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Screening and characterization of pathogenic fungi for possible control of Coptotermes formosanus

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SCREENING AND CHARACTERIZATION OF PATHOGENIC FUNGI FOR POSSIBLE CONTROL OF *COPTOTERMES FORMOSANUS*

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ABSTRACT

Coptotermes formosanus was used as "bait" to isolate pathogenic fungi from soil. Fifteen pathogenic fungal isolates were obtained, all either *Metarhizium anisopliae* (six isolates) or *Beauveria bassiana* (nine isolates). There were no differences in numbers of fungal isolates from the three sampling locations. However, significantly more isolates were found in woodlands (eleven) than in pastures (four). Median lethal doses (LD_{50}s) of these fungal species to *C. formosanus* were interspersed, indicating that fungal isolates rather than species had the greatest effect on virulence. *In vitro* growth characteristics were significantly correlated with virulence against termites, suggesting that fungal virulence might be predicted *in vitro* rather than by bioassay. Conidial production on termite cadavers increased significantly over 11 days post-death. Effects of isolates of *M. anisopliae* and *B. bassiana* on *in vivo* sporulation were significant. *B. bassiana* isolates could be categorized into a group with high total sporulation (day 11) and low quick sporulation (on days 2 and 3), while *M. anisopliae* isolates fell into another group with high quick sporulation and low total sporulation. Conidial production was significantly higher *in vitro* than *in vivo*. Correlation between *in vivo* and *in vitro* conidial production was positive and significant. This may allow preliminary *in vitro* screening of a large number of isolates for high *in vivo* sporulation.

Fungal species and isolate significantly affected disease prevalence in termite populations. Sporulation of *M. anisopliae* played a more important role than virulence in producing epizootics in termites, but this was not the case for *B. bassiana*. Isolates characterized by quick sporulation (day 2 after death) did not produce better epizootics in termites than those with high total sporulation (day 11 after death) in either fungal species.

An isolate of *M. anisopliae* ranked highly in all three categories (virulence, quick sporulation, total sporulation) produced better epizootics than an isolate that was inferior in all three characteristics. High temperature (35ºC) significantly reduced fungal germination rates, leading to significant reduction of epizootics. Thus, fungal characteristics other than virulence must be taken into account for the seasonal colonization approach to termite microbial control.

CHAPTER 1

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), has been a serious pest in China and Japan for centuries (Tamashiro, *et al*., 1987). For almost as long, *C. formosanus* was restricted to the area around its native home, which is thought to be southern China (Kistner, 1985). It moved into some of the Pacific islands in the late 19th century and in the last 50 years has expanded its distribution into the southern United States (Henderson, 2001; Su and Tamashiro, 1987). In the continental United States, populations have been found in Alabama, California, Florida, Georgia, Louisiana, Mississippi, South Carolina, North Carolina, Tennessee, and Texas (Haagsma *et al*., 1995). In Louisiana, Twenty-nine parishes are infested with *C. formosanus* (Henderson, 2001). This is a dramatic increase from the 5 or 6 infested parishes reported by La Fage (1987) (Henderson, 2001). A mature colony of *C. formosanus* can cause a tremendous amount of damage in a short time. Unprotected homes newly built over strong colonies have been almost destroyed in two years (Tamashiro, *et al*., 1987). The damage to wood structures by this termite is significant and can exceed $3 billion annually (Hamer, 1985; Su, 1994; Su and Scheffrahn, 1990; Su and Scheffrahn, 1998).

The management of *C. formosanus* generally involves preventive measures and remedial control should prevention fail (Lewis, 1997; Su and Scheffrahn, 1998). Soil treatments are often used to protect structures from *C. formosanus* nesting in the ground (Logan *et al.,* 1990). If an infestation occurs, remedial control measures must be initiated. However, remedial control is more difficult and must be tailored to the particular infestation. Although currently registered termiticides have undergone rigorous field-testing, efficacy results have been mixed (Su and Scheffrahn, 1998). Some termiticides are expensive and less persistent in soil, leading to reduced longevity and the failure of the chemical barrier (Su and Scheffrahn, 1998). In addition, large

quantities of persistent insecticides are raising concerns about applicator safety, environmental contamination and possible deleterious effects on non-target animals. The limitations with current soil termiticides have provided the impetus to look for alternatives in recent years (Henderson, 2001; Su and Scheffrahn, 1998).

As an alternative, biological control is generally perceived as providing both long-lasting insect control and having less potential for damage to the environment or non-target organisms than chemical interventions (Grace, 1997; Hokkanen and Lynch, 1995; Howarth, 1991; Khetan, 2001). The use of biological control agents to suppress subterranean termites within their hidden galleries is appealing (Grace, 1997; Logan *et al*., 1990). The many social interactions within a subterranean termite colony and their dark, damp habitat in which they live would seem to favor survival and distribution of pathogenic microorganisms (Grace, 1997). Entomogenous fungi usually invade via external cuticle of the insects by contact (Tanada and Kaya, 1993; Khetan, 2001), and have great potential for disease epizootics in a termite colony due to their self-replicating nature. One important advantage of a fungal pathogen over chemical pesticides is the potential to recycle and augment the pathogen load (Fuxa, 1987).

During the past 20 years, insect-pathogenic fungi have been the major focus of research on microbial control of termites. Much of this research has been focused on hyphomycetes: *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch.) Sorokin, two fungi believed to be well-adapted to the soil ecosystem (Keller and Zimmerman, 1989). The Formosan subterranean termite is susceptible to a wide range of isolates of *M. anisopliae* and *B. bassiana* derived from a variety of insects and from soil (Ko, 1982; Milner *et al*., 1998b; Wells *et al*., 1995). Milner (1998b) reported that 93 isolates of *M. anisopliae*, most derived from a survey of termite material, showed activity against termites. Selection of fungal isolates based on their

virulence on termites in laboratory bioassays has received particular attention (Almerida *et al*., 1997; Bao and Yendol, 1971; Hanel, 1982a; Ko, 1982; Lai, 1977; Wells *et al*., 1995; Zoberi and Grace, 1990). However, field trials lacked effectiveness because the fungi failed to initiate epizootics in the termite populations (Milner and Pereira, 2000).

The Formosan subterranean termite, as a soil-dwelling social insect, poses some special problems in the field for biological control with pathogenic fungi (Jackson *et al.*, 2000, Milner, 1997). These problems include: (1) the very large colonies with millions of individuals; 2) the hidden and extensive underground galleries that may encompass more than 100 m radius (Su *et al*., 1982; Su and Tamashiro, 1987); (3) satellite nests, which are common, and are difficult to find (Su and Tamashiro, 1987); (4) portions of a colony may be a cut-off from the main colony (Su and Tamashiro, 1987), which may cause the failure of the microbial control with only a one time application; and (5) colony defensive behaviors (e.g. avoidance, walling off cadavers, etc.) against fungal treatments (Logan *et al*., 1990; Reese, 1971; Su *et al*., 1982; Wood and Sands, 1978; Zoberi, 1995). Obviously, knowledge on the traits that make a fungus best adapted for termites in their environment is needed.

A crucial first step for biological control of termites is the selection of a suitable isolate. There are three main sources of isolates: culture collections such as the USDA-ARS Collection of Entomopathogenic Fungi (Ithaca, NY); isolation from the environment in which the target insect lives; and direct isolation from the target insect itself (Milner, 1992). The warm moist conditions within termite nests are conducive to the growth of pathogenic fungi (Thomas, 1987; Milner *et al.*, 1998b; Zoberi, 1979). Hendee (1933) found 25 genera and 8 unidentified fungi associated with the nests of the western subterranean termite, *Reticulitermes hesperus* Banks. However, isolation of fungal entomopathogens from termites is uncommon, despite frequent

anecdotal observations of various fungal growths associated with weak termite colonies in the laboratory (Beard, 1974). The soil environment serves as an important reservoir of entomopathogens (Carruthers and Hural, 1990; Fuxa and Kunimi, 1997) and many new isolates or species of entomopathogens have been found directly or indirectly through soil sampling (Ko, 1982; Milner *et al*., 1998a; Sajap *et al*., 1997). More frequently, however, the most effective isolate is obtained from an epizootic in the target pest under natural conditions (Milner, 1992). Collecting entomogenous fungi from mycosed cadavers in the field is laborious and inefficient. Semi-selective media has commonly been used to detect entomogenous fungi from soil (Milner *et al*., 1998b; Sajap *et al*., 1997). Some of the most successful microbial control agents have been obtained from host insects rather than by selective media or from existing culture collections (Jackson *et al*., 2000). The '*Galleria* bait method', by which insects are used as bait to detect and isolate fungal pathogens from the soil, may possibly be the most successful way to detect pathogenic fungi specifically for termites (Ko, 1982; Zimmermann, 1986). The termite worker of *C. formosanus* was used in our research as bait to detect and isolate termite fungal pathogens.

A seasonal colonization approach, in which a pathogen recycles and suppresses two or more pest generations but must be re-introduced (Fuxa, 1987; Fuxa and Tanada, 1987), may be useful for control of termites with pathogenic fungi. This approach requires a pathogen with high rates of reproduction and transmission (Fuxa, 1987).

High conidial production on cadavers is a desirable characteristic for inoculative augmentation approach (seasonal colonization) (Fuxa, 1987; Fuxa and Tanada, 1987). This approach may be useful for delivering pathogenic fungi through attractant-baited traps (Stimac and Pereira, 1997). Therefore, sporulation characteristics of a fungus may play a role for the initiation of epizootics in termites (Hanel and Watson, 1983; Pereira and Stimac, 1992).

However, fungal virulence has been considered as the most important criterion for selecting fungal species or isolates for termite microbial control (Almeida *et al*., 1997; Bao and Yendol, 1971; Hanel, 1982a; Lai, 1977; Lai *et al*., 1982; Wells *et al*., 1995; Zoberi and Grace, 1990). This has not resulted in successful field trials of fungi against termites (Kramm *et al*., 1982; Sajap *et al*., 1997; Zoreri, 1995). In one study (Milner *et al*., 1998a), the *M. anisopliae* isolate most effective against termites in laboratory and field experiments was not the most virulent. It is possible that aspects of sporulation are an important criterion for selection of fungal isolates in termite control (Hanel and Watson, 1983). Hall (1984) stated that a problem with selecting isolates on the basis of the usual bioassay tests (virulence tests) is that this type of test does not assess the epizootic potential under field conditions (Fuxa, 1987; Fuxa and Tanada, 1987; Fuxa *et al*., 1998; Milner *et al*., 1998a). Control of subterranean termite has to depend on the infection of termites visiting a bait station or other infected area and transmission of the fungus to the nest. This transmission is possible through transfer of conidia among interacting termites, or due to secondary infection derived from sporulating cadavers (Almeida *et al.*, 1997; Fuxa, 1989; Milner, 1997). Thus, evaluating the effects of the fungal sporulation characteristics and the fungal virulence on development of epizootics in the termite populations seems to be critical in fungal isolate selections.

To be most effective in the field, fungal isolates of *M. anisopliae* or *B. bassiana* should successfully recycle in the termite's environment (Fuxa, 1995; Hanel, 1982b; Milner *et al*., 1998a). Another factor that could be as important as sporulation in selecting fungal isolates for termite control is tolerance to high temperatures. Formosan subterranean termites usually maintain their colonies at 30-35°C (Greaves, 1964; Li, 1984), which is above the optimum temperature for conidial germination and growth of most isolates of *M. anisopliae* and *B.*

bassiana (Milner *et al*., 1998a). This suggests that most isolates of these two fungal species would not be able to reproduce, spread, and cause disease epizootics in *C. formosanus* colonies (Fargues *et al*., 1997; Hywel-Jones and Gillespie, 1990; Milner *et al*., 1998a). The effect of the high temperature (35°C) on conidial germination and on fungal epizootics in termite microcosms was examined in this research.

It is likely that termite social behaviors are important in spreading conidia (Grace and Zoberi, 1992; Kramm and West, 1982; Kramm *et al*., 1982; Robsengaus and Tranillo, 1997). However, termites have defensive mechanisms that help isolate diseased nestmates (Logan *et al*., 1990; Reese, 1971; Rosengaus *et al*., 1998b; Su *et al*., 1982; Wood and Sands, 1978; Zoberi, 1995). Also termite excretions and volatiles are suggested to reduce the fungal growth (Chen *et al*., 1998; Rosengaus *et al*., 1998a; Wright *et al*., 2000). The capability of a fungus to cope with a field colony for initiating disease epizootics must be taken into account in selecting fungal isolates. The best fungal pathogens against termite colony must be adapted for the living environment (Fuxa, 1989; Fuxa *et al*., 1998; Goettel *et al*., 2000; Milner, 1997).

The specific purposes of this research were as follows:

1. To screen entomogenous fungi from soil collected in Louisiana;
2. To characterize fungal pathogens against *C. formosanus*;
3. To determine whether fungal sporulation, or virulence of *B. bassiana* and *M. anisopliae* contributed more to development of epizootics in termites held in microcosms;
4. To examine the effect of high temperature (35°C) on conidial germination and on fungal epizootics in termite microcosms.

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CHAPTER 2

VIRULENCE AND IN VITRO CHARACTERISTICS OF PATHOGENIC FUNGI ISOLATED FROM SOIL BY BAITING WITH *COPTOTERMES FORMOSANUS*

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), is a promising candidate for control with entomogenous fungi. Its social behavior, preference for humid conditions and contact with soil are factors that favor fungal pathogens (Milner, 1997; Wells *et al*., 1995). A mature colony of *C. formosanus* can cause great damage in a short time. Unprotected homes built over strong colonies have been almost destroyed in two years (Tamashiro *et al*., 1987). Entomogenous fungi are safe to humans and other non-target organisms (Laird *et al*., 1990), which is important in the urban setting where termites are often pests. The use of fungi as biological agents against termites is an attractive alternative to present technology. Among the many entomogenous fungi, *Metarhizium anisopliae* and *Beauveria bassiana* are potentially the most useful in termite biocontrol. They are distributed worldwide in soil where termites are usually found (Ko, 1982; Milner and Lutton, 1976; Milner *et al*., 1998a; 1998b). Many isolates are virulent against termites (Jones *et al*., 1996; Ko, 1982; Lai *et al*., 1982; Milner *et al*., 1998a; Wells *et al*., 1995), and they are relatively inexpensive to grow in quantity (Delate *et al*., 1995; Grace, 1997; Jackson *et al*, 2000; Milner, 1992).

A crucial first step for biological control of termites is the selection of a suitable isolate for further development. Because soil is an important reservoir of entomopathogens (Fuxa and Kunimi, 1997), new isolates or species of entomopathogens often are found directly or indirectly through soil sampling (Ko, 1982; Milner *et al*., 1998a; Sajap *et al*., 1997). Isolations of fungal entomopathogens from *C. formosanus* are relatively rare, despite frequent anecdotal observations of various fungal growths associated with weak termite colonies in the laboratory (Beard, 1974). Frequently, the

most effective isolate is obtained from an epizootic in the target pest under natural conditions (Milner, 1992). In the case of termites, there are no confirmed reports of *M. anisopliae* or *B. bassiana* causing natural mortality in the field (Milner *et al*., 1998a), and thus previous studies have mostly involved isolates obtained from other, often unrelated, insects (Hanel, 1981; Lai *et al*., 1982; Kramm and West, 1982). Semi-selective media have been used to detect entomogenous genera, such as *Metarhizium*, *Beauveria*, and *Conidiobolus* (Milner *et al*., 1998a; Sajap *et al*., 1997). However, most successful microbial control agents have been obtained from host insects rather than by selective medium or from existing culture collections (Jackson *et al*., 2000).

In order to more effectively detect fungi that are specifically pathogenic to termites, *C. formosanus* was used in our research as a "bait" to detect and isolate fungal pathogens from soil. The soil samples were collected from different locations and ecosystems in Louisiana. Six isolates (four of *M. anisopliae* and two of *B. bassiana*) were obtained from the USDA-ARS Collection of Entomopathogenic Fungi (Ithaca, NY). A second objective was to compare the virulence and *in vitro* growth of these fungal isolates to *C. formosanus* and to select fungal isolates for possible further development as microbial insecticides.

MATERIALS AND METHODS

Sampling Ninety soil samples were taken from woodlands and pastures in the vicinities of Baton Rouge, New Orleans, and Lake Charles, Louisiana, in 1999. At each location, three sites were selected from woodlands and pastures respectively; from each site, five samples were collected by core sampler (15 cm diameter, 10-15 cm depth), approximately 3 m apart in a transect. Each soil sample was stored in an individual

plastic bag (Whirl-Pak sterile sample bag 15×22 mm); and if necessary, the samples were stored at 4-6°C up to two weeks prior to processing.

Soil pH and moisture Soil pH was measured in three sub-samples, 10 g each, taken from each soil sample (Corning pH meter, Corning Science Products, Corning, New York, USA). A subsample of 50 g soil from each sample was saturated with distilled water. A soil moisture meter probe (Lincoln Irrigation Inc. Lincoln, NE) was then applied to the saturated soil, and its indicator needle was calibrated to the maximum reading (100% water content). Water content was then determined for the original sample and sterile water was added, if necessary, so that all soil samples were adjusted to 70% water content before fungal isolation.

Fungal isolation and culture Formosan subterranean termites were used as a bait to isolate pathogenic fungi from soil. Termite workers were collected from a colony infesting trees in Lake Charles. One hundred termite workers were released into a 100 g subsample from each soil collection in a Pyrex® storage dish (100mm diameter, 80mm high) and covered with a lid. After 24 hours incubation at 27°C, a piece of wet filter paper (100 mm diameter) was placed on top of the soil for 10-20 minutes. The termites trapped on the filter paper then were transferred into an empty Petri dish (100×15 mm) for surface decontamination of the termites, which is essential for removal of saprophytes on the body surface. All termites from each test jar were placed in 1% NaClO, plus 0.005% of Tween 100, for 1 min, and they then were briefly rinsed in 2-3 changes of sterile water. All washed termites were put on sterile filter paper to dry and transferred to four Petri dishes (100×15mm), 25 termites/dish, with a wet piece of filter paper in each dish as the source of food and moisture. Petri dishes were incubated at 27 °C without

light for up to 12 days, and mortality was recorded daily. Dead termites were surface decontaminated as above, except that they also were immersed in 70% alcohol for 5 seconds before NaClO treatment. The termites then were placed on a general culture medium, Sabouraud dextrose agar + yeast (SDAY), and incubated up to 12 days at 27 °C. After prolific conidiogenesis was observed, the fungus was transferred onto SDAY agar. All isolates were stored at -20 °C in silica gel (Smith, 1993).

USDA fungal collections Six fungal isolates were obtained from the USDA-ARS Collection of Entomopathogenic Fungi (Ithaca, NY), including four isolates of *M. anisopliae* (F3045 from *C. formosanus* in Hawaii; F724 from a chrysomelid beetle in Brazil, F683 from a scarab beetle in China; and F794 from leaf litter in Wisconsin) and two isolates of *B. bassiana* (F714 from a delphacid in China and F356 from an acridid in Australia). One isolate of *M. anisopliae*, Bio-blast® (biological termiticide), was obtained from the Ecoscience Corporation (Worcester, MA).

Virulence bioassay Our techniques were adapted from those of Wells *et al*. (1995). The fungi were grown on SDAY at 27 °C. Conidia were harvested under sterile conditions by flooding the plate with sterile distilled H_2O and then scraping the colony with forceps. Conidia were stirred into suspension for 20 min in 200 ml 0.005% Triton X-100 and distilled H_2O, which was then filtered with through cheesecloth. The concentration of conidia in each suspension was determined with a counting chamber (Petroff-Hausser, Hausser Scientific Partnership, Horsham, PA). All suspensions were stored at 6°C until use. Viability of conidia at the time of treatment was measured by mixing a drop of suspension into a drop of Sabouraud broth on a microscope slide and

incubating under high humidity for 24 h at 27°C. All suspensions displayed > 98% germination of conidia.

For the bioassay, termites were anesthetized with a frozen refrigerant pack (20.32×17.78×3.81mm), which was positioned under the Petri dish with the termites to be tested for one min, and 0.5μl of suspension was applied to the ventral surface of each termite with a micro-dispenser (Drummond Scientific Co., Broomall, PA). The bioassay of each fungal isolate included five fungal doses plus a control, with the range of doses determined in a preliminary test. Four replications of ten insects each were treated with each dose, and 0.005% Triton X-100 in distilled water served as the control. After inoculation, each replicate of ten termites was placed in a 100×15 mm Petri dish with a piece of wet filter paper (Whatman #1, diameter 90mm), on which two pieces of wet balsa wood (approximately 50×30×1.5 mm) were provided as food and shelter. The dishes were all sealed with parafilm and incubated at 27°C. The insects were examined daily; dead individuals were removed and incubated under the same conditions for 5 d to allow for external fungal growth. After 10 d, the number of live termites was recorded; a few were occasionally missing and presumed cannibalized. Mortality due to the pathogen was calculated as the number of termite cadavers that grew the appropriate fungal species (some cadavers grew saprophytes, particularly *Aspergillus* spp.) divided by the number of individuals that could be accounted for.

Statistical analysis The χ^2 statistic determined the differences in number of pathogenic fungi isolated among the different locations (cities) and the differences between two ecosystems at each location. Logistic regression analysis (SAS Institute, 1996) was used to determine the effects of soil pH and water content on the number of

detected fungal isolates. Probit analysis of fungal isolates was performed with the POLO-PC program (Russell *et al*., 1977). Differences in virulence between two categories of *in vitro* fungal characteristics on agar and between isolates from two soil ecosystems were analyzed by analysis of variance (ANOVA) (SAS Institute, 1996).

RESULTS

A total of fifteen isolates of *M. anisopliae* and *B. bassiana* were detected from ninety soil samples (Table 2.1). *M. anisopliae* was obtained from 6.7% of all samples, while 10% of samples contained *B. bassiana*. There were no differences among the numbers of fungal isolates from Baton Rouge, New Orleans, and Lake Charles, Louisiana (χ^2 = 0.000, df = 2, P = 1.000). However, significantly more isolates were found in woodlands than in pastures (χ^2 = 3.920, df = 1, P = 0.048). *Metarhizium anisopliae* was not isolated from pasture soils.

Soil pH ranged from acid to neutral for all sampling sites in three locations, and there was no significant pH effect on the number of fungal isolates (Table 2.2, χ^2 = 0.205, df = 1, P = 0.650). Soil samples collected from different sites in different locations varied greatly in water content, ranging from 5-80%. There was no significant impact of soil water content on the number of detected fungal isolates (Table 2.2, χ^2 = 0.642, df = 1, P = 0.423).

Table 2.3 summarizes the results of virulence bioassays, which indicated that both *M. anisopliae* and *B. bassiana* were virulent to *C. formosanus*. The ranks of LD_{50}s (median lethal doses) from these two fungi were interspersed, showing that fungal strains rather than species had greater effect on virulence. On the basis of the LD_{50}s, USDA culture

Table 2.1 Entomopathogenic fungi isolated by baiting soil with *C. formosanus*

Sampling locations in Louisiana	Sampling environments for each location	Sampling site in each environment [a]	Date of soil samples	Number of fungal isolates	Fungal species	Assigned isolate numbers
New Orleans	Woodland	1	6/17/1999	1	*M. anisopliae*	G-6170
		2	8/2/1999	1	*B. bassiana*	W-8021
		3	8/18/1999	1	*B. bassiana*	W-8181
	Pasture	1	6/17/1999	0	/	/
		2	8/2/1999	1	*B. bassiana*	W-8022
		3	8/18/1999	1	*B. bassiana*	W-8182
Baton Rouge	Woodland	1	6/29/1999	2	*B. bassiana*	W-6291
					M. anisopliae	G-6292
		2	7/2/1999	0	/	/

(Table con'd.)

		3	7/15/1999	2	*B. bassiana*	W-7151
					M. anisopliae	G-7152
	pasture	1	6/29/1999	0	/	/
		2	7/2/1999	0	/	/
		3	7/15/1999	1	*B. bassiana*	W-7150
Lake Charles	Woodland	1	6/18/1999	1	*M. anisopliae*	G-6180
		2	7/19/1999	2	*B. bassiana*	W7191
					M. anisopliae	G-7192
		3	8/3/1999	1	*M. anisopliae*	G-8032
	pasture	1	6/18/1999	0	/	/
		2	7/19/1999	0	/	/
		3	8/3/1999	1	*B. bassiana*	W-8031
TOTAL				15	2	

[a] Five soil samples in each sampling site

Table 2.2 Physical characteristics of soil from which fungi were isolated

Sampling locations in Louisiana	Sampling Environments for each location [a]	Soil pH (mean±SE)	Soil water content (% ±SE)
New Orleans	Woodland	6.12±0.76	40.67±14.62
	pasture	6.34±0.47	15.33±5.49
Baton Rouge	Woodland	5.78±1.16	31.67±17.69
	pasture	6.02±0.76	34.67±26.49
Lake Charles	Woodland	3.81±0.51	8.00±2.54
	pasture	5.77±1.27	43.33±32.16

[a] Total 15 soil samples in each location

Table 2.3 Log-dose-probit parameters and colony growth characteristics on SDAY for the isolates of *M. anisopliae* and *B. bassiana* against *C. formosanus*

Fungal Species	Isolate number [a]	Slope±SE (ld-p line)	LD_{50} (90%CL) [b] (conidia/termite)	Chi-Square Value [c]	Geographical origin [d]	Original host species	Category of fungi [f]
M. anisopliae	F 3045	1.30±0.12	0.08 (0.05-0.12)	4.7	Hawaii (Oahu)	*C. formosanus*	1
"	F 724	1.87±0.19	0.56 (0.49-0.65)	0.5	Brazil	*Cerotoma arcuata*	1
"	F 683	1.46±0.08	0.43 (0.31-0.57)	11.8 *	P. R. CHINA	Coleoptera: Scarabaeidae	1
"	F 794	1.18±0.08	0.30 (0.20-0.43)	10.6 *	Wisconsin	Leaf litter(sugar maple)	2
B. bassiana	F 714	1.04±0.92	0.05 (0.04-0.07)	1.7	P. R. CHINA	*Nilaparvata lugens*	1
"	F 356	3.00±0.18	0.80 (0.70-0.92)	7.1	Australia	Orthoptera: Acrididae	1
M. anisopliae	Bioblast	1.30±0.01	0.09 (0.07-0.13)	3.2	commercial	unknown	1
"	G7192	2.10±0.19	0.68 (0.50-0.85)	4.5	Lake Charles(W)	*C. formosanus* [e]	1
"	G7152	1.34±0.11	0.70 (0.59-0.84)	2.0	Baton Rouge(W)	"	1

(Table con'd.)

″	G6292	1.63±0.13	0.73 (0.63-0.83)	3.5	Baton Rouge(W)	"	1
″	G6180	2.37±0.23	1.08 (0.95-1.22)	1.3	Lake Charles(W)	"	2
″	G6170	1.27±0.09	1.20 (0.99-1.44)	0.3	New Orleans(W)	"	2
″	G8032	2.40±0.17	1.22 (1.09-1.35)	2.8	Lake Charles(W)	"	2
B. bassiana	W7150	2.06±0.18	0.67 (0.50-0.86)	6.2	Baton Rouge(P)	"	1
″	W8022	1.65±0.14	0.90 (0.61-1.27)	7.5	New Orleans(P)	"	1
″	W8182	1.31±0.09	1.67 (1.39-2.00)	1.7	New Orleans(P)	"	1
″	W8181	3.88±0.35	2.72 (2.56-2.87)	3.5	New Orleans(W)	"	2
″	W8021	1.53±0.11	2.39 (2.09-2.73)	2.4	New Orleans(W)	"	2
″	W6291	1.33±0.19	3.99 (3.33-5.02)	2.6	Baton Rouge(W)	"	2
″	W7191	1.15±0.09	4.65 (3.56-6.25)	3.1	Lake Charles(W)	"	2
″	W7151	1.63±0.13	4.69 (4.02-5.43)	3.1	Baton Rouge(W)	"	2
″	W8031	1.26±0.12	4.96 (3.24-10.17)	9.0 *	Lake Charles(P)	"	2

(Table con'd.)

[a] F = USDA/ARS Collection of Entomopathogenic Fungi; G and W =fungi from bating isolation

[b] Conidia × 10^5/insect, POLO-PC calculates confidence limits only when g < 0.5

[c] * = data heterogeneous

[d] (P) = pasture, (W) = woodlands

[e] *C. formosanus* was used as "bait" when isolated from soil

[f] 1 = Compact growth with flat elevation and sporulation > 50% of total colony growth area on agar after 12 days at 27°C
2 = Filamentous growth with raised elevation and sporulation < 50% of total growth area on agar after 12 days at 27°C

F714, isolated from a delphacid in China, was the most virulent isolate we tested. The most virulent Louisiana fungus was *B. bassiana* isolated from a pasture near Baton Rouge. There was no difference between fungal virulence in fungi isolated from woodland versus pasture ecosystems ($F = 0.020$; $df = 1, 13$; $P = 0.888$).

Two groups of fungal isolates could be categorized based on their colony characteristics. Category 1 had a compact growth colony with flat elevation and >50% of the surface area of the fungal colony sporulated after 12 days of growth. Category 2 had filamentous growth with raised elevation and < 50% sporulation after 12 days. The fungal isolates in category 1 were more virulent to termites (mean $LD_{50} = 6.11 \pm 0.443 \times 10^4$ conidia/insect) than those in category 2 (mean $LD_{50} = 2.72 \pm 1.746 \times 10^5$ conidia/insect) ($F = 16.380$; $df = 1, 20$; $P < 0.001$).

DISCUSSION

Termites seem to be very susceptible to a wide range of isolates of *Metarhizium* and *Beauveria* derived from a variety of soil samples and insects (Almeida *et al*., 1997; Delate *et al*., 1995; Hanel, 1981; Lai *et al*., 1982; Milner *et al*., 1998a; Wells *et al*., 1995). This also was the case in the current study, although virulence differed by as much as 16X among *M. anisopliae* isolates and 100X among *B. bassiana* isolates (Table 2.3). Similarly, a large number of isolates of *M. anisopliae* from termite colonies or from termite derived material in Australia were virulent to termites (Milner *et al*., 1998a). However, termites are rarely infected naturally in the field (Milner, 1997; Milner *et al*., 1998a; Roberts and Humber, 1981), which suggests that their defensive behavior may play a major role in defending fungal attack.

Fungal *in vitro* growth characteristics (Table 2.3) were correlated with virulence against termites ($R^2 = 0.450$, $P = 0.001$). This suggests that isolates of *M. anisopliae* or *B. bassiana* potentially may be categorized in a preliminary screening into different virulence groups, based visually on their colony characteristics on a culture medium instead of virulence bioassays. In addition to their virulence, the greater production of conidia by category 1 isolates might make them more suitable than category 2 isolates for commercial production. Thus, these fungal characteristics may allow for efficient screening of a large number of isolates against termites.

Using termites as a "bait" to detect pathogens in soil was an effective way to isolate fungi and is more efficient than isolation of pathogens from dead, field-collected termites (Ko 1982). Separating bait termites from their nest significantly increased their chances to be infected, because termite nests are physically and chemically different from adjacent soils (Badawi *et al*., 1982). Termites modify soil by differential selection of soil particles, incorporation of saliva and excreta into the lining of galleries, and modification of organic matter during its passage in the termite gut (Lee and Wood, 1971). Also, fecal pellets and fumigation of the nest with volatiles significantly decreased germination rates of fungal spores and colony growth (Rosengaus *et al*., 1998; Wright *et al*., 2000). The baiting technique used in this study had the additional advantage of enabling detection and isolation of *C. formosanus* pathogens with little interference from saprophytic organisms. Baiting also may prove effective in fungal ecology. For example, we isolated more fungi from woodlands than from pastures (Table 2.1), although soil pH and water content did not affect the number of isolates (Table 2.2). For practical reasons, it may be advantageous to search for suitable strains of pathogens in the environment of the target

pest (Milner *et al*., 1998b), where ecological factors have selected for fungal survival, reproduction, and transmission in populations of the target insect. It is not known why baiting in the current study did not detect pathogenic nematodes, which also are common in soil (Tanada and Kaya, 1993), possibly worker termites are relatively unsusceptible and too active to be infected (Mauldin and Beal, 1989; Mix, 1985).

The common occurrence of fungi pathogenic to *C. formosanus* in soil (Table 2.1) suggests that they might be important to natural mortality of termites founding new colonies. The success rate of colony founding pairs in a soil environment is generally very low (Fei, 2000; McMahan, 1962; Rosengaus and Traniello, 1993), although *C. formosanus* appears to be relatively free of serious fungal diseases in its nests (Milner, 1997; Milner *et al*., 1998a; Roberts and Humber, 1981). A possible reason for this low success rate is the presence of *M. anisopliae* and *B. bassiana* (Ko, 1982; Jackson *et al*., 2000), which are distributed in soils worldwide (McCoy *et al*., 1988). More than 16% of soil samples collected from Louisiana in the current research contained fungi pathogenic to Formosan subterranean termites, primarily *M. anisopliae* and *B. bassiana*. As many as 75% of soil samples collected from Kamuela in Hawaii contained factors pernicious to termites, mainly entomogenous fungi (Ko, 1982); *C. formosanus* is an important pest of wood structures in Hilo and Kona on the island of Hawaii, but it has never been observed in Kamuela (Tamashiro *et al*., 1980).

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CHAPTER 3

SPORULATION OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* ON *COPTOTERMES FORMOSANUS* AND *IN VITRO*

INTRODUCTION

Biological control with pathogenic fungi is a promising alternative to chemical control against the Formosan subterranean termite, *Coptotermes formosanus*, (Grace, 1997; Milner *et al*., 1998a; 1998b; Wells *et al*., 1995). Extensive use of chemicals can cause environmental hazards and induce resistance. Biological control with pathogenic fungi might provide long-lasting insect control without damage to the environment or non-target organisms (Hokkanen and Lynch, 1995; Howarth, 1991; Khetan, 2001). Also, social interactions within a subterranean termite colony and its dark, damp habitat would seem to favor survival and distribution of pathogenic fungi (Grace, 1997; Grace and Zoberi, 1992).

The Deuteromycetes *Metarhizium anisopliae* and *Beauveria bassiana* are common soil-borne entomopathogenic fungi that occur worldwide (Fuxa and Kunimi, 1997; McCoy *et al*., 1988) and co-exist with many kinds of termites, including subterranean termites (Jackson *et al*., 2000; Milner *et al*., 1998b). Both species infect *C. formosanus* (Ko, 1982; Lai *et al*., 1982; Sun *et al*., 2001; Wells *et al*., 1995) but are not known to cause natural epizootics in termites (Jackson *et al*., 2000; Milner, 1997). Therefore, a seasonal colonization approach, in which a pathogen recycles and suppresses two or more pest generations but must be re-introduced (Fuxa, 1987; Fuxa and Tanada, 1987), may be useful for control of termites with pathogenic fungi. This approach requires a pathogen with high rates of reproduction and transmission (Fuxa, 1987). Large termite populations with millions of individuals in a colony and galleries that may stretch more than 100 m (Su and Tamashiro, 1987) make it impractical to inoculate fungal spores directly onto most colony members.

Most research for selecting the best species or isolates for termite control has concentrated on virulence (Almeida *et al*., 1997; Bao and Yendol, 1971; Hanel, 1982; Lai. *et al*., 1982; Wells *et al*., 1995; Zoberi and Grace, 1990), although host specificity, temperature tolerance, repellency to termites, nest volatile responses, and conidia production on artificial media also have been investigated (Fargues *et al*., 1997; Hegedus *et al*., 1990; Hywel-Jones and Gillespie, 1990; Ko, 1982; Milner *et al*., 1998a; Staples and Milner, 2000; Wright *et al.,* 2000). Such research has not resulted in successful field trials of fungi against termites (Kramm *et al*., 1982; Sajap *et al*., 1997; Zoberi, 1995).

In selecting fungal strains for termite control, it seems logical to concentrate on their production of conidia on the termite cadavers (Fuxa, 1987; Fuxa *et al*., 1998). An isolate that produces large numbers of propagules or produces them quickly might overcome termite defense behaviors of burying infected colony members or isolating them in walled off parts of the nest (Logan *et al*., 1990; Reese, 1971; Su *et al*., 1982; Wood & Sands, 1978; Zoberi, 1995). Such a fungus could be transmitted from infected termites to unexposed nest-mates via social contacts. The production of conidia on *C. formosanus* may vary considerably among different fungal isolates or species. *In vivo* production of conidia has not been investigated previously in *C. formosanus* and has seldom been researched in other insect-fungus systems (Arthurs and Thomas, 2001; Cooper and Sweeney, 1986; Kalsbeek *et al*., 2001).

The purposes of the current study were to estimate *in vivo* conidia production of isolates of *M. anisopliae* and *B. bassiana* on *C. formosanus* and to compare this with their production and growth rates *in vitro*.

MATERIALS AND METHODS

Termites Worker termites of *C. formosanus* were collected from a colony infesting trees in Lake Charles, Louisiana. Collected termites were reared at room temperature in plastic containers with sand and wetted corrugated cardboard until use.

Fungal species and isolates A total of twenty-two isolates of *M. anisopliae* and *B. bassiana* were used in the experiments. Four isolates of *M. anisopliae* (F3045 from *C. formosanus* in Hawaii; F724 from a chrysomelid beetle in Brazil, F683 from a scarab beetle in China; and F794 from leaf litter in Wisconsin) and two isolates of *B. bassiana* (F714 from a delphacid in China and F356 from an acridid in Australia) were obtained from the USDA-ARS Collection of Entomopathogenic Fungi (Ithaca, NY). One isolate of *M. anisopliae*, Bio-blast® biological termiticide, was obtained from the EcoScience Corporation (Worcester, MA). Six isolates of *M. anisopliae* (G6170, G6292, G7152, G6180, G7192, G8032) and nine isolates of *B. bassiana* (W7150, W8031, W8022, W8182, W8021, W8181, W6291, W7151, W7191) came from baiting soil samples from Louisiana with live *C. formosanus* (Sun *et al*., 2001, see chapter 2).

The fungi were grown on Sabouraud dextrose agar + yeast (SDAY) at 27 °C (Wells *et al*. 1995). Conidia were harvested under sterile conditions by flooding the plate with sterile distilled H_2O and then scraping the colony with forceps. Conidia were stirred into suspension for 20 min in 200 ml 0.05% Triton X-100 and distilled H_2O, which was then filtered with through cheesecloth. The concentration of conidia in each suspension was determined with a hemocytometer (Petroff-Hausser, Hausser Scientific Partnership, Horsham, PA).

Conidia production *in vivo* Individual termites were inoculated by topical application of conidia. The termites were immobilized for one min with an ice brick (refrigerant pack, 20.32×17.78×3.81 mm), which was positioned under a Petri dish (100×15mm) containing ten termites, and 1μl of conidial suspension was applied to the ventral surface of each termite with a micro-dispenser (Drummond Scientific Co., Broomall, PA). The inoculated dose (conidia/insect) for each isolate was the dosage killing 80% (see chapter 2), and ten control termites for each isolate were inoculated with 0.05% Triton X-100 in distilled water. After inoculation, each group of ten termites was caged in a Petri dish (100 ×15 mm) with a piece of wet filter paper (Whatman #1, diameter 90mm), on which two pieces of wet balsa wood (approximately 50×30×1.5 mm) were provided as food and shelter. The dishes were sealed with parafilm and incubated at 27°C without light for eight days. Termite mortality was checked daily, and each dead termite was carefully transferred to a single 1.5 ml sterile micro-centrifuge tube (Brinkmann Instruments, Inc., Westbury, NY), containing a wetted slip of filter paper for moisture, in such a way that the paper did not touch the cadaver. Each dead termite was randomly assigned to be examined for conidia at 2, 3, 4, 5, 8 or 11 days after death and was then incubated at 27°C until that day. On the assigned day, each tube was transferred to a freezer at -85°C to stop fungal development. There were ten termites (replications) for each examination day for each isolate. Eighty termites were inoculated with each isolate in order to provide enough termites to compensate for the LD_{80} and death from other causes.

Removal and suspension of conidia Each cadaver was examined initially under a dissecting microscope for possible contaminants or death from other factors; such cadavers were discarded. For cadavers exhibiting *B. bassiana* or *M. anisopliae*, 0.2 ml

sterile distilled water with 0.05% Triton X-100 was added to each tube to immerse the termite. The tube then was vortexed three times, 1 min each, with a Fisher Vortex-Genie2 (Scientific Industries, Inc. Bohemia, NY) to wash conidia from the cadaver, and the cadaver was removed from the tube. In order to break down clumps of conidia for accurate counting, the suspension in the tube was agitated with a sterile micro-pestle (Tissue Grinder, Pellet Pestle® Motor, VWR Corp., OH). In order to save possible residual spores, the used pestle was washed into another micro-centrifuge tube with 0.3ml of the 0.05% Triton X-100. The rinsings were transferred immediately into the first tube to form a total of 0.5 ml spore suspension as the stock for counting of conidia.

Quantification Each stock suspension underwent a preliminary count with a hemocytometer (Petroff-Hausser, Hausser Scientific Partnership, Horsham, PA). If a sample had fewer than 20 propagules per counting chamber (all 25 cells) (Lacey, 1997), then each tube from that sample day was centrifuged (Centrifuge 5417 C/R, Eppendorf®, Brisckmann Instruments, Inc. Wesburry, NY) for 5 minutes (14000 rmp, 20°C). If a preliminary sample had more than 100 propagules per counting chamber (Lacey, 1997), each stock suspension from that day was serially diluted. After all suspensions were brought into the proper range, the final hemocytometer count was replicated three times.

Colony growth rate and conidial production on agar In order to compare *in vitro* growth with *in vivo* conidia production, all isolates were grown on SDAY. Twenty µl of spore suspension at 10^3 conidia/ml were inoculated onto SDAY in a Petri dish (100×15mm). The inoculum was spread over the agar with a sterile spreader (30mm wide) on an inoculating turntable (114mm, VWRbrand®, VWR Corporation, OH). The dishes were incubated at 27°C for 30 hr, and a single germinated conidium was excised

from the agar by micro-scalpel and transferred to the center of a new SDAY Petri dish. This dish was sealed with parafilm and cultured at 27°C without light for 14 d. There were five dishes (replications) for each fungal isolate. The diameter of each colony, or the largest diameter if not circular, was measured daily from the bottom of the dish. The colony growth rate was calculated by averaging the colony growth (mm/day) over 14 d.

Four isolates of *M. anisopliae*, F3045, F794, G6180, and Bio-blast®, and four isolates of *B. bassiana*, F714, F356, W8031, and W7191, were selected to estimate conidia production *in vitro*. The fungal suspension for each isolate was prepared as above. The inoculated concentration (conidia/ml) for each isolate was the concentration killing 80% of termites (Sun *et al*. 2001), as in the topical application above. One µl of conidial suspension was applied with a micro-dispenser (Drummond Scientific Co., Broomall, PA) onto the center of a Petri dish (60 ×15 mm) with 5 ml SDAY agar. All dishes were incubated at 27°C for 11 days without light. There were four replications for each isolate. Conidia were harvested under sterile conditions by flooding the plate with 2 ml sterile 0.05% Triton X-100 and then scraping the colony with forceps. The resulting suspension was transferred by pipette to a 10 ml sterile polyethylene tube (Elkay Inc., MA). In order to harvest all possible conidial residuals from the dishes, each dish was rinsed three times with 2 ml sterile 0.05% Triton X-100. The rinsings were added to the initial tube, and the final suspension then was filtered through cheesecloth after it was agitated with a sterile micro-pestle to separate the conidial clumps. The concentration of conidia in each stock suspension was determined as above. After all suspensions were brought into the proper range of concentration, the final hemocytometer counts were replicated three times.

Data analysis For each fungal isolate, an intrinsically linear model $Y = b_0 e^{b1X}$ (where Y = number of conidia/insect, and X = number of days after insect death) was used to estimate average percentage increase in sporulation per day *in vivo* by the PROC REG procedure of SAS (SAS Institute, 1996). For all isolates, sporulation data *in vivo* and *in vitro* were subjected to analysis of variance in the general linear model procedure, with the Tukey HSD test among means (SAS Institute, 1996). The slice statement of SAS was used to detect significant differences in sporulation by day for interactions of day by species, day by dose, and day by species by dose. Vegetative growth rates (mm/day) were subjected to a general linear model analysis (SAS Institute, 1996). Based on sporulation characteristics of each isolate (quick sporulation and total sporulation), a complete linkage cluster analysis (SAS Institute, 1996) was performed, and two groups of isolates were classified from the resulting dendrogram (a DISTANCE macro was used to compute the Euclidean distance among isolates in SAS).

RESULTS

Based on our observations of all twenty-two isolates, mycelium usually was observed 1-2 days post-death on termites treated with *M. anisopliae* and *B. bassiana*. Mycelium initially emerged around the worker termite mouthparts, near the base of antennae, and on the cervix. Growth from other intersegmental membranes and around the legs soon followed. Eventually, the cadavers were totally covered with mycelia and then conidia. Isolates F356, F683, and G6180 exhibited exceptional mycelial development, which was extensive and quickly radiated out from the cadavers. Most isolates of *M. anisopliae* produced conidia in spots on the cadaver surface before it was completely covered with mycelia. Insects treated with *B. bassiana* usually produced

conidia after mycelia fully colonized and covered the surface of the cadaver. The mean vegetative growth rate (4.7 mm/day) of *M. anisopliae* on agar was significantly higher than that of *B. bassiana* (2.6 mm/day) ($F = 226.31$; $DF = 1, 108$; $P < 0.0001$ (Table 3.1). *M. anisopliae* isolates had a greater variation in growth rate (1.9 to 5.5 mm/day) than did *B. bassiana* (2.4 to 3.1 mm/day) (Table 3.1).

Sporulation of all fungal isolates on termite cadavers increased significantly over time ($F = 433.81$; $DF = 5, 1239$; $P < 0.0001$). The range of average percentage increase in sporulation per day was 41-159% in *M. anisopliae* and 5-122% in *B. bassiana* (Table 3.1). Fungal species was not directly correlated with sporulation ($F = 3.40$; $DF = 1, 1239$; $P = 0.0653$), indicating that fungal isolate had a greater effect than species on sporulation.

Sporulation characteristics were different between the two fungal species. *M. anisopliae* had greater variation in conidia production *in vivo* than *B. bassiana*. Sporulation on day 2 differed by as much as 9X and 88X among isolates of *B. bassiana* and *M. anisopliae*, respectively. Similarly, sporulation on day 3 differed by as much as 26X and 216X among isolates of *B. bassiana* and *M. anisopliae*, respectively. "Quick sporulation" in Table 3.2 was defined as that occurring by day 2 or 3. Sporulation on day 11, termed "total sporulation," differed up to 15X and 21X among isolates of *B. bassiana* and *M. anisopliae*, respectively. Although the effect of fungal species on *in vivo* sporulation was not significant, the interactions of fungal species with quick sporulation and with total sporulation were significant ($P < 0.05$, Table 3.2), indicating that the two species have different sporulation patterns. *B. bassiana* isolates could be categorized by cluster analysis or graphically into a group with high total sporulation and low quick

Table 3.1 Sporulation of isolates of two species of entomogenous fungi on *C. formosanus* at different times after death, and conidia production and colony growth rate *in vitro*

Isolate	conidia × 10^5 / insect (days after termite-death) (± SE) [a]						mean % increase in conidia/day [b]	Inoculated conidia /insect [c]	Growth on agar (±SE) (mm/day) [d]	Conidia (± SE) *in vitro* [e]
	2	3	4	5	8	11				
M. anisopliae										
F3045	6.14 ± 2.12 c	137.90 ± 47.11 b	169.26 ± 29.06 cd	477.78 ± 90.23 d	488.10 ± 168.50 bc	471.09 ± 100.52 cd	45.8	0.35	5.1 ± 0.0	13466.67 ± 2025.76 bc
F724	2.27 ± 0.67 ab	7.30 ± 2.49 a	20.97 ± 8.96 ab	35.42 ± 9.38 ab	29.82 ± 6.24 a	54.27 ± 10.40 ab	40.6	1.58	5.2 ± 0.1	/
F683	0.34 ± 0.06 a	2.58 ± 1.76 a	8.93 ± 2.96 ab	27.47 ± 4.71 ab	44.54 ± 5.28 ab	58.58 ± 5.59 b	80.9	1.62	4.8 ± 0.0	/
F794	1.43 ± 0.17 ab	1.40 ± 0.08 a	1.85 ± 0.50 a	5.89 ± 1.11 a	48.83 ± 5.09 ab	40.67 ± 6.27 a	58.2	1.54	1.9 ± 0.0	9416.67 ± 348.94 b
Bioblast	0.27 ± 0.10 a	17.69 ± 9.11 a	103.84 ± 11.44 bc	119.12 ± 9.30 abc	569.55 ± 34.06 c	833.21 ± 21.91 e	116.8	0.42	5.0 ± 0.1	18200.00 ± 2188.52 c
G7192	1.68 ± 0.63 ab	72.23 ± 16.83 ab	149.17 ± 23.75 cd	320.67 ± 20.90 c	357.25 ± 31.74 bc	339.00 ± 28.84 cd	52.7	1.71	4.9 ± 0.1	/
G7152	4.62 ± 2.24 b	71.74 ± 12.90 ab	196.67 ± 18.50 d	233.58 ± 28.34 bc	292.67 ± 31.60 abc	269.83 ± 26.79 c	45.0	2.98	4.5 ± 0.2	/

(Table con'd.)

G6292	5.57 ± 1.19	40.89 ± 15.44	59.45 ± 26.33	175.67 ± 16.46	338.92 ± 18.42	361.85 ± 69.08	67.7	2.38	4.9 ± 0.1	/
	bc	a	ab	bc	bc	cd				
G6180	0.35 ± 0.04	9.64 ± 3.89	66.64 ± 9.01	154.17 ± 30.25	243.08 ± 34.07	221.00 ± 22.69	89.9	2.46	4.2 ± 0.1	1242.90 ± 94.59
	a	a	abc	bc	abc	bc				a
G6170	0.24 ± 0.10	25.30 ± 9.10	90.22 ± 17.38	108.42 ± 16.08	278.42 ± 30.29	310.75 ± 24.29	93.0	5.50	5.5 ± 0.1	/
	a	a	bc	abc	abc	cd				
G8032	0.07 ± 0.02	0.64 ± 0.28	6.23 ± 2.49	34.34 ± 6.48	265.28 ± 29.83	364.43 ± 29.91	159.0	2.74	5.5 ± 0.0	/
	a	a	ab	ab	abc	cd				
Mean	1.95 ± 0.35	35.68 ± 6.39	78.21 ± 7.82	152.05 ± 15.62	273.13 ± 23.20	301.99 ± 24.34	77.2 ± 10.91	2.12 ± 0.42	4.7 ± 0.1	10581.56 ± 1741.67
F	12.78	13.30	20.91	59.25	56.62	73.09	/	/	/	117.12
df	10, 92	10, 95	10, 98	10, 97	10, 96	10, 92	/	/	/	3,12
P>F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	/	/	/	<0.0001
B. bassiana										
F714	6.57 ± 0.40	9.10 ± 0.57	65.58 ± 4.26	127.02 ± 6.73	727.50 ± 51.64	1097.50 ± 46.83	81.3	0.32	3.1 ± 0.1	30866.67 ± 2155.78
	c	a	a	bc	bc	c				d
F356	1.50 ± 0.22	6.20 ± 1.92	3.86 ± 1.04	32.14 ± 6.54	53.61 ± 9.53	72.68 ± 20.24	49.9	1.53	2.6 ± 0.1	6450.00 ± 436.84
	ab	a	A	a	a	a				b
W7150	0.70 ± 0.13	1.65 ± 0.48	13.64 ± 9.82	60.94 ± 17.29	296.41 ± 142.40	602.09 ± 78.61	121.5	1.69	2.6 ± 0.1	/
	a	a	a	ab	ab	b				

(Table con'd.)

W8022	2.66 ± 0.84	4.50 ± 0.68	8.04 ± 1.35	166.50 ± 32.60	649.36 ± 101.67	916.93 ± 87.79	109.5	2.90	2.5 ± 0.0	/
	ab	a	a	bc	b	bc				
W8182	3.69 ± 0.28	9.15 ± 1.54	124.75 ± 47.91	101.00 ± 21.67	533.75 ± 109.26	977.50 ± 60.39	81.9	7.35	2.5 ± 0.1	/
	ab	a	a	abc	b	bc				
W8181	2.35 ± 0.98	43.33 ± 6.70	66.15 ± 9.91	152.50 ± 28.71	477.70 ± 106.83	580.29 ± 72.77	67.9	4.48	2.7 ± 0.1	/
	ab	b	a	bc	b	b				
W8021	6.42 ± 0.69	9.48 ± 1.10	121.38 ± 91.19	319.59 ± 19.99	416.09 ± 25.52	709.09 ± 75.97	66.9	8.50	2.5 ± 0.1	/
	c	a	a	d	b	bc				
W6291	2.49 ± 0.14	9.42 ± 0.75	137.92 ± 61.94	89.84 ± 6.92	1031.68 ± 45.08	761.68 ± 32.35	87.4	17.10	2.6 ± 0.1	/
	ab	a	a	abc	c	bc				
W7191	6.07 ± 0.51	5.38 ± 0.06	17.88 ± 1.58	27.07 ± 0.82	178.17 ± 11.57	613.50 ± 15.76	71.6	24.95	2.4 ± 0.1	2866.67 ± 438.04
	c	a	a	a	ab	b				a
W7151	5.78 ± 0.36	8.60 ± 0.75	16.45 ± 1.14	277.50 ± 11.53	356.84 ± 10.89	795.84 ± 54.72	77.4	15.45	2.4 ± 0.1	/
	bc	a	a	d	ab	bc				
W8031	2.76 ± 0.19	4.10 ± 0.44	11.21 ± 2.21	104.67 ± 6.91	312.67 ± 40.01	570.67 ± 68.27	87.4	23.10	2.6 ± 0.1	19275.00 ± 861.56
	ab	a	a	abc	ab	b				c
Mean	3.77 ± 0.24	9.53 ± 1.11	54.04 ± 11.95	132.77 ± 10.06	461.17 ± 33.02	707.89 ± 30.23	82.06 ± 6.00	9.76 ± 2.70	2.6 ± 0.0	14864.58 ± 2908.58
F	19.79	24.12	21.37	23.74	21.06	35.02	/	/	/	139.20
df	10, 96	10, 95	10, 95	10, 94	10, 97	10, 96	/	/	/	3,12
P>F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	/	/	/	<0.0001

(Table con'd.)

[a] One-way ANOVA of SAS was used to analyze the sporulation of each fungal species and isolate. Means (conidia/insect) in each column within each fungal species followed by the same letter did not differ significantly at P = 0.05 (Tukey, HSD).

[b] The percentage increase in conidia/day was defined as 100(b1), where b1 derives from the model $Y= b_0 e^{b1X}$ (where the e constant = 2.7182, X = number of days after insect death, and Y = conidia/insect).

[c] Conidia $\times 10^5$ / insect, Topically applied at the LD_{80}.

[d] Average colony size increase in two weeks from a single spore at 27°C.

[e] Conidia production ($\times 10^5$) growing on agar SDAY at 27°C on day 11 after inoculation; the inoculum of each isolate was the same amount of conidia suspension used on a single termite. Means in each column within each fungal species followed by the same letter did not differ significantly at P = 0.05 (Tukey, HSD).

Table 3.2 Analysis of interactions of day by species, day by dose, and day by dose by species affecting *in vivo* sporulation characteristics of *M. anisopliae* and *B. bassiana* [a]

Sporulation Characteristic	Day of sporulation	Parameters	Factors that interacted with day of sporulation		
			Species	Dose [b]	Species by Dose
Quick sporulation	2	F value	54.76	5.37	24.45
		P> F	<0.0001	0.0048	<0.0001
		df	1, 1239	2, 1239	5, 1239
	3	F value	4.24	20.35	11.85
		P> F	0.0398	<0.0001	<0.0001
		df	1, 1239	2, 1239	5, 1239
Total sporulation	11	F value	8.41	22.59	17.73
		P> F	0.0038	<0.0001	<0.0001
		df	1, 1239	2, 1239	5, 1239

[a] GLM analysis of variance (SAS Institute, 1996) of data in Table 1. The slice statement of SAS was used to detect the significant difference by days post death for interactions of day by species, day by dose, and day by species by dose.

[b] The inoculation doses for all isolates were categorized into three groups with the Ln (LD_{80}) transformation: L (low dose), Ln (LD_{80}) < 0; M (medium dose), 0 < Ln (LD_{80}) < 1; H (high dose), Ln (LD_{80}) > 1.

sporulation, while *M. anisopliae* isolates generally fell into another group with high quick sporulation and low total sporulation (Fig. 3.1).

Every isolate tested produced more conidia *in vitro* on day 11 after inoculation than *in vivo* on day 11 after termite death ($F = 489.95$; $DF = 1, 100$; $P < 0.0001$). *In vitro* and *in vivo* sporulation differed by as much as 88.73X and 231.5X among the selected isolates of *B. bassiana* and *M. anisopliae*, respectively (Table 3.1). The correlation of *in vivo* and *in vitro* conidial production was positive and significant ($R^2 = 0.50$, $P < 0.0001$).

DISCUSSION

Fungal isolates of *B. bassiana* and *M. anisopliae* differed greatly in their capability to sporulate *in vivo* (Table 3.1). *In vivo* sporulation of these two fungi has not been quantified previously, even though total sporulation of an entomogenous fungus *in vivo* is likely to be an important characteristic in the seasonal colonization approach to microbial control. Pathogen replication in target insects can enhance fungal population density in host environments and the likelihood of conidial transmission or spread in the host population (Fuxa, 1987; Fuxa *et al.*, 1998). Entomogenous fungi in soil, such as *B. bassiana* and *M. anisopliae*, are generally present at low densities (Jackson and O'Callaghan, 1997). An isolate characterized by increased total sporulation *in vivo* may have the advantage of increased density in soil. The variation in total sporulation (day 11) by *M. anisopliae* (21X) and by *B. bassiana* (15X) indicates some potential to select improved isolates for microbial control (St. Leger *et al*, 1992). Rath *et al*. (1995) recorded a 10-fold increase in *M. anisopliae* conidia in soil after an epizootic in *Adoryphorus coulonii*. Isolates that can recycle and provide enough propagules for effective control have been useful in the seasonal colonization approach (Almeida *et al*., 1997; Fuxa *et al*., 1998).

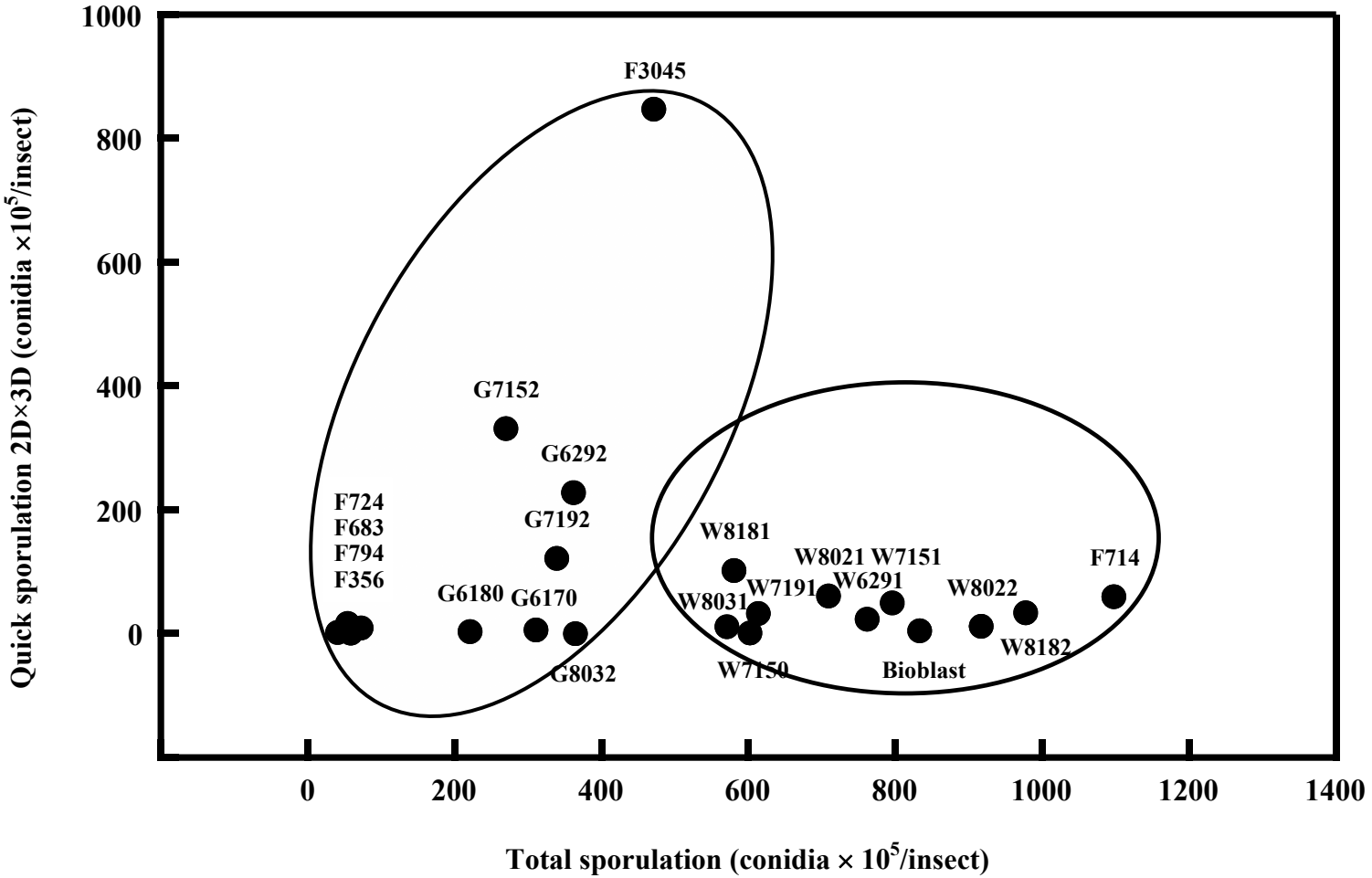

Fig. 3.1 Patterns of quick sporulation and total sporulation by *M. anisopliae* and *B. bassiana* on *C. formosanus* (2D and 3D represent sporulation at day 2 and day 3, and quick sporulation was calculated as 2D × 3D), based on cluster analysis (SAS Institute, 1996). The left oval contains only isolates of *M. anisopliae* except Bioblast; and the right oval contains only isolates of *B. bassiana* except F356. See Table 3.1 for isolate designations.

Another parameter measured and analyzed in the current study (Table 3.1, 3.2) was quick sporulation, which may play a more important role in social insects than total sporulation. *C. formosanus* nests can contain millions of individuals (Su and Tamashiro, 1987) which are rarely infected naturally in the field because of their defensive behavior of walling off or isolating infected colony members (Logan *et al*., 1990; Reese, 1971; Su *et al*, 1982; Wood and Sands, 1978; Zoberi, 1995). Therefore, early or quick sporulation of a pathogenic fungus on its termite host may be advantageous, leading to spore transmission or spread by termite social contacts before unexposed individuals can isolate the spore-covered termite cadavers. In this respect, *M. anisopliae* may provide a better choice of isolates due to its higher level of genetic variation associated with quick sporulation than *B. bassiana* (88X vs. 9X on day 2, 216X vs. 26X on day 3, respectively, Table 3.1) (St. Leger *et al*, 1992). In previous research of this parameter on vine weevil larvae, *Otiorhynchus sulcatus*, one isolate of *M. anisopliae* sporulated nearly twice as quickly as another isolate (Moorhouse *et al*., 1993).

Thus, *M. anisopliae* might have evolved for rapid vegetative growth (Table 3.1) and quick sporulation (Tables 3.1, 3.2, Fig. 3.1), whereas *B. bassiana* has developed to more slowly produce a total large number of conidia. In attempts to induce the epizootics in populations of soil-dwelling insects, *M. anisopliae* seemingly should be better adapted than *B. bassiana* to insects with social defensive behaviors due to its high growth rate and quick sporulation on cadavers. On the other hand, *B. bassiana* might be better adapted to non-social insects, because its high total sporulation on cadavers should enhance population densities of fungal propagules in soil (Rath *et al.*, 1995).

In vitro sporulation of *B. bassiana* was 5 – 89X greater among different isolates than *in vivo*, and the difference was 6 – 232X in *M. anisopliae*. Nutrient-rich agar apparently provided a large surface area on which conidia yield was greater than in vivo, even though the same amount of inoculum was applied on both substrates (Table 3.1). The quality of nutrients also may be an important factor affecting sporulation, because all components of a termite cadaver may not be easily utilized by fungus without the help of a secondary enzyme system (Huxham *et al*., 1989). Among the fungal isolates, sporulation *in vitro* generally was correlated with conidial production *in vivo*. This may allow preliminary *in vitro* screening of a large number of isolates for high *in vivo* sporulation. Of course, a high level of *in vitro* sporulation is a valuable characteristic in itself, allowing for mass production on a commercial scale.

An interesting continuation of this research would be to compare the capability of isolates with good quick and total sporulation versus those with high virulence (Fuxa and Tanada, 1987; Sun *et al*., 2001, see chapter 2) in producing epizootics in *C. formosanus* populations. This would help answer fundamental questions about the relative importance of virulence in entomopathogen epizootics and microbial control.

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CHAPTER 4

EFFECTS OF VIRULENCE, SPORULATION, AND TEMPERATURE ON *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TRANSMISSION IN *COPTOTERMES FORMOSANUS* LABORATORY MICROCOSMS

INTRODUCTION

Deuteromycete entomogenous fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, have major advantages for insect biological control. The primary reasons for interest in these fungi (Fuxa, 1997; McCoy *et al*., 1988) are their entry by contact, replication in target insects (Ferron, 1978; McCoy *et al*., 1988; Roberts and Humber, 1981), safety to non-target organisms (Hokkanen and Lynch, 1995; Howarth, 1991), numerous strains (St. Leger *et al*., 1992), and *in vitro* mass-culture (Jackson *et al*., 2000). They are a promising alternative to chemical control for termites (Grace, 1997). However, the Formosan subterranean termite, *Coptotermes formosanus*, poses special problems in biological control with these fungi as a soil-dwelling, social insect (Jackson *et al*., 2000). These termites are hard to detect until the sudden appearance of damage (Su and Tamashiro, 1987), and it is problematic to inoculate pathogens directly onto most members of a colony that may contain millions of individuals (Su and Scheffrahn, 1998; Stimac and Pereira, 1997).

For these reasons, an inoculative augmentation approach may be promising for termite control with fungi (Fuxa, 1987; 1995). A fungus introduced through attractant-baited traps (Stimac and Pereira, 1997) has the potential to replicate and spread to produce epizootics in termite population (Fuxa *et al*., 1998; Fuxa and Tanada, 1987). Termite grooming behavior and proctodeal trophallaxis could hasten the spread of fungal disease through a termite colony (Grace and Zoberi, 1992; Kramm and West, 1982; Kramm *et al*., 1982; Rosengaus and Tranillo, 1997).

Fungal virulence has been used as the most important criterion for selecting fungal species or isolates for termite microbial control (Almeida *et al*., 1997; Bao and Yendol,

1971; Hanel, 1982a; Lai, 1977; Lai *et al*., 1982; Wells *et al*., 1995; Zoberi and Grace, 1990). This has not resulted in successful field trials of fungi against termites (Kramm *et al*., 1982; Sajap *et al*., 1997; Zoberi, 1995). In one study (Milner *et al*., 1998), the *M. anisopliae* isolate most effective against termites in laboratory and field experiments was not the most virulent.

It is possible that sporulation could be an important criterion for selection of fungal isolates for termite control. Termite colonies are rarely infected naturally (Hajek and St. Leger, 1994; Milner, 1997; Milner and Pereira, 2000; Roberts and Humber, 1981), mainly because they wall off or isolate the infected colony members (Logan *et al*., 1990; Reese, 1971; Su *et al*., 1982; Wood and Sands, 1978; Zoberi, 1995). Quick sporulation after an infected termite dies may be advantageous in overcoming such defense behavior. A fungus that sporulates quickly might be transmitted from mycosed termites to unexposed nestmates when the latter attempt to isolate the spore-covered cadavers. The total number of conidia produced on a termite cadaver is another sporulation parameter that could pertain to fungal epizootics (Fuxa *et al*., 1998) but has received little attention in research (Hanel, 1982a).

Another factor that could be as important as virulence in selecting fungal isolates for termite control is tolerance of high temperatures. Termites usually maintain their colonies at 30-35°C, which is above the optimum temperature for conidial germination and growth of most isolates of *M. anisopliae* and *B. bassiana* (Greaves, 1964; Li, 1984; Milner *et al*., 1998). This suggests that most isolates of these two fungal species would not be able to reproduce, spread, and cause disease epizootics in termite colonies (Fargues, *et al*., 1997; Hywel-Jones and Gillespie, 1990; Milner *et al*., 1998).

Our purposes were to test whether quick sporulation, total sporulation, or virulence of *B. bassiana* and *M. anisopliae* contributed more to development of epizootics in termites held in laboratory microcosms. We also examined the effect of the high temperature (35°C) on conidial germination and on fungal epizootics in termite microcosms.

MATERIALS AND METHODS

Termites Worker and soldier termites of *C. formosanus* were collected from a colony infesting trees in Lake Charles, Louisiana. Collected termites were reared at room temperature in plastic containers with sand and wetted corrugated cardboard until use.

Conidial suspension Our techniques were adapted from those of Wells *et al*. (1995) for fungal growth and suspension. The fungi were grown on Sabouraud dextrose agar + yeast (SDAY) at 27 °C. Conidia were harvested under sterile conditions by flooding the plate with sterile distilled H_2O and then scraping the colony with forceps. Conidia were stirred into suspension for 20 min in 100 ml 0.05% Triton X-100 and distilled water, which was then filtered through cheesecloth. The concentration of conidia was determined with a hemocytometer (Petroff-Hausser, Hausser Scientific Partnership, Horsham, PA). The stock suspension of each isolate was diluted to the LD_{99} (see chapter 2).

Fungal isolates Eleven isolates each of *M. anisopliae* and *B. bassiana* were considered for experiments in the current study based on their quickness of sporulation and total sporulation on *C. formosanus* (see chapter 3) and on their virulence (LD_{50}) (see chapter 2). Quick sporulation and total sporulation were defined by the number of conidia per dead insect at 2 days and at 11 days after termite death, respectively. Within each species, pairs of isolates were chosen to provide at least one pairing to test each of four hypotheses: 1) that quick sporulation was more important to epizootics than virulence; 2) that total sporulation

was more important than virulence; 3) that quick sporulation was more important than total sporulation; and 4) that an isolate superior in all three parameters would better produce epizootics than an inferior isolate. This resulted in seven isolates of *M. anisopliae* and five isolates of *B. bassiana* being chosen for the experiments; these are characterized from previous research (see chapters 2 and 3) in Table 4.1.

Effect of fungal isolate on transmission Each replication at each treatment (fungal isolate) was tested in a laboratory microcosm. Each microcosm consisted of a transparent plastic box (18×8×4 cm, Pioneer Packaging Co., North Dixon, KY), which had three identical chambers (5.6×8.0×4.0 cm) with small openings (0.5cm diam.) at the bottoms of each of the two inner walls between the chambers. Twenty grams of #4 sterilized fine blasting sand moistened with 4.6 ml sterile distilled water were pressed lightly into the first and third chambers. The central chamber contained a sterile cotton pad (55×80 mm) (VWR Scientific, Media, PA) moistened with 9 ml sterile water on the bottom of the chamber. Four pieces of moist balsa wood (*Ochroma lagopus* Swartz), each measuring 8×55×1.5 mm, were placed on-end on the cotton pad to provide termite food and shelter. The balsa-wood pieces were dried in an oven (Model 19200, Thermolyne Corp., Dubuque, Iowa) at 150°C for two hours and then weighed before being moistened and placed in the chamber.

One hundred and fifty termites including 15 soldiers were introduced into the first chamber, and another 15 termite workers treated with fungal suspension by topical application were introduced into the third chamber. For topical application, termites were anesthetized for 1 min by placing them in a Petri dish set on an ice pack (20×17×4 mm),

Table 4.1 Characteristics of fungal isolates used in the experiments [a]

Fungal isolate	Virulence (LD_{50}, conidia$\times 10^5$ per insect)	Sporulation as conidia$\times 10^5$/insect (days post-death)	
		Quick (2d)	Total (11d)
M. anisopliae			
F3045	0.08 d	6.14 a	471 b
G7152	0.70 b	4.62 a	269 bc
F724	0.56 b	2.27 a	54 d
Bioblast	0.09 d	0.27 b	833 a
G8032	1.22 a	0.07 b	364bc
G6180	1.08 a	0.35 b	220 c
F794	0.30 c	1.43 a	40 d
B. bassiana			
F714	0.05 c	6.57 a	1097 a
F356	0.80 b	1.50 c	72 c
W7191	4.65 a	6.07 a	613 ab
W8031	4.96 a	2.76 b	570 b
W7150	0.67 b	0.70 d	602 b

[a] Fungal isolates in a species were selected to maximize the differences in virulence, quick sporulation, and total sporulation for testing effects on disease epizootics in termites. Means in each column of each fungal species followed the same letter are not significantly different at $P < 0.05$ (see Chapter 2 and 3).

and 1 μl of suspension of conidia at the LD_{99} was applied to the ventral surface of each termite with a micro-dispenser (Drummond Scientific Co., Broomall, PA). The treated termites were released into the third chamber of each experimental unit after the droplet dried. Each microcosm was then covered with its lid, the edges of which were sealed with parafilm. The microcosm was incubated at 27°C without light for15 d.

The survival of termite workers and soldiers was checked for each microcosm after 15 d incubation, when the balsa wood pieces in each container were collected and dried at 150°C in an oven (Model 19200, Thermolyne Corp., Dubuque, Iowa) for two hours and weighed to determine food consumption. Additionally, cadavers encased in sand cemented with termite excreta were found on the surface of the foraging arena, and these were counted. Control microcosms for each fungal species were identical to treatment microcosms except that 0.05% Triton X-100 in sterile water was the inoculum. There were four replications (microcosms) for each fungal isolate and control.

Effects of Temperature on transmission Conidial suspensions of each fungal isolate were prepared as above and serially diluted to 10^5 conidia/ml. One hundred μl of spore suspension was inoculated onto SDAY in a Petri dish (100×15mm) and spread over the agar with a sterile spreader (30mm wide) on an inoculating turntable (114mm, VWRbrand®, VWR Co., OH). Each isolate of each fungal species in Table 1 was grown at two different temperatures, 27°C and 35°C, and at two different incubation times, 24 h and 48 h. There were four plates or replications for each temperature/time combination for each isolate. After incubation for the designated time and temperature, each plate was fixed and stained with lactophenol and cotton blue (Becnel, 1997), which terminated spore development. After the fixative was absorbed into medium, three 2×5-cm pieces of

agar in each plate were excised with a micro-scalpel and placed separately on glass slides with coverslips. These were examined for spore germination, which was defined as emergence of a germ-tube at least half of the conidial length. At least 100 spores were sampled from each slide.

Based on the observed germination rates at 35ºC for 24 h incubation, two isolates of *M. anisopliae*, F3045 and Bio-blast, and four isolates of *B. bassiana*, F714, F356, W7150, and W7191, were then tested in a disease transmission experiment. Within each species, these isolates had the greatest differences in germination to test effects on epizootics. All subsequent procedures were the same as above except that the microcosms were incubated at 35°C. Sterile distilled water was added to all three chambers of each microcosm every other day to compensate for increased water loss at the high temperature.

Data analysis The effects of fungal isolates on germination rate, colony mortality, food consumption, and numbers of encased cadavers were compared statistically by analysis of variance through the SAS General Linear Model Procedure (one-way ANOVA) with the Tukey HSD test (SAS Institute, 1996). The effects of fungal species, isolate, and incubation temperature on termite mortality were analyzed by the SAS General Linear Model Procedure with the sliced effect for interactions (SAS Institute, 1996). Percentage mortality and percentage germination were log-odds transformed prior to data analysis.

RESULTS

Fungal isolate experiment The defensive behavior of isolating dead termites in walled-off parts of the nest (necrophoresis) was observed in every microcosm with treated termites. Three to four days after fungal treated termites were added to each

microcosm, cadavers coated with sand or chewed wood cemented with termite excreta began appearing in piles. These piles were formed in all three chambers of the microcosm but were most commonly found in the central, food chamber. Cadavers on which conidia had formed were never found singly encased with sand during the course of experiments.

Isolates of *M. anisopliae* ($F = 111.35$; $df = 7, 24$; $P < 0.0001$) and *B. bassi*ana ($F = 40.71$; $df = 5, 18$; $P < 0.0001$) significantly affected total termite mortality (Table 4.2). Total mortality ranged from 24.2 to 64.3% in *M. anisopliae* treatments and 25.8 - 37.6% with *B. bassiana* (Table 4.2). Worker mortality was significantly higher for every fungal treatment than for controls. Only one isolate (*M. anisopliae* isolate F724) caused significantly more soldier mortality than in the control. Termites treated with *M. anisopliae* had significantly higher total mortality than those treated with *B. bassiana*.

No isolate reduced food consumption compared with controls, but termites treated with *M. anisopliae* consumed more wood than those treated with *B. bassiana* (Table 4.2).

Fungus-treated termites formed significantly more encased cadaver piles than in untreated controls ($P < 0.05$, Table 4.2) after treatment with every isolate except *B. bassiana* F714 ($P < 0.05$, Table 4.2). This parameter did not differ significantly between the two fungal species.

Temperature experiment The effects of temperature on conidial germination at 24 h were significant in *M. anisopliae* ($F = 22.47$; $df = 1, 14$; $P = 0.0003$) and *B. bassiana* ($F = 29.44$; $df = 1, 30$; $P < 0.0001$). Similarly, the germination rates at 48 h were also affected by temperature in *M. anisopliae* ($F = 132.36$; $df = 1, 14$; $P < 0.0001$) and *B. bassiana* ($F = 605.64$; $df = 1, 30$; $P < 0.0001$). High temperature (35°C) caused low germination rates

Table 4.2 Effects of *M. anisopliae* and *B. bassiana* isolates on mortality, food consumption, and numbers of encased cadavers of *C. formosanus* contained in laboratory microcosms [a]

Fungal isolate	Number of cadaver piles (± SE)	Food consumed (mg ± SE)	% Mortality (± SE)		
			Workers	Soldiers	Total
M. anisopliae					
F3045	3.3±0.25 d	65.5±4.19 ab	68.2±0.57 a	25.0±5.69 ab	64.2±0.43 a
G7152	6.5±0.29 ab	78.0±4.95 a	67.8±3.57 a	23.3±4.30 ab	63.8±3.56 a
F724	4.0±0.41 cd	48.3±5.60 b	54.5±2.38 b	35.0±5.00 a	52.7±3.93 ab
Bioblast	6.8±0.48 ab	50.5±7.27 ab	56.0±0.47 b	10.0±1.92 b	51.8±0.39 b
G8032	7.5±0.29 a	65.5±4.77 ab	31.3±2.62 c	11.7±4.19 b	29.5±2.31 c
G6180	4.3±0.25 cd	71.0±7.11 ab	29.2±2.56 c	13.3±4.71 ab	27.7±2.09 c
F794	5.3±0.25 bc	70.3±9.54 ab	25.0±1.23 c	20.0±7.20 ab	24.2±1.37 c
Control	1.0±0.41 e	55.0±7.77 ab	6.8±0.57 d	8.3±5.00 b	7.0±0.91 d
Mean	4.8±0.51	63.0±2.57 *	42.4±3.82 *	18.3±2.18	40.1±3.56 *
B. bassiana					
F714	3.5±0.50 cd	45.3±10.64 a	39.8±1.83 a	10.0±4.30 a	37.6±1.78 a
F356	8.3±0.63 a	46.5±8.61 a	35.0±4.43 ab	41.7±8.33 a	33.8±3.98 ab
W7191	5.3±0.48 bc	66.8±1.49 a	35.2±1.97 ab	20.0±11.55 a	33.5±2.21 ab
W8031	6.8±0.48 ab	53.5±3.59 a	29.0±1.00 ab	10.0±7.93 a	27.3±1.38 ab
W7150	4.0±0.41 c	44.0±1.68 a	10.0±5.77 b	25.8±1.89 a	25.8±1.89 b
Control	1.3±0.63 d	43.8±5.17 a	7.7±3.19 c	11.7±3.19 a	8.5±0.55 c
Mean	4.8±0.37	49.9±2.82	28.9±2.34	17.2±3.59	27.7±2.13

[a] Data were subjected to a GLM one way ANOVA for each species. Means in each column followed by the same letter did not differ significantly at $P < 0.05$, Tukey HSD. Overall parameter means within each species did not include the control, and overall means with an asterisk indicate significant differences between two fungal species at $P < 0.05$.

Table 4.3 Effects of fungal isolate on spore germination (preliminary experiment) and on mortality, food consumption, and numbers of encased cadavers of *C. formosanus* contained in laboratory microcosms at different temperatures [a]

Fungal Isolate	Temperature (°C)	% Germination [b]		Number of cadaver piles (± SE)	Food consumed (mg ± SE)	% Mortality (± SE)		
		24 h	48 h			Workers	Soldiers	Total
M. anisopliae								
F3045	27	30.1 ±1.30 A	89.0 ± 1.03 B	3.3 ± 0.25 B	65.5 ± 4.19 A	68.2 ± 0.57A	25.0 ± 5.69 A	64.2 ± 0.43 A
Bioblast	27	35.4 ± 1.22 A	97.1 ± 0.60 A	6.8 ± 0.48 A	50.5 ± 7.27 A	56.0 ± 2.32 B	10.0 ±1.92 B	51.8 ±0.39 B
Control	27	/	/	1.0 ± 0.41 C	55.0 ± 7.77 A	6.8 ± 0.57 C	8.3 ± 4.99 B	7.0 ± 0.91 C
F3045	35	12.6 ± 0.96 b	29.7 ± 1.30 b	0.3 ± 0.25 b	22.8 ±2.72 a	16.5 ± 0.74 b	20.0 ±2.72 a	16.8 ± 0.83 b
Bioblast	35	24.4 ± 1.22 a	35.4 ± 0.92 a	3.0 ± 0.41 a	23.8 ±2.53 a	29.8 ± 2.49 a	23.3 ±7.93 a	29.4 ± 2.69 a
Control	35	/	/	0.5 ± 0.29 b	33.0 ±5.07 a	15.0 ± 1.11 b	3.3 ±1.92 b	13.9 ± 1.08 b
B. bassiana								
F356	27	24.3 ± 1.13 B	90.9 ± 0.25 B	8.3 ± 0.632 A	46.5 ± 8.61 A	35.0 ± 4.43 AB	41.7 ± 8.33 A	33.8 ±3.98 AB
F714	27	36.5 ±1.13 A	93.6 ± 1.08 B	3.5 ± 0.50 BC	45.3 ±10.64 A	39.8±1.83 A	10.0 ± 4.30 B	37.6 ±1.78 A
W7150	27	29.4 ±1.43 A	96.9 ± 0.71 A	4.0 ± 0.41 B	44.0 ± 1.68 A	27.0 ± 2.59 B	10.0 ± 5.77 B	25.8 ± 1.89 B
W7191	27	30.2 ± 0.89 A	97.1 ± 0.32 A	5.3 ± 0.48 B	66.8 ± 1.49 A	35.2 ± 1.97 AB	20.0 ±11.55 AB	33.5 ±2.22 AB
Control	27	/	/	1.3 ± 0.63 C	43.8 ± 5.17 A	7.7 ± 0.79 C	11.7 ± 3.19 B	8.5 ± 0.55 C

(Table con'd.)

F356	35	15.9 ± 1.77 bc	32.1 ± 1.29 a	1.0 ± 0.41 a	22.8 ± 2.49 a	22.7 ± 4.70 a	16.7 ± 3.33 a	22.1 ± 4.18 a
F714	35	18.9 ± 1.73 b	26.4 ± 1.17 b	1.8 ± 0.25 a	27.0 ± 4.45 a	25.0 ± 2.74 a	16.7 ± 7.93 a	24.2 ± 2.65 a
W7150	35	10.7 ± 0.97 c	25.3 ± 0.68 b	1.0 ± 0.41 a	20.3 ± 2.56 a	18.8 ± 2.06 a	16.7 ± 1.92 a	18.6 ± 2.02 a
W7191	35	27.4 ± 0.83 a	26.7 ± 1.67b	0.5 ± 0.29 a	18.3 ± 1.55 a	22.7 ± 3.34 a	20.0 ± 6.09 a	22.4 ± 3.20 a
Control	35	/	/	0.5 ± 0.29 a	31.0 ± 4.78 a	14.0 ± 1.83 a	8.3 ± 1.67 a	13.5 ± 1.61 a

[a] Data were subjected to one way ANOVA in GLM. Means in each column within each temperature and within fungal species followed by the same letter (capital letters for 27°C, lowercase letters for 35°C) did not differ significantly at $P < 0.05$, Tukey HSD.
[b] Conidia ($n \geq 100$) forming a germ tube at least half of the length of the conidium.

Table 4.4 Analysis of variance for total termite mortality as dependent variable in the temperature experiment [a]

Tested effects	DF	F	Pr > F	Sliced effect for interaction
Species	1, 36	50.8	<0.0001	/
Isolate (species)	4, 36	3.3	0.0219	/
Temperature	1, 36	176.6	<0.0001	/
Temperature by Species	1, 36	40.8	<0.0001	b
Temperature by Isolate (species)	4, 36	5.9	<0.0010	c
Overall effects	11, 36	24.9	<0.0001	/

[a] GLM was used for analysis of data represented in Table 3.
[b] Temperature effect within each species was significant at $P < 0.05$
[c] Temperature effect within each isolate was significant at $P < 0.05$

of conidia in both fungal species at 24 h and 48 h (Table 4.3).

Temperature also affected termite total mortality (Tables 4.3, 4.4). Correlations between conidial germination rates (at 24 h and 48 h) and total termite mortality were significant and positive for *M. anisopliae* ($R^2 = 0.671$, n = 16, P = 0.0001 at 24 h; $R^2 = 0.841$, n = 16, P < 0.0001 at 48 h) and *B. bassiana* ($R^2 = 0.385$, n = 32, P = 0.0002 at 24 h; $R^2 = 0.432$, n = 32, P < 0.0001 at 48 h). Most notably, *M. anisopliae* isolate Bioblast had a higher germination rate at 35°C and higher termite total mortality than isolate F3045 (Table 4.3). Sliced effects for interactions between temperature and fungal species, as well as between temperature and fungal isolates, were significant (P < 0.05, Table 4.4), indicating that temperature effects between two fungal species or among isolates of each species were significant. Significant differences in termite total mortality and numbers of encased cadaver piles were detected among isolates of *M. anisopliae* at 27°C and 35°C. *B. bassiana* isolates caused significant differences in total mortality, food consumption, and numbers of encased cadaver piles at 27°C but not at 35°C (Table 4.3).

DISCUSSION

The current research was based on an assumption that factors other than pathogen virulence are important to fungal epizootics and microbial control of termites (Fuxa, 1987; 1989; Fuxa *et al.,* 1998). In order to analyze and compare the effects of different fungal characteristics on disease epizootics in colonies of *C. formosanus* held in laboratory microcosms, the isolates tested in the experiments for each species (Table 4.1) were chosen in pairs such that at least one pair tested each of four hypotheses: 1) that total sporulation is more important to epizootics than virulence, 2) that quick sporulation is more important than virulence, 3) that quick sporulation is more important than total sporulation,

and 4) that an isolate superior in all three parameters would produce better epizootics than an inferior isolate. However, testing four hypotheses within each species resulted in experiments with numerous isolates and multiple parings relating to each hypothesis. All isolate pairs relating to each hypothesis must be considered in the current discussion, so a summary of pertinent isolate pairs is presented in Table 4.5.

Sporulation characteristics of *M. anisopliae* may have a greater effect than virulence on disease epizootics in colonies of *C. formosanus* held in the microcosms. Isolate pairs 1 and 6 strongly supported hypotheses 1 and 2, respectively. The other isolate pairs (2, 3, 4, 5, 7, and 8) that differed significantly in the pertinent pair of characters permitted no clear conclusion due to the third, confounding fungal characteristic (Table 4.5). These results suggest that high virulence might not be the most important criterion for selecting pathogens in insect microbial control, although pathogen virulence is interrelated with disease transmission in host populations (Fuxa, 1987). Fungal isolates producing more conidia or producing them more quickly may simply counteract the advantage of a fungal isolate with greater virulence by getting more conidia to the target termite (Fuxa *et al*., 1998; Kramm *et al*., 1982; Milner, 1997).

Fungal virulence has been the most used criterion for selecting fungal species or isolates in termite microbial control (Almeida *et al*., 1997; Bao and Yendol, 1971; Hanel, 1982a; Lai, 1977; Lai *et al*., 1982; Wells *et al*., 1995; Zoberi and Grace, 1990). *B. bassiana* produced epizootics in a field population of grass grub (*Costelytra zealandica*) at Matangi, New Zealand (Townsend *et al*., 1995); the fungal isolate collected from this location, however, was not as virulent as other isolates in laboratory bioassays. Similarly, a *M. anisopliae* isolate most effective against termites in laboratory and field experiments was not the most virulent (Milner *et al*., 1998). High virulence may be a disadvantag

Table 4.5 Fungal characteristics relating to hypotheses regarding epizootics of *M. anisopliae* and *B. bassiana* in *C. formosanus* held in a laboratory microcosm [a]

Isolate pair number	Isolates [b]	Difference between isolates [c]			Conclusion about hypothesis [g]
		Virulence [d]	Quick sporulation [e]	Total sporulation [f]	
M. anisopliae		**Hypothesis 1: Total sporulation effect > virulence**			
1	G7152 *	**Low**	no significant	**High**	A
	F794	**High**	difference	**Low**	
2	F724 *	**High**	High	**Low**	C
	G8032	**Low**	Low	**High**	
3	F724 *	**High**	High	**Low**	C
	G6180	**Low**	Low	**High**	
4	F794	**High**	High	**Low**	C
	G8032	**Low**	Low	**High**	
5	F794	**High**	High	**Low**	C
	G6180	**Low**	Low	**High**	
		Hypothesis 2: Quick sporulation effect > virulence			
6	G7152*	**Low**	**High**	Low	A
	Bioblast	**High**	**Low**	High	
7	F724	**Low**	**High**	Low	B
	Bioblast	**High**	**Low**	High	
8	Bioblast *	**High**	**Low**	High	C
	F794	**Low**	**High**	Low	
		Hypothesis 3: Quick sporulation effect > Total sporulation			
9	F3045 *	no significant	**High**	**Low**	A
	Bioblast	difference	**Low**	**High**	
10	G7152 *	Low	**High**	**Low**	A
	Bioblast	High	**Low**	**High**	
11	F724 *	High	**High**	**Low**	C
	G8032	Low	**Low**	**High**	

(Table con'd.)

12	F724 *	High	**High**	**Low**	C
	G6180	Low	**Low**	**High**	
13	F794	High	**High**	**Low**	D
	G8032	Low	**Low**	**High**	
14	F794	High	**High**	**Low**	D
	G6180	Low	**Low**	**High**	
15	F724	Low	**High**	**Low**	B
	Bioblast	High	**Low**	**High**	
16	Bioblast *	High	**Low**	**High**	C
	F794	Low	**High**	**Low**	
		Hypothesis 4 : High sporulation/virulence effect > low			
17	F3045	**High**	**High**	**High**	A
	G6180	**Low**	**Low**	**Low**	
B. bassiana		**Hypothesis 1 : Total sporulation effect > virulence**			
1	F356	**High**	Low	**Low**	E
	W8031	**Low**	High	**High**	
2	F356	**High**	Low	**Low**	E
	W7191	**Low**	High	**High**	
		Hypothesis 2 : Quick sporulation effect > virulence			
3	W7150	**High**	**Low**	no significant	F
	W7191	**Low**	**High**	difference	
4	W8031	**Low**	**High**	no significant	F
	W7150	**High**	**Low**	difference	
5	F356	**High**	**Low**	Low	E
	W8031	**Low**	**High**	High	
6	F356	**High**	**Low**	Low	E
	W7191	**Low**	**High**	High	
		Hypothesis 3 : Quick sporulation effect > Total sporulation			
7	F356	no significant	**High**	**Low**	F
	W7150	difference	**Low**	**High**	

(Table con'd.)

		Hypothesis 4 : High sporulation/virulence effect > low			
8	F714 * W7150	**High** **Low**	**High** **Low**	**High** **Low**	A
9	F714 F356	**High** **Low**	**High** **Low**	**High** **Low**	E
10	F714 W8031	**High** **Low**	**High** **Low**	**High** **Low**	E

[a] Four hypotheses were proposed to test the relative effects of fungal characteristics on epizootics of entomogenous fungi in termite colonies; for example, hypothesis 1 is that an isolate with better total sporulation will cause greater disease prevalence than an isolate with greater virulence.

[b] See text for explanation. The asterisk (*) indicates significantly higher total termite mortality ($P < 0.05$) from Table 4.2.

[c] "Low" and "high" denote a significant difference; bold lettering designates the fungal characteristics pertinent to that particular hypothesis.

[d] Determined by LD_{50} (conidia/ termite) (see chapter 2).

[e] *In vivo* fungal sporulation (number of conidia/termite cadaver) on day 2 after death (see chapter 3).

[f] *In vivo* fungal sporulation (number of conidia/termite cadaver) on day 11 after death (see chapter 3).

[g] Lettering indicates the following conclusions: A) supports the tested hypothesis; B) weakly supports hypothesis; C) results not inconsistent with hypothesis, but confounding characteristic permits no clear conclusion; D) support the alternative hypothesis; E) weakly support alternative hypothesis; F) Inconclusive.

in transmission, because less severely infected termites live longer, are more ambulatory, and have a greater tendency to spread spores (Kramm *et al*., 1982). In a study of an entomopathogenic virus in greenhouse microcosms, a recombinant isolate with improved virulence could not compete for insect hosts with an isolate having lower virulence but a greater reproduction rate (Lee *et al*., 2001).

The results with *M. anisopliae* did not consistently support the hypothesis, or its alternative, that quick sporulation was more important total sporulation in causing epizootics in the termite colonies (9 through 16, Table 4.5). An isolate such as F794, which produced low mortality in the microcosms (Table 4.2), may have certain untested features, such as repellency. Staples and Milner (2000) reported that termites often retreated from a sporulated substrate after initial contact and sealed off tunnels by plugging with sand, due to fungal repellency, thereby preventing further contact with the fungal conidia. Perhaps the quickly sporulating isolate F794 initiates such behavior if it is repellent, whereas other isolates of *M. anisopliae* (e. g. F3045, G7152) with quick sporulation may be less repellent to termites, leading to spore transmission by termite social contacts before unexposed individuals can isolate the spore-covered cadavers. It is clear that further research on the effects of characteristics other than virulence and sporulation on epizootics is necessary.

Pair 17 (Table 4.5) demonstrated that high virulence and sporulation in the same isolate (F3045) was advantageous in epizootics. This isolate produced the greatest total mortality of any *M. anisopliae* isolate, significantly higher than all but two others (Table 4.2). Thus, the combination of quick sporulation, total sporulation, and virulence may be desirable for fungi used in the seasonal colonization approach to control of termites (Fuxa, 1987; Fuxa *et al*., 1998; Almeida *et al*., 1997).

Unlike *M. anisopliae*, the results with *B. bassiana* did not clearly support any of the four hypotheses (Table 4.5). This indicates that other characteristics of this species might play a defining role in epizootics. Evidence is accumulating that termite behavior (Staples and Milner, 2000) and defensive chemicals (Rosengaus, *et al*., 1998a; Wright, *et al*., 2000) limit the spread of fungal spores in a termite colony.

The significantly higher termite mortality caused by *M. anisopliae* than by *B. bassiana* (Table 4.2) may be due to different *in vivo* sporulation patterns (see chapter 3) or different levels of fungal repellency between *B. bassiana* and *M. anisopliae* (Staples and Milner, 2000).

A common phenomenon in *C. formosanus*, as with many termite species, is to defend the colony by isolating infected nestmates from healthy ones in encased cadaver piles. This significantly reduces disease transmission, particularly in a soil substrate (Boucias *et al*., 1996; Page, 1967; Roberts and Humber, 1981; Rosengaus *et al.*, 1998b). Fungal isolates in the current research differed in the degree to which they evoked this defensive response (Table 4.2), but the cues that might have triggered that difference are unknown (Prestwich, 1984). It might be expected that quick sporulating isolates would induce isolation of fewer cadaver piles than slow isolates, but this was not consistently true in the current research (Tables 4.1, 4.2).

In selecting fungal isolates for use against termites, it is important to take into account high nest temperatures (30-35ºC) maintained by *C. formosanus* (Li, 1984), because high temperatures adversely affect entomogenous fungi (Arthurs and Thomas, 2001; Hanel, 1982b; Hanel and Watson, 1983; Hywel-Jones and Gillespie, 1990; Milner, 1997). High temperature in general reduced fungal germination rates and disease epizootics in the

termite microcosms (see Results and Tables 4.3, 4.4), although the *M. anisopliae* isolate Bioblast at 35ºC was the only case in which a significantly higher germination rate was associated with higher termite mortality (Table 4.3). Conidial germination of *M. anisopliae* also was associated with mortality of mosquito larvae, *Culex pipiens pipiens* (Daoust and Roberts, 1982). The significant temperature effects between fungal species and among isolates (Table 4.4) suggest that fungi can be chosen to improve termite control at high temperatures. Strains of *M. anisopliae* generally have a higher temperature profile for germination than *B. bassiana* (Inglis *et al*., 1997; Milner, 1997).

Thus, the current research supports the idea that fungal characteristics other than virulence, such as sporulation and temperature tolerance, can determine the potential success of seasonal colonization agents for *C. formosanus* and other insects. However, it is clear that further research is necessary to elucidate all of the interactions between this termite and fungal pathogens.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

The purpose of this research was to screen and characterize entomogenous fungi for possible control of the Formosan subterranean termite, *Coptotermes formosanus*. The major research of this dissertation was focused on 1) detecting fungi that are specifically pathogenic to *C. formosanus* by using this termite as a "bait" from collected soil samples; 2) estimating *in vivo* sporulation of isolates of *M. anisopliae* and *B. bassiana* and their virulence against *C. formosanus*; 3) determining whether fungal sporulation, or virulence of *M. anisopliae* and *B. bassiana* contributed more to development of epizootics termites held in microcosms; 4) examining the effects of high temperatures on spore germination and fungal epizootics in termite colony.

Using termites as a "bait" to detect pathogens in soil was an effective way to isolate fungi and is more efficient than isolation of pathogens from dead, field-collected termites. Separating bait termites from their nest significantly increased their chances to be infected, because termite nests are physically and chemically different from adjacent soils. More than 16% of the soil samples collected from Louisiana contained fungi pathogenic to Formosan subterranean termites, either *M. anisopliae* or *B. bassiana*. There were no differences in numbers of fungal isolates from the three sampling locations (New Orleans, Baton Rouge, and Lake Charles). However, significantly more isolates were found in woodlands (eleven) than in pastures (four).

Median lethal doses (LD_{50}s) of these fungal species to *C. formosanus* were quantified, indicating that fungal isolates rather than species had the greatest effect on virulence. Among nine Louisiana and two USDA isolates (from an acridid in Australia and from a delphacid in China) of *B. bassiana*, LD_{50}s ranged from 4.95×10^3 to 4.96×10^5 conidia/termite, a difference of 100X. LD_{50}s of six Louisiana and four USDA isolates of

M. anisopliae (from *C. formosanus* in Hawaii, a chrysomelid in Brazil, a scarab in China, and leaf litter in Wisconsin) ranged from 7.89×10^3 to 1.22×10^5 conidia/termite. USDA culture number F714 of *B. bassiana*, which was isolated from a delphacid in China, was the most virulent isolate we tested. The most virulent Louisiana fungus isolated from soil was a strain of *B. bassiana* found in a pasture near Baton Rouge. *In vitro* growth characteristics were significantly correlated with virulence against termites, suggesting that fungal virulence might be predicted based on colony morphology rather than by bioassay.

Fungal isolates of *B. bassiana* and *M. anisopliae* differed greatly in their capability to sporulate *in vivo*. Conidial production increased significantly over 11 days post-death. Effects of isolates of *M. anisopliae* and *B. bassiana* on *in vivo* sporulation were significant. Although the overall effects of fungal species on *in vivo* sporulation were not significant, the interactions between fungal species and certain times post-death were significant, indicating different sporulation patterns between the two fungal species. *B. bassiana* isolates could be categorized into a group with high total sporulation (day 11) and low quick sporulation (on days 2 and 3), while *M. anisopliae* isolates fell into another group with high quick sporulation and low total sporulation. This could give *M. anisopliae* an advantage over *B. bassiana* in termite microbial control due to termite defensive social behaviors. Conidial production was significantly higher *in vitro* than *in vivo*. *In vitro* and *in vivo* sporulation differed by as much as 89X and 232X among the selected isolates of *B. bassiana* and *M. anisopliae*, respectively. Correlation between *in vivo* and *in vitro* conidial production was positive and significant. This may allow preliminary *in vitro* screening of a large number of isolates for high *in vivo* sporulation.

Fungal sporulation characteristics *in vivo* were significantly associated with producing epizootics in a termite colony. After fungus-inoculated workers (9.1% of colony) were placed in a microcosm with uninfected termites, termite mortality increased significantly than that of an untreated colony, which ranged from 24.2 to 64.3% in *M. anisopliae* and 25.8-37.6% in *B. bassiana*. Fungal species and isolate significantly affected disease prevalence in termite populations due to different fungal characteristics. Sporulation in *M. anisopliae*, rather than virulence of a fungus, played a more define role in producing epizootics in termite colony. The quick sporulation of *M. anisopliae* did not consistently show the better epizootics in termites than total sporulation among different isolates. But quick sporulation may be advantageous in coping with termite defensive behavior (necrophoresis) than total sporulation. For possible success in termite microbial control, the sporulation characteristics of a fungus should be considered as an important criterion in selecting fungal isolates.

In selecting fungal isolates for use against termites, it is important to take into account high nest temperature (30-35ºC) maintained by *C. formosanus*. High temperature (35ºC) significantly reduced fungal germination rates of conidia, and led to significant reduction of disease epizootics in termite colonies. The correlations between conidial germination rates (at 24 h and 48 h) and total termite mortality were significant and positive for *M. anisopliae* and *B. bassiana*. The significant temperature effects within different fungal species or isolates indicate that fungi can be chosen to improve termite control at high temperatures.

This research supports the hypothesis that fungal characteristics, other than high virulence, such as sporulation and temperature tolerance, can determine the potential

success of seasonal colonization agents for *C. formosanus* and other insects. *M. anisopliae* seems better adapted than that of *B. bassiana* in producing high prevalence of fungal diseases and coping with high temperature. However, it is clear that further research is necessary to elucidate all of the interactions between this termite and fungal pathogens.

VITA

Jianzhong Sun, the second son in the family, was born on October 2, 1958, in Nantong, People's Republic of China. He obtained his Bachelor of Science degree in plant protection from Nanjing Agricultural University, Nanjing, China, in 1982. In the same year, he received a research assistantship sponsored by Jiangsu Academy of Agricultural Sciences to attend the graduate program under the direction of Dr. Zhengwen Du. He completed the Master of Science degree in entomology from Nanjing Agricultural University, Nanjing, China. During 1985 to 1996, he worked at the Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, China. Jianzhong also was with the Department of Entomology, Auburn University, as a visiting scientist from 1991 to1992 and with the Department of Entomology, Louisiana State University, as a senior visiting scientist from 1996 to 1997. He began his doctoral studies in entomology in 1998 at Louisiana State University, Agricultural Center, under the direction of Dr. James R. Fuxa and Dr. G. Henderson. Mr. Sun is expected to receive the degree of Doctor of Philosophy in May 2002.