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# Improved mortality of the Formosan subterranean termite by fungi, when amended with cuticle-degrading enzymes or eicosanoid biosynthesis inhibitors

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**Abstract** Formosan subterranean termites (FST) were exposed to strains of *Beauveria pseudobassiana* (Bpb) and *Isaria fumosorosea* (Ifr) to determine virulence of the fungi. Once lethality was determined, sublethal doses of Bpb were combined with enzymes capable of degrading the insect cuticle to measure the potential to enhance fungal infection. Bpb applied to FST in combination with proteinases and a chitinase caused increased mortality over the fungus alone. Mortality was enhanced when Ifr was applied to FST in combination with a chitinase isolated from *Serratia marcesans*. A lipase isolated from *Pseudomonas cepacia*, when combined with Ifr, also resulted in greater mortality than all control treatments. FST were also exposed to the eicosanoid biosynthesis inhibitors (EBIs) dexamethasone (DEX), ibuprofen (IBU), and ibuprofen sodium salt (IBUNA), in combination with Ifr. Combining Ifr with IBUNA caused significantly increased mortality on days 6, 7, and 9. Cuticle-degrading enzymes and EBIs may have potential to enhance the pathogenic effect of a fungal control agent against the Formosan subterranean termite.

## Introduction

The Formosan subterranean termite (FST, *Coptotermes formosanus* Shiraki) is a pest insect that causes significant expenditure in prevention and repair costs in the USA annually. Microbial species have been investigated as potential control measures to complement chemical control methods (Shimizu and Yamaji 2002; Su 2002). *Isaria fumosorosea* (Ifr) has been shown to control FST in the laboratory (Wright et al. 2003). Species of *Paecilomyces* sect. *Isarioidea* are synonymous with *Isaria* (Luangsa-Ard et al. 2005). Keratin hydrolysate was previously found to be a biocompatible surfactant with foaming properties that allows delivery of the fungus into the nest environment of FST in trees and other difficult-to-reach void spaces (Dunlap et al. 2007). With a foam carrier system available to deliver Ifr into the void spaces of termite nests, augmentation of the virulence of this and other entomopathogenic agents is especially worthy of further investigation. Combining Ifr with compounds such as cuticle-degrading enzymes can increase the pathogenic potential of the treatment in the field. A novel strain of *Metarhizium anisopliae* isolated from an FST alate has been shown to cause mortality of FST alates and worker termites in the lab and in the field (Wright et al. 2005). *Beauveria* species have been demonstrated as microbial control agents of insect pests (Rehner and Buckley 2005; Ghikas et al. 2010). In order for entomopathogens to kill termites, they must have the capability of bypassing termite pre-penetration defense mechanisms and also survive passage to nestmates through allogrooming (Chouvenc et al. 2011). Combining entomopathogens with enzyme or chemical applications may enhance the pathogenic effect (Bulmer et al. 2009)

Cuticle-degrading enzymes isolated from three bacteria were studied for their ability to increase effectiveness of Ifr against FST. The enzymes chitinase and lipase have been isolated from entomopathogenic fungi and have been correlated with enhanced virulence (Hegedus and Khachatourians 1988; Mendonsa et al. 1996; Hernandez-Torres et al. 2004; Nahar et al. 2004; Dahiya et al. 2006). Selection of an appropriate chitinase for use with fungal entomopathogens is critical

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because chitinases are thought to be expressed by plants to protect against pathogenic fungi (Taira et al. 2002) and are used to control plant pathogens in agricultural applications (Dahiya et al. 2006; Karasuda et al. 2003). Bacterial chitinases aid host nutrition and parasitism, and act as antibacterial agents and immunoenhancers (Wen et al. 2002; Dahiya et al. 2006).

Chitinases may increase penetration of pest insects by entomopathogens by degredation of chitin (St. Leger et al. 1996). This effect may lead to an increase in virulence (El-Sayed et al. 1989; Gupta et al. 1994). A mutant of the fungus *I. fumosorosea* that overexpresses chitinases caused a significant increase in mortality of whitefly compared to the parental strain (Hernandez-Torres et al. 2004). Conversely, a mutant strain of the fungus *Nomuraea rileyi*, with decreased chitinase expression, showed a comparative decrease in mortality of cabbage looper larvae (El-Sayed et al. 1989). *Paecilomyces lilacinus* is used as a biological control agent of plant parasitic nematodes and its chitinase activity correlates with that of *Trichoderma* spp. and *M. anisopliae* (Khan et al. 2003).

It has been proposed that chitinases will increase penetration of target insects by pathogenic fungi through degradation of structural chitin (St. Leger et al. 1996) with the potential to increase virulence (El-Sayed et al. 1989; Gupta et al. 1994). A stable mutant of *I. fumosorosea* that over-produces chitinases displayed twofold greater mortality of whitefly than the parental strain (Hernandez-Torres et al. 2004). By contrast, a mutant strain of the fungus *N. rileyi* with decreased chitinolytic activity compared to the parental strain also caused a decreased mortality rate among cabbage looper larvae (El-Sayed et al. 1989). *P. lilacinus* is a biological control agent for use against plant parasitic nematodes. Chitinase activity isolated from this species correlates with that found in *Trichoderma* spp. and *M. anisopliae* (Khan et al. 2003).

Eicosanoids are biologically active, oxygenated C20 polyunsaturated fatty acid metabolites. They are involved in human immune responses in the form of prostaglandins and leukotrienes. In addition, they have been found to protect invertebrates from infection by bacteria (Miller et al. 1994, 1996; Stanley 1998). Eicosanoid biosynthesis inhibitors (EBI) were found to increase insect mortality when applied in combination with the bacterium *Serratia marcesans* to tobacco hornworms, *Manduca sexta* (L.) (Stanley-Samuelson et al. 1991). Insects form nodules in an active defensive response to bacterial infection. Nodule formation was inhibited when *M. sexta*, the tenebrionid beetle, *Zophobas* sp., and crickets, *Gryllus assimilis* (F.) were injected with both an EBI and bacteria (Howard et al. 1998; Miller et al. 1996, 1999).

Connick et al. (2001) exposed FST to combinations of the bacterium *S. marcesans* and the EBIs ibuprofen (IBU), ibuprofen sodium salt (IBUNA), and dexamethasone (DEX). Both IBU and DEX significantly increased mortality of FST workers versus mortality caused by *S. marcesans* alone, with DEX showing the greater effect on mortality. Based on other studies, it is believed that the increased mortality resulted from the suppression of the FST immune system by the EBIs (Stanley-Samuelson et al. 1991; Miller et al. 1994, 1999; Howard et al. 1998). IBUNA in combination with *S. marcesans* caused a lower mortality than the bacterium alone (Connick et al. 2001). IBUNA, unlike IBU, is water soluble and this factor may have affected the termite or the bacterium through dilution in the moist environment in which termites live and were tested. None of the EBIs, when used alone, caused significantly higher mortality than a peptone control. This was the first report of a pharmaceutical effect on insects exposed to EBIs through contact and normal feeding rather than injection. Cuticle-degrading enzymes and EBIs are here combined with entomopathogenic fungi to determine their potential to increase the rate of FST mortality.

*I. fumosorosea* (Ifr) has been developed as a biocontrol agent for whitefly control (Jackson et al. 1997). Methods to produce Ifr blastospores in a stable, wet-able powder formulation have been developed (Jackson 1999; Jackson et al. 2003). Screening of Ifr against the Formosan and native subterranean termite workers revealed that it was pathogenic to termites (Wright et al. 2003). Additional work with Ifr demonstrated that a 5 min exposure of termites to Ifr spores is sufficient to cause 100 % mortality in 4 to 7 days (Wright et al. 2003). EBIs are believed to suppress insect immune systems thereby aiding infection by microbial pathogens (Connick et al. 2001). A synergistic effect from the application of EBIs and the bacterium *S. marcesans* showed that DEX and IBU both enhanced the pathogenic effect of the bacterium (Connick et al. 2001).

## Materials and methods

### Microbial strains

*Beauveria pseudobassiana* strain ARSEF 8046 was obtained from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) and was originally isolated from a deer tick. The culture was inoculated onto potato dextrose agar (PDA) plates and incubated at 30 °C for 7 days. After incubation, fungal spores were scraped from the plates into a sterile tube using 100 μL of sterile 0.01 % aqueous Triton X-100 and an inoculating loop. Spore concentrations were quantitated using a Levy hemacytometer (0.1 mm deep; VWR, West Chester, PA, USA). For initial mortality assays, the following concentrations were prepared: $2\times10^3$, $2\times10^6$, and $2\times10^9$ spores/mL. For assays in combination with enzymes, a concentration of $2\times10^7$ spores/mL was prepared.

*I. fumosorosea* (Ifr) strain 3581, isolated from *Bemisia argentifolli* in McAllen, Texas, was formulated in Hyflo

Super-Cel (Celite Corp., Lompoc, CA). The formulation was produced by a previously reported method (Jackson et al. 1997) and provided by Dr. Mark Jackson of the USDA-ARS-NCAUR, Peoria, IL, USA. Stock cultures were grown on potato dextrose agar (PDA; DIFCO, Detroit, MI, USA) for 3 weeks at room temperature, cut into 1-$mm^2$ agar plugs and stored in 10 % glycerol at −80 °C. Conidial plates were produced by inoculating PDA dishes with a conidial suspension from frozen stock cultures and incubating at 25 °C for 2–3 weeks. The spores were diluted in Hyflo to a final spore count of approximately $1.6\times10^{10}$ spores/g for combination with chitinase from *Streptomyces griseus*, or to a final spore count of approximately $6\times10^{8}$ spores/g for combination with the chitinase from *S. marcesans* and lipase from *Pseudomonas cepacia*.

### Termites used for mortality bioassays

FST workers, third instar or older based upon size, and soldiers were provided from bucket traps (Su and Scheffrahn 1986) located in New Orleans, LA, USA. Termites from four different colonies were selected to prevent colony vitality biasing of data. Each colony represented one replicate. Each treatment was applied independently to 18 workers and 2 soldiers from each replicate. Only one half of the termites in each experimental sample were directly exposed to the fungus to confirm that the previously demonstrated transfer of fungal spores from termites to their nestmates occurred. Varying mortality rates of controls are attributed to seasonal changes in termite viability. All termites for each individual experiment were collected at the same time.

### Cuticle-degrading enzymes

Proteinase A (ProA) was isolated from *Aspergillus melleus* and proteinase B (ProB) is from a bacterial source. One unit of each will hydrolyze casein to produce color equivalent to 1.0 μmol (181 μg) of tyrosine/min at pH 7.5 at 37 °C. Lipase A (LipA) was isolated from *Thermomyces lanuginosus* and lipase B (LipB) was isolated from *P. cepacia*. One unit of each liberates 1 μmol oleic acid/min at pH 8.0 and 40 °C. Chitinase A (Chi A) was isolated from *S. griseus*, and chitinase B (Chi B) was isolated from *S. marcesans*. One unit of each will liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin/h at pH 6.0 at 25 °C in a 2-h assay. All enzymes were obtained from Sigma (St. Louis, MO, USA). Each was prepared at a concentration of 2 units of enzyme/mL in water. A 500 μL aliquot (1 unit) of the enzyme was used to wet the filter paper, with an equal volume of water applied to filter paper in the control replicates.

### *B. pseudobassiana* and cuticle-degrading enzymes

Twenty FST from each colony were placed into a 2 mL conical microcentrifuge tube containing 0.5 mL of a suspension containing the stated concentration of spores in sterile, deionized water for 2 min, independent of termites from the other colonies. Tubes were agitated by hand during the incubation time to ensure that the termites were submerged in the liquid. The termites were then transferred to a 90 mm disc of filter paper (Whatman, Maidstone, England) in the lid of a 100× 15 mm Petri dish where they were allowed to air dry. Control termites were exposed as described above, but the microcentrifuge tube contained sterile water only. These termites were then transferred to a 55 mm Whatman filter in the lid of a 60×15 mm Petri dish with an equal number of untreated nestmates. The paper disc served as a moisture and nutrient source. For control and fungus-only assays, the filter paper was wetted with water. For enzyme-only or fungus with enzyme assays, the filter paper was wetted with the appropriate enzyme solution at a concentration of 1 unit per filter disc in water. Bioassays were incubated at 25 °C and 85 % humidity, optimized for fungal and termite viability, while mortality was monitored. For initial mortality assays mortality was monitored on days 7, 14, and 21. For assays with combined fungal and enzyme treatments, mortality was monitored on day 7.

### *I. fumosorosea* and cuticle-degrading enzymes

Chi A and Chi B were diluted to working concentrations of 0.06 and/or 0.6 mg/mL in water. For enzyme-only treatments, 20 termites from each colony were placed on a 55-mm disc of filter paper, wetted with 0.5 mL of the appropriate enzyme, in the lid of a 60×15 mm Petri dish as enzyme controls. For fungus-only treatments, 10 termites from each colony were placed in a 100×15 mm Petri dish containing 2 g of diluted Ifr spores and were allowed to walk on the spores for 5 min, independently of termites from other colonies. The termites were then transferred to a 55 mm disc of filter paper, wetted with 0.5 mL of water, in the lid of a 60×15 mm Petri dish along with an equal number of nestmates not exposed to the fungus. For the control, 20 termites from each colony were placed on a 55 mm disc of filter paper, wetted with 0.5 mL of water, in the lid of a 60×15 mm Petri dish. Combined treatments exposing 20 termites from each colony to both Ifr and the respective enzyme concentration were prepared as described in the following steps: a 55 mm disc of filter paper, in the lid of a 60×15 mm Petri dish, was wetted with 0.5 mL of one of the chitinase solutions. For fungus-only treatments, 10 termites from each colony were exposed to Ifr spores as described. The termites were then transferred to a filter paper disc, wetted with 0.5 mL of the appropriate concentration of chitinase, along with an equal number of nestmates not directly exposed to the fungus.

Lip A and Lip B were diluted to working concentrations of 2 and 10 mg/mL. For both enzyme control and combined enzyme/Ifr treatments, 20 FST from each colony were placed into a 2-mL microcentrifuge tube containing 0.5 mL of the appropriate lipase solution for 2 min independent of termites from the other colonies. The termites were then transferred to a 90-mm disc of Whatman filter paper and allowed to air dry. Termites exposed to an enzyme only were transferred to a 55-mm disc of filter paper, wetted with 0.5 mL of water. Water control termites were exposed as described as above, but the microcentrifuge tube contained water rather than an enzyme. For combined treatments, half of the termites were subsequently exposed to Ifr spores as described above. Ten termites exposed to both the lipase and the fungus were placed onto a filter paper, wetted with 0.5 mL of water, along with 10 nestmates which were exposed to the lipase only. All Petri dishes containing termites were incubated at 25 °C and 90 % humidity while mortality was monitored for up to 21 days.

### *I. fumosorosea* and eicosanoid biosynthesis inhibitors

IBU and DEX (Sigma Chemical Co., USA) solutions were made in ethanol to a final concentration of 50 μg/mL and a 50 μg/mL solution of IBUNA (Sigma Chemical Co., USA) was made in sterile water. All solutions were used immediately. To prevent the ethanol from affecting the termites, 1 mL of the DEX and IBU solutions were pipetted onto Whatman #4 filter paper discs and allowed to dry. The filter paper was then wetted with 1 mL of sterile water. FST were exposed to the EBI when they walked on and consumed the filter paper.

Ten FST workers from each of four colonies were allowed to walk on a sporulated fungal culture on a PDA plate for 2.5 min. The exposed FST workers were then transferred to Petri dishes (Falcon, Franklin Lakes, NJ, USA) which contained 10 of their nestmates and a Whatman #4 filter paper disc. The filter paper was dampened with either 1 mL of sterile water (Solution 2000 Water Purification System, Solution Consultant Inc., Jasper, GA, USA), or 1 mL of a 50 μg/mL solution of one of the EBIs. The Petri dishes were then incubated at 25°C and ~99 % humidity for the duration of the experiment. Controls included exposure of termites allowed to walk on Ifr alone, exposed to one of the EBIs alone, or exposed to 1 mL of sterile water only.

### Data analysis

All data were analyzed using analysis of variance (ANOVA) and least significant difference (LSD) at $p \leq 0.05$ (Cody and Smith 1997). One hundred percent mortality represents an absolute value of 80 termites. Treatments with the same letter on the same day are not significantly different. All analyses were run using the SAS System Software (Cody and Smith 1997).

### Human and animal rights

No humans or other animals were subjects in this study.

## Results

### *B. pseudobassiana* and cuticle-degrading enzymes

On day 7, the mortality rate for termites exposed to the control treatment which contained only water was 3.8 %. On the same day, termites exposed to both $10^3$ and $10^6$ spores/mL had mortality rates of 2.5 %. Termites exposed to $10^9$ spores/mL reached a mortality rate of 100 % by day 7 (Fig. 1). By day 14, 7.5 % of the control termites had died, while the mortality rate for the $10^3$ and $10^6$ treatment levels was 3.8 and 11.3 % respectively. By day 21, the $10^6$ spore concentration treatment mortality rate equaled that of the control at 12.5 %, while the $10^3$ treatment was 10.0 % on the same day.

Once it was determined that *B. pseudobassiana* caused mortality of FST, the potential for increased mortality with enzymes capable of degrading termite cuticle was investigated. Termites were exposed to $1 \times 10^7$ spores/mL of *B. pseudobassiana*, and 1 unit each of two proteinases, one lipase and one chitinase. These concentrations were chosen because they did not cause significant mortality individually, and would allow identification of an increase in mortality caused by synergism. Each enzyme was then combined with the fungus for determination of potential increased virulence on day 7. None of the individual treatments were significantly different from one another. The combined treatments of the fungus and the lipase were not significantly different from the individual treatments. Bpb + Lip A caused a mortality rate of 10.0 %, while exposure of termites to Bpb + Lip B resulted in a mortality rate of 13.8 % (Fig. 2). The combination of *Beauveria* with Pro A caused a 21.3 % mortality, while the combination of the fungus with Pro B resulted in a mortality rate of 22.5 %. Pro A is from the fungus *Aspergillus* and Pro B is from a bacterium. Both caused mortality significantly higher than that of the control, the *Beauveria* strain alone, or either Pro A or Pro B alone. The combination of *Beauveria* with chitinase resulted in a mortality rate of 32.5 %, the highest observed in this study.

### *I. fumosorosea* and eicosanoid biosynthesis inhibitors

To measure potential synergism between EBIs and a fungal biocontrol agent, FST workers were exposed to Ifr for 2.5 min in this experiment, half the time previously found to be effective. Through day 5 of the experiment, none of the chemical treatments enhanced the pathogenicity of Ifr (Figs. 3, 4, and 5). From day 6 through day 9, the Ifr + Dex (Fig. 3) and Ifr + IBUNA (Fig. 4) treatments showed increased mortality over

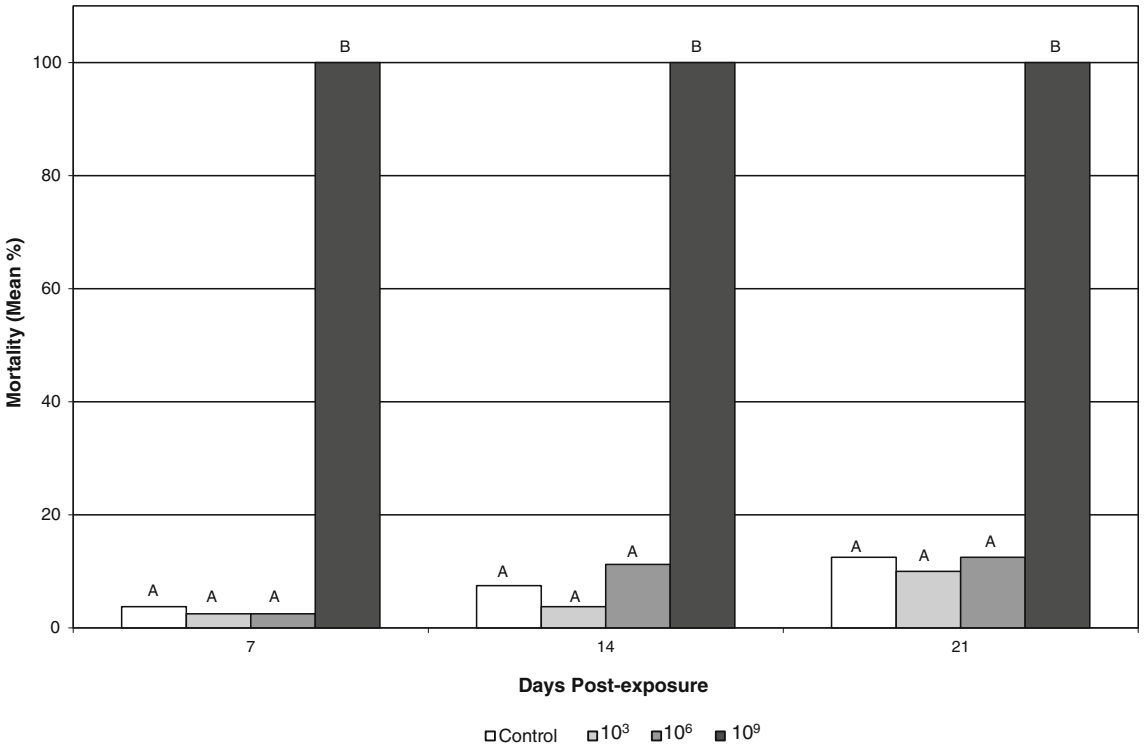


**Fig. 1** Mortality (mean %) of FST by *B. pseudobassiana* strain ARSEF 8046 at concentrations of $10^3$, $10^6$, and $10^9$ spores per mL on 7, 14, and 21 days after exposure; termites were exposed to the stated concentration of the fungal strain in liquid, and incubated on moistened filter paper; mortality was determined weekly

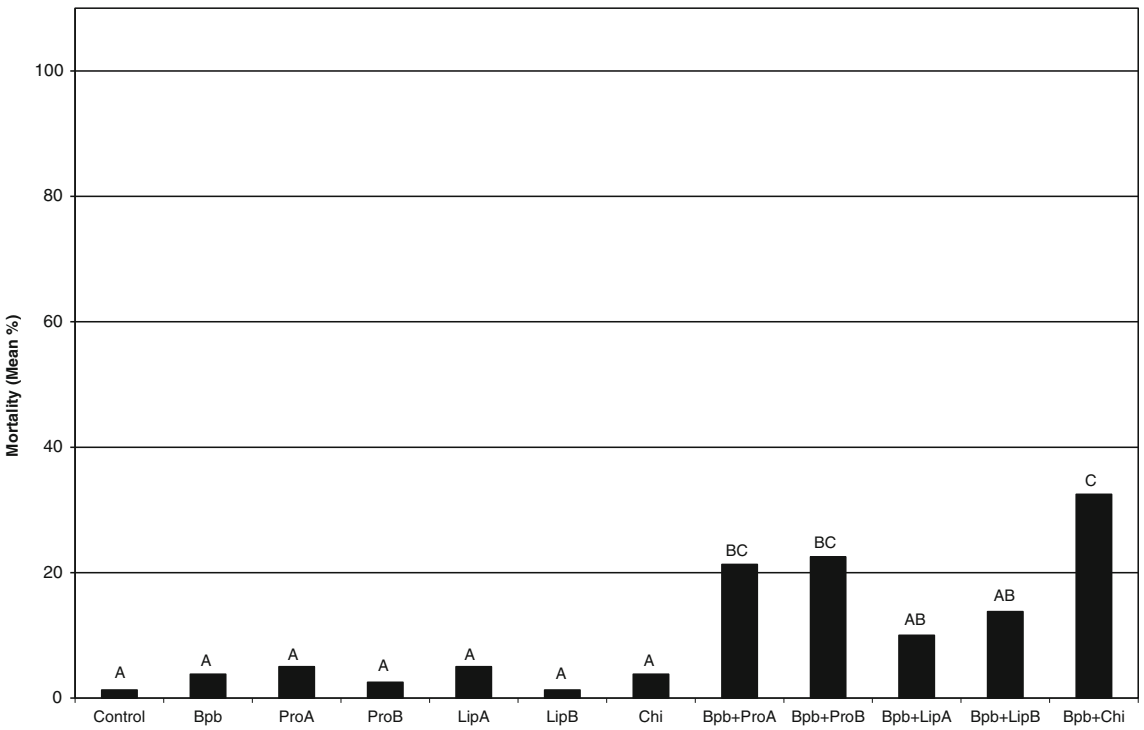


**Fig. 2** Mortality (mean %) of FST by *B. pseudobassiana* strain ARSEF 8046 (*Bpb*) at a concentration of $10^7$ spores/mL on day 7 in combination with proteinase (*Pro*), lipase (*Lip*), and chitinase (*Chi*) enzymes; termites were exposed to the fungal strain and enzymes in liquid, then incubated on moistened filter paper; mortality was determined weekly

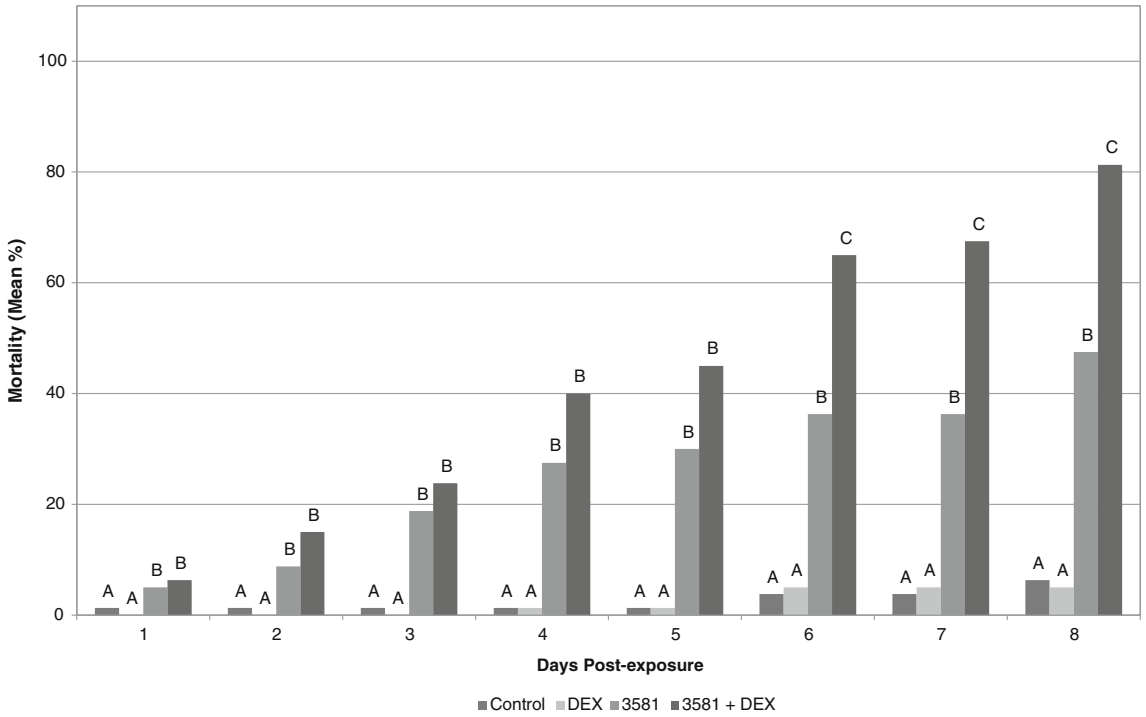


**Fig. 3** Comparative mortality of FST workers exposed to dexamethasone (*Dex*) and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

Ifr alone. Both Ifr alone and Ifr with the Dex supplement reached 100 % mortality by day 12 (Fig. 3). Mortality in excess of 50 % with termites exposed to Ifr alone confirms previous studies that the fungus was transferred among nestmates. Ifr with the IBUNA supplement reached 100 % mortality by day 16 (Fig. 4). IBU, which was shown to enhance pathogenicity of *S. marcesans* (Connick et al. 2001), did not have a positive effect on the pathogenicity of Ifr. The

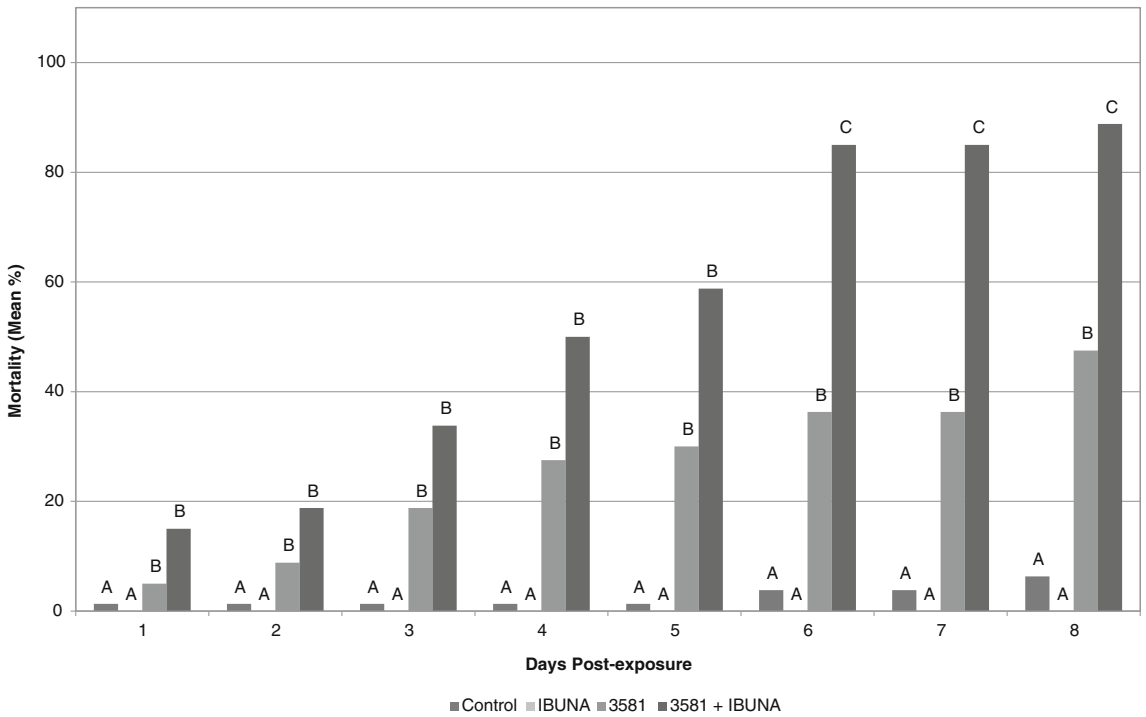


**Fig. 4** Comparative mortality of FST workers exposed to ibuprofen sodium salt (*IBUNA*) and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

bacterium and the IBU were delivered as liquid solutions while the termites were exposed to the fungus and IBU separately. This may have changed any solubility effects between the chemical and the microbes. At no point did the mortality rate of the Ifr + IBU combination exceed that of Ifr alone (Fig. 5). On day 6, when the DEX and IBUNA treatments exceeded the Ifr treatment, Ifr + IBU had killed only 26 % of the termites compared to 48 % killed by Ifr alone. On day 16, the Ifr, Ifr + IBU, and IBU treatments had reached 100, 99, and 6 % mortality, respectively (Fig. 5).

### *I. fumosorosea* and cuticle-degrading enzymes

On day 7, the mortality rate for termites exposed to the control treatment which contained only water was 2.5 %. On the same day, termites exposed to Ifr alone had a mortality rate of 22.5 % and reached a maximum of 63.8 % mortality on day 21.

Ifr spores in combination with Chi A at 0.06 mg/mL caused 43.8 % mortality at day 7, 70.0 % at day 14 and 88.8 % at day 21 (Fig. 6). The same enzyme at a concentration of 0.6 mg/mL when combined with Ifr spores caused 26.3 % mortality at day 7, 50.0 % at day 14 and 68.8 % at day 21. Ifr alone caused 41.3, 80.0, and 85.0 % mortality on days 7, 14, and 21, respectively. The mortality rates of the fungus in combination with the enzyme were not significantly different from those of the fungus alone. In fact, the values for the 0.6 mg/mL chitinase are somewhat, although not statistically, lower than those of the Ifr only treatment. On days 7 and 14, these treatments were also statistically not different from the untreated and enzyme-only controls. On day 21, all Ifr treatments were significantly different from the water control which showed only 21.3 % mortality. The combined Ifr + 0.06 mg/mL chitinase treatment, with 85.0 % mortality, was significantly different from the chitinase only controls, which at 0.06 mg/mL caused 20.0 % mortality, and at 0.6 mg/mL caused 15.0 % mortality.

Ifr spores when combined with 0.6 mg/mL of Chi B caused significantly greater mortality, 17.5 %, than the fungus alone, 3.8 %, as early as day 4 post-exposure (Fig. 7). By day 16, the combined treatment had caused 100 % mortality. The maximum mortality on day 16 was 56.3, 47.5 and 15.0 % for the fungus alone, enzyme alone and water control, respectively. Each value was significantly different from the combined treatment.

Ifr spores when combined with lipase at a concentration of 2 mg/mL caused significantly greater mortality than the fungus alone as early as day 2 post-exposure, 38.8 % mortality vs. 1.3 % mortality (Fig. 8). A rate of 100 % mortality in the combined treatment was reached at day 14. The 10 mg/mL treatment in combination with the fungus caused significantly greater mortality by day 4 and reached a maximal mortality of 97.5 % at the end of the experiment on day 16. The water control, 2 mg/mL lipase and 10 mg/mL lipase controls reached maximal mortality rates of 28.8, 56.3, and 51.3 % on day 16. All were significantly lower than the mortality rates of the combined treatments.

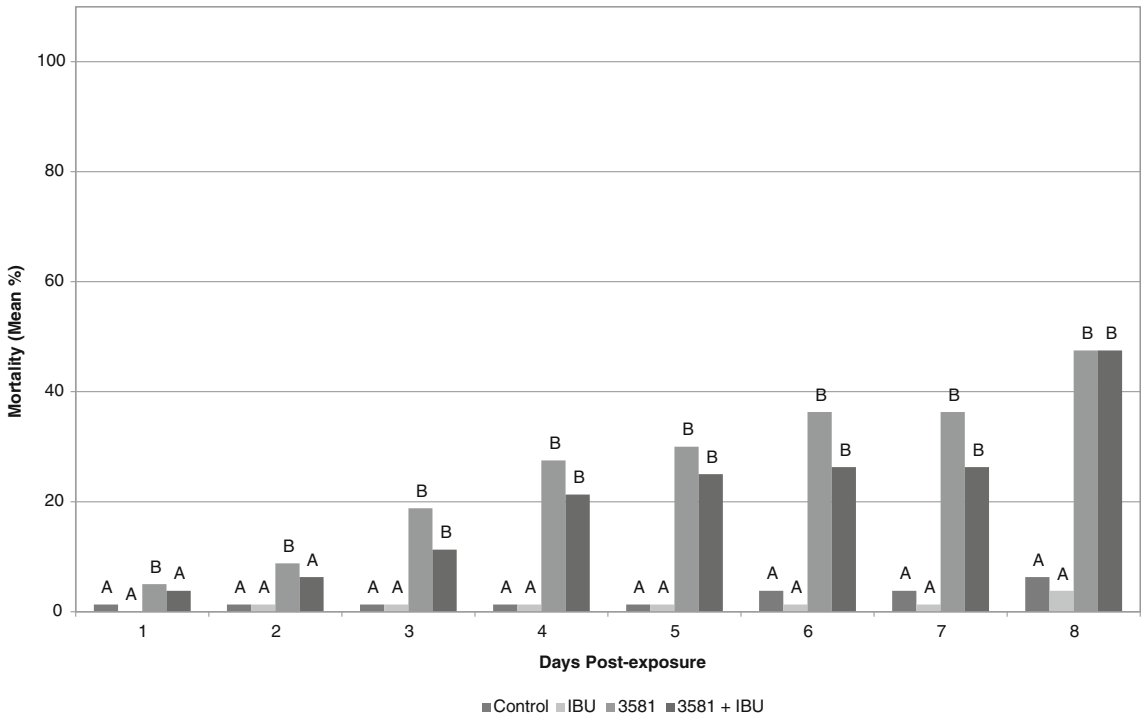


**Fig. 5** Comparative mortality of FST workers exposed to ibuprofen (*IBU*) and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

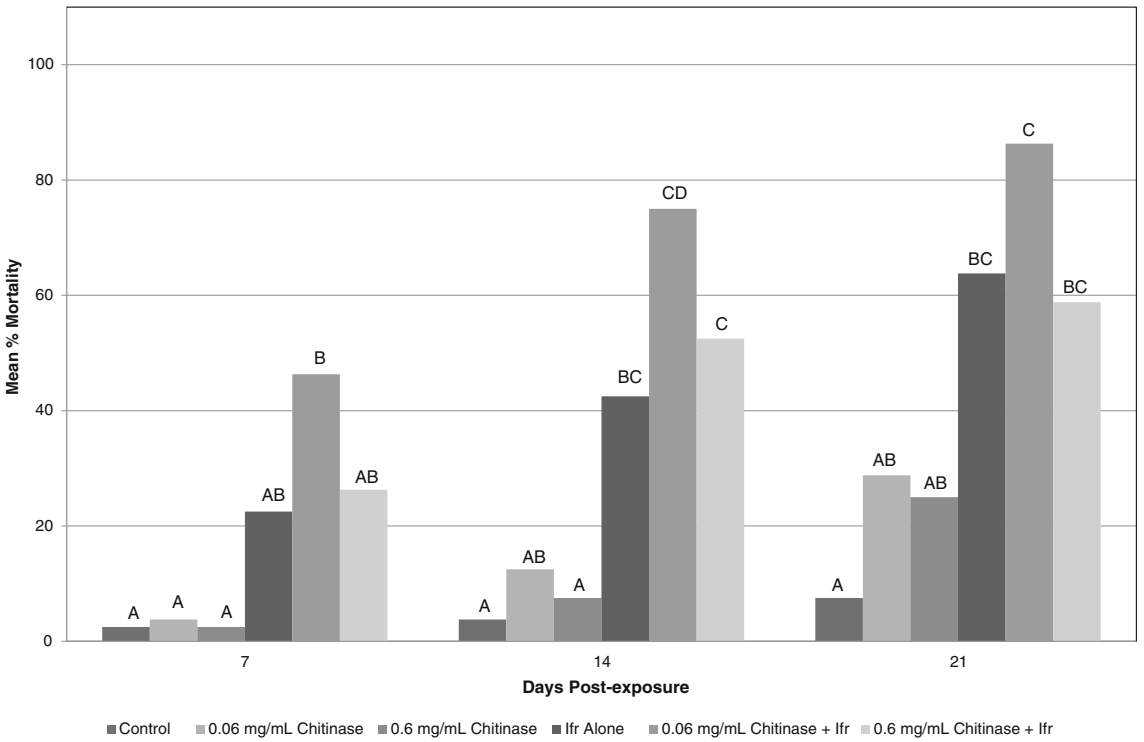


**Fig. 6** Comparative mortality of FST workers exposed to chitinase from *S. griseus* and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

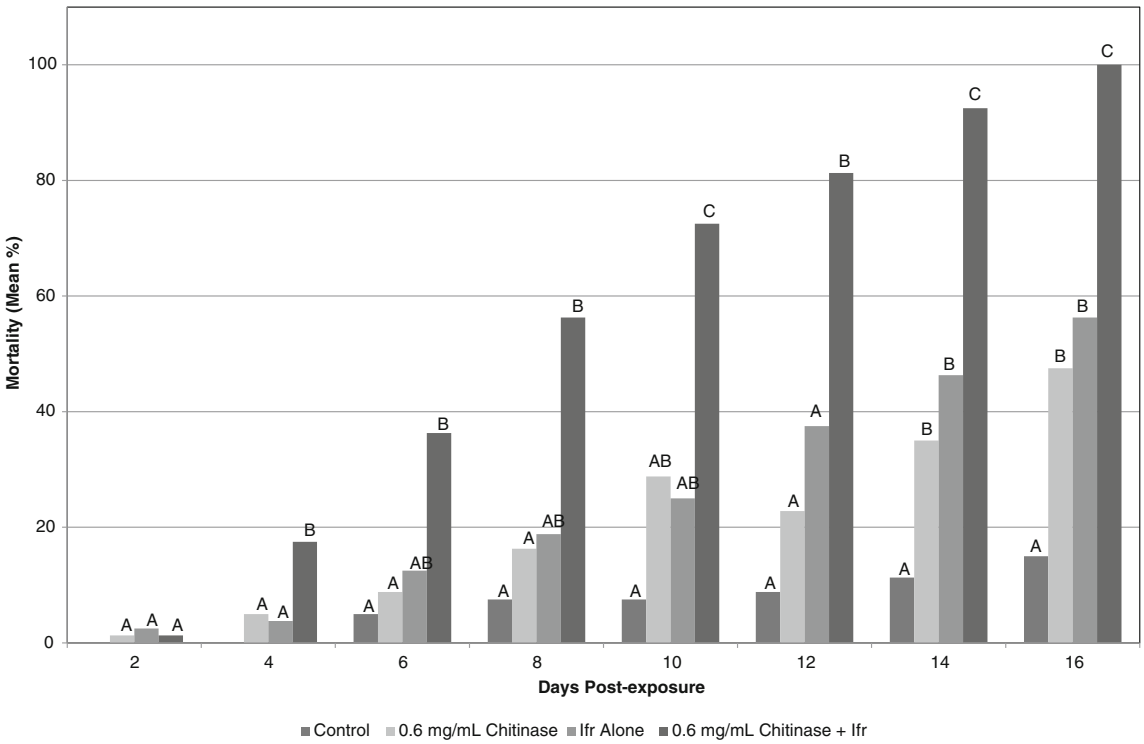


**Fig. 7** Comparative mortality of FST workers exposed to chitinase from *Serratia marcesans* and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

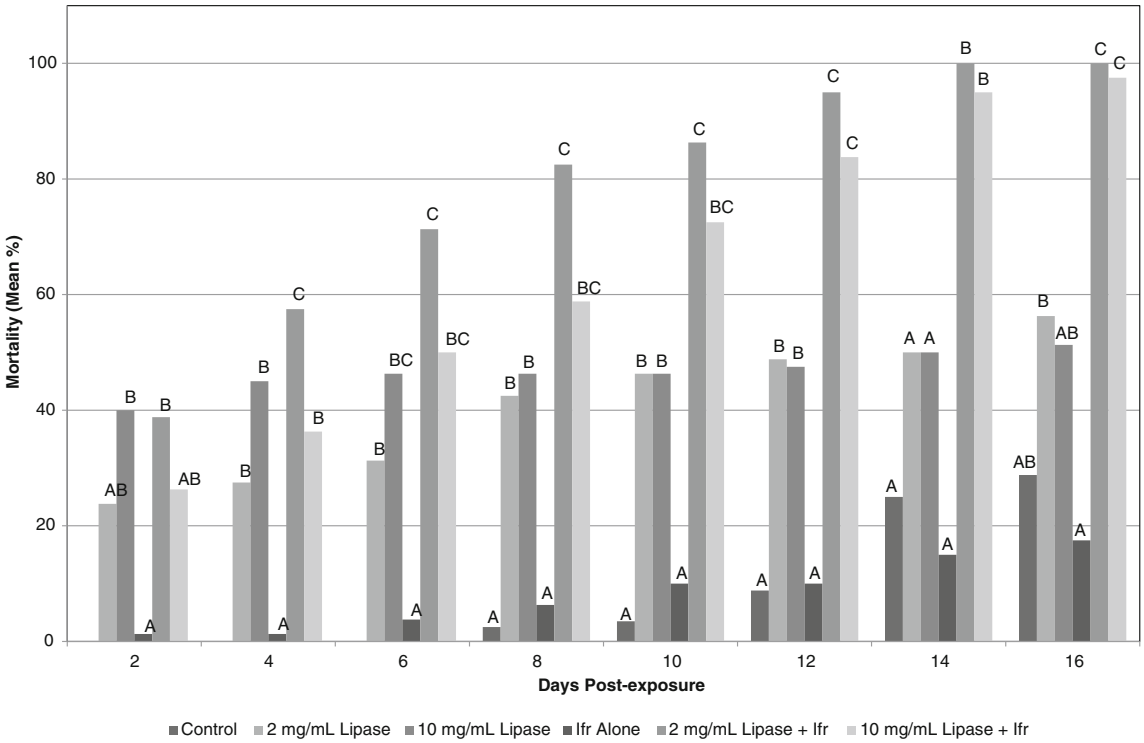


**Fig. 8** Comparative mortality of FST workers exposed to lipase and/or *I. fumosorosea* (*3581*), only half the termites were directly exposed to the fungus

## Discussion

Use of cuticle-degrading enzymes to enhance virulence of entomopathogenic fungi must be balanced with selection of enzymes that do not inhibit the biological control agent. Fungal inhibition would be evident by decreased activity when compared to the activity of the pathogen alone. Appropriate combinations can result in synergism, utilizing lower concentrations of each component than if they were applied individually. These results demonstrate an increased mortality rate as early as day 7 between *B. pseudobassiana* strain 8046 and both proteinases, as well as the chitinase. The lipase tested here did not produce an effect that was significantly different from the control. These data suggest that the proteinase and chitinase enzymes have potential use with this fungus. Further investigation of interactions between entomopathogenic fungi and cuticle-degrading enzymes may yield increased FST control conditions.

The desire to utilize cuticle-degrading enzymes to enhance virulence of entomopathogenic fungi must be tempered with selection of enzymes that do not negatively impact the biological control agent. Proper selection has the potential to generate enzyme/fungus combination treatments that control the target insect utilizing less of each component than if they were applied individually. These results demonstrate effect on mortality by chitinase from *S. griseus* and suggest a negative influence on the fungus' pathogenic properties by this enzyme. The other two enzymes tested, chitinase from *S. marcesans* and lipase from *P. cepacia*, did increase the mortality rate of the FST when applied with Ifr when compared to application of the fungus or enzyme alone. These data suggest that these two enzymes have potential for use with Ifr in particular, and that further investigation of interactions between other entomopathogenic fungi and cuticle-degrading enzymes may yield compatible pairings. Further research is needed to determine if other treatments can improve control, and to optimize the ratio of enzymes to fungal spores.

Termites are cryptic pests that are difficult to detect and treat. Control methods will ideally affect colony suppression to prevent re-infestation. Termites will remove dead termites from the main body of the colony and can detect and avoid some chemical and biological control agents. Biological and chemical control agents have the potential to enhance the effect of each other. Of particular interest are chemicals that inhibit the immune system of termites making them more susceptible to microbial infection. As with successful chemicals, the selected microbe must not be repellent to termites or it will be avoided before a sufficient inoculum can be delivered. The microbe must also be delivered to nest members that do not forage to the delivery site. Inoculated foraging termites must also live long enough to travel back to the nest and transfer the microbe to nestmates for the colony to be suppressed. The EBIs DEX and IBUNA are shown here to enhance pathogenicity of Ifr. These EBIs can potentially be delivered to termites in the field in a foam or gel by injection into an infested tree or wood structure, or they could be

impregnated into a solid substrate such as paper or wood in a bait station. Pathogenicity of fungal spores delivered simultaneously would then be enhanced. These studies and previous work with EBI and a bacterium indicate that further study in this area is warranted to yield ideal integrations of biological and chemical control agents.

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## References

Bulmer MS, Bachelet I, Raman R, Rosengaus RB, Sasisekharan R (2009) Targeting an antimicrobial effector function in insect immunity as a pest control strategy. PNAS 106(31):12652–12657

Chouvenc T, Su NY, Grace JK (2011) Fifty years of attempted biological control of termites—analysis of a failure. Biol Cont 59:69–82

Cody RP, Smith JK (1997) Applied statistics and the SAS programming language, 4th edn. Prentice-Hall, Inc., Upper Saddle River

Connick WJ Jr, Osbrink WLA, Wright MS, Williams KS, Daigle DJ, Boykin DL, Lax AR (2001) Increased mortality of *Coptotermes formosanus* (Isoptera: Rhinotermitidae) exposed to eicosanoid biosynthesis inhibitors and *Serratia marcesans* (Eubacteriales: Enterobacteriaceae). Environ Entomol 30(2):449–455

Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbio and Biotech 71: 773–782

Dunlap CA, Jackson MA, Wright MS (2007) A foam formulation of *Paecilomyces fumosoroseus*, an entomopathogenic biocontrol agent. Biocontrol Sci and Technol 17:513–523

El-Sayed GN, Coudron TA, Ignoffo CM, Riba G (1989) Chitinolytic activity and virulence associated with native and mutant isolates of an entomopathogenic fungus *Nomuraea rileyi*. J Invert Pathol 54: 394–403

Ghikas DV, Kouvelis VN, Typas MA (2010) Phylogenetic and biogeographic implications inferred by mitochondrial intergenic region analyses and ITS1-5.8S-ITS2 of the entomopathogenic fungi *Beauveria bassiana* and *B. brogniartii*. BMC Microbiol 10:174–189

Gupta SC, Leathers TD, El-Sayed GN, Ignoffo CM (1994) Relationships among enzyme activities and virulence parameters in *Beauveria bassiana* infections of *Galleria mellonella* and *Trichoplusia ni*. J Invert Pathol 64:13–17

Hegedus DD, Khachatourians GG (1988) Production of an extracellular lipase by *Beauveria bassiana*. Biotech Lett 10:637–642

Hernandez-Torres I, Iracheta M, Galan-Wong LJ, Hernandez C, Contreras J, Jackson M, Pereyra-Alferez B (2004) A *Paecilomyces fumosoroseus* mutant over-producing chitinase displays enhanced virulence against *Bemisia tabaci*. World J Microbiol Biotech 20: 207–210

Howard RW, Miller JS, Stanley DW (1998) The influence of bacterial species and intensity of infections on nodule formation in insects. J Insect Physiol 44:157–164

Jackson MA (1999) Method for producing dessication tolerant *Paecilomyces fumosoroseus* spores. US Patent 5:968,808

Jackson MA, Cliquet S, Iten LB (2003) Media and fermentation processes for the rapid production of high concentrations of stable blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Biocontrol Sci and Technol 13:23–33

Jackson MA, McGuire MR, Lacey LA, Wraight SP (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycol Res 101:35–41

Karasuda S, Tanaka S, Kajihara H, Yamamoto Y, Koga D (2003) Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. Biosci Biotech and Biochem 67:221–224

Khan A, Williams K, Molloy MP, Nevalainen H (2003) Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. Prot Express and Purif 32:210–220

Luangsa-Ard JJ, Hywel-Jones NL, Manoch L, Samson RA (2005) On there lationships of *Paecilomyces* sect. *Isarioidea* species. Mycol Res 109:581–589

Mendonsa ES, Vartak PH, Rao JU, Deshpande MV (1996) An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedes aegypti* mosquito. Biotechnol Lett 18:373–376

Miller JS, Nguyen T, Stanley-Samuelson DW (1994) Eicosanoids mediate insect nodulation responses to bacterial infections. Proc Nat Acad Sci USA 91:12418–12422

Miller JS, Howard W, Nguyen T, Nguyen A, Rosario RMT, Stanley-Samuelson DW (1996) Eicosanoids mediate nodulation responses to bacterial infections in larvae of the tenebrionid beetle, *Zophobas atratus*. J Insect Phys 42:3–12

Miller JS, Howard RW, Rana RL, Tunaz H, Stanley DW (1999) Eicosanoids mediate nodulation reactions to bacterial infections in adults of the cricket, *Gryllus assimilis*. J Insect Phys 45:75–83

Nahar P, Ghormade V, Deshpande MV (2004) The extracellular production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomopathogenic fungi in the biological control of insect pests. J Invert Pathol 85:80–88

Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. Mycologia 97:84–98

Shimizu S, Yamaji M (2002) Pathogenicity of entomopathogenic fungi to the termite, *Reticulitermes speratus*. Jap J Appl Entomol Zool 46: 89–91

St Leger RJ, Joshi L, Bidochka MJ, Rizzo MW, Roberts DW (1996) Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviridae* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl and Environ Microbiol 62:907–912

Stanley DW (1998) Eicosanoids mediate insect cellular immune reactions to bacterial infections. Adv Experimental Med and Biol 433:359–362

Stanley-Samuelson DW, Jensen E, Nickerson KW, Tiebel K, Ogg CL, Howard RW (1991) Insect immune response to bacterial infection is mediated by eicosanoids. Proc Natl Acad Sci USA 88:1064–1068

Su N-Y (2002) Novel technologies for subterranean termite control. Sociobiol 40:95–101

Su N-Y, Scheffrahn RH (1986) A method to access, trap, and monitor field populations of the Formosan subterranean termite (Isoptera: Rhinotermitidae) in the urban environment. Sociobiol 12:299–304

Taira T, Ohnuma T, Yamagami T, Aso Y, Ishiguro M, Ishihara M (2002) Antifungal activity of rye (*Secale cereale*) seed chitinases: the different binding manner of class I and class II chitinases to the fungal cell wall. Biosci Biotechnol and Biochem 66:970–977

Wen CM, Tseng CS, Cheng CY, Li YK (2002) Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. Biotechnol and Appl Biochem 35:213–219

Wright MS, Connick WJ, Jackson MA (2003) Use of *Paecilomyces spp*. as pathogenic agents against subterranean termites. U.S. Patent 6, 660,291.

Wright MS, Raina AK, Lax AR (2005) A strain of the fungus *Metarhizium anisopliae* for controlling subterranean termites. J Econ Entomol 98:1451–1458