

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**ABSTRACT** Susceptibility of *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) larvae to the entomopathogenic fungus *Metarhizium anisopliae* (Metschinkoff) Sorokin (Ma79) (Hypocreales: Clavicipitaceae) was evaluated at two different temperatures. The ability of the fungus to reinfect healthy sand flies was followed up for ~20 wk and the effect of in vivo repassage on the enhancement of its virulence was assessed. The fungus reduced the adult emergence at 26 ± 1°C when applied to larval diet. Six spore concentrations were used in the bioassays ranging from 1 × 10^6 to 5 × 10^8 spores/ml. Mortality decreased significantly when the temperature was raised to 31 ± 1°C at all tested concentrations. Fungus-treated vials were assayed against sand fly larvae at different time lapses without additional reapplication of the fungus in the media to determine whether the level of inocula persisting in the media was sufficient to reinfect healthy sand flies. Twenty weeks postapplication, there were still enough infectious propagules of Ma79 to infect 40% of *P. papatasi* larvae. A comparison between the infectivity of 10 subsequent in vitro cultures and the host-passed inocula of the fungus against sand fly larvae was conducted. Mortalities of *P. papatasi* larvae changed significantly when exposed to inocula passed through different insects. Presented data can provide vector control decision makers and end users with fundamental information for the introduction and application of *M. anisopliae* as an effective control agent against the main cutaneous leishmaniasis old-world vector *P. papatasi*.

**KEY WORDS** sand flies, *Metarhizium anisopliae*, biological control, fungal persistence

Leishmaniasis, a vector-borne zoonotic disease, is prevalent in 98 countries, affecting an estimated 12 million people, with ~0.2–0.4 million visceral leishmaniasis and 0.7–1.2 million cutaneous leishmaniasis cases per year (World Health Organization [WHO] 2008, Alvar et al. 2012). Phlebotomine sand flies are the vectors of at least 20 species of leishmania (WHO 2008). *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) is the proven vector of zoonotic cutaneous leishmaniasis, and *Leishmania major* is the etiological agent associated throughout North Africa, the Middle East, and Southwest Asia.

The evolutionary selection of insect strains that are resistant to chemical agents has been repeatedly observed (Mwangala and Galloway 1993, Guglielmone et al. 2001). Furthermore, these chemicals are often toxic to humans as well as other nontarget organisms and can lead to environmental damage. Therefore, the entomopathogenic fungi are currently under intensive study as a promising alternative to insecticides (Warburg 1991, Reithinger et al. 1997, Amóra et al. 2009, 2010). Few attempts have been approached to use *Metarhizium anisopliae* (Metch.) Sorok (Hypocreales: Clavicipitaceae) against phlebotomine sand flies (Ngumbi et al. 2011, El-Shazly et al. 2012).

The assessment of the postapplication persistence of a biological control agent is important with respect to its biocontrol potential and performance (Bidochka 2001). Most biological control agents have a threshold population level needed for biocontrol; therefore, survival of the fungus for a certain period is necessary for efficient pest control (Paulitz 2000). Furthermore, fungal pathogens have the potential to develop on insect cadavers and reintroduce additional inocula to the environment (Thomas et al. 1996). Accordingly, experiments were conducted to evaluate reinfestation ability of *M. anisopliae* against the sand fly larvae, using the treated vials from previous bioassays at different time intervals without the addition or renewal of the fungal dose in the media.

Several commercial products of entomopathogenic fungi are available for insect control, owing to the
relative technical ease of spore mass production. However, the continual production of inoculum on artificial culture media may finally lead to instability and attenuation of virulence of many entomopathogenic fungi (Fargues and Robert 1983, Morrow et al. 1989, Butt and Goettel 2000, Butt et al. 2006). In several studies, virulence is restored by passaging attenuated inocula through a suitable host (Fargues and Roberts 1983, Morrow et al. 1989, Prenerova 1994). Therefore, in the present work, M. anisopliae was subcultured in vitro 10 times and then repassaged twice in vivo through P. papatasi third instar, Spilosetthus pandurus (Scopoli) (Heteroptera: Lygaeidae) fifth instar, and Spodoptera littoralis (Boisdval) (Lepidoptera: Noctuidae) third instar to evaluate the in vivo passed M. anisopliae virulence against P. papatasi larvae.

The specific objectives of the current study were: 1) to evaluate the efficacy and pathogenicity of M. anisopliae against P. papatasi larvae under laboratory conditions at two different temperatures; 2) to determine the persistence of conidia of M. anisopliae over time under controlled conditions, measured as infectivity against P. papatasi; and 3) to determine changes in virulence of M. anisopliae against P. papatasi associated with in vitro and in vivo serial passage.

Materials and Methods

Insect Colony. P. papatasi larvae were kindly provided from the insectaries of the Vector Biology Research Program at U.S. Naval Medical Research Unit No. 3 (NAMRU-3), Cairo, Egypt. The colony was initially collected from Sinai, Egypt, and established at NAMRU-3. A laboratory colony of sand flies was then maintained at the Department of Entomology, Faculty of Science, Cairo University according to the technique described by Modi and Tesh (1983).

Fungal Cultures. M. anisopliae (Ma79), the fungal isolate used in this study, was isolated from Agrotis sp. (Lepidoptera: Noctuidae) in Germany. This was kindly obtained from Dr. G. Zimmermann (Institute for Biological Control, Darmstadt, Germany), cultured, and adapted to different insect hosts in the Department of Entomology laboratory since 2000 until current. The isolate was grown on Sabouraud dextrose yeast agar (SDAY). The medium was autoclaved at 120°C for 20 min, poured in petri plates (9 cm diameter × 1 cm), and kept sterile under room temperature. Multispore cultures were plated out and then repassaged twice in vivo through P. papatasi third instar, Spilosetthus pandurus (Scopoli) (Heteroptera: Lygaeidae) fifth instar, and Spodoptera littoralis (Boisdval) (Lepidoptera: Noctuidae) third instar to evaluate the in vivo passed M. anisopliae virulence against P. papatasi larvae.

The Change in Spore Content of M. anisopliae at Two Different Temperatures. Fungal infection bioassays were performed under two different temperature regimens (26 ± 1 and 31 ± 1°C) in 40-ml plastic vials (3.5 cm in diameter, 6.5 cm in height) with a 1-cm-thick basal layer of plaster of Paris extending through a hole drilled on the bottom of the vial. The plaster was saturated with distilled water before the experiment and was blotted with filter paper to remove standing water immediately before use. Approximately 0.15 g of finely ground larval diet, composed of rabbit feces, rabbit chow, and beef liver powder (Modi and Tesh 1983), was mixed with 0.5 ml of fungal suspension and added to each vial. In control groups, 0.5 ml of sterile water immediately before use. Approximately 0.15 g of finely ground larval diet, composed of rabbit feces, rabbit chow, and beef liver powder (Modi and Tesh 1983), was mixed with 0.5 ml of fungul suspension and added to each vial. In control groups, 0.5 ml of sterile water was used at each interval. Mortality calculated was based on the percentage of adult emergence.

Reinfection Ability and Persistence of the Fungus. The fungus-treated vials from the previously completed bioassays, which were carried out at 26 ± 1°C, contained mycosed pupal cadavers, larval food, and feces. Control vials contained exuviae of immature stages, larval food, and feces. The experiment was carried out by adding 10 second-instar larvae and =0.15 g of larval diets in each of these vials without addition or renewal of the fungal dose at six different time intervals (1, 2, 4, 6, 8, and 16 wk), which started from the emergence of the last survived adult of the previously completed bioassay. The vials were placed in tightly covered plastic boxes (20 cm wide × 28 cm long × 10 cm high). Wet cotton was kept inside each box to maintain high humidity. Plastic boxes were held in an illuminated incubator maintained at 75–85% relative humidity (RH) and a photoperiod of 14:10 (L:D) h regimen. Mortality calculated was based on the percentage of adult emergence.

The Infectivity of M. anisopliae to P. papatasi larvae were kindly provided from the insectaries of the Vector Biology Research Program at U.S. Naval Medical Research Unit No. 3 (NAMRU-3), Cairo, Egypt. The colony was initially collected from Sinai, Egypt, and established at NAMRU-3. A laboratory colony of sand flies was then established at the Department of Entomology, Faculty of Science, Cairo University according to the technique described by Modi and Tesh (1983).

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The change in initial spore content of *M. anisopliae* was detected by following up the treated vials at different week periods (2, 4, 8, 11, and 16 wk), starting with the emergence of the last survived adult. The contents of each vial (mycelial outgrowth pupae, larval food, and feces) were ground in a microcentrifuge tube (1.5 ml) using a Teflon micropestle and were then homogenized in 5 ml sterile distilled water containing 0.05% Tween 80. The suspension was then serially diluted (2, 4, 8, 16 and 32×) in twofold dilution series. In the last dilution (32×), the number of spores was counted using the previously mentioned counting method. These procedures were replicated three times for each concentration at each period.

### In Vivo Passage of *M. anisopliae* (Ma79). This test was to improve infectivity of the fungus after the passage in different insect hosts. Third instar larvae of *S. littoralis*, the Egyptian cotton leaf worm; third instar larvae of *P. papatasi*, and fifth instar nymphs of the hemipteran seed bug *S. pandurus* were incubated with conidiospores of the 10th subculture of *M. anisopliae* (Ma79) in the dark for 48–96 h at 25 ± 1°C. To distinguish between infectious spores that succeeded to penetrate the insect bodies and spores that passively attached to the cuticle, surface sterilization process was meticulously conducted. Larvae and nymphs that might be infected with *M. anisopliae* (Ma79) were surface sterilized with 3 ml 70% ethanol, 3 ml 4% sodium hypochlorite, and 3 ml sterile distilled water each for 3 min. Insects were then gently placed on SDAY medium, and were incubated in the dark at 25 ± 1°C until adequate growth of fungus was observed. The fungus was transferred to fresh SDAY medium and incubated for ~14 d under the same conditions. On sporulation, microscopic examination of the resulting fungus was done for species confirmation. Passage procedure was repeated two times.

Conidia were harvested from the 10th in vitro subculture (Ma79) and 10th (second in vivo) subcultures (MaSpod, MaPh, and MaSpilo), counted, diluted, and assayed twice against second instar *P. papatasi* using the same concentrations and methodology described previously. The corrected larval mortality resulting from the 10th subcultures was compared with that resulting from the second in vivo cultures. Five replicates per concentration were done.

### Results

The Infectivity of *M. anisopliae* at Two Different Temperatures. Observations showed that most of mortalities occurred in the pupal stage. Most of larval mortalities took place in high concentrations. Some adults died on emergence in case of low concentrations. The entomopathogenic fungus *M. anisopliae* (Ma79) caused significant reduction in the percentage of adult emergence when applied to larval diets. An increase in Ma79 concentration was associated with a decrease in percentage of adult emergence, and the total mortality at 26 ± 1°C ranged from 30.9 to 83.9%. Percentage mortality decreased when the temperature was raised from 26 ± 1°C to 31 ± 1°C at all concentrations. For example, at the lowest concentration, it decreased from 30.9 to 15.3% (*t* = 3.58, df = 16, *P* = 0.003), and from 83.9 to 51.3% (*t* = 5.29, df = 14, *P* < 0.001) at the highest concentration. Thus, there was a difference in fungus-induced mortality rates between temperature treatments, with virulence significantly higher at 26 ± 1°C than 31 ± 1°C (Table 1).

The LC90s of the fungus at the lower and higher temperatures were 1.7 × 10⁸ spores/ml (CI = 1.2–2.4 × 10⁸; df = 52; *P* < 0.05) and 2.8 × 10⁸ spores/ml (CI = 1.4–7.2 × 10⁸; df = 52; *P* < 0.05), respectively, suggesting that the probability of a sand fly dying soon after infection was greater at 26 ± 1°C than at 31 ± 1°C.

### Reinfestation Ability and Persistence of the Fungus

The experiments were carried out using the treated vials from previous bioassays at different time lapses without the addition or renewal of the fungal dose in the media. We assayed fungus-treated vials against healthy sand fly larvae to determine whether the level of inocula persisting in their breeding media was sufficient to reinfect healthy sand flies, and when it would start to deplete. The fungus was persistent and able to reinfect the larvae in the bioassay vials up to 20 wk postapplication (4.3 wk in the first bioassay ±16 wk in
the second bioassay) at all concentrations. The overall mean period from the zero day of fungal application until the emergence of the last survived adult of the previous bioassays was 30.40 ± 3.89 d ± SD (4.3 wk). The infectivity of sand fly larvae at all tested concentrations followed the same pattern of decline over time; it increased until it became significantly higher than that of the previous bioassay (day 0) on the second week (44 d after application) (P < 0.05), except for the lowest and highest concentrations, where it increased from 30.9 and 53.9% to 46.5 and 97.6% (P > 0.05), respectively, and then mortality rates began to decrease gradually until it became significantly lower in the 16th wk (142 d after application) (P < 0.05), except for 5 × 10⁸ and 1 × 10⁹ spores/ml, where it did not differ significantly. However, sufficient inocula persisted and were able to infect 5–40.6% of sand fly larvae 142 d after spore application. The reduction in the percentage mortality of sand flies after 20 wk, compared with initial mortality in the previous bioassays, ranged from 17.9 to 43.3% (Table 2).

The Change in Spore Content of M. anisopliae. Spore counting of M. anisopliae (Ma79) was carried out after finishing the bioassay at different periods over ≈20 wk after application at two concentrations. For both spore concentrations (2.5 × 10⁸ and 2.5 × 10⁹) increased to reach the maximum values, 1.85 × 10¹⁰ and 3.6 × 10¹⁰, respectively, at 44 d (second week) after application, and then decreased gradually to reach the minimum values, 2.2 × 10⁷ and 3.3 × 10⁷, respectively, 142 d (16th wk) after application. In general, the spore content densities decreased over time from the second week, where the ratio of final or initial spore content was 7.6 and 1.4 for 5 × 10⁸ and 5 × 10⁹ spores/ml, respectively. Then it significantly declined, and the ratio of final or initial spore content became 0.88 and 0.13 (P < 0.001), respectively, at the 16th wk for both concentrations. Despite the different initial spore content, the final spore contents of both concentrations were not significantly different at all week periods (P > 0.05), except the final spore content at the second week (t = −5.05, df = 4, P = 0.012; Table 3).

In Vivo Passage of M. anisopliae (Ma79). Infectivity of the fungus was amplified after in vivo passage. There was no significant difference between the percentages of the emergence in sand flies treated with 2 × host-passed conidia either in P. papatasi third instar or S. pandurus fifth instar (Maph, MaSpilo), whereas both passages have significantly reduced percentage of adult emergence when compared with the 10× in vitro inoculated M. anisopliae conidia (Ma79) at all tested concentrations (P ≤ 0.01). However, the conidia of M. anisopliae after passage through the third instar S. littoralis (MaSpod) did not significantly increase mortality of sand flies. There was a direct pro-

<table>
<thead>
<tr>
<th>Periods a</th>
<th>5 by 10⁸ spores/ml a</th>
<th>Ratio of final or initial spore content</th>
<th>5 by 10⁹ spores/ml b</th>
<th>Ratio of final or initial spore content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final spore content</td>
<td></td>
<td>Final spore content</td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>1.85 by 10⁸ ± 1.7 × 10⁷</td>
<td>7.6 ± 0.69a</td>
<td>3.6 by 10⁹ ± 2.8 × 10⁷</td>
<td>1.4 ± 0.11a</td>
</tr>
<tr>
<td>4 wk</td>
<td>1.3 × 10⁹ ± 1.5 × 10⁷</td>
<td>5.2 ± 0.55ab</td>
<td>2 by 10⁹ ± 3.4 × 10⁷</td>
<td>0.80 ± 0.13b</td>
</tr>
<tr>
<td>8 wk</td>
<td>1.1 by 10⁸ ± 1.6 × 10⁶</td>
<td>4.7 ± 0.64b</td>
<td>1.85 by 10⁸ ± 2.5 × 10⁷</td>
<td>0.74 ± 0.10b</td>
</tr>
<tr>
<td>11 wk</td>
<td>5 by 10⁷ ± 5.7 × 10⁶</td>
<td>2.0 ± 0.23c</td>
<td>7 by 10⁸ ± 1.1 × 10⁷</td>
<td>0.28 ± 0.04c</td>
</tr>
<tr>
<td>16 wk</td>
<td>2.2 by 10⁶ ± 3.4 × 10⁶</td>
<td>0.88 ± 0.13c</td>
<td>3.3 by 10⁷ ± 7.5 × 10⁷</td>
<td>0.13 ± 0.02c</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE of three replicates. Means followed by the same letter within each column are not significantly different (ANOVA, Tukey test, P > 0.05). a Initial spore content = 2.5 by 10⁸ (0.5 ml of 5 by 10⁸ spore/ml) added to a bioassay vial containing 10-s instar P. papatasi and about 0.15 g food, 15 vials. b Initial spore content = 2.5 by 10⁹ (0.5 ml of 5 by 10⁸ spore/ml) added to a bioassay vial containing 10-s instar P. papatasi and about 0.15 g food, 15 vials.
portion between the fungal concentrations and the percentage mortality of sand fly; the percentage mortality of sand flies was 30.9, 31.5, 57.7, and 59.5% ($F = 9.12; df = 3; P < 0.01$) at the lowest spore concentration and 83.9, 83.3, 93.6, and 95.4% ($F = 4.61; df = 3; P < 0.05$) at the highest concentration of Ma79, MaSpod, Maph, and MaSpilo applications, respectively (Table 4).

Table 4. Infectivity of *M. anisopliae* (Ma79) to second instar *P. papatasi* after passage in *S. littoralis* and *P. papatasi* third instars and in fifth instar *S. pandurus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fungal concn DN/ml</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma79</td>
<td>1 by 10^8</td>
<td>1.7 ± 1.1a</td>
</tr>
<tr>
<td></td>
<td>1 by 10^9</td>
<td>3.3a</td>
</tr>
<tr>
<td>MaSpod</td>
<td>1 by 10^8</td>
<td>1.0 ± 0.9a</td>
</tr>
<tr>
<td></td>
<td>1 by 10^9</td>
<td>1.6a</td>
</tr>
<tr>
<td>Maph</td>
<td>1 by 10^8</td>
<td>2.7 ± 0.7b</td>
</tr>
<tr>
<td></td>
<td>1 by 10^9</td>
<td>3.0b</td>
</tr>
<tr>
<td>MaSpilo</td>
<td>1 by 10^8</td>
<td>3.0 ± 2.6b</td>
</tr>
<tr>
<td></td>
<td>1 by 10^9</td>
<td>3.7b</td>
</tr>
</tbody>
</table>

Data are corrected percentage mortality and are presented as mean ± SE of five replicates per concn, 10 larvae per replicate, six concn per each one of two assays. Means followed by the same letter within each column are not significantly different (ANOVA; Tukey test; $P > 0.05$). Mean control mortality = 7.2 ± 1.7%.

MaSpod, MaPh, and MaSpilo: *Spodoptera littoralis* third instar larvae, *Phlebotomus papatasi* third instar larvae, and *Spilostethus pandurus* fifth instar nympha-passed conidia, respectively.

Discussion

The efficiency of *M. anisopliae* (Ma79) at the highest concentration on larvae of *P. papatasi* (84%) was similar to that of *M. anisopliae* variety *acridum* on larvae of *Lutzomyia longipalpis* (87%) (Amóra et al. 2010). Superior results were observed when another entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill (Hypocreales: Cordycipitaceae) was tested on *L. longipalpis* larvae, obtaining an efficiency of 100% mortality (Amóra et al. 2009). It was shown that the effect of fungus-contaminated diet on the susceptibility of *P. papatasi* larvae in this study was more efficient than offering a diet contaminated with *Bacillus thuringiensis* variety *israelensis* (Bacillales: Bacillaceae), as in the earlier study of Wahba et al. (1999).

Table 5. Variation of LC$_{50}$ in the second instar *P. papatasi* because of host passage of *M. anisopliae* (Ma79)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n^a$</th>
<th>Slope ± SE</th>
<th>LC$_{50}$ (95% CI)</th>
<th>$x^b$</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma79</td>
<td>1,080</td>
<td>0.335 ± 0.022</td>
<td>1.7 × 10$^7$ (1.2-2.4 × 10$^7$)</td>
<td>81.11</td>
<td>52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MaSpod</td>
<td>600</td>
<td>0.320 ± 0.019</td>
<td>1.2 × 10$^6$ (8.8 by 10$^5$-1.4 by 10$^6$)</td>
<td>71.24</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Maph</td>
<td>600</td>
<td>0.356 ± 0.028</td>
<td>1.0 × 10$^6$ (2.0 by 10$^5$-2.6 by 10$^6$)</td>
<td>102.97</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MaSpilo</td>
<td>600</td>
<td>0.428 ± 0.029</td>
<td>8.9 × 10$^6$ (2.6 by 10$^6$-1.9 by 10$^6$)</td>
<td>91.73</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values followed by the same letter not different, as judged by overlapping 95% CI.

$^a$ The fungus was subcultured in vitro 10 times.

$^b$ Ten larvae per replicate, five replicates per concn (nine replicates incase of Ma79), six concn per treatment, two assays for each treatment.

In addition, low infection rates of the cytoplasmic polyhedrosis virus against different geographical strains of *P. papatasi* larvae were determined experimentally by Warburg (1991) and it was concluded that the Egyptian *P. papatasi* were mostly refractory.

Temperature may be a limiting factor for using entomopathogenic fungi (Carruthers et al. 1985, Inglis et al. 1996). The results of the current study showed that lower mortality values were observed at higher temperature (31 ±1°C), whereas the highest mortalities of *P. papatasi* larvae, infected with *M. anisopliae*, occurred at 26 ±1°C (mean 84%); this temperature corresponds to the optimal temperature for in vitro growth of several isolates of the fungus *B. bassiana* (Fargues et al. 1997). Temperatures closer to the thermal optimum would encourage faster spore germination and mycelial growth, which would result in more rapid fungal infection and host death. High temperatures adversely affect entomogenous fungi (Hywel-Jones and Gillespie 1990, Arthurs and Thomas 2001) and in general, they reduce fungal germination rates and disease transmission. Thus, our results support the suggestions of Fuxa (1987, 1995) and Fuxa et al. (1998), who have demonstrated that factors other than pathogen virulence, such as temperature tolerance, are important to pathogen epizootics and microbial control of insects.

During this study, persistence of conidia was determined by larval mortality and the change in spore content of *M. anisopliae* at different week periods. The reduction in sand fly percentage mortality may have reflected a declining fungal density and not conidial...
viability. A positive correlation between estimates of fungal density and sandfly mortality was observed. The latter may indicate the recycling of the fungal inocula in the sand fly cadavers. This was followed by a significant decline in the spore content within 20 wk postfungal application, correlated with a decline in mortality among sand fly larvae. However, the initial spore content was reduced to 9 and 11 fold in the case of the respective initial spore concentrations 5 × 10^7 and 5 × 10^6 spores/ml; mortality was still observed among sand flies. Similarly, Daoust and Pereira (1986) found out that B. bassiana conidia on cadavers of curculionid and chrysomelid pests of cowpeas remained viable for at least 16 wk (=112 d) when stored outside in protected conditions.

The negative effect of time on spore content and fungal survival may be because of the consumption of supplements in the vial contents, which may affect the fungal saprophytic activity. Additionally, the natural microbial competition among large number of fungal propagules for limited nutrients and space has negatively influenced the fungal survival. Microorganisms in such an environment may out-compete the fungus for available resources. Cadavers of fungus-killed insects are considered natural and persistent reservoirs for numerous entomopathogenic fungi (Sprenkel and Brooks 1977, Daoust and Pereira 1986, Thomas et al. 1996, Sawyer et al. 1997). The latter fact may explain how conidia remain viable for extended periods. The long-term establishment of fungi has been hypothesized to depend on the availability of conidia to recycle through the hosts, which provide the fungus with organic matter.

The ability of an entomopathogen to persist in the habitat of its host is important for the effectiveness of naturally occurring and introduced pathogens (Jacques 1983). The high persistence of M. anisopliae that was observed in this study indicates that the fungus may survive well in the host habitat for at least 4 mo. Consequently, these data suggest that M. anisopliae (Ma79) has the potential to control sand fly larvae for one growing season with a single application. In particular, fungal persistence after application must be substantial to minimize the logistical challenges and cost of retreatment (Scholte et al. 2007).

A decline in virulence was detected after passaging Nomuraea rileyi (Farlow) Samson (Hypocreales: Clavicipitaceae) and B. bassiana 10 and 16 times, respectively, on artificial growth media (Aizawa 1971; Morrow et al. 1989). For most entomopathogenic fungi, repeated subculturing on artificial medium results in a decline in the virulence of the conidia, which can be enhanced through host passage (Brownbridge et al. 2001; Quesada-Moraga and Vey 2003; Shah et al. 2007). Some earlier studies also strengthened our findings and suggested that virulence is restored irrespective of whether the culture is passed through the highly susceptible larvae of the wax moth (Galleria mellonella) or the flour beetle (Tenebrio molitor) (Shah et al. 2005). The virulence of Paecilomyces farinosus toward the English grain aphid, Sitobion avenae (F.) (Hemiptera: Aphididae), increased after a single passage (and continued for up to nine passages), as well as when the fungus was grown in the medium containing S. avenae cuticle as the only nutrient source (Hayden et al. 1992). The enhancement of virulence is well documented, but little is known about the underlying mechanisms of virulence. However, some studies attributed this to appressorium formation and/or the increment of cuticle-degrading enzymes activities. For example, Nahar et al. (2008) reported that appressorium formation in M. anisopliae decreased because of repeated in vitro subculturing on potato dextrose agar. Interestingly, appressorium formation increased because of in vivo passage in Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). Furthermore, Nahar et al. (2008) observed a gradual decrease in the induced activities of chitinase, chitosanase, and protease with repeated in vitro subculturing of M. anisopliae. These activities were seen to increase after insect passage. In addition, attenuated culture may not differentiate into the form adapted for the hemocoel or produce the metabolites that normally suppress the host defense. Wang et al. (2003) reported that attenuated cultures did not produce destruxins, the secondary metabolites harmful to the host’s immune system. These explanations may clarify why host passage has improved the pathogenicity of M. anisopliae in this study.

Strains of hyphomycetous fungi may become adapted to additional heterologous hosts with forced passage (Ferron 1985). It has been hypothesized that the most virulent fungal strains are isolated from the test organism or a closely related species (Soares et al. 1983, Poprawski et al. 1985). M. anisopliae isolate used in this study was not isolated from the test organism, although it showed high infectivity against the sand fly larvae.

Our study supports the hyphomycetous entomopathogen M. anisopliae to be effectively launched in the control of the cutaneous leishmaniasis primary old-world vector P. papatasi. Immature stages (larvae and pupae) are found in organically enriched soil in a variety of habitats (e.g., animal burrows, tree holes, and animal pens). Both sand fly immature stages and M. anisopliae naturally occur in soil, providing maximum opportunity for interaction and contact. The utility of soil-borne fungus against insect species that breed in organic-rich soil has been previously suggested. A significant reduction of adult emergence has been obtained by applying water suspensions of the same isolate inside rodent burrows, the breeding sites of the immature sand fly stages, in Accra, Ghana, and North Sinai, Egypt (A.Z. et al., unpublished data). The reinfection ability and persistence of the fungus together with its aptitude to be amplified through other hosts provide the vector control decision makers, officials, and end users with crucial application information.

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References Cited


Sorok. and Beauveria bassiana (Bals.) Vuill. to adult Phlebotomus duboscqi (Neveu-Lemaire) in the laboratory. J. Vector Borne Dis. 48: 37–40.


SPSS Statistics. 2008. SPSS Statistics software for windows, release 17.0. SPSS Statistics, Chicago, IL.


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