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Potential Nontarget Effects of *Metarhizium anisopliae* (Deuteromycetes) Used for Biological Control of Ticks (Acari: Ixodidae)

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ABSTRACT The potential for nontarget effects of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin, when used for biological control of ticks, was assessed in laboratory trials. Fungal pathogenicity was studied against convergent ladybird beetles, *Hippodamia convergens* Guérin-Méneville, house crickets, *Acheta domesticus* (L.), and the milkweed bugs *Oncopeltus fasciatus* (Dallas). Fungal spores applied with a spray tower produced significant mortality in *H. convergens* and *A. domesticus*, but effects on *O. fasciatus* were marginal. Placing treated insects with untreated individuals resulted in mortality from horizontal transmission to untreated beetles and crickets, but not milkweed bugs. Spread of fungal infection in the beetles resulted in mortality on days 4–10 after treatment, while in crickets mortality was on day 2 after treatment, suggesting different levels of pathogenicity and possibly different modes of transmission. Therefore, *M. anisopliae* varies in pathogenicity to different insects. Inundative applications can potentially affect nontarget species, but *M. anisopliae* is already widely distributed in North America, so applications for tick control generally would not introduce a novel pathogen into the environment. Pathogenicity in lab trials does not, by itself, demonstrate activity under natural conditions, so field trials are needed to confirm these results and to assess methods to minimize nontarget exposure.

KEY WORDS *Metarhizium anisopliae*, pathogenicity, nontarget effects, *Hippodamia convergens*, *Acheta domesticus*, *Oncopeltus fasciatus*

ENTOMOPATHOGENIC FUNGI ARE used as microbial control agents for insect pests (Burge 1988), and considerable research is devoted to development of new fungal strains for biological control (Hajek and St. Leger 1994). Most of this effort is currently directed at agricultural and structural pests, but studies have also investigated fungal pathogenicity to medically important arthropods, including mosquitoes (e.g., Fetter-Lasko and Washino 1983), tsetse flies (e.g., Kaaya and Munyinyi 1995), and ticks (e.g., Kaaya et al. 1996).

Laboratory trials have demonstrated that the fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin is highly pathogenic to the black-legged tick, *Ixodes scapularis* Say (Zhioua et al. 1997). This tick species is the primary vector of Lyme disease in the eastern United States (Fish 1993), and has also been implicated as a vector of babesiosis (Spielman et al. 1985) and human granulocytic ehrlichiosis (Schwartz et al. 1997). Current management options for this tick species include habitat manipulation and chemical control, which are variably effective (Wilson and Deblinger 1993) and can have negative effects on wildlife populations (Ginsberg 1994).

Natural enemies of ticks such as *M. anisopliae* can potentially provide effective management of tick populations with relatively low potential for negative environmental effects. However, *M. anisopliae* has been isolated from a wide variety of insect species (Humber 1992), is pathogenic to diverse arthropods (Zimmerman 1993, Genthner et al. 1997), and could potentially cause mortality in populations of nontarget species. Flexner et al. (1986) reviewed effects of microbial pesticides on a variety of arthropods and found that most trials showed only weak pathogenicity to nontarget species. Nevertheless, application of concentrated solutions of *M. anisopliae* spores to obtain effective mortality in tick populations (Zhioua et al. 1997) increases the potential for pathogenicity to nontarget organisms when this fungus is used for tick control. This possibility is of special concern in natural areas such as national parks, where protection of nontarget species is an important mandate. Therefore, even though *M. anisopliae* is widely distributed, the potential for negative effects on nontarget populations needs to be assessed for applications of concentrated spore solutions to natural areas.

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In this study we carried out pathogenicity trials in the laboratory with *M. anisopliae* against nontarget insects from distantly related orders (Orthoptera, Hemiptera, and Coleoptera). We tested direct pathogenicity to insects sprayed with fungal spores, as well as the potential for horizontal transmission of fungal infection from treated to untreated individuals.

Materials and Methods

Test Insects. Initial samples taken using various techniques (including flagging, sweep netting, and beating vegetation) from habitats with high tick abundance in southern Rhode Island, captured numerous species of arthropods from several orders (including Coccinellidae, litter-dwelling arthropods, and plant-feeding Hemiptera). Therefore, we selected three species of insects that were available in culture and that represented diverse orders for testing for fungal pathogenicity. Convergent ladybird beetles, *Hippodamia convergens* Guérin-Méneville (Coccinellidae), were obtained from Berkshire Biological (Westhampton, MA). House crickets, *Acheta domesticus* (L.) (Gryllidae) were obtained from a local pet store (supplier: Armstrong's Cricket farm, West Monroe, LA). The milkweed bugs, *Oncopeltus fasciatus* (Dallas) (Lygaeidae), were obtained from a culture maintained at the University of Rhode Island. Isolates of *M. anisopliae* were not previously recorded from these insect species in USDA/ARS databases (Humber 1992, Humber and Hansen 2001, SBML 2001).

Test insects were placed in plastic chambers (150 mm diameter by 25 mm high) (Falcon, Integrid Tissue Culture dishes, Becton Dickinson Labware, Franklin Lakes, NJ), with a floor of Whatman #2 filter paper (Whatman, Hillsboro, OR) saturated with distilled water. Beetles (all adults) were treated on 16 September 1998, supplied with a vial of 10% sucrose with a cotton wick, and observed from 17 September through 17 October 1998. Crickets (adults and nymphs) were treated on 25 April 2000, supplied with a vial of distilled water with a cotton wick and dry dog food, and observed from 21 to 30 April 2000. The milkweed bugs (nymphs) were treated on 27 February 2001, supplied with a vial of distilled water with a cotton wick along with dry milkweed seeds, and observed from 28 February through 22 March 2001.

Pathogenicity Trials. The insects were sprayed with a Potter Precision Laboratory Spray Tower (Burkard, Rickmansworth Hertfordshire, England) to allow precise dosage of fungal spore exposure. This apparatus puffs a measured concentration of spores in an aqueous solution onto the target specimens, with dosages calibrated by initial trials. *H. convergens* and *A. domesticus* were sprayed with 10^8 spores per milliliter, and *O. fasciatus* was sprayed with 10^9 spores per milliliter for both direct and indirect pathogenicity trials (the higher dosage was used in the last set of trials because of increased estimates of dosages needed for field effectiveness against ticks based on contemporaneous studies).

For the direct pathogenicity trials, 10 chambers were prepared with 10 insects in each chamber. Control insects were sprayed with distilled water before placement in five "untreated" chambers, while test insects were sprayed with an aqueous spore solution of *M. anisopliae* strain ESC1 (Bio-Blast, Ecoscience, East Brunswick, NJ) before placement in the five "treatment" chambers (total = 100 individuals). For the indirect pathogenicity trials, 10 untreated insects were placed in each chamber, and an additional five insects were added to each. The five additional insects were sprayed with distilled water for the five control chambers, while the five insects added to the treatment chambers were sprayed with the fungal spore solution (total = 150 specimens).

The chambers were held in incubators at 23°C and a photoperiod of 12:12 (L:D) h (except for the beetles in the direct exposure trial, which were held in dark because of problems with the incubator lighting system), and observed daily. The numbers of live and dead insects were recorded, as well as the numbers of insects with overt signs of fungal infection (visible hyphae), each day until all specimens were dead.

Analysis. Trends in the numbers of insects dead were compared between treated and untreated groups with Kolmogorov-Smirnov two-sample tests. The Kolmogorov-Smirnov Test is sensitive to significance of the largest difference at any point between the two cumulative mortality distributions being compared (Siegel 1956). In the indirect pathogenicity trials, the number of insects that died from indirect fungal exposure (epizootic spread) was estimated by subtracting the number of directly exposed insects from the number dead, then determining the number of initially unexposed insects that died each day (=the number that died incrementally each day after the number directly exposed had died). The number of initially unexposed insects that died in the treated chambers each day was multiplied by the proportion of insects surviving in the unexposed controls to correct for natural mortality of unexposed insects (this is the proportion that would have been alive without fungal exposure). The result gave an estimate of the number of initially unexposed insects that died each day from horizontal transmission of *M. anisopliae.*

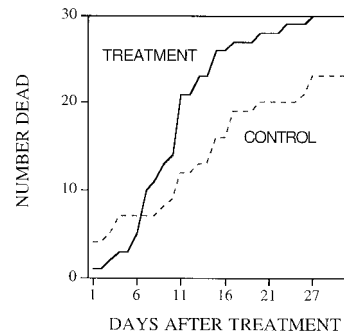

Fig. 1. Cumulative mortality of *Hippodamia convergens* exposed to spores *of Metarhizium anisopliae.*

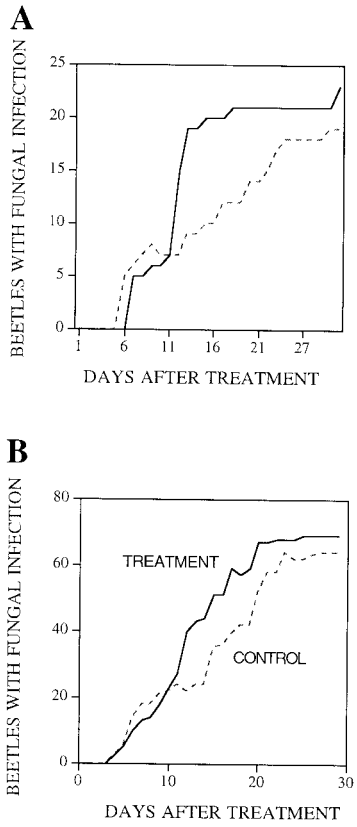

Fig. 2. Fungal growth on *H. convergens* exposed to spores of *M. anisopliae.* (A) Direct exposure to fungal spores. (B) Indirect exposure trial.

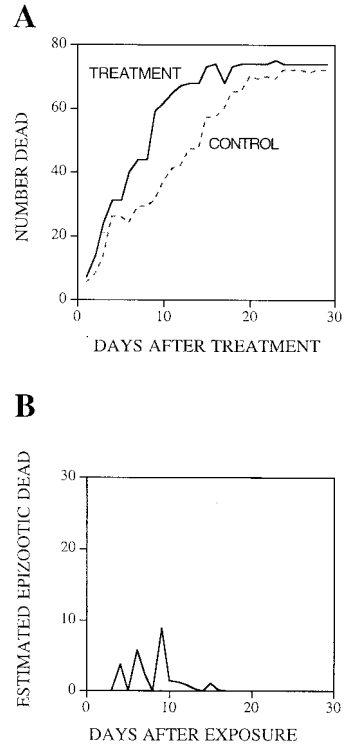

Fig. 3. Mortality of *H. convergens* in indirect exposure trials. (A) Cumulative mortality in treated and untreated cultures. (B) Estimated daily mortality of beetles from indirect exposure to *M. anisopliae.*

Results

Mortality of *H. convergens* directly exposed to *M. anisopliae* spores, along with untreated controls, is shown in Fig. 1. The trend of mortality in directly treated beetles (Fig. 1) was significantly different from untreated beetles (Kolmogorov-Smirnov test, one-tailed, $D_{\max} = 0.333, N = 30, P < 0.05$). Similarly, fungal growth on treated beetles (Fig. 2A) was greater than on untreated controls ($D_{\max} = 0.333, N = 30, P < 0.05$). The chambers were not opened until the end of the experiment, so fungi were not isolated from individual cadavers. Some of the fungi growing on controls were green (like *M. anisopliae*), suggesting that this fungus was present in some beetles, either naturally or due to contamination. However, many were white, probably a saphrophytic fungus growing on the cadavers, and the significant difference between treatment and controls confirms the increased mortality from experimental exposure to fungal spores. In the indirect exposure trials, mortality in beetle cultures where some of the beetles were treated (Fig. 3A) was greater than in cultures with untreated beetles ($D_{\max} = 0.373, N = 75, P < 0.001$). Fungal growth on beetles in the indirect trial (Fig. 2B) was significantly greater in the treated than in the untreated cultures ($D_{\max} = 0.267, N = 75, P < 0.01$). Beetle mortality from spread of *M. anisopliae* (Fig. 3B) was greatest from days 4 to 10 after initial exposure of the treated beetles to fungal spores.

Cricket mortality was more rapid than beetle mortality, with all crickets dead by day 9 after fungal exposure (Fig. 4). Mortality among treated crickets was significantly greater than controls (Kolmogorov-Smirnov test, one-tailed, $D_{\max} = 0.26, n_1 = n_2 = 50, P < 0.05$). However, very little evidence of fungal growth was detected. Cricket mortality in the indirect exposure trial (Fig. 5A) was also significantly greater in

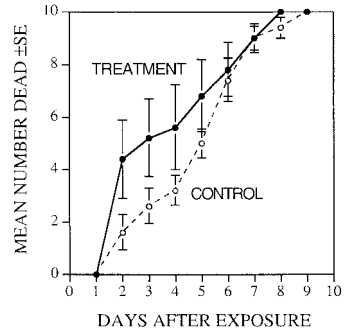

Fig. 4. Cumulative mortality of *Acheta domesticus* exposed to spores of *Metarhizium anisopliae.*

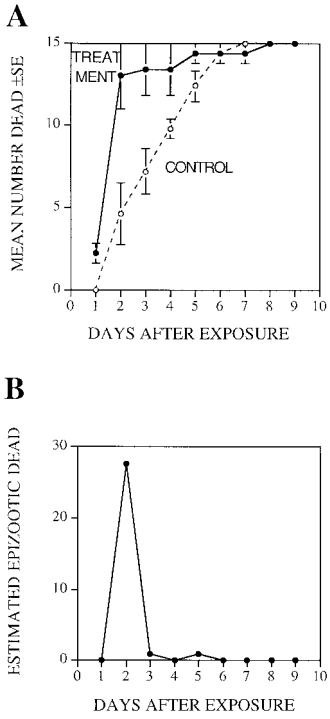

Fig. 5. Mortality of *A. domesticus* in indirect exposure trials. (A) Cumulative mortality in treated and untreated cultures. (B) Estimated daily mortality of crickets from indirect exposure to *M. anisopliae.*

treated cultures than in untreated controls (D_{max} = 0.56, $n_1 = n_2 = 75$, $P < 0.001$) In this case, mortality from indirect exposure was rapid (Fig. 5B), concentrated on day 2 after initial exposure to fungal spores.

Mortality of milkweed bugs (Fig. 6) differed only marginally between treated and untreated controls (Kolmogorov-Smirnov test, one-tailed, $D_{max} = 0.24$, $n_1 = n_2 = 50$, $P = 0.06$), again with little evidence of fungal infection. In the indirect mortality experiment (Fig. 7), mortality in treated cultures did not differ significantly from controls ($D_{max} = 0.17$, $n_1 = n_2 = 75$, $P = 0.11$). However, the small amount of mortality potentially attributable to epizootic spread occurred on days 5–15, similar to the timing in beetles.

Discussion

Pathogenicity of *M. anisopliae* to immature *I. scapularis* requires sufficient concentrations of fungal spores. Concentrations up to 10^6 spores per milliliter showed little pathogenicity, while 10^7 spores per milliliter produced considerable mortality and 10^8 spores per milliliter produced 100% tick mortality in laboratory trials (Zhioua et al. 1997). A similar phenomenon has been described for pathogenicity of *M. anisopliae* to insects, including weevils (Schabel 1978) and wasps (Harris et al. 2000). Preliminary trials (Zhioua and associates, unpublished) suggest that even higher concentrations might be required for effective tick control in the field. Our results demonstrate that at such high concentrations *M. anisopliae* is pathogenic to taxonomically disparate groups of insects, including beetles (Fig. 1) and crickets (Fig. 4). However, the relatively low pathogenicity to milkweed bugs (Fig. 6) shows that insects vary in their susceptibility to this fungus.

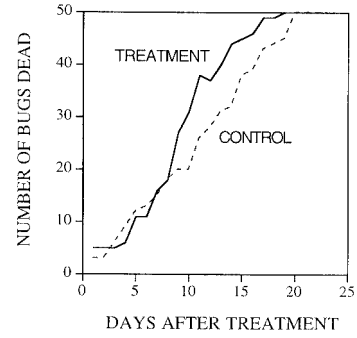

Fig. 6. Cumulative mortality of *Oncopeltus fasciatus* exposed to spores of *Metarhizium anisopliae.*

The indirect pathogenicity trials demonstrate diversity in the potential, and mode, of horizontal transmission of the fungus. Spread of fungal infection from treated to untreated insects was substantial in beetles and crickets, but not in milkweed bugs. Mortality from spread of fungal infection from infected to untreated ladybird beetles occurred mostly on days 4–10 (Fig. 3B), when there was considerable fungal growth on the treated beetles (Fig. 2B). Treated crickets, however, showed little evidence of fungal growth, and mortality from horizontal transmission occurred primarily on day 2 after initial exposure of treated individuals (Fig. 5B). This result suggests that spread was by transfer of spores from the cuticles of treated to untreated crickets, either directly by jostling, or indirectly on the substrate. Similar transfer has been described for *Beauveria bassiana* (Balsamo) Vuillemin between vespid wasps (Harris et al. 2000).

Despite variability in the susceptibility of different insects to *M. anisopliae*, our data show that some species are susceptible to this fungus, and that spread from infected to uninfected insects can occur under

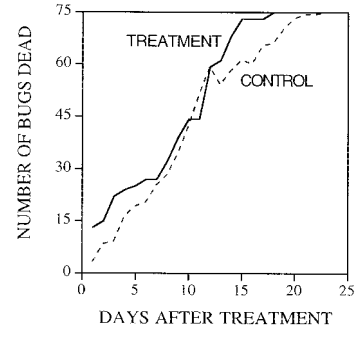

Fig. 7. Cumulative mortality of *O. fasciatus* in indirect exposure trials.

laboratory conditions. These results call for caution in the use of *M. anisopliae* for tick management, at least until nontarget effects are assessed under natural conditions. The high pathogenicity in these trials is not necessarily indicative of similar levels of pathogenicity in the field. Indeed, Hajek et al. (1996) reported pathogenicity of *Entomophaga maimaiga* Humber, Shimazu & Soper to a far narrower range of insect species in nature than would be predicted on the basis of laboratory trials. Under conditions that are ideal for fungal growth, even species that are usually saprophytic can sometimes act as pathogens (Tanada and Kaya 1993). In contrast, under field conditions, fungal spore concentrations decline with distance from the applicator, and the effects of environmental conditions such as low humidity and temperature can compromise fungal activity (e.g., Ramoska 1984, Milner et al. 1997, Sosa-Gomez and Moscardi 1998, Boucias and Pendland 1998). Therefore, our laboratory results, though cautionary, do not necessarily indicate risk of nontarget effects in nature. Recent reports of sinusitis in humans caused by *M. anisopliae* (Revankar et al. 1999) are also cautionary of potential vertebrate effects, although such reports are rare. Natural isolates of *M. anisopliae* have been collected from the field in Rhode Island (M. Browning, personal communication) but neither broadscale epizootics nor vertebrate involvement have, to our knowledge, been reported. Thus, presence of the fungus does not, by itself, indicate a risk of negative effects on populations of nontarget species. The next step in assessing the potential for nontarget effects of *M. anisopliae* used for tick control, would be field trials targeted at insect species that occur in areas likely to be treated for ticks.

Several approaches to pest management with entomopathogenic fungi could minimize the likelihood of nontarget effects. In the case of management of *I. scapularis*, the fungus is best applied to the adult stage of the tick, because adults are more susceptible to fungal attack than immatures (Zhioua et al. 1997), and because adults seek hosts in vegetation above the ground (Ginsberg and Ewing 1989), where they are easier to target than the leaf-litter-dwelling immatures. Application of fungal spores in early spring or late fall, when adult *I. scapularis* are active, could minimize any nontarget effects because relatively few other arthropod species are active at those times of year. Furthermore, application to tick hosts, such as deer, can target spore applications to minimize exposure of nontarget arthropods. Applications to immature stages, which would entail granular applications or ground-level sprays, and thus increased exposure of nontarget species, could be restricted to areas that are already highly manipulated (e.g., residential areas).

However, applications in natural areas with relatively undisturbed invertebrate faunas (e.g., national parks and wildlife refuges) have greater potential for adverse effects on nontarget species. One approach to minimize the likelihood of such negative effects is to use these entomopathogens only in areas where the fungus is already present. Furthermore, applications in natural areas should be targeted at specific areas within habitat-types where humans are likely to encounter ticks (Ginsberg and Ewing 1989, Maupin et al. 1991, Siegel et al. 1991, Carroll et al. 1992), or at critical tick hosts, such as deer or mice (Lane et al. 1991, Wilson and Deblinger 1993). This approach would minimize the likelihood of negative environmental effects because the fungal pathogen is already present in the environment. The management approach is to change the distribution of the fungus so as to efficiently target the pest species. However, this approach requires preliminary screening for entomopathogens (e.g., Zhioua et al. 1999), which might be plausible for a large preserve or park, but would not be practical for many applications.

Metarhizium anisopliae is widely distributed and attacks a diversity of insects (Zimmerman 1993), with different strains varying in pathogenicity to different insect species (Tanada and Kaya 1993). This fungus is potentially widely applicable for tick control, but knowledge of local distribution is important to assess the risk of nontarget effects. Our results demonstrate the potential for nontarget effects of *M. anisopliae* when used for tick control, but they do not provide an accurate assessment of the true risk in nature. Future research is needed on the effects of this fungus on nontarget species under controlled field conditions, and on methods to deliver fungal spores to ticks that minimize exposure of nontarget arthropods.

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