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Entomopathogenic fungi to control bark beetles: a review of ecological recommendations

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Abstract

There is considerable interest in applying entomopathogenic fungi as a biological control to limit insect populations due to their low environmental and human applicator impacts. However, despite many promising laboratory tests, there are few examples where these fungi were successfully applied to manage bark beetles. Here, we explore how environmental conditions unique to bark beetle habitats may have limited previous entomopathogenic fungus applications, including variable temperatures, ultraviolet light, bark beetle symbiotic microorganisms, tree phytochemicals, and cryptic bark beetle behaviors. Based on the existing literature, we provide a framework for interpreting the pathogenicity of entomopathogenic fungi to bark beetles, with emphasis on both standardizing and improving laboratory approaches to enhance field applications. Our synthesis indicates that most previous laboratory evaluations are conducted under conditions that are not representative of actual bark beetle systems; this may render fungal isolates functionally non-pathogenic in field settings. We recommend that future studies place particular effort into understanding entomopathogen response to the presence of bark beetle symbiotic microorganisms, plant phytochemicals, and potential as a tree endophyte. Additionally, field application methods should aid entomopathogens in overcoming stressful conditions and allow the fungus to infect multiple bark beetle life stages.

Supporting information may be found in the online version of this article.

Keywords: *Beauveria bassiana*; biological control; environment; forest insects; insect behavior; *Metarhizium anisopliae*; mycoinsecticide; virulence

1 INTRODUCTION

Insect-infecting fungi are ubiquitous natural enemies of arthropod populations worldwide. Several 'mycoinsecticide' products from entomopathogenic fungi have been developed to treat a variety of pests due to their relative ease of commercial-scale production and ability to infect many target organisms.[1] Scientists have published more than one paper each day on fungal potential as plant endophytes and biocontrol agents of arthropods since 2015. Hence, the vast amount of recent literature coupled with a potential for future growth in microbial insecticide use warrants direction to ensure that knowledge gaps are addressed with limited redundant research effort.

Bark beetles are part of a diverse subfamily of insect pests that can cause substantial mortality to forest landscapes mostly by attacking vulnerable conifer trees in temperate regions around the world. These tree mortality events are often driven by population 'outbreaks' and garner significant public concern. In general, bark beetles emerge as adults from phloem galleries during the late spring or early summer and search for susceptible host trees with the help of aggregation and anti-aggregation semiochemicals.[2] Many beetles are also intimately associated with a diverse assemblage of symbiotic microorganisms that impact beetle fitness in a variety of ways. These communities are transported via specialized secretory structures (such as mycangia) that facilitate the acquisition and transmission of symbiotic fungi to new environments.[3] During the colonization of new host trees, both beetles and their associated microorganisms are exposed to resins and significant quantities of phytochemicals, especially monoterpene hydrocarbons and phenols, that serve as constitutive and inducible anti-herbivory defenses.[4] If beetle populations are successful at overcoming these defenses, females form species-specific gallery morphologies within the phloem where they lay their eggs. The collective challenge to host trees from the combination of adult tunneling, larval feeding on cambial tissues and the growth of associated microorganisms often results in tree death.[2]

Strains of entomopathogenic fungi are promising microbial control agents for population management of bark beetles, with little or no adverse effects on plants or vertebrates, including human applicators.[5,6] Several *Cordyceps* and *Ophiocordyceps* anamorphs, such as *Beauveria*, *Hirsutella*, *Isaria*, *Lecanicillium*, and

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Metarhizium species, have been used to control bark beetle populations (Supporting Information, Table S1). These fungi are easy to culture, produce ample spores for potential commercial use, and have been isolated from a multitude of sources such as interior and exterior plant surfaces, insects, soil, and forest environments worldwide. Additionally, the ability to infect arthropod hosts by direct penetration of the exoskeleton gives these fungi an advantage over other entomopathogenic organisms that only kill their host through an existing wound or after ingestion.[1]

Despite decades of research on fungi as biological control agents of insects, a gap in our knowledge remains: Why do promising laboratory evaluations often lead to unsuccessful field applications? Consequently, an improved understanding of the biotic and abiotic factors that contribute to the persistence and efficacy of entomopathogenic fungi *in situ* could promote more efficient and cost-effective applications for target pests. Herein, we analyze the research regarding the use of entomopathogenic fungi as biological control agents to limit bark beetle populations. We will focus on bark beetles *sensu stricto*, phloem breeding tree-killers because of their unique behavior and managerial needs in temperate coniferous forest ecosystems; though will use examples from other insects when appropriate. In this review, we (i) examine the abiotic and biotic factors specific to bark beetle habitats that entomopathogenic fungi must overcome, and (ii) discuss a framework for progressing the study of entomopathogenic fungi as biological control agents of bark beetles.

2 ENTOMOPATHOGENIC FUNGI AND BARK BEETLE ENVIRONMENTS

Entomopathogenic fungi are predominantly subterranean species that require some degree of physical protection from adverse environmental conditions and primarily rely on arthropods for medium- to long-distance spore dispersal. Since bark beetles occupy complex aboveground habitats, there is the potential that inundative application of entomopathogenic fungi will have difficulty tolerating environmental factors that are unique to these systems. Accordingly, a variety of abiotic and biotic factors most relevant to bark beetle environments such as (i) temperature, (ii) ultraviolet light exposure, (iii) competition with other microorganisms, and (iv) plant secondary metabolites, can inhibit or promote fungal pathogenicity. The effects of each of these factors on fungal growth, reproduction, and virulence are explored in detail below.

2.1 Temperature

Ambient temperature strongly affects entomopathogenic fungi as biological control agents. Despite considerable phenotypic diversity among isolates, entomopathogenic fungi are mesophilic species overall. Growth and germination rates are typically maximized under 'room conditions' at around 25 °C.[7,8] Optimal temperatures for pathogenicity generally range from 15 to 35 °C, with maximum virulence often within 20 to 25 °C.[8,9] Osmotic imbalance and tissue damage resulting from the production of reactive oxygen species contributes to temperature intolerance in these fungi and may restrict fungal growth to a narrow temperature range.[10]

Isolate response to diurnal temperature fluctuations, as opposed to multiple stable temperatures typical of chamber studies, is an understudied characteristic of natural environments. While trees moderately insulate against ambient temperatures, bark beetle habitats are frequently characterized by variable temperatures,[11] and very few studies explicitly consider diurnal fluctuation as a limitation of isolate virulence to bark beetles. Isolates grow and germinate best at a constant temperature near 25 °C and fare incrementally worse as temperature variability increases.[12] Therefore, greater consideration of fungal response to daily temperature fluctuations could enhance the application in bark beetle systems and researchers should focus testing on isolates and inoculation methods that are responsive over a range of targeted temperatures.

In many cases, fungi must also overcome high temperatures (>30 °C) because application methods usually rely on field stability during the bark beetle flight period, which coincides with the warmest part of the year. While these isolates may be relatively uncommon because entomopathogen density decreases during the summer months in a conifer forest,[13] we speculate that one way to overcome temperature intolerances is by isolating fungi from areas that match future site conditions. For example, one study found that *Beauveria bassiana* collected from the hottest areas are the most tolerant in terms of growth, sporulation, and germination at high temperatures.[7] Another study demonstrated that continuous culturing of *Metarhizium anisopliae* at high temperatures increases growth rate at these conditions.[14] Additionally, an important fitness tradeoff did not take place and the isolates retained pathogenicity towards *Melanoplus sanguinipes*.

2.2 Ultraviolet light

Ultraviolet light (UV) exposure inhibits growth, sporulation, and pathogenicity for practically every entomopathogenic fungus examined and is considered the most significant hurdle to overcome for successful biocontrol in many systems.[8] Many entomopathogenic species such as *Beauveria*, *Isaria*, and *Metarizium* are not well-adapted to resist UV exposure; and as with temperature stress, UV radiation leads to oxidative stress and cell damage.[14]

In the coffee berry borer, *Hypothenemus hampei,* habitats *B. bassiana* isolated from *H. hampei* germinates well in the shade. However, once exposed beetles exit the shade of *Coffea* plants and enter into direct sunlight, they can avoid *B. bassiana* infection through UV inactivation.[15] A similar 'sanitation' technique was described in *Boisea rubrolineata*, where, after exposure to sunlight, the insects produced monoterpenes that inhibited germination of a commercial *B. bassiana* strain.[16] Despite spending most of their time below the surface of a tree, the application of fungi to control bark beetles should nonetheless take the effects of sunlight into account because application methods usually rely on beetle exposure during dispersal (i.e., during the beetle flight period). Thus, several methods could optimize the efficacy of entomopathogenic fungi in settings exposed to sunlight. The first is selecting isolates, however rare, which are most resistant to UV damage. Oil- and clay-based emulsions may also protect from UV exposure[17] and are discussed in detail within Section 4. Additionally, application during times and in locations with indirect sunlight, especially as an endophyte, could help the fungus avoid UV damage.[18]

Finally, genetic modification has proven to be a viable option for overcoming UV damage in *Beauveria* and *Metarhizium* species where sunlight has been a limiting abiotic factor. Overexpression of either a protease or chitinase gene, or both simultaneously, increases virulence which decreases the amount of time the fungus needs to tolerate stressful environmental conditions. Researchers have also improved UV-resistance directly by expressing photolyases for DNA repair. Overexpression of superoxide dismutase, which detoxifies reactive oxygen species, is another option. To overcome the lack of melanin, pigment production activation has increased both UV-resistance and virulence against multiple insects.[14]

2.3 Bark beetle-associated microorganisms

Bark beetles are associated with a consistent community of symbiotic microorganisms that aid in beetle development and the ability to colonize host trees.[19] Knowledge of the interactions among bark beetles, entomopathogenic fungi, and bark beetle-symbionts is important for successful fungal application. For instance, the bark beetle immune system differs in response to mutualistic versus antagonistic fungi,[20] and *B. bassiana* from a bark beetle environment modifies the gut microbiome to increase virulence.[21] Research on direct fungus to fungus competition is, however, much more prevalent than investigations that incorporate beetle response within the greater microbiome.

A common method for examining competition between entomopathogenic fungi and bark beetle-associated microorganisms is through dual culture Petri dish assays that allow researchers to compare primary aspects of resource capture, or growth rates, and secondary aspects of competition, such as the ability to overtake or inhibit a competing fungus. On malt extract agar, *Leptographium abietinum*, a symbiont of the bark beetle *Dendroctonus rufipennis*, is a slightly faster grower than commercial and sympatric *B. bassiana* isolates and can more rapidly exploit uncolonized resource space; though both fungi can exclude one another from colonized areas.[19] The same deadlock is found in the fungi *Ambrosiella roeperi* and *A. grosmanniae*, associated with *Xylosandrus* ambrosia beetles, when ambrosia and entomopathogenic fungi are simultaneously introduced into the same resource-limited environment. However, when the ambrosia fungi are inoculated onto the substrate first, to mimic secondary colonization by commercial strains of *B. bassiana* and *Metarhizium brunneum*, ambrosia fungi capture primary resource space and grow over the entomopathogenic fungi.[22] This suggests that priority effects may occur among fungal species *in situ* and that early application is important in spraying and autodissemination management strategies. Interactions among entomopathogenic fungi and bark beetle-associated microbes can also be indirect and occur via volatile interactions. For instance, volatiles produced by the *D. brevicomis*-symbiotic yeast *Ogataea pini*, inhibit radial growth of a commercial *B. bassiana* strain but enhance radial growth of a mutualistic nutritional fungus.[23]

2.4 Plant secondary metabolites

The coniferous trees colonized by bark beetle species are usually well-defended by chemically rich resins. Upon boring into tree phloem, beetles and their symbionts are exposed to significant quantities of monoterpene hydrocarbons that the beetles can often tolerate at constitutive concentrations.[24] Since entomopathogenic fungi take multiple days to parasitize their hosts, the fungi inevitably come into contact with tree secondary metabolites regardless of the biocontrol application method.

Insects vary in susceptibility to *Isaria fumosorosea* and *B. bassiana* after feeding on certain agricultural plants. Reduced efficacy of the pathogens could be due to insect immunity after ingesting phytochemicals that entomopathogenic fungi are not extremely tolerant of such as tomatine[25] or through direct inhibition by plant volatiles.[26] In two cases where endophytic entomopathogenic fungi benefit plant secondary metabolism, tomatoes and *Echinacea purpurea* upregulate phytochemicals in response to *B. bassiana* inoculation.[18] These processes aid the plants by reducing herbivory and increasing plant growth, respectively.

The effects of entomopathogenic fungi on conifer secondary metabolism are unknown, though conifer monoterpenes can have detrimental effects on *B. bassiana* efficacy.[9] While constitutive levels of conifer monoterpenes can enhance the growth rate of a bark beetle symbiotic fungus,[27] many *B. bassiana* isolates appear to be inhibited by even low amounts of conifer phytochemicals. The growth rate of *B. bassiana* isolates from conifer forests was reduced by over 90% versus a negative control when exposed to induced concentrations of monoterpenes common in *Picea engelmannii*.[9] In another study, *B. bassiana* isolates from various sources were able to tolerate full-strength *Pinus contorta* monoterpenes, better than a 100-fold dilution of *P. banksiana* phytochemicals.[28] Since conifer monoterpenes appear to limit many of the *B. bassiana* isolates that have been tested, we speculate that adult colonization of conifer phloem could 'sanitize' the beetle from certain *B. bassiana* isolates.

3 ENTOMOPATHOGENICITY AND INSECT BEHAVIOR

Insects explore and evaluate their environment for potential dangers when obtaining refuge, food, and mates. In nature, there is often a combination of external abiotic and biotic factors that influence insect avoidance, attraction, or 'non-avoidance' behavior.[29] Pathogenic and non-pathogenic fungal isolates produce different volatile blends, which are likely detected by insects to influence avoidance behavior.[30] Understanding how insects can respond to the presence of their pathogens is critical to the use of entomopathogens as a biological control agent of bark beetles.

Within tree galleries, the bark beetle *Dryocoetes confusus* appears to 'wall off' fungal-infected individuals so that the remaining offspring do not become contaminated.[31] This behavior is an example of bark beetles' ability to detect and avoid an entomopathogenic fungus, something that could have severe management implications in the future application of the fungus if not taken into consideration. Contrary to the 'walling off' behavior observed in some bark beetles, other laboratory studies have revealed that there is potential for vertical and horizontal transmission in bark beetle systems.[32,33] Bark beetle response to microbial volatile organic compounds allows us to further speculate how the presence of *B. bassiana* may influence bark beetle behavior. For example, hexanol (a typical 'green leaf volatile') is a prominent component of both pathogenic and non-pathogenic isolates of *B. bassiana*[30] and acts as a repellent to the bark beetles *D. ponderosae* and *D. frontalis*.[34] This repellent behavior may be detrimental for the prospects of *B. bassiana* as a direct control for bark beetles, but perhaps inundative application before bark beetle attack could prevent colonization.

The processes and mechanisms behind insect detection, attraction, and avoidance behaviors in response to entomopathogenic fungi are generally unclear and a greater understanding of these effects could substantially improve the application of entomopathogenic fungi to control bark beetles. Collectively, these studies indicate that matching fungal traits to environmental conditions alone may not be sufficient as a means to control bark beetles; a suite of insect behaviors can also strongly impact the efficacy of entomopathogens in the field and much is still unknown about how bark beetles respond to these fungi.

4 FUTURE DIRECTIONS FOR CONTROL OF BARK BEETLES WITH ENTOMOPATHOGENIC FUNGI

4.1 Improvements to laboratory evaluations

Bark beetle population control is complicated because the beetles have a cryptic lifecycle, live in a dynamic environment, and mainly attack chemically well-defended hosts across large natural landscapes. As a whole, literature documenting that entomopathogenic fungi exist in natural systems is vast; with isolations coming from the majority of studied bark beetle species (Supporting Information, Table S1). This is an important first step (Fig. 1), especially when marketing these fungi as an augmentative biological control for a pathogen that is already present in that pest's natural environment. Isolating strains from various environments is also beneficial for understanding isolate to isolate variation and answering basic biological questions such as 'how host-specific are entomopathogenic fungi?'; 'what determines host specificity?'; and 'how do strains from certain geographical locations and hosts differ in their response to certain environments?' While there is ample documentation on the presence of entomopathogenic fungi in bark beetle systems, prevalence measurements are more nuanced due to different sampling, culturing, and sequencing methods. Supporting Information, Table S1 contains details on entomopathogenic fungus abundance in bark beetle habitats and indicates that isolation rates are generally at or below 5%, but range from 0% to 43% overall. A large-scale ecological study on the relative abundance of entomopathogenic fungi present in bark beetle systems, especially throughout an outbreak, is lacking and could elucidate the role of pathogens as natural enemies of bark beetle populations.

Screening potential fungal isolates for the tolerance of system-specific environmental conditions should be the next step when selecting strains for potential bark beetle field application. Evaluations of fungal abiotic and biotic tolerance have not been conducted in the context of most studied bark beetle species (Supporting Information, Table S1), which could explain laboratory to field discrepancies. Future research must address how potential biological control isolates respond to fluctuating temperatures, the role of entomopathogens in the bark beetle holobiont, and mechanisms underlying conifer secondary metabolite tolerance. Additionally, studies should address the ability of isolates to endure multiple stressful environmental conditions simultaneously, as growth, reproduction, and tolerance tests typically take place under 'room conditions,' or at around 25 °C with presumed humidity levels between 30% and 60% (Supporting Information, Table S1). Bark beetles live in complex environments and a multivariate approach is necessary to enhance our understanding of which fungal traits can be matched with habitat conditions.

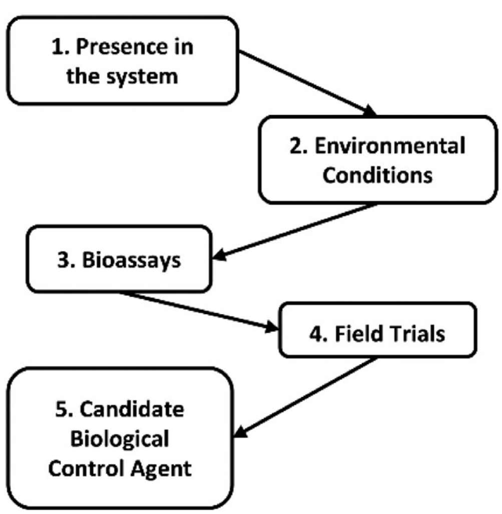

Figure 1. Framework for improved isolate selection of entomopathogenic fungi as biological control agents of bark beetles. Once isolates have met *in vitro* standards, researchers should conduct controlled field trials to test suitable inoculation methods and the potential for non-target effects. If a candidate isolate does not emerge from field trials, isolate selection should be refined in additional laboratory evaluations. Black lines and numbers signify progressing through the proposed evaluation process.

Laboratory bioassays under representative environmental conditions that take insect behavior into account are the next step in evaluating potential strains for control of bark beetles. Bioassays have been completed on the majority of bark beetle species that have been studied in association with entomopathogenic fungi (Supporting Information, Tables S1 and S2). Gaps in the literature most likely exist due to difficulty capturing a sufficient supply of individuals while controlling for insect age and life history, or research objectives other than developing management strategies to control bark beetles. Almost every bioassay conducted on bark beetles, so far, has taken place under 'room conditions' (Supporting Information, Table S2). The rate of bark beetle mortality increases as temperature increases,[9] which highlights the potential impacts of variable environments on the interpretation of isolate virulence.

The use of tree-based bioassay substrate has been extremely common throughout the entomopathogenic fungus-bark beetle literature (Supporting Information, Table S2). A substrate containing tree phloem is an important step when evaluating potential bark beetle biological controls, as filter paper containing spore solution can have inconsistent results with other substrates.[9] A proper substrate also provides shelter and nutrients for beetles while exposing the beetles and fungi to other microorganisms and tree phytochemicals. Additionally, phloem-based bioassays allow investigators to observe beetles and to take insect behavior into account when screening potential isolates. Very little is presently known about potential 'detection and avoidance' behaviors or even horizontal and vertical transmission that may occur inside bark beetle galleries after a pathogen is introduced.

It is important to match the method of inoculation in a laboratory setting with the method that practitioners plan to use in the field. Direct application, such as dipping or applying drops of spore solution directly to the insect, is desirable when it is necessary to standardize for spore concentration, such as an initial pathogenicity evaluation. It can also be beneficial if the planned method of field inoculation is by releasing infected bark beetles with the goal of horizontal or vertical transmission through populations. Indirect techniques, such as inoculating the substrate before adding insects to the arena, can be a more accurate representation of future field applications, especially with bark beetles who can only become exposed to an augmentative application during their summer flight period.

4.2 Application as a spray with a protectant

A simple way to spread entomopathogenic fungi for the treatment of bark beetles is by directly spraying standing or downed logs with a spore solution. This method may be especially useful for preventing an attack on certain high-value trees and is an application style that managers could use with little additional equipment. Unfortunately, spraying has not been consistently successful in field application against bark beetles (Supporting Information, Table S3), potentially due to the lack of protection that this method provides from environmental factors discussed earlier in this paper.

Including a protectant that allows for increased viability and does not reduce virulence is important when spraying entomopathogenic fungi. Application with a polymeric matrix in a bark beetle system allows conidia to remain viable more than 30 days after application. Additionally, this formulation does not affect virulence to *Ips typographus* under laboratory conditions.[35] Natural products can also aid in entomopathogen survival against UV-exposure. Of the 12 additives tested for UV-protection, which ranged from plant oils to clays, humic acid most prolonged spore survival under laboratory and field conditions. In the field, colony-forming unit counts were 7.8 times higher for *B. bassiana* added to humic acid than the control after one week.[17]

4.3 Application with semiochemical traps

Many practitioners and scientists evaluate using 'assisted autodissemination' systems to introduce entomopathogenic fungi into field environments.[29] These traps attract an insect into an inoculation device to become contaminated with the entomopathogen before returning to its host environment to infect other pests either horizontally or vertically. Previous field trials using semiochemical-baited traps have resulted in the most successful application against bark beetles (Supporting Information, Table S3), however, these studies are often preliminary with a relatively small sample size and short evaluation period which may not adequately address population reduction. Formulations that kill eggs, larvae, pupae, and adults will be the most effective for bark beetle management applications. Once the adult enters the tree and reproduces it has already succeeded. Thus, a method that solely targets adults will not effectively reduce populations unless the beetles die before reproducing. Dissemination methods that allow for vertical transmission of entomopathogenic fungi from adults to young, during birth or soon after, may be especially challenging because bark beetles are not highly social insects and larvae only feed in uncontaminated areas of the phloem.[36]

However, if horizontal or vertical transmission does occur, autodissemination devices can be valuable for bark beetle systems because spraying an entire forest is not feasible and both male and female aggregations are easily incited with the deployment of semiochemicals. Additionally, a dissemination chamber can protect fungi from UV which may be particularly limiting for foliar and bark application. New technologies are being developed in bark beetle systems to eliminate the detrimental effects of rainfall,[35,37] though high temperatures (>40 °C) inside traps may still be a concern.[35]

4.4 Colonization as an endophyte

Since entomopathogenic fungi are largely soil-dwellers, these species may be able to colonize trees via their root system. *Beauveria bassiana* has been isolated from pines in areas that are susceptible to the bark beetles *Hylastes ater* and *Hylurgus ligniperda*, both of which are invasive species in New Zealand.[38] Research on the use of fungi as an endophyte to control insects and plant pathogens is still relatively young, with the majority of the research taking place on how to best inoculate plants for endophytic establishment. While entomopathogenic fungi have been isolated from mature trees, studying the effects of fungi as an endophyte will take considerable time and effort because young trees are not susceptible to bark beetle attacks and many colonization methods occur as plants are seeds or seedlings.[18] In pines, endophytic establishment has occurred through seed coating and root dip coatings.[38] Phloem colonization may be another obstacle because some entomopathogenic fungal isolates are relatively intolerant of conifer phytochemicals.

Perhaps managers should reframe how entomopathogens are utilized as a management tool against bark beetles and shift from a focus on producing beetle mycosis to the use of fungi as conifer endophytes. We hypothesize that endophytic fungi can aid trees in phytochemical upregulation, similar to what has been observed in agricultural plants.[18] Thus, indirectly protecting the tree from bark beetles and ultimately achieving the same management goal.

4.5 Host-specificity

Finally, we recommend studying the potential adverse effects of applying entomopathogenic fungi to a largely unmanaged natural environment. Studies should evaluate how a fungal strain changes as it passes through the environment over many generations, even if applying for short-term population reduction. *Beauveria*, *Isaria*, and *Metarhizium* species are considered generalist pathogens overall and many arthropods that occupy trees, understory vegetation, and soil in forest environments may be susceptible to fungal infection. Fortunately, promising prospects for entomopathogen host-specificity have arisen where *B. bassiana* isolates from various sources were virulent against bark beetles but not pathogenic to bark beetle predators[39] or honeybees.[28]

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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