ASPERGILLUS SCLEROTIORUM FUNGUS IS LETHAL TO BOTH WESTERN DRYWOOD (*INCISITERMES MINOR*) AND WESTERN SUBTERRANEAN (*RETICULITERMES HESPERUS*) TERMITES.

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- Entomopathogenic
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- Pest management
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- *Incisitermes minor*
- Termites

ABSTRACT

Termite control costs $1.5 billion per year in the United States alone, and methods for termite control usually consist of chemical pesticides. However, these methods have their drawbacks, which include the development of resistance, environmental pollution, and toxicity to other organisms. Biological termite control, which employs the use of living organisms to combat pests, offers an alternative to chemical pesticides. This study highlights the discovery of a fungus, termed "APU strain," that was hypothesized to be pathogenic to termites. Phylogenetic and morphological analysis showed that the fungus is a strain of *Aspergillus sclerotiorum*, and experiments showed that both western drywood (*Incisitermes minor*) and western subterranean (*Reticulitermes hesperus*) termites die in a dose–dependent manner exposed to fungal spores of *A. sclerotiorum* APU strain. In addition, exposure to the *A. sclerotiorum* Huber strain elicited death in a similar manner as the APU strain. The mechanism by which the fungus caused termite death is still unknown and warrants further investigation. While these results support that *A. sclerotiorum* is a termite pathogen, further studies are needed to determine whether the fungal species has potential as a biological control agent.

INTRODUCTION

Termites are a common structural and economic pest worldwide. While estimates vary considerably, termites are responsible for between $500 million and $1 billion of wood damage and cost $1.5 billion in control measures per year in the United States alone (7,32). An array of problems have been associated with the use of chemical pesticides to control pests like termites, such as the development of resistance in target organisms, environmental pollution, and toxicity to non–target organisms, including humans (25). Avoidance behavior to such chemicals is also a significant challenge in controlling termite populations (33). Because of such complications, biological termite control agents that are natural insect pathogens have been proposed, including parasitoids, viruses, bacteria, protozoa, nematodes, and fungi (7).

Fungi, especially those that occur naturally in termite habitats, are promising candidates for use as biological control agents because they are well–adapted to survive in such environments (33). Entomopathogenic fungal species such as *Beauveria bassiana*,

Paecilomyces fumosoroseus, and *Metarhizium anisopliae* have been suggested as potential termite control agents, with the latter being the best studied and most effective of the three (17,19,28). There are limitations, however, to the effectiveness of fungi as termite control agents in their native habitats, due to specific termite behaviors such as allogrooming (6), spore avoidance (22), and vibrational alarm signals in response to fungal spores (22,29). In addition, naturally occurring antimicrobial compounds in termite feces and alimentary canals have been described (6, 23).

In this current study, we hypothesize that a novel isolated fungus is entomopathogenic to termites. The research objectives are to genetically and morphologically identify the APU strain, experimentally infect two different species of termites, *Incisitermes minor* and *Reticulitermes hesperus*, with APU strain, and determine if dose has any effect on termite survival.

MATERIALS AND METHODS

TERMITE COLLECTION

Incisitermes minor and *Reticulitermes hesperus* termites were obtained either from decomposing firewood in Arcadia, CA, or from branches found in the foothills of the San Gabriel Mountains in Glendora, CA. Termite-containing wood was stored in plastic bins in a cool, dark room until used in experiments. To collect termites, wood was broken apart and termites were picked up with lightweight forceps or paintbrushes, and placed in Petri dishes with a brown paper towel as food, with additional moisture for *R. hesperus*. Termites were left two or more days in Petri dishes after collection and before the start of an experiment to ensure that healthy, uninjured insects were used.

FUNGAL CULTURES, DRY SPORE COLLECTION, AND QUANTIFICATION.

A strain of fungus believed to have eliminated an *I. minor* colony at Azusa Pacific University in 2009 (called "APU strain") was isolated from a deceased termite and cultured on Difco Sabouraud Dextrose Agar (SDA) (BD Diagnostics, Franklin Lakes, NJ). Fungus was repeatedly subcultured on SDA plates to ensure a pure culture. *Aspergillus sclerotiorum* strain Huber (16892, American Type Culture Collection, Rockville, MD) was used as a comparison strain. For spores that were used to infect termites, cultures of both strains were grown in the dark on SDA plates with penicillin-streptomycin (100 U/ml penicillin and 100 mg/ml streptomycin, diluted from 100X stock solution, HyClone Laboratories, Logan, UT) at 25°C for approximately 3 weeks, or until a confluent layer of yellow spores was present. Each plate was then either used at 3 weeks or later to infect termites directly.

To collect spores, confluent plates were inverted and placed atop a 0.45 mm sterile vacuum filter, then gently and repeatedly tapped to dislodge spores. Spores were then left in the filter apparatus under vacuum to dry for approximately 3 hours. The resulting dry spore powder was quantified by resuspending in a solution of 0.1% (v/v) Tween-20 and counting in a hemocytometer. Live spore quantification was determined by standard plate count on SDA in duplicate or triplicate for each dilution, and fungal colonies were counted on days 2–4, depending on the growth.

ITS REGION AND β-TUBULIN GENE POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

The species identification of the strain of fungus that was presumed to kill a colony of *I. minor* termites in 2009 (called "APU strain") was initially performed by PCR amplification of the internal transcribed spacer region (ITS) of fungal ribosomal DNA isolated using ZR Fungal/Bacterial DNA miniprep kit (Zymo, Irvine, CA) according to the manufacturer's protocol, with the following additional step: prior to DNA extraction, fungal samples suspended in water were frozen at –20°C. The primer pair made for amplification of the fungal ITS region was ITS1 and ITS4 (sequences as previously described) which amplifies a region that includes the entire 5.8S rRNA gene (31). PCR was also performed using primers (Bt2a and Bt2b), designed to amplify *Aspergillus* β–tubulin, as previously described (10). PCR products were purified using the Wizard SV Gel and PCR Clean–up kit (Promega, Madison, WI), and sequenced at the DNA Sequencing Core Lab (City of Hope, Duarte, CA).

PHYLOGENETIC ANALYSIS OF ITS AND β-TUBULIN GENE REGIONS OF APU STRAIN

The APU strain ITS sequence was subjected to a megablast search using the fungi RefSeq ITS database in BLAST (National Center for Biotechnology Information). The top eight sequences ranked by maximum score were used. *Aspergillus tanneri*, the 8th sequence, was used as an outgroup, similar to a previous phylogenetic study (27). For ITS, sequences were aligned along with the APU strain sequence using ClustalW in MEGA6 software. Default settings were used for the alignment. Under the pairwise alignment and multiple alignment sections, the gap opening penalties were set to 15, and the gap extension penalties were set to 6.66. Under the multiple alignment section, the gap opening penalty was set to 15 and the gap extension penalty to 6.66. The DNA weight matrix was set to IUB. The transition weight was 0.5. The use of a negative matrix was turned off. The delay of divergent cutoff was set to 30%. After alignment, the sequences were trimmed to 562bp, and used to construct maximum likelihood phylogenetic trees using MEGA6. The Tamura 3–parameter model was selected, as well as gamma distributed with invariant sites (G + I), as done previously (27). The number of discrete gamma categories was 5. Gaps and missing data were subject to complete deletion. The ML Heuristic method was set to Nearest–Neighbor–Interchange, and the initial tree was set to Default – NJ/BioNJ. The branch swap filter was set to very strong. The number of threads selected was 1. Bootstrap analysis of 1000 replicates was performed.

The sequence of a region of the β–tubulin gene, a commonly sequenced gene in fungi, was also subjected to a megablast using BLAST. The first 14 strains of unique species similar to APU strain were chosen for phylogenetic tree construction. The *Aspergillus tanneri* β–tubulin sequence was acquired from NCBI as an outgroup, as done previously (27). A total of 15 sequences of β–tubulin regions were acquired and aligned along with the APU strain sequence in MEGA6 using the same parameters as mentioned above for the ITS region. After alignment, the sequences were trimmed to 579bp. A maximum likelihood phylogenetic tree was constructed in MEGA6. All selected options were the same as done for the ITS region as described earlier except that the Kimura 2–parameter was used as done

previously (27). In addition, the first, second, third, and noncoding sites were selected. Bootstrap analysis was performed with 1000 replicates.

METHODS FOR MORPHOLOGICAL STUDIES AND MICROSCOPY

For light microscopy analysis, fungal microcultures were set up by inoculating each of the 4 edges of a 1 cm x 1 cm square of Sabouraud dextrose agar (SDA) on top of a microscope slide, and covering the inoculated medium with a sterile glass microscope cover slip. The slide culture was set on a small platform in a Petri dish, and extra water was put in the bottom of the dish below the level of the slide to allow proper moisture. Fungi were grown in the dark at 25°C for 3–4 days. To view, cover slips were carefully removed and placed on top of clean microscope slides with lactophenol cotton blue stain (Medical Chemical Corp., Torrance, CA) and initial photographs were taken using a Leica DM70 microscope. For measuring APU strain features, fungus was inoculated onto a SDA square but grown directly on a micrometer, and viewed using a Nikon TMS microscope. To view macroscopic fungal colony growth, fungi were inoculated onto Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA) plates and grown in the dark at 25°C for 7 days, as previously described (14).

An electron micrograph was taken by directly putting a sample on a conductive double-sided adhesive (PELCO Image Tab™, Ted Pella, Inc, Redding, CA) and viewed using a Hitachi TM-1000 tabletop scanning electron microscope.

I. MINOR TERMITE INFECTION WITH APU STRAIN OR HUBER STRAIN

An initial study was conducted to assess the lethality of APU strain to *I. minor*. Thirty *I. minor* termites of differing developmental stages were individually inoculated on the back by inoculation loop with APU strain from an overgrown SDA culture plate. Thirty control termites were untreated. Each group was placed in a Petri dish with 90 mm Whatman No. 3 paper for food. Live termites were counted each day for 7 days.

To compare the effect of APU strain to *A. sclerotiorum* Huber strain on *I. minor* termites, 40 termites per group were placed on a SDA plate overgrown with either the APU strain or Huber strain and allowed to walk around for 2 minutes, during which they became covered in fungal spores. Forty control termites were removed from the same colony as the experimental group and were left untreated. Each group of 40 was then placed on a piece of 90 mm Whatman No. 3 paper in a Petri dish, and live termites were counted daily except on day 5 for the duration of the experiment.

DOSE RESPONSE OF *I. MINOR* TERMITES TO APU STRAIN

The response of *I. minor* termites to increasing doses of *A. sclerotiorum* APU strain was determined for 4 different doses (150 mg, 70 mg, 30 mg, and 3 mg) by inoculating n=3 groups containing 25 termites each of differing developmental stages with each dose. The 3 mg doses were prepared by adding 9 parts powdered sugar to 1 part fungal spores, and using 30 mg of the sugar/fungal mixture per Petri dish. Each dose of fungal spores were uniformly spread on a 90 mm disk of Whatman No. 3 filter paper in a Petri dish,

where termites were kept throughout the experiment and thus exposed to spores for the duration of the experiment. The 0 mg control groups (*n*=2) were prepared in the same manner, except spread with 75 mg of powdered sugar instead of fungal spores. The termite groups were kept in the dark, and the number of live individuals was determined daily (approximately every 24 hours) over 9 days. Each mg of spores corresponded to 1.07 x 10^8 spores by hemocytometer estimation. Viable counts were not performed. A one way ANOVA followed by a TukeyHSD test (RStudio Version 0.99.441) was performed in order to determine the significance of the doses on the number of living termites on days 5 and 9 of the experiment.

DOSE RESPONSE OF *R. HESPERUS* TERMITES

Ten or 25 mL of a 1.5% (w/v) agar solution (Bacto–agar, BD Difco Franklin Lakes, New Jersey) was first pipetted into glass Petri dishes and allowed to solidify to provide enough moisture for subterranean termites and inhibit further fungal growth (33). Whatman No. 3 filter paper disks (90 mm) were placed on top of the solidified agar as a food source. Various quantities of dry APU strain *A. sclerotiorum* spores were evenly spread on top of the paper. The following quantities of spores were used: 25, 50, 75, 100 mg. Each mg corresponded to 1.05 x 10^8 total spores as counted by hemocytometer, and 4.86 x 10^5 viable spores by plate count determination. For the control groups, 75 or 100 mg of powdered sugar was spread on the filter paper so as to mimic the powdery nature of the spores. Termites were separated into groups of 35 termites of differing developmental stages. The groups were then introduced into dishes containing either fungal spores or powdered sugar (*n*=2 for 100 mg spores; *n*=3 for all other dosages, where data were pooled from two separate experiments). The termites lived in the dishes for the duration of the experiment, and their survival was recorded daily for 9 days. All of the dishes were stored in the dark and only exposed to light when survivability was recorded. A one way ANOVA followed by a TukeyHSD test (RStudio Version 0.99.441) was performed in order to determine the significance of the doses on the number of living termites on day 9 of the experiment.

RESULTS

FUNGUS ISOLATE EXHIBITS *I. MINOR* TERMITE PATHOGENICITY

Initially, *I. minor* termites were exposed to APU strain spores and the results of this experiment showed that the number of live termites inoculated with the APU strain declined throughout one week of observation, beginning with the first inoculated termite death on day 3 (Fig. 1A). By day 7, all 30 of the inoculated termites were dead. By contrast, all 30 uninoculated control termites were alive until day 6, and on day 7, there were still 27 live control termites. Inoculated termites that died were examined using light microscopy for signs of fungal infection, such as shown in Fig. 1B, and upon transfer to fungal media (Sabouraud Dextrose Agar), grew fungus that appeared very morphologically similar (data not shown). Scanning electron microscopy of dead inoculated termites indicated fungal growth, seemingly of one type that, due to its unenclosed spores, belonged to the phylum Ascomycota (Fig. 1C).

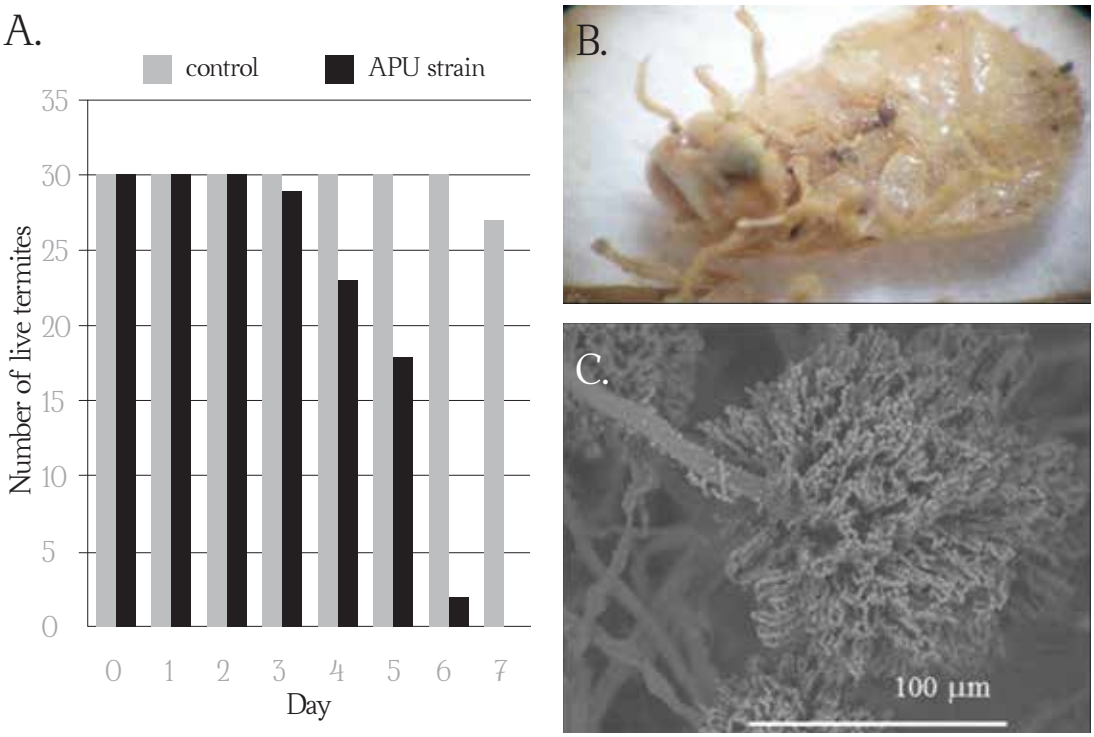


Figure 1: Healthy *Incisitermes minor* termites inoculated with APU strain show decreased viability over time. A. Survivability of infected termites over time. Control termites were unaffected. B. Light micrograph of a representative deceased *I. minor* termite after inoculation with APU strain with characteristic yellow spores visible. C. Scanning electron micrograph of fungal fruiting body found on deceased termite (800x magnification).

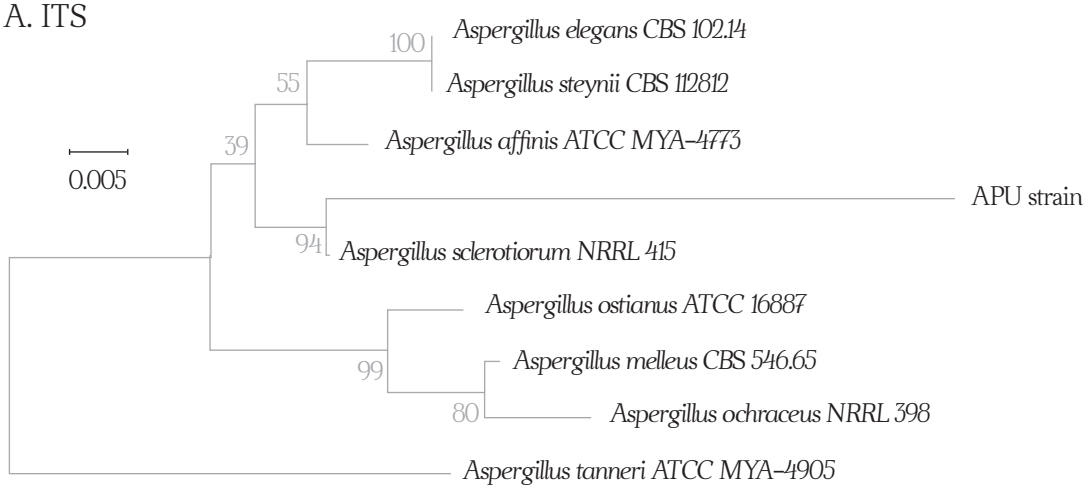
IDENTIFICATION OF APU STRAIN AS *ASPERGILLUS SCLEROTIORUM*

In order to determine if the APU strain was a known insect or termite pathogen, it was necessary to identify the species of fungus. Once the ITS sequence of the APU strain was determined, and a BLAST comparison using validated reference sequences (RefSeq, National Centers for Biotechnology Information) was performed, a phylogenetic tree was constructed consisting of 8 species with the most similar ITS sequences (Fig 2A). *A. sclerotiorum* had the closest sequence to the APU strain ITS region, making it the probable identity of the APU strain. Identification of APU strain was confirmed to be *A. sclerotiorum* by an independent lab (Accugenix, Newark, DE) that analyzed a smaller region of ITS (ITS2).

To corroborate these results, a region of the β-tubulin gene from APU strain was chosen to be sequenced as a means of species identification. Similar to the ITS region, the sequence of the β-tubulin gene showed closest identity to *A. sclerotiorum* (Fig. 2B).

Since sequence results of both ITS and β-tubulin regions suggested *A. sclerotiorum* was the APU strain species, the APU strain was compared microscopically to a known reference strain of *A. sclerotiorum* (Huber strain). Microscopic examination of APU

A. ITS



B. β-tublin

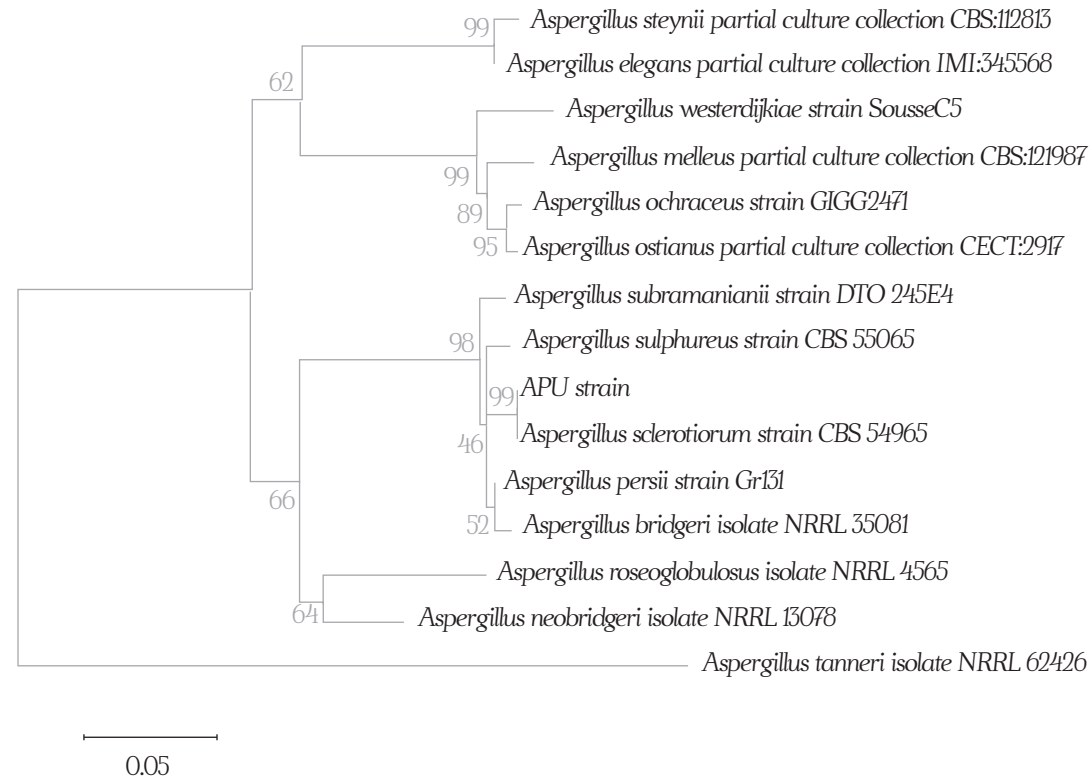
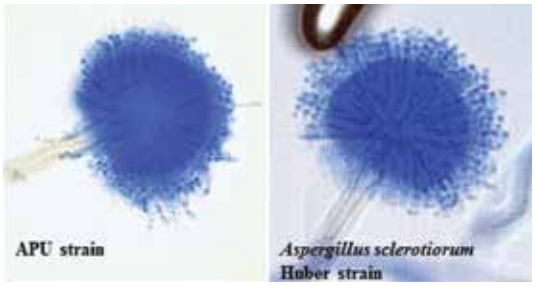


Figure 2. Phylogenetic trees based on ITS sequences and β-tubulin gene sequences of APU strain indicate identity is *Aspergillus sclerotiorum*. The phylogenetic tree for A. ITS and for B. β-tubulin genetic regions of APU strain were each constructed using the Maximum Likelihood method in MEGA6. Bootstrapping percentages are shown near the branch they refer to. A strain of *Aspergillus tanneri* was used as an outgroup for each tree.

A.



B.

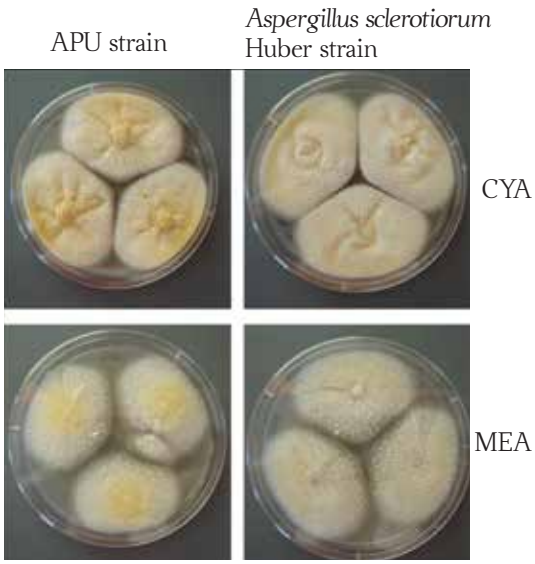


Figure 3. Microscopic and macroscopic comparison of APU strain and *A. sclerotiorum* Huber strain show similarities. A. Microscopic examination of fruiting body structures using white light microscopy. B. Fungal colony growth at day 7 on either Czapek Yeast Agar (CYA) or Malt Extract Agar (MEA) medium, as indicated.

Table 1. APU strain morphological features compared to those of *Aspergillus sclerotiorum*

Fungus	Metulae	Phialides	Vesicle		Spores	
			Shape	Diameter	Texture	Diameter
A. sclerotiorum[a]	7–12 µm	6–8 µm	pyriform/ spherical	17–35 µm	Smooth/finely roughened	2.5–3.0 µm
APU strain	8 µm	8 µm	spherical	23 µm	smooth	3.0 µm

[a]*all values for A. sclerotiorum are as previously described (14)*

and Huber strains showed very similar morphological features, such as the shape of the conidial head structures, which displayed similar overall spherical shapes with spherically shaped vesicles, as shown in Fig. 3A. Additionally, the vesicles of both fungi have biseriate appendages (made up of metulae and phialides) leading to the spherical conidia (spores). Various other fungal structures of the APU strain were observed or measured by light microscopy and compared to those of *A. sclerotiorum* from Klitch's key to identifying *Aspergillus* species (14). All structures observed or measured on the APU strain were found to fall within the range of structures found in *A. sclerotiorum* (Table 1), although they were not unique to *A. sclerotiorum.*

APU and Huber strains of fungus were also grown on media to compare colony morphologies. On both Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA), colonies of APU and Huber strains looked nearly identical in size, indicating a similar growth rate, as well as colony shape and texture (Fig. 3B). Both species displayed white mycelia and had liquid colony exudate. The major difference in colony appearance between the two fungal strains

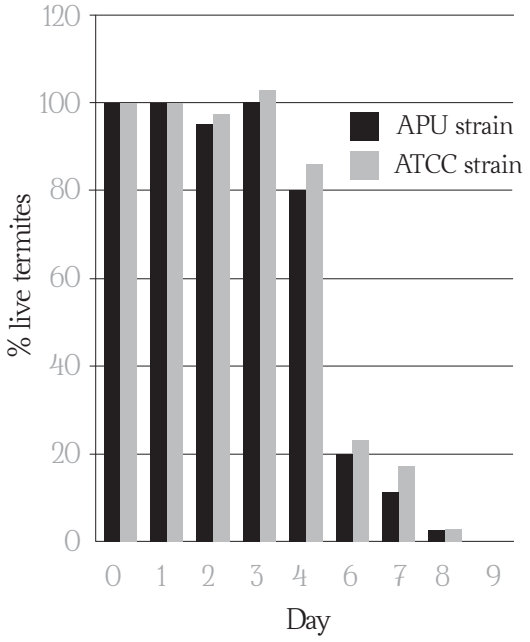


Figure 4. Comparison of survival of *Incisitermes minor* drywood termites over time infected with two different strains of *Aspergillus sclerotiorum*. *I. minor* termites were exposed to APU strain ("APU strain," black bars) or Huber strain ("ATCC strain," grey bars). Control termites were unexposed. Each group had 40 individuals from the same colony. Data are expressed as mean percent of the uninfected control live termites on each day.

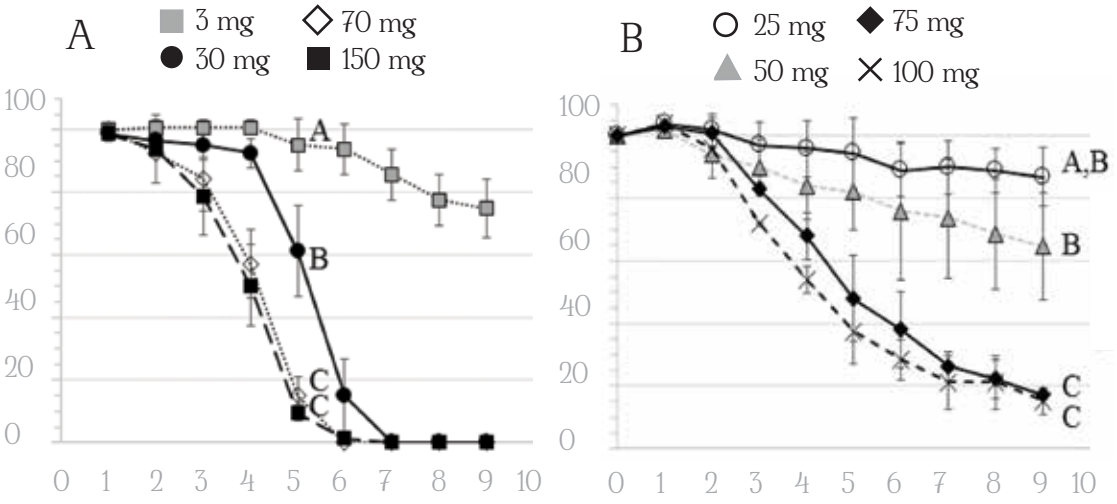


Figure 5. APU strain *Aspergillus sclerotiorum* affects *I. minor* and *R. hesperus* termites in a dose–dependent manner.

Termites were exposed to various total mg quantities of dried fungal spores as indicated. Data are expressed as mean percent ± S.D of the uninfected control live termites on each day. Control termites were exposed to powdered sugar. A. *I. minor* termites were placed into groups of 25 and exposed to each mg quantity of fungus (n=3) or 75 mg powdered sugar (n=2). Each mg contained 1.07 x 10^8 total spores by hemacytometer count. Viable counts were not performed. Different letters on day 5 indicate statistically significant differences ($p<0.05$) with the control belonging to group A. B. *R. hesperus* termites were placed into groups of 35 and exposed to each mg quantity of fungus (n=3 or n=2) or 100 or 75 mg powdered sugar (n=3) . Each mg contained 1.05 x 10^8 total spores by hemocytometer count, and 4.86 x 10^5 viable spores. Different letters on day 9 indicate statistically significant differences ($p<0.05$) with the control belonging to group A.

was the spore color: APU strain spores were bright yellow–orange in color, while the spores of Huber strain were beige (visible in Fig. 3B).

INCISITERMES MINOR TERMITES DIE AFTER EXPOSURE TO BOTH APU AND HUBER STRAINS OF *A. SCLEROTIORUM*

I. minor termites exposed to either APU or Huber strains showed very similar survivability kinetics over time, and all exposed termites died by day 9 (Fig. 4). Initially, for the first several days after fungal exposure, there was little termite death seen. A large decrease in live termites then occurred from day 4 to day 6 in both experimental groups.

BOTH *I. MINOR* AND *RETICULITERMES HESPERUS* TERMITES DIED AFTER EXPOSURE TO APU STRAIN IN A DOSE-DEPENDENT FASHION.

Since initial experiments involving *I. minor* termites walking on overgrown plates of APU strain (Fig 1A and Fig 4) or Huber strain (Fig 4) resulted in fewer live termites over time compared to controls, larger experiments were conducted with termites exposed to varying doses of fungal spores. The results shown in Fig. 5 clearly demonstrate that the numbers of live *I. minor* and *R. hesperus* termites decreased over time as the dose of dry fungal spores they were exposed to increased, in the range of 3 to 150 mg for *I. minor* (Fig. 5A), and 25 to 100 mg for *R. hesperus* (Fig. 5B). For *I. minor* termites, all individuals per group died by day 7 when exposed to 30 mg or higher doses (Fig. 5A), but on day 5, a statistical difference in the average percent of live termites ($p<0.05$) was seen for each dose except for 70 and 150 mg doses. Statistical analysis confirmed that there was a significant effect of dose in relation to *I. minor* termite survival on day 5 (ANOVA, $F = 66.64$, $Pr(>F) = 1.1e{-}06$), as well as on day 9 (ANOVA, $F= 289.4$, $Pr(>F) = 1.7e{-}09$). For *R. hesperus* termites infected with varying mg dose quantities of APU strain, there was a more gradual decline in the numbers of live termites over the 9 day course of the experiment, with no dose resulting in the death of all members in the groups. At day 9, however, 18% of termites treated with the highest dose of the spores survived compared to the controls. 86% of those treated with the lowest dose survived. Significantly different groups are shown in Fig. 5B ($p<0.05$), and statistical analysis confirmed that there was a significant effect of dose in relation to *R. hesperus* termite survival on day 9 (ANOVA, $F =36.2$, $Pr=1.49e{-}05$).

DISCUSSION

In 2009, a colony of *Incisitermes minor* western drywood termites housed at Azusa Pacific University (APU) died mysteriously, and dead termites were inspected and found to be covered in a fungus with yellow spores, which was subsequently isolated. It was possible that either the fungus was feeding off of termites that had died of other causes or it contributed to the termites' demise. Therefore, *I. minor* termites were initially infected with the purified APU strain, all of which died by day 7. This indicated that the fungus contributed to termite death.

To see if APU strain was a novel termite pathogen, it was identified by genetic analysis. APU strain was found to be most genetically similar to *Aspergillus sclerotiorum* fungus based on both its internal transcribed spacer (ITS) region sequence within the ribosomal RNA gene loci and its β-tubulin gene sequence (Fig. 2). This ITS region has been shown to be the most accurate "DNA bar coding" region of fungal genomes for species identification to date (24) and has the most numerous fungal sequence submissions to Genbank (approximately 800,000). External independent corroboration of the APU strain as *A. sclerotiorum* was also determined by sequence examination of a smaller ITS region, ITS2 (Accugenix). A second region of the APU strain genome, a portion of the β-tubulin gene, was also sequenced, since this region has been shown to be polymorphic and useful in identifying filamentous fungi within phylum Ascomycota (10). The species used to construct the β-tubulin tree (Fig. 2B) that were genetically similar to *A. sclerotiorum* were different from those used in the ITS region tree (Fig. 2A) because the β-tubulin gene sequences were used from the entire Genbank database, rather than selected ITS reference strain sequences in the RefSeq database. However, this β-tubulin phylogenetic tree shows similar species relationships to a phylogenetic tree previously published based on three DNA regions: β-tubulin gene, calmodulin gene, and ITS region (27). These three genetic analyses all confirm that APU strain belongs to *A. sclerotiorum*.

APU strain also had similar morphological features to a reference strain of *A. sclerotiorum* Huber when examined microscopically (Fig. 3A). More extensive observation of its microscopic morphology and measurement of several of its features (Table 1) fit within published parameters for *A. sclerotiorum* (14), although they did not rule out many related *Aspergillus* species that have similar features. This was helpful because it did not contradict the genetic analyses performed. The colony morphologies of APU and Huber strains when grown on solid media also appeared similar (Fig. 3B). The major difference in appearance between the two strains was the color of the spores, however, it is not unusual for strains of the same *Aspergillus* species to have a range of spore pigmentation (14). Taken together, these genetic and morphological approaches to species identification indicated that the identity of APU strain was *A. sclerotiorum*. This was especially interesting since *A. sclerotiorum* has not generally been studied for its entomopathogenicity.

Infection of western drywood termites with two different strains of *A. sclerotiorum* (APU and Huber) resulted in decreased viability of termites over time, and both strains displayed similar kinetics (Fig. 4). These similar kinetics further suggest that the two belong to the same species. This was not necessarily an expected result, as different strains of the same species of entomopathogenic fungi may show selective pathogenicity, depending on the source of their isolation (13,30). Since the APU strain of *A. sclerotiorum* was isolated from dead termites, and the Huber strain was originally isolated from a decaying apple in 1933, they could have had very different effects on drywood termites (12). Interestingly, they were both lethal to termites when termites were exposed to each fungus in the same manner, which suggests an inherent pathogenicity for termites. In addition, to our knowledge, it is the first demonstration of *A. sclerotiorum* as an *I. minor* termite pathogen.

The APU strain of *A. sclerotiorum* was not only lethal to western drywood termites (family Kalotermitidae), but also to western subterranean termites (family Rhinotermitidae). Groups of termites of both species infected with *A. sclerotiorum* had fewer live termites than control termites over time, and this decrease in viability was dose-dependent (Fig 5). In this set of experiments, each group of termites as a whole was exposed to a particular fungal dose; this was preferable to individually exposing termites because it has been shown that the drywood termite *Incisitermes schwarti* is more susceptible to death by fungal infections when in isolation, rather than in groups of 10 or 25 (3). Additionally, in these experiments, control termites were exposed to powdered sugar, an inert but particulate substance, in order to establish that the fungus was entomopathogenic and not causing or contributing to termite death in some non-specific way, for instance, by physically blocking respiratory structures. The apparent greater lethal effect that the 30 and 70 mg doses of spores had on *I. minor* compared with the 25 and 75 mg doses with *R. hesperus* may either be due to a greater susceptibility that *I. minor* has for the fungus, or that there were more viable spores per mg in the *I. minor* experiments than in the *R. hesperus* experiments, although the total amounts of spores per mg were very similar. This second possibility is likely, because the experiments with *R. hesperus* were done much later than the *I. minor* experiments with the same preparation of spores, and it very likely lost viability in that time, although it is not possible at this point to rule out the first possibility. Low doses of fungal spores resulted in greater termite viability than higher doses, which suggests that both termite species have antifungal defenses to protect them from the lower doses of fungus, although their immune systems are largely uncharacterized. One complicating factor, however, is that the 3 mg dose of spores used to infect *I. minor* contained powdered sugar. This could possibly have given an immune advantage to the *I. minor* as has previously been reported in *Odontotermes formosanus* (8), and may explain why this dose was not significantly different from the control uninoculated termites on either day 5 or 9. In any case, this is the first study indicating that *A. sclerotiorum* is pathogenic to both of these species of termite pests, which are among the five important termite pest species out of 45 total termite species in the United States (26).

The mechanism for *A. sclerotiorum*'s infection of and entomopathogenicity to termites is unknown. Spores could be ingested and germinate in the alimentary tract, as seen in termites infected with *Beauveria bassiana*, or they could invade the termite by secreting cuticle-degrading enzymes, as seen in termites infected with *Metarhizium anisopliae* (4,15). *A. sclerotiorum* may then cause disease by one or more of its known excreted metabolites, such as the insecticidal aspochracin molecules (27). One aspochracin molecule previously isolated from *Aspergillus ochraceus* has been shown to be toxic to silkworm larvae (21). Control termites in the dose-dependent experiments in our study (Fig. 5) were treated with powdered sugar to try to control for the possibility that spores might be blocking termite respiratory spiracles or interfering with other aspects of their physiology as particles, and not by some fungus-specific mechanism. However, the powdered sugar is not uniformly sized compared to the spores, and so using inert particulates of the same size as the fungal spores, perhaps pollen, would make a better control. Finally, it is also possible that *A. sclerotiorum* pathogenesis is related to the termite's immune response to the fungus. Both the route of infection and the mechanism for pathogenesis of *A. sclerotiorum* in termites are currently being explored.

POTENTIAL USE OF *A. SCLEROTIORUM* AS A TERMITE BIOLOGICAL CONTROL AGENT

The use of microbiological agents as biological controls for pests like termites includes the use of the fungi *M. anisopliae* and *B. bassiana*. Any fungus introduced into an environment to control termites must be horizontally transmissible to other nest mates, since in many termite species, direct treatment of the nest may not be possible (22). Whether *A. sclerotiorum* is transmissible from infected to uninfected termites is currently under investigation, but seems likely since it was found on many individuals from the same colony upon initial isolation. If it proves to be transmissible, then infecting individuals and introducing them into nests or other approximations of termite nests, such as termite planar arenas, would be necessary to determine the feasibility of *A. sclerotiorum* as a termite control agent (5,6).

Biological control agents must also be benign to humans and other inhabitants of the area in which they are applied. As a candidate species for termite biological control, *A. sclerotiorum* shows minimal pathogenicity to humans. There have been only a few documented human cases of *A. sclerotiorum* disease, including nail infection (onychomycosis) and ear canal infection (otomycosis) (1,9,11). However, like most fungi, it could potentially pose a more serious threat to a person with underlying immunodeficiency. Less well-known are the effects of *A. sclerotiorum* on other insect species that may occupy similar niches as termites. *M. anisopliae*, the most extensively studied entomopathogenic fungus, has been shown to be lethal to all species of termites tested which is part of what makes it such a promising control for termites (20). However, *M. anisopliae* is also lethal to a number of other non-related insects, including locusts and grasshoppers (18), ticks (2), and mosquitoes (25), just to name a few. Whether *A. sclerotiorum* is less harmful to other insects than it is to termites is not well-established; to date, it has only reportedly been tested on mosquitoes. In that study, two species of mosquito larvae (*Culex quinquefasciatus* and *Aedes fluviatilis*) infected with low doses of *A. sclerotiorum* (4.5×10^5 or 1.75×10^5, respectively) displayed 0% or 18% mortality rates, respectively, by day 10 post-infection, which was lower than many other different species of *Aspergillus* tested in the same study (16). It will be interesting to see if *A. sclerotiorum* is less pathogenic to other insect types, as well. If it is less pathogenic, it may prove to be a better choice of fungal species for use in termite biological control.

1. Amri, M., Gorcii, M., Essabbah, N., Belhajali, H., Letscher-Bru, V., Zili, J., Azaiez, R., & Babba, H. 2010. *Aspergillus sclerotiorum*: About a case of onychomycosis in Tunisia. *J. Mycol. Med.* 20:128–132.

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REFERENCES

2. Benjamin, M. A., Zhioua, E., & Ostfeld, R. S. 2002. Laboratory and field evaluation of the entomopathogenic fungus *Metarhizium anisopliae* (Deuteromycetes) for controlling questing adult *Ixodes scapularis* (Acari: Ixodidae). *J. Med. Entomol.* 39:723–728.

3. Calleri, D. V., 2nd, Rosengaus, R. B., & Traniello, J. F. 2010. Disease resistance in the drywood termite, *Incisitermes schwarzi*: does nesting ecology affect immunocompetence? *J. Insect Sci.* 10:1–12.

4. Charnley, A. K. (2003). Fungal pathogens of insects: Cuticle degrading enzymes and toxins. In J. A. Callow (Ed.), *Advances in Botanical Research* (Vol. 40, pp. 241–321): Academic Press.

5. Chouvenc, T., & Su, N.–Y. 2012. When subterranean termites challenge the rules of fungal epizootics. PLoS ONE 7:e34484.

6. Chouvenc, T., Su, N.–Y., & Robert, A. 2009. Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. *J. Invertebr. Pathol.* 101:130–136.

7. Culliney, T. W., & Grace, J. K. 2000. Prospects for the biological control of subterranean termites (Isoptera : Rhinotermitidae), with special reference to *Coptotermes formosanus*. *Bull. Entomol. Res.* 90:9–21.

8. Dong, H., Lei, Q., & Xue, C. 2005. Food choice of the underground termite, *Odontotermes formosanus*. *Scientia. Silvae Sinicae* 41:91–95.

9. Garcia–Martos, P., Guarro, J., Gene, J., Mira, J., Linares, M., & Ortoneda, M. 2001. Onychomycosis caused by *Aspergillus sclerotiorum*. *J. Mycol. Med.* 11:222–224.

10. Glass, N. L., & Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323–1330.

11. Harima, N., Inoue, T., Kubota, T., Okada, O., Ansai, S., Manabe, M., Ichinoe, M., & Kasai, T. 2004. A case of otomycosis caused by *Aspergillus sclerotiorum*. *J. Dermatol.* 31:949–950.

12. Huber, G. A. 1933. *Aspergillus sclerotiorum*, n. sp., and its relation to decay of apples. *Phytopathology* 23:306–308.

13. Jones, W. E., Grace, J. K., & Tamashiro, M. 1996. Virulence of Seven Isolates of *Beauveria bassiana* and Metarhizium anisopliae to Coptotermes formosanus (Isoptera: Rhinotermitidae). *Environ. Entomol.* 25:481–487.

14. Klitch, M. A. 2002. *Identification of Common Aspergillus Species*. Netherlands: Centraalbureau Voor Schimmelcultures.

15. Kramm, K. R., & West, D. F. 1982. Termite pathogens: effects of ingested *Metarhizium, Beauveria,* and *Gliocladium* conidia on worker termites (*Reticulitermes* sp.). *J. Invertebr. Pathol.* 40:7–11.

16. Lage de Moraes, A. M., Da Costa, G. L., De Camargo Barcellos, M. Z., De Oliveira, R. L., & De Oliveira, P. C. 2001. The entomopathogenic potential of *Aspergillus* spp. in mosquitoes vectors of tropical diseases. *J. Basic Microbiol.* 41:45–49.

17. Lenz, M. (2005). Biological control in termite management: the potential of nematodes and fungal pathogens. Paper presented at the Fifth International Conference on Urban Pests, Singapore.

18. Lomer, C. J., Prior, C., & Kooyman, C. 1997. Development of *Metarhizium* spp. for the control of grasshoppers and locusts. *Mem. Entomol. Soc. Can.* 129:265–286.

19. Meikle, W. G., Mercadier, G., Rosengaus, R. B., Kirk, A. A., Derouané, F., & Quimby, P. C. 2005. Evaluation of an entomopathogenic fungus, *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Deuteromycota: Hyphomycetes) obtained from Formosan subterranean termites (Isop., Rhinotermitidae). *J. Appl. Entomol.* 129:315–322.

20. Milner, R. J., & Staples, J. A. 1996. Biological control of termites: results and experiences within a CSIRO project in Australia. *AGRIS.* 6:3–9.

21. Myokei, R., Sakurai, A., Chang, C.–F., Kodaira, Y., Takahashi, N., & Tamura, S. 1969. Structure of aspochracin, an insecticidal metabolite of aspergillus ochraceus. *Tetrahedron Lett.* 10:695–698.

22. Rath, A. C. 2000. The use of entomopathogenic fungi for control of termites. *Biocontrol Sci. Technol.* 10:563–581.

23. Rosengaus, R. B., Guldin, M. R., & Traniello, J. F. A. 1998. Inhibitory effect of termite fecal pellets on fungal spore germination. *J. Chem. Ecol.* 24:1697–1706.

24. Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., & Consortium, F. B. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U.S.A.* 109:6241–6246.

25. Scholte, E. J., Njiru, B. N., Smallegange, R. C., Takken, W., & Knols, B. G. J. 2003. Infection of malaria (*Anopheles gambiae* s.s) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*. *Malar. J.* 2:1–8.

26. Su, N. Y., & Scheffrahn, R. H. 1990. Economically important termites in the United State and their control. *Sociobiology.* 17:77–94.

27. Visagie, C. M., Varga, J., Houbraken, J., Meijer, M., Kocsubé, S., Yilmaz, N., Fotedar, R., Seifert, K. A., Frisvad, J. C., & Samson, R. A. 2014. Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). *Stud. Mycol.* 78:1–61.

28. Wang, C., & Powell, J. E. 2002. Isolation and evaluation of *Beauveria bassiana* for control of *Coptotermes formosanus* and *Reticulitermes flavipes* (Isoptera : Rhinotermitidae). *Sociobiology.* 41:369–381.

29. Wang, C. L., & Powell, J. E. 2004. Cellulose bait improves the effectiveness of *Metarhizium anisopliae* as a microbial control of termites (Isoptera : Rhinotermitidae). *Biol. Control.* 30:523–529.

30. Wells, J. D., Fuxa, J. R., & Henderson, G. 1995. Virulence of four fungal pathogens to *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *J. Entomol. Sci.* 30:208–215.

31. White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White (Eds.), *PCR Protocols: A Guide to Methods and Applications.* (pp. 315–322). San Diego: Academic Press.

32. Wright, M. S., Osbrink, W. L. A., & Lax, A. R. (2004). Potential of entomopathogenic fungi as biological control agents against the Formosan subterranean termite. In W. M. Wilson (Ed.), *Agricultural Applications in Green Chemistry* (pp. 173–188). Washington, D.C.: American Chemical Society.

33. Zoberi, M. H. 1995. *Metarhizium anisopliae*, a fungal pathogen of *Reticulitermes flavipes* (Isoptera, Rhinotermitidae). *Mycologia.* 87:354–359.