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Combined Effect of Microbial and Chemical Control Agents on Subterranean Termites

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Termite mortality was measured when fungi were combined with bacteria or a chemical termiticide to determine whether a synergistic effect occurred. The fungus *Beauveria bassiana* was combined with the non-repellant chemical termiticide imidacloprid. Of the three *B. bassiana* strains tested one, *B. bassiana* ATCC 90519, was sufficiently pathogenic on its own that the advantage of a supplementary chemical treatment was marginal. The mortality caused by another fungal strain, *B. bassiana* ATCC 26037, was improved in combination with imidacloprid at both of the tested chemical concentrations over the first 14 days. The remaining fungal strain, *B. bassiana* ATCC 90518, demonstrated an overall mortality rate in combination with imidacloprid of 82.5%, versus a rate of 65.0% for the fungus alone. The fungus *Isaria fumosorosea* (Ifr) was combined with the bacterium *Bacillus thuringiensis* (Bt). On day 5, Ifr, Bt, and the combined treatment at a 10^6 spores or cells/ml dosage caused 8.8%, 22.5%, and 15.0% mortality, respectively. The Bt and combined mortality rates are not significantly different. Control mortality on day 5 was 5.0%. On day 13 the combined 10^6 treatment mortality rate was 91.3%, which was significantly higher than all other treatments: control at 17.5%, Ifr at 36.3% and Bt at 35.0%. When Ifr and Bt were applied at a 10^9 spores or cells/ml dosage, Ifr alone caused a mortality rate of 97.5% as early as day 5. The combination with Bt could not significantly increase the effectiveness of this dosage. These data demonstrate the potential for synergistic effects of fungal and chemical treatment methods, thereby broadening the use of microbial control agents and reducing the quantity of chemical agents necessary to effect control.

Keywords: *Bacillus*, *Beauveria*, *Coptotermes*, imidacloprid, *Isaria*

Introduction

Subterranean termites (ST) are destructive insect pests that damage trees and structures composed of wood. In the United States ST are estimated to cause $1 billion in damage annually, including prevention and repair costs. A predominant invasive species of ST, the Formosan subterranean termite (FST), *Coptotermes formosanus* (Shiraki), has become an economically significant pest in the United States. FSTs are damaging pests because of the size of their colonies, the fact that they attack several species of living trees, and their high rate of reproduction. Novel non-repellant chemicals and bait technology have been developed to control termite infestations. In order for these techniques to work they must not repel termites, must be easily transferrable in or on termite bodies, and have delayed toxicity which allows transfer from foraging workers to members of the termite colony that do not forage (Su and Scheffrahn, 1996, 1998). The focus of this study is the potential synergistic effect of biological fungal and bacterial agents to control termites, when applied together or with chemical control agents.

Fungi exhibit qualities which can make them ideal for this application, including a slow-acting nature similar to that of successful chemicals, the ability to self-replicate, and the ability of fungal spores to be spread by termite social behavior (Grace and Zoberi, 1992). The pathogenic effect of strains of *Beauveria bassiana* (Balsamo) Vuillemin have been demonstrated in laboratory colonies of *C. formosanus* (Delate *et al*., 1995; Wells *et al*., 1995; Wright *et al*., 2002). Jones *et al.* discovered in 1996 that small numbers of *Beauveria bassiana* spores can be spread throughout a *C. formosanus* colony without being detected by the termites. Conditions in a termite nest, moderate temperature and high humidity, are conducive to the growth of fungal species and are important factors in fungal survivability and propagation (Kramm *et al*., 1982; Ignoffo, 1992).

The three *B. bassiana* strains selected for testing were isolated from either the soil or from insects other than termites. They have been previously shown to cause mortality in termites, have been transferred between termites that forage and nestmates that do not forage, and they have been recovered post-mortem from infected termites (Wright *et al*., 2002). Two strains, ATCC 26037 and ATCC 90519, were equally pathogenic to termites, causing 100% mortality at day 7. Strain ATCC 90518 reached a maximal mortality rate of 96% at 21 days. By comparison, two strains of another entomopathogenic fungal species, *Metarhizium anisopliae*, reached maximal mortality rates of 100% and 25% mortality at day 21 (Wright *et al*., 2002). In this study FST were exposed to the three previously tested *B. bassiana* strains alone, and in combination with sublethal doses of imidacloprid.

Isaria fumosorosea (Ifr) is an entomopathogenic fungus that has been previously shown to cause significant mortality

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to Formosan subterranean termites (FSTs) (Wright *et al*., 2003, 2008). Ifr is formulated in a stable, wettable powder (Jackson *et al*., 1997) compatible with a keratin foam dispersant (Dunlap *et al*., 2012). Species of *Paecilomyces* sect. *Isarioidea* are synonymous with *Isaria* (Luangsa-Ard *et al*., 2005).

Bacillus thuringiensis (Bt) is a spore-forming bacterium that has been used to control insect pests. Most Bt-based insecticides include toxic spore-crystals and the expression of these toxins in plants targets insect pests that chew and bore into agricultural plants (Bravo *et al*., 2011). Bt is evaluated here for its ability to cause mortality of termites, and for potential synergism with Ifr.

Determining effective treatments of pathogen combinations will allow lower dosages of each pathogen to be used, and consequently lower the cost of treatment. Combined treatments will also reduce the likelihood that termites will immunologically limit the effectiveness of individual microorganisms.

Materials and Methods

Microbial strains

Three strains of *B. bassiana* were obtained from the American Type Culture Collection (ATCC, USA). *B. bassiana* strain ATCC 26037 was isolated from the Colorado potato beetle in Czechoslovakia; *B. bassiana* strain ATCC 90518 was isolated from the soil in Oregon; and *B. bassiana* strain ATCC 90519 was isolated from the Japanese beetle. All cultures were grown in Petri dishes containing potato dextrose agar (PDA; BD, USA) in a 25°C incubator for 7 to 14 days.

Isaria fumosoroseus strain ARSEF 3581 blastospores were generously provided by Dr. Mark Jackson (USDA-ARS-NCAUR, USA) as a wettable powder. Spores were rehydrated in 0.01% aqueous Triton X-100 (Sigma-Aldrich, USA).

Bacillus thuringiensis strain ATCC 33679 was obtained from ATCC, and was originally isolated from diseased insect larvae. It was selected for this study after causing mortality of FST in a broad screening of several *Bacillus* strains. The culture was inoculated onto Luria agar (LA; BD) plates and incubated at 25°C for 2 days. After incubation, a single colony was used to inoculate 10 ml of Luria broth (LB; BD). The culture was incubated at 28°C and 225 rpm for approx 6 h, and then used to inoculate 100 ml of LB which was incubated at 28°C and 225 rpm overnight. The bacterial mass was harvested by centrifugation and the pellet was resuspended in water. Cell and spore concentrations were quantified using a Levy hemacytometer (0.1 mm deep; VWR, USA).

Termites

Termites were collected from City Park, New Orleans, LA, USA using bucket traps (Su and Scheffrahn, 1986). Four colonies were used for each treatment, with each colony representing one replicate, to prevent colony vitality biasing of data. Twenty *C. formosanus* workers of at least 3rd instar (as determined by size), including 10% soldiers, were used in each of the 4 replicates.

Exposure of termites to *Beauveria* and recovery of the fungus

For exposure of termites to *B. bassiana*, ten Formosan subterranean termites from each of four colonies were allowed to walk on *B. bassiana* cultures in Petri dishes for 10 min. These workers were then transferred to Petri dishes containing Whatman #4 filter paper dampened with sterile water and containing 10 unexposed termites from the same colony. Petri dishes containing both the exposed and unexposed termites were then placed in an unlit incubator at 25°C and 99% humidity. Replicated control plates were incubated as described above and contained 20 termites from a single colony, none of which had been exposed to fungal cultures.

At each observation for termite mortality, dead termites were counted, removed from the filter paper-lined Petri dish and transferred to another Petri dish containing potato dextrose agar to allow propagation of fungi associated with the cadaver. The plates were incubated in an unlit 25°C incubator and were observed daily for evidence of *B. bassiana* growth.

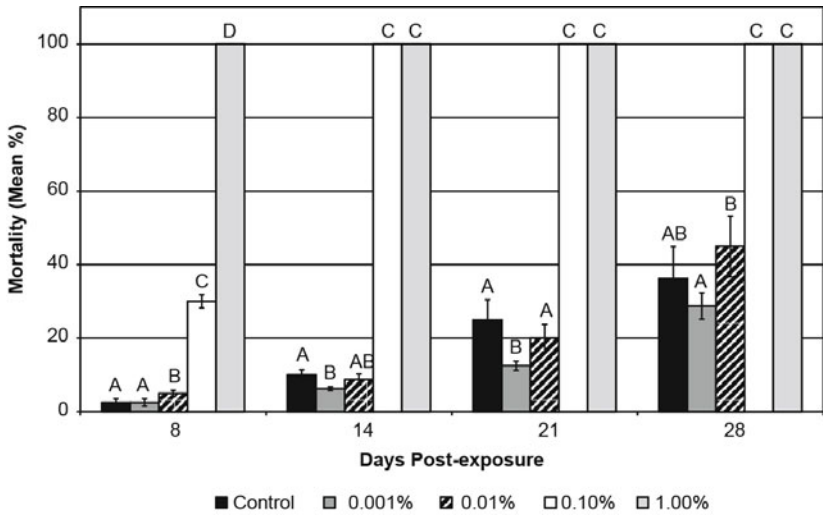

Fig. 1. Mortality of FST exposed to varying concentrations of imidacloprid. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

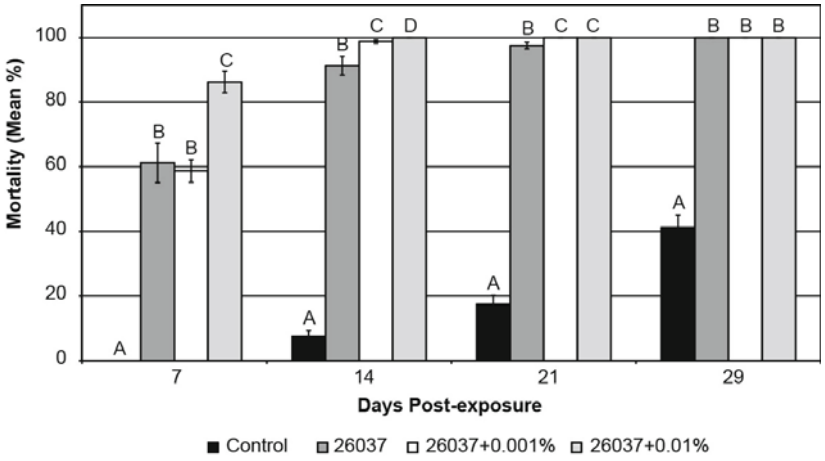

Fig. 2. Mortality of FST exposed to *B. bassiana* strain ATCC 26037 and imidacloprid. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

Exposure of termites to imidacloprid alone and in combination with *Beauveria*

For exposure of termites to the chemical termiticide, twenty Formosan subterranean termites from each of four colonies were placed in Petri dishes with filter paper dampened with the stated concentration of imidacloprid (Sigma-Aldrich, USA). The imidacloprid was dissolved in acetone. A 0.5 ml aliquot was dispensed onto the filter paper and allowed to dry completely before the filter paper was rewetted with 0.5 ml of water. Controls received 0.5 ml of acetone only, which was allowed to dry before the filter paper was rewetted with 0.5 ml water. The Petri dishes were then placed in an unlit incubator at 25°C and 99% humidity. Replicated control plates were incubated in the same manner and contained 20 termites from a single colony placed on filter paper wetted with water only. For exposure of termites to both the *B. bassiana* and imidacloprid, termites were exposed to fungal cultures as described above, and were incubated on filter paper wetted with the stated concentration of imidacloprid.

Exposure of termites to *Isaria* and *Bacillus*

For exposure of termites to the two fungi Ifr and Bt, ten termites from each of four colonies were exposed to 2 g of a dry Ifr spore formulation with a concentration of 10^6 and 10^9 spores/g for 5 min, then transferred to Petri dishes. The dishes contained filter paper wetted with Bt at concentrations of 10^6 or 10^9 cells/ml and an equal number of termite nestmates that were unexposed to the fungus. As controls, termites were exposed to each fungus alone, and to water only. The Petri dishes were incubated at 25°C and 99% humidity while mortality was monitored.

Statistical analysis

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.

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Results

In preliminary studies, exposure to *B. bassiana* strains ATCC 26037 and ATCC 90519 resulted in 100% mortality of FST

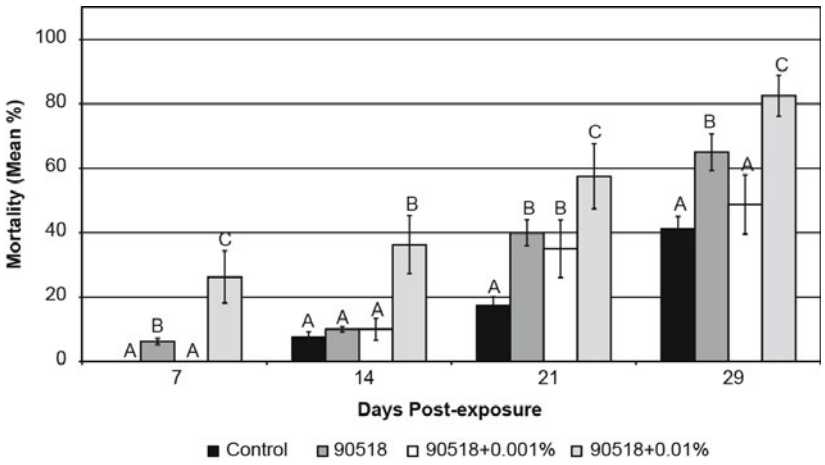

Fig. 3. Mortality of FST exposed to *B. bassiana* strain ATCC 90518 and imidacloprid. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

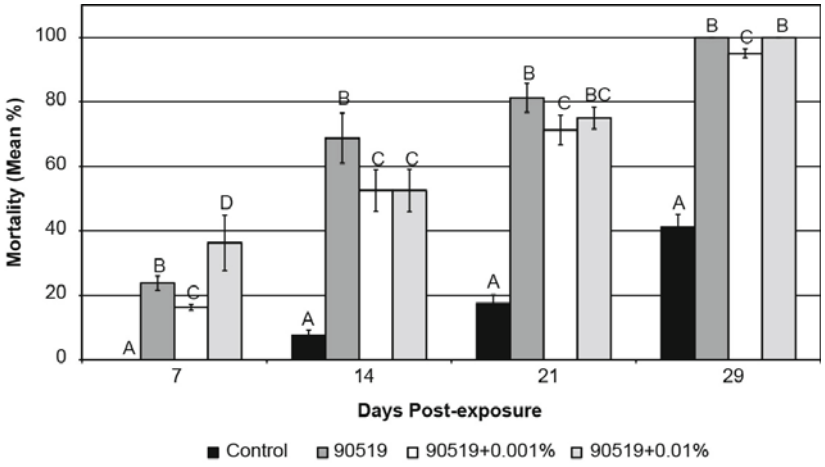

Fig. 4. Mortality of FST exposed to *B. bassiana* strain ATCC 90519 and imidacloprid. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

on day 7. The third *B. bassiana* strain, ATCC 90518, caused 43% mortality on day 7, and 96% mortality at the conclusion of the experiment, day 21. By comparison, the control replicates had an average mortality rate of 18%. Dead termites were transferred to PDA plates and incubated to measure recovery of *Beauveria*. The average recovery rates were 32% for termites exposed to strain ATCC 90518, 74% for strain ATCC 90519 and 85% for strain ATCC 26037.

The chemical termiticide imidacloprid at concentrations of 0.1% and 1.0% caused 100% mortality of FST by day 14 (Fig. 1). Concentrations of 0.001% and 0.01% imidacloprid caused mortality rates of 28.8% and 45.0%, respectively on day 28. Both rates were similar to the control mortality rate of 36.3% over the 28 day experimental period.

After determining the mortality of the *Beauveria* strains and imidacloprid alone, FST were exposed to the microbial and chemical agents in combination. Both combined treatments and the ATCC 26037 control exceeded 50% mortality on day 7 indicating that the fungus was transferred to nestmates (Fig. 2). The combination of strain ATCC 26037 and 0.01% imidacloprid caused 100% mortality on day 14. On day 21 the ATCC 26037 and 0.001% imidacloprid combination also reached 100% mortality. Strain ATCC 26037 reached 97.5% mortality on day 21. Also on day 21 the control in which termites were not exposed to either the biological agent or the chemical agent had reached only 17.5%. In combination with 0.001% imidacloprid, strain ATCC 90518 caused a mortality rate equal to or less than that of the fungal strain alone (Fig. 3), on days 14, 21, and 29. However, the mortality rate of strain ATCC 90518 was increased when the fungal strain was combined with 0.01% imidacloprid, resulting in a maximal mortality rate of 82.5% mortality on day 29 versus a rate of 65.0% for the fungus alone on the same day. When *B. bassiana* strain ATCC 90519 was tested the only synergistic effect was observed in combination with 0.01% imidacloprid on day 7, 36.3% mortality for the combination versus 23.8% for the fungus alone (Fig. 4). On days 14 and 21 the fungus alone caused a mortality rate higher than, or equal to, any of the combinations.

FST were also exposed to a combination of a fungus and a bacterium. At a dosage of 10^6 spores/ml of the fungus Ifr or 10^6 cells/ml of the bacterium Bt on day 5, the Bt treatment caused 22.5% mortality which was greater than that caused by Ifr (8.8%) and the combined treatment (15.0%) (Fig. 5). At 9 days post-exposure the combined treatment caused 35.0% mortality, which was significantly higher than the mortality caused by Ifr alone, 12.5%, but not by Bt alone, 27.5%. A combined effect was observed when termites were exposed to Ifr in combination with Bt at 13 days at a dosage of 10^6 spores or cells/ml, respectively. At this time point the

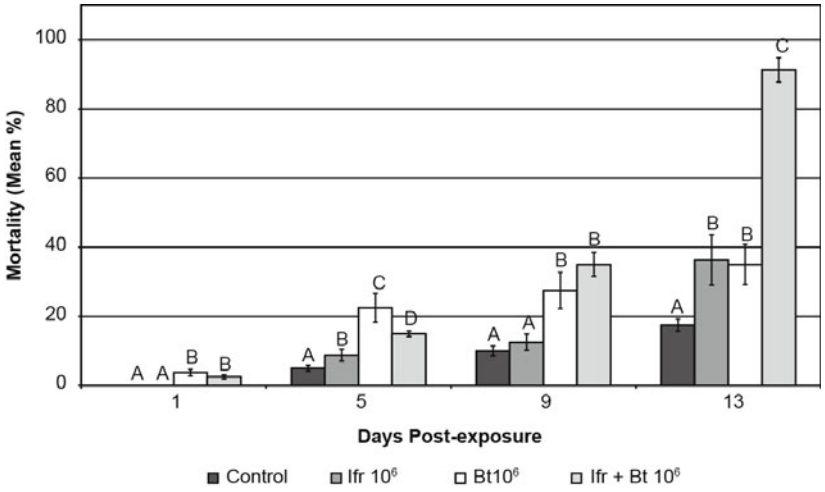

Fig. 5. Mortality of FST exposed to 10^6 spores of *I. fumosorosea* and/or 10^6 cells of *B. thuringiensis*. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

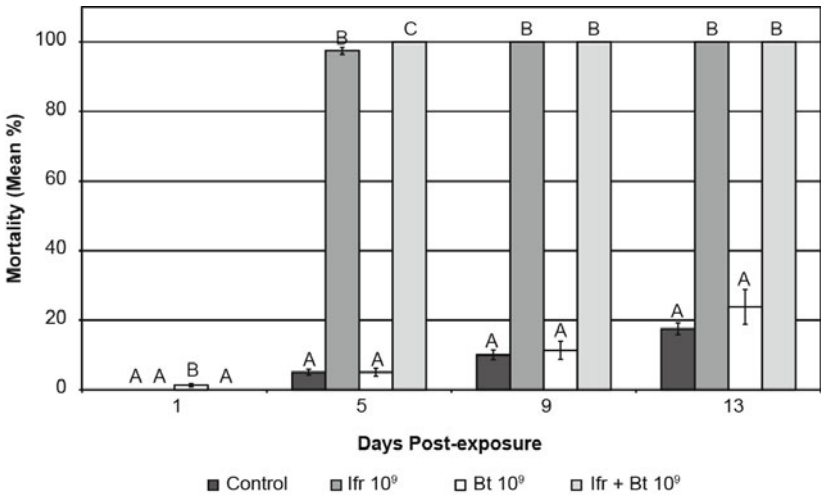

Fig. 6. Mortality of FST exposed to 10^9 spores of *I. fumosorosea* and/or 10^9 cells of *B. thuringiensis*. Treatments with the same letter on the same day are not significantly different. $P \leq 0.05$

combined treatment caused 93.1% mortality. This mortality rate was significantly higher than the 36.3% mortality caused by exposure to Ifr alone and the 35.0% mortality caused by exposure to Bt alone.

No synergistic effect was observed when termites were exposed to the fungus Ifr in combination with the bacterium Bt at a dosage of 10^9 spores or cells/ml, at any of the timepoints. At this dosage the Ifr treatment alone resulted in a significantly high mortality rate, 97.5%, as early as day 5 (Fig. 6).

Discussion

Biological control and chemical control are two research avenues being pursued to limit populations of destructive subterranean termites, especially the Formosan subterranean termite. Individual treatments have advantages that can make them more suited to particular types of infestation. For example, a chemical that can be foamed is well suited to filling the void space in an infested tree, and a fungus that can be delivered as a powder can have long-term shelf stability, but become active when introduced to the moist environment of a termite nest. It is, however, difficult to effect complete control with a single microbial agent (Chouvenc *et al.*, 2011). Testing for potential synergistic effects can help to address this difficulty. In this study sub-lethal doses of fungal pathogenic agents that are known to cause mortality in termites and other insects were applied in combination with a chemical agent and a bacterium.

Beauveria bassiana strains 26037, 90518, and 90519 were selected for this study based on their ability to cause mortality of FST in preliminary studies. Recovery of these strains from cadavers was also measured to determine transfer among nestmates. Only 50% of the termites were directly exposed to the fungi, so the resulting mortality rates greater than 50% confirm that the pathogens were passed among nestmates.

Prior to exposing termites to combinations of the biological and chemical agents, mortality rates were determined for the agents individually. This also allowed confirmation of transmission of the fungus from termite to termite, which has not been previously shown for these strains of *Beauveria*. One factor that is critical to the success of any biological control agent is its ability to be spread among termites. Colony members that forage must be able to transfer a microbe that they encounter while foraging to nestmates that do not forage. Without transfer between termites colony control will not be successful. Transfer of strains of *B. bassiana* was measured by directly exposing only half of the termites in each replicate. Therefore a mortality rate greater than 50% represents transfer from directly exposed termites to nestmates.

Termites live in an environment that is conducive to the growth of microorganisms. Several of the microorganisms that termites encounter have the potential to cause termite mortality. To confirm that the fungal strains introduced in these experiments, which had not been previously shown to cause termite mortality, were the causative agents. *B. bassiana* was recovered from dead termites by placing the cadavers on an agar medium to allow growth of associated microbes.

Baseline mortality rates were also measured for the chemical control agent. Using the testing protocol that was used for the combined treatments, termites were exposed to imidacloprid at a concentration of either 0.001%, 0.01%, 0.1% or 1.0% v/v in acetone. To observe a synergistic effect between the fungus and imidacloprid it was desirable to expose the termites to a sub-lethal dose of the chemical in combination with the fungus. This provided an opportunity to determine if the chemical enhances the mortality rate compared to that of the fungus alone. The data indicated that the 0.1% and 1.0% concentrations are not ideal for observation of a synergistic effect, since they cause significant mortality rates on their own. The 0.001% and 0.01% concentrations were chosen for further studies in combination with the fungus.

Half of the termites in each replicate were first exposed to a culture of *B. bassiana* strain ATCC 26037, ATCC 90518, or ATCC 90519, and were then incubated with an equal number of nestmates on filter paper wetted with either 0.001% or 0.01% imidacloprid. In the fungal controls termites were incubated on filter paper wetted with water and mortality varied by strain. The other control contained termites that were not exposed to either the fungus or imidacloprid and none caused significant mortality. While strain ATCC 26037 caused significant mortality alone its effect was initially en-

hanced in combination with imidacloprid, especially at the 0.01% concentration during the first two weeks of the experimental period. *B. bassiana* strain ATCC 90518 was synergistically enhanced in combination with 0.01% imidacloprid on day 14. On day 21 the combination of strain ATCC 90518 and imidacloprid at both concentrations was significantly higher than either treatment alone. The combination of *B. bassiana* strain ATCC 90519 and 0.01 imidacloprid was significantly higher than all other treatments on day 7. On all other days the mortality rate of strain ATCC 90519 alone exceeded or equaled the combined treatments.

The developments of microbial agents that are pathogenic to Formosan subterranean termites provide additional treatment options in the effort to control this significant pest. The fungus *I. fumosorosea* has been previously shown to cause mortality of termites (Wright *et al*., 2003). A biologically compatible foaming agent has been developed to effectively deliver *Isaria* and other microbial agents into the void spaces formed by termites in their nest environment, thereby enhancing the probability of termites encountering the microbial agents carried in the foam. The isolation of additional microbes for delivery with the foaming agent will be advantageous (Dunlap *et al*., 2007). Bt when combined with Ifr at the lower concentration was found to have a synergistic effect, but once the mortality caused by Ifr alone increased the effect was nullified. Bt when combined with Ifr at the higher concentration had no synergistic effect due to the high rate of mortality caused by Ifr alone. Ifr has been successfully applied in combination with the foam. The results reported here demonstrate the potential to deliver fungi in combination with a bacterium to control termites.

The data reported here indicate the potential for a synergistic effect when fungal entomopathogens and sub-lethal doses of imidacloprid are used as a combined treatment, but different fungal strains respond at different rates. An integrated regimen may ultimately reduce the fungal and chemical dosage rates necessary for effective treatment. Also, strains that do not appear to cause significant mortality on their own may have other features that make them more suitable for treatment than a strain that causes a high rate of mortality, such as lack of repellency. Chemicals in termite nests and termite defense mechanisms designed to control microbial blooms may inhibit the growth of fungi that otherwise seem to be ideally suited to control termite infestations. It is possible that a fungal strain with a low mortality rate may be better able to tolerate conditions in a particular nest or infested area and, in combination with a chemical agent, may provide better control than a strain that caused high mortality but is inhibited by nest conditions.

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