Isolation and Evaluation of *Beauveria bassiana* for Control of *Coptotermes formosanus* and *Reticulitermes flavipes* (Isoptera: Rhinotermitidae)

by

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ABSTRACT

Six *Beauveria bassiana* (Balsamo) Vuillemin isolates were obtained from *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki in the U.S. and China. These isolates, plus the *B. bassiana* isolate 26037 from American Type Culture Collection that was isolated from Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) were compared against *C. formosanus* and *R. flavipes* in the laboratory. Most *B. bassiana* isolates caused termite mortality between 4-8d after treatment. The isolates obtained from termites were not significantly more virulent than the standard strain. The relative virulence between the 6 *B. bassiana* isolates and 2 *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates against *R. flavipes* were compared. *Beauveria bassiana* was found to be much less virulent than *M. anisopliae* against *R. flavipes*.

Key Words: *Reticulitermes flavipes*, *Coptotermes formosanus*, *Beauveria bassiana*, *Metarhizium anisopliae*, termites, biological control.

Termites are important urban pests which can cause a tremendous amount of damage to homes and structures. Prevention of termite damage has been a challenge because of their large populations and cryptic behavior. Various methods in termite control were explored in the past including physical, cultural, chemical, and biological methods (Pearce 1997). Among them, biological control is one that received great interest among researchers (Milner & Staples 1996, Grace 1997).

Beauveria bassiana (Balsamo) Vuillemin is a naturally occurring entomopathogenic fungus with a very wide host range (Tanada & Kaya 1993). Comparisons among different *B. bassiana* isolates and with other entomopathogenic fungi (Lai *et al.* 1982, Delate *et al.* 1995, Jones *et al.* 1996, Neves & Alves 2000) revealed differences in isolate virulence against termites. *Beauveria bassiana*, when applied in combination with imidacloprid in the field, effectively controlled *Cornitermes cumulans* (Kollar) colonies and, therefore, reduce the cost and quantities of

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chemical insecticide needed for control of termites (Neves & Alves 1999). Based on its pathogenicity, growth characteristics, and safety, *B. bassiana* was suggested for potential use as a biological control agent against termites (Jones *et al.* 1996, Neves & Alves 2000).

Coptotermes formosanus Shiraki and *Reticulitermes flavipes* (Kollar) are 2 of the most important urban pests in the U.S. (Snyder 1954, Su & Scheffrahn 1988). In the past there were few studies on pathogens associated from these termites (Zoberi & Grace 1990, Weste *et al.* 2001). In an effort to find more virulent *B. bassiana* isolates for control of these termites, we isolated *B. bassiana* associated with dead termites in Mississippi, U.S. and in Hunan and Guangdong provinces, China. This paper reports results of isolation of new *B. bassiana* isolates from termite cadavers and comparison of their virulence and pathogenicity against *C. formosanus* and *R. flavipes* under various conditions. Comparative virulence between *B. bassiana* isolates and *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates also was studied.

MATERIALS AND METHODS

Isolation of Pathogens from Termites. *Reticulitermes flavipes* was collected from dead trees and stumps in MS, U.S. during 1999-2000. *Coptotermes formosanus* was collected in Hunan and Guangdong provinces, China during 1999-2000. A total of 396 collections of *C. formosanus* and 77 collections of *R. flavipes* were made. The termites along with the nesting material or soil were put in plastic containers and were observed in the laboratory for 1-2 months. The rearing containers were either round or rectangular with or without lids. The size of the containers ranged from 0.03 - 0.07m^2. Water was added to the containers every 3-7 days to maintain moisture. The dead termites that appeared in the containers were picked up every 2-4 days and were put in sterile centrifuge vials and stored at –16°C. The bottom of each centrifuge vial was filled (1/3 full) with Drierite™ desiccant (W.A. Hammond Drierite Co. Xenia, OH) to avoid moisture buildup in the centrifuge vials. The dead termites were separated from the dessicant by a piece of sterile filter paper. Isolation of pathogens were conducted within 2 months of collection of the dead termites. Fungal mycelia on the surface of dead termites was touched by a sterile needle and streaked onto potato dextrose agar (Difco, Becton Dickinson and Company) dishes. The dishes were incubated in an unlit incubator (30° C, 82% RH) for 7-14d. Pure cultures were obtained through a series (2-3) of subcultures of the original inoculation on potato dextrose agar. Pure fungal cultures were deposited in the Fungal Collection, USDA ARS, Southern Regional Research Center, New Orleans, LA. Six isolates

of *Beauveria bassiana* and 1 isolate of *M. anisopliae* were obtained from *C. formosanus* and *R. flavipes* (Table 1). The *B. bassiana* isolate 26037 from the American Type Culture Collection, and *M. anisopliae* isolate FI610 from the CSIRO Insect Pathology Culture Collection, Australia were included as standard isolates for comparison purposes. These isolates were previously tested and showed high virulence against termites among other strains of the same fungus (M. Wright, personal communication, Milner *et al.* 1998).

Table 1. Collection records of *B. bassiana* and *M. anisopliae* isolates.

Fungal species	Isolate	Source insect of the isolate	Collection site and date
Beauveria bassiana	2514	*Coptotermes formosanus*	Guangzhou, Guangdong, China; 19-VIII-1999
Beauveria bassiana	2554	*Coptotermes formosanus*	Xifengdu village, Chenzhou, Hunan, China; 2-VI-2000
Beauveria bassiana	2556	*Coptotermes formosanus*	Xiangxiang, Hunan, China; 19-V-2000
Beauveria bassiana	2557	*Coptotermes formosanus*	Zhujiangqiao village, Chenzhou, Hunan, China; 1-VI-2000
Beauveria bassiana	2562	*Reticulitermes flavipes*	Stoneville, MS, USA; 1-II-1999
Beauveria bassiana	2564	*Coptotermes formosanus*	Lumberton, MS, USA; 3-IV-2000
Beauveria bassiana	26037	*Leptinotarsa decemlineata* (Say)	Ivanka pri Dunaji, Czechoslovakia; VI-1965
Metarhizium anisopliae	2563	*Reticulitermes flavipes*	Stone Co., MS, USA; X-2000
Metarhizium anisopliae	FI610	*Coptotermes lacteus* (Froggatt)	Brown mountain, Australia

Preparation of Conidia Suspension. Cultures of all isolates were grown at 30°C in the dark for 7-14d. Conidia suspensions were made by lightly scraping the fungal culture surface with a sterile "L" shaped cell spreader into a 60ml volume plastic container. The conidia clumps were suspended in 40ml 0.01% Tween 80 (ICI Americas Inc.). The suspensions were agitated for 5 min using a Vortex-Genie 2™ laboratory mixer (Daigger & Co., Inc.) to dissociate conidia clumps. The suspensions were then filtered through 1 layer of cloth (36×36 threads/cm^2, threads of 0.3-0.4mm thick) to remove conidia clumps and mycelial debris. Concentrations of the suspensions were determined by counting on a Neubauer hemocytometer under phase-contrast microscopy. Conidia concentrations were diluted to a range of 1×10^2 to 5×10^7/ml. Conidia suspensions were stored at 5°C and used within one day of preparation.

Collection and Preparation of Termites. *Reticulitermes flavipes* was collected from cardboard bait buried near trees in the field in

Stoneville, MS. *Coptotermes formosanus* was collected from cardboard bait buried in the field in New Orleans, Louisiana. The termite colonies were kept in large plastic containers in the laboratory at room temperature (25-28°C) for 0-3 months. One day prior to testing, termite workers (numbers varied according to experiments) of 3rd instar and older were counted and transferred to 90×15mm plastic petri dishes lined with wet filter paper (82cm diameter). Dead and weak termites were replaced with healthy termites immediately before the test. Termite weight was measured in 3 groups of 30 workers by a Sartorius LA 2308 balance (Sartorius North America Inc., New York) 1d before the treatment.

Experimental Design. The virulence of the *B. bassiana* and *M. anisopliae* isolates was compared by a randomized complete block design with each block representing a termite colony. Each experiment had a factorial treatment structure. The factors considered were isolates, conidia concentration (rate), and colony. All experimental units were kept in an unlit incubator at 25°C and 82% RH.

Experiment 1. Comparative Virulence of 6 *B. bassiana* Isolates against Individual *C. formosanus* workers in 96-well Plates. This method was proposed by Grace (1994). *Beauveria bassiana* isolates 2554, 2556, 2557, 2562, 2564, and 26037 were tested against *C. formosanus* workers placed individually on filter paper disks (Whatman grade 1 paper) in the wells of disposable flat-bottomed 96-well polystyrene plates (Daigger & Co., Inc). The well size was 7mm in diameter and 11mm deep. A 14-d-old fungal culture grown at 30°C was used. A droplet of 10ul *B. bassiana* conidia suspension was applied by a pipet to the filter paper disk in each well. The concentrations of the conidia suspensions were 5×10^2, 1×10^3, 5×10^3, 1×10^4, 1×10^5, and 1×10^6/ml. The control was 10ul of 0.01% Tween 80. Each treatment was applied to 3 different colonies of 30 workers. Termite weight of the 3 colonies were (mean ± SE): 2.54 ± 0.02, 3.56 ± 0.06, and 3.02 ± 0.04mg per worker, respectively. Termite mortalities were recorded every day for 15d. The criteria for dead termites were lack of movement when prodded by a sterile needle or only slight movement of antennae and legs after probing.

Experiment 2. Virulence of *B. bassiana* Isolate 2556 against Individual *R. flavipes* in 96-well Plates. This experiment was to compare the dose-mortality responses to *Beauveria bassiana* treatment between *C. formosanus* and *R. flavipes*. Similar to Experiment 1, workers of *R. flavipes* were placed individually on filter paper disks in the wells of disposable flat-bottomed 96-well tissue culture plates. A 7-d-old fungal culture grown at 30°C was used. The concentrations of the conidia suspensions were 2×10^2, 5×10^2, 1×10^3, 5×10^3, 1×10^4, and 1×10^5/

ml. Each treatment was applied to 3 different colonies of 30 workers. Termite weight of the 3 colonies were: 2.45 ± 0.06, 2.47 ± 0.06, and 3.10 ± 0.20mg per worker, respectively. Termite mortalities were recorded every day for 14d.

Experiment 3. Comparative Virulence of 6 *B. bassiana* Isolates against Groups of *C. formosanus* in Petri Dishes. The test was conducted in 50 × 11 mm plastic petri dishes with one 46 ×1 mm filter paper disk per dish (Fisher Scientific, Pittsburgh, PA). Six *B. bassiana* isolates were tested at the rate of 1×10^7 / ml equivalent to 6×10^5 / cm^2 expressed by number of conidia per cm^2 of the filter paper. A 14-d-old fungal culture grown at 30°C was used. One-ml of conidia suspension was applied to the filter paper evenly in 10-15 drops. Thirty termite workers were then added to each petri dish. Each treatment was applied to 4 different colonies. Termite mortality was recorded at 14d.

Experiment 4. Comparative Virulence of the 7 *B. bassiana* Isolates against Groups of *R. flavipes* in Containers with Vermiculite/Sand Medium. The test was conducted in round plastic containers of 5.1cm diameter by 3.5cm high. A southern yellow pine (*Pinus* sp.) block (1.9 × 1.9 × 1.9 cm) was buried at the center of the each container. Then each container was filled with 30cm^3 *B. bassiana* treated medium. The medium was composed of vermiculite:sand:conidia suspension at 16:14:9 by volume. A 13-d-old fungal culture grown at 30°C was used. The concentration of the *B. bassiana* conidia suspension was 4×10^7 / ml equivalent to 1.2×10^7 / cm^3 in the medium. The medium was mixed with *B. bassiana* suspension thoroughly by a sterile plastic stick. The medium in the control was mixed with 0.01% Tween 80. Eighty *R. flavipes* workers were added to each container. Each treatment was applied to 4 different colonies. Termite mortality was recorded at 20d.

Experiment 5. Comparative Virulence of the 6 *B. bassiana* Isolates and 2 *M. anisopliae* Isolates against Groups of *R. flavipes* in Containers with Vermiculite/Sand Medium. Similar to Experiment 4, 6 isolates of *B. bassiana* and 2 isolates of *M. anisopliae* were tested against *R. flavipes* in containers with vermiculite and sand rearing medium. The treatment rate was 1×10^7 / ml equivalent to 3×10^6 / cm^3. This rate was lower than Experiment 4 because preliminary tests showed *M. anisopliae* would cause 50-100% termite mortality at this rate. Each treatment was applied to 4 colonies. Eighty *R. flavipes* workers were added to each container. *Beauveria bassiana* conidia were harvested from an 8-d-old culture at 30°C. *Metarhizium anisopliae* conidia were harvested from an 11-d-old culture. Termite mortality was recorded at 22d.

Statistical Analysis. For tests on individual termites, the median lethal dose causing 50% mortality (LD_{50}) and their 95% fiducial limits

(FL) were determined by probit regression analysis (PROC PROBIT) (SAS Institute 2000). The criteria for a significant difference was non-overlapping fiducial limits of the LD50 values. Mortality data in experiments 3-5 were transformed (arcsine of the square root) and analyzed using Analysis of Variance (ANOVA) for comparison among isolates. PROC MIXED in the SAS software was used (SAS Institute 2000). Means were compared by the Least Significant Difference (LSD) after ANOVA (SAS Institute 2000). One-tailed tests were used for comparisons between isolates and control. Two-tailed tests were used for comparisons among isolates.

RESULTS

Comparative Virulence of the 6 *B. bassiana* Isolates against Individual *C. formosanus* and *R. flavipes* in 96-Well Plates. *Coptotermes formosanus* treated with *B. bassiana* began to exhibit mortality at 4-5d after treatment (Fig. 1). The time-mortality response curves showed that the mortality of the termites caused by *B. bassiana* plateaued at 5-9d for all tested isolates, with higher rates plateauing faster than lower rates. Most of the *C. formosanus* mortality caused by *B. bassiana* occurred between 4-8d. The dose-response patterns of the 6 strains are similar. Comparison of the LD_{50} values of different isolates did not show any significant differences among the isolates with available LD_{50} values (Table 2). In Experiment 2, individual *R. flavipes*-treated *B. bassiana* 2556 showed similar time-response curves as *C. formosanus* (Fig. 2). *Reticulitermes flavipes* began to exhibit mortality at 4d after treatment. The LD_{50} of one colony (weight per worker: 3.1 ± 0.2 mg) at 10 d was 31 (FL: 5-100). Estimates on LD_{50} of the other 2 colonies were not available because of lack of fit to the probit regression model ($P < 0.05$).

Comparative Virulence of 6 *B. bassiana* Isolates against Groups of *C. formosanus* in Petri Dishes. At the rate of 1×10^7/ml, all of the *B. bassiana* isolates except 2556 caused significantly higher mortality to *C. formosanus* than control ($F = 8.2$; df = 6, 18; $P < 0.01$) ($P < 0.10$, LSD) (Fig. 3). Isolate 2556 was significantly less virulent than isolates 2554, 2557, 2562, and 26037. Isolate 2564 was less virulent than isolates 26037 ($P < 0.05$, LSD).

Comparative Virulence of 7 *B. bassiana* Isolates against Groups of *R. flavipes* in Containers with Vermiculite/Sand Medium. At a rate of $1.2 \times 10^7/cm^3$, all *B. bassiana* isolates except 2557 and 2564 caused significant mortality to *R. flavipes* after 20 d ($F = 2.4$; df = 7, 21; $P = 0.05$) ($P < 0.10$, LSD) (Fig. 4). There was not significant differences among the isolates ($P > 0.05$, LSD).

Comparative Virulence of the 6 *B. bassiana* Isolates and 2 *M. anisopliae* isolates against Groups of *R. flavipes* in Containers with Vermiculite/Sand Medium. None of the 6 *B. bassiana* isolates caused significant mortality to *R. flavipes* at a rate of 3×10^6/cm^3 (F = 32.3; df = 8, 24; $P < 0.01$) ($P > 0.10$, LSD). Whereas both of the *M. anisopliae* isolates caused significantly higher mortality of *R. flavipes* than the *B. bassiana* isolates ($P < 0.05$, LSD) (Fig. 5).

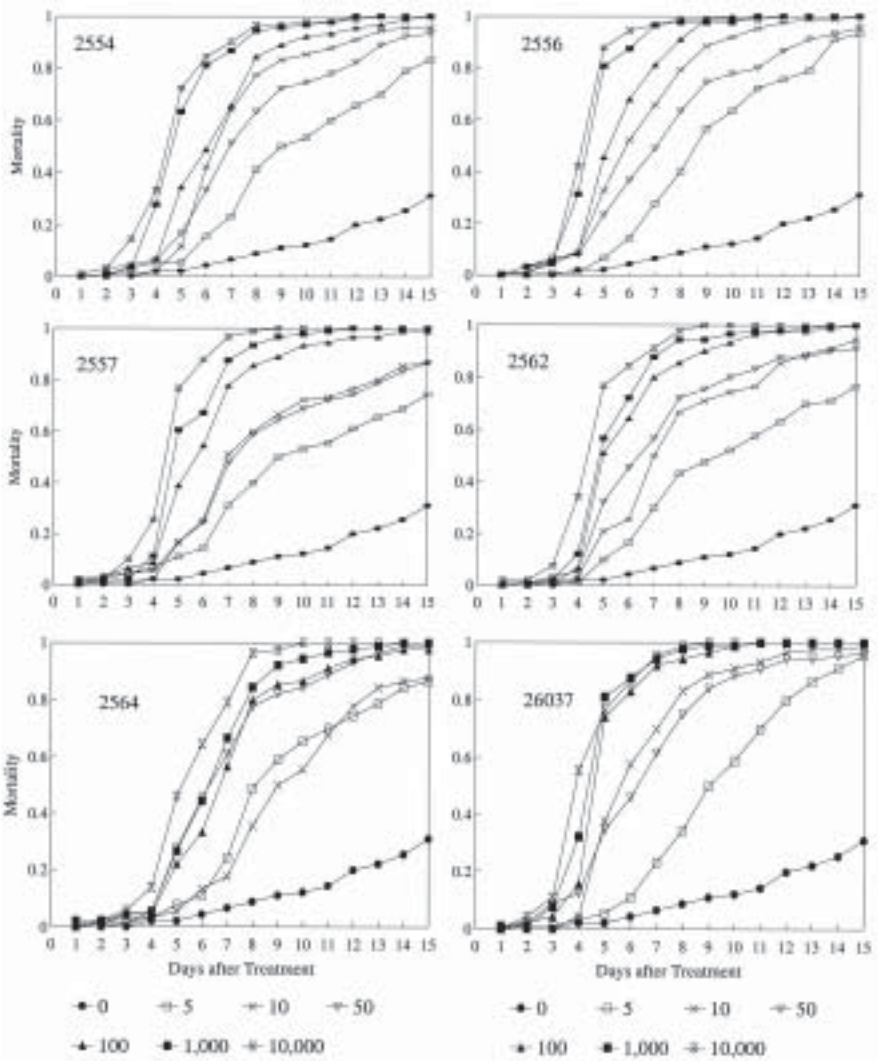

Fig.1. Effect of 6 *B. bassiana* isolates against individual *C. formosanus* in 96-well plates. Mean of 3 colonies with 30 workers in each colony. The rate is the number of conidia per termite.

Table 2. LD_{50} (conidia/termite) and their 95% fiducial limits of 6 *B. bassiana* isolates against individual *C. formosanus* in 96-well plates.

Colony	Isolate[1]	LD_{50} (mean and 95% fiducial limit)	n	Slope	SE of Slope	χ^2	*P*
1	2554	1 (0-3)	7	0.72	0.24	9.0	< 0.01
	2562	2 (0-4)	7	1.46	0.5	8.4	< 0.01
	26037	2 (0-4)	7	1.24	0.37	11.2	< 0.01
2	2562	33 (11-76)	7	0.81	0.15	30.7	< 0.01
	2564	27 (1-145)	7	0.88	0.23	15.2	< 0.01
3	2556	1 (0-5)	7	1.28	0.34	14.1	< 0.01
	2557	15 (5-23)	7	1.12	0.23	24	< 0.01
	2562	9 (3-16)	7	1.11	0.23	22.8	< 0.01
	2564	3 (0-36)	7	0.50	0.17	8.2	< 0.01

[1] Only those isolations with good fit of the probit model are included.

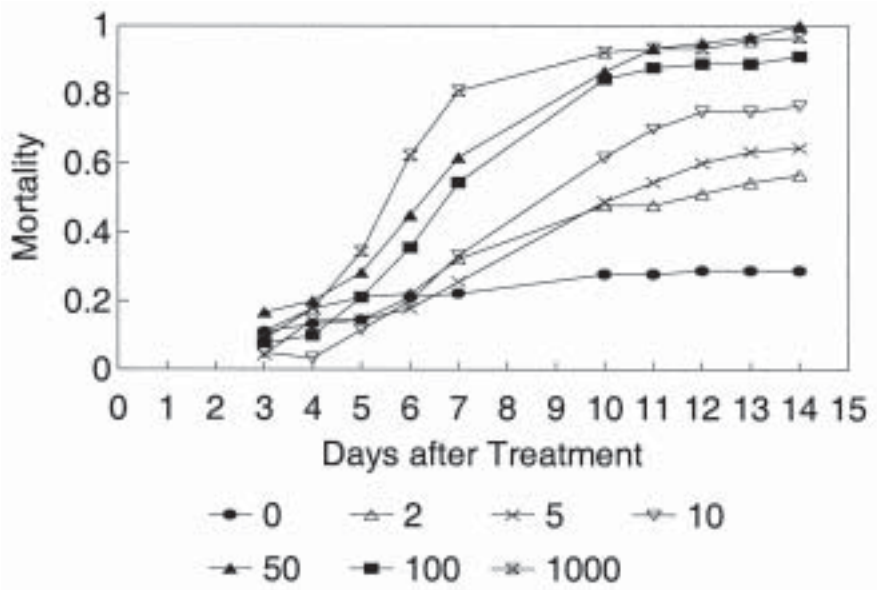

Fig. 2. Effect of *B. bassiana* isolate 2556 against individual *R. flavipes* in 96-well plates. Mean of 3 colonies with 30 workers in each colony. The rate is the number of conidia per termite.

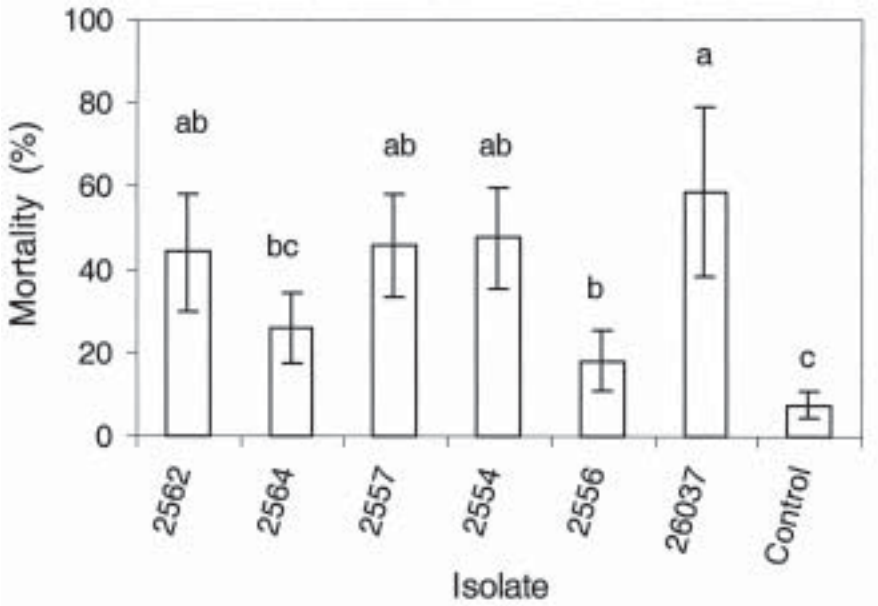

Fig. 3. Comparative virulence of *B. bassiana* isolates against *C. formosanus* in petri dishes at the rates of 1×10^7/ml. Bars with different same-case letters are significantly different ($P < 0.05$, LSD). Mean ± SE of 4 colonies with 30 workers in each colony.

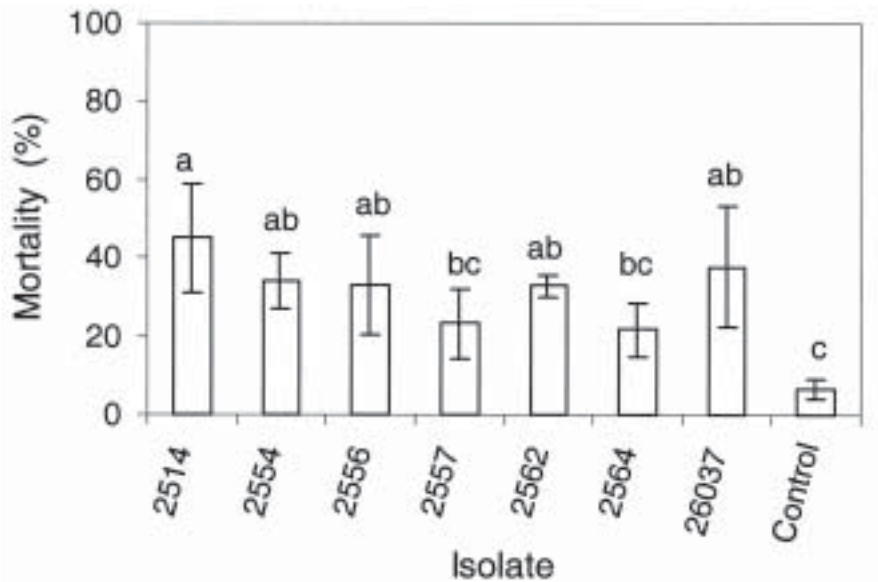

Fig. 4. Comparative virulence of 7 *B. bassiana* isolates against *R. flavipes* in containers with vermiculite/sand medium at a rate of $1.2 \times 10^7/cm^3$. Bars with different letters are significantly different ($P < 0.05$, LSD). Mean ± SE of 4 colonies with 80 workers in each colony.

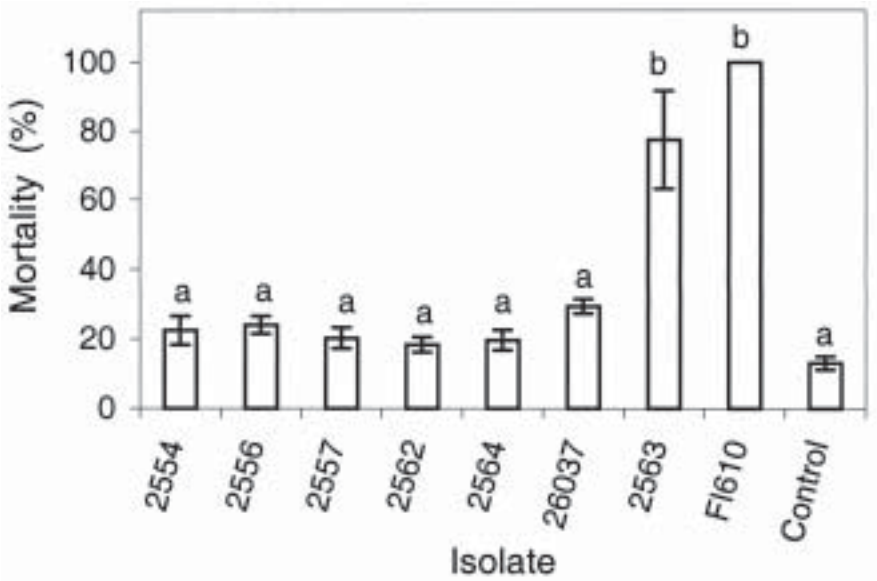

Fig. 5. Comparative virulence of *B. bassiana* and *M. anisopliae* against *R. flavipes* in containers with rearing medium at a rate of $3 \times 10^6/cm^3$. Bars with different letters are significantly different ($P < 0.05$, LSD). Mean ± SE of 4 colonies with 80 workers in each colony.

DISCUSSION

Results from bioassays of individual termites in 96-well plates showed that all of the *B. bassiana* isolates were highly virulent against *C. formosanus*. However, they showed much lower virulence against termites in petri dish tests with groups of termites. In petri dish assays against *C. formosanus*, 1 ml of conidia solution per dish at a rate of 1×10^7/ml or $6 \times 10^5/cm^2$ caused less than 50% mortality (corrected by Abbott (1925)). Bioassays of *B. bassiana* in round containers filled with medium and a conidia concentration of $3 \times 10^6/cm^3$ did not cause significant mortality of *R. flavipes*. Termites reared in treated petri dishes and round containers filled with medium walked freely and therefore were constantly in direct contact with *B. bassiana*. The much lower virulence to groups of termites versus individual termites relates to the social behavior of termites (Boucias *et al.* 1996). As indicated by a previous study, termite-grooming behavior can effectively remove fungal conidia from the termite body (Lai 1977). Therefore, individual termite bioassays with pathogens produced abnormally higher results of efficacy and should not be used for testing microbial pest control agents on termites.

Fungal isolates associated with termites are suggested to likely be more virulent than isolates obtained from other hosts (Milner & Staples 1996). Based on our tests with the isolates obtained from dead termites, none of the 6 newly isolated strains from termites were more virulent than the standard isolate. The best possible reason is that the isolates from termites might represent saprophytic isolates that existed in the termite-nesting environment and grew on dead termites. Wells *et al.* (1995) only found slight differences in virulence between *B. bassiana* isolates from Isoptera and those from other insect orders. Almeida *et al.* (1997) found that *B. bassiana* isolates from termites were more virulent than isolates obtained from soil samples. High virulence and pathogenicity is essential for *B. bassiana* to control termites. Until now, little effort has been devoted to explore more virulent isolates obtained from termites (Zoberi & Grace 1990, Almeida *et al.* 1997). Hence, isolation of pathogens from termites, especially naturally dead termites, should be further studied.

This study showed that *B. bassiana* was much less virulent to termites than *M. anisopliae*. Similar results were recorded by Lai *et al.* (1982) and Delate *et al.* (1995). Research conducted in Australia also revealed *M. anisopliae* to be the most effective fungal pathogen to termites (Milner *et al.* 1998). *Metarhizium anisopliae* is easy to mass produce and has been successful in field colony control of mound building termites (Milner & Staples 1996). These results suggest that *M. anisopliae* might be a more promising pathogen than *B. bassiana* for practical control of termites. However, contrary results were reported from studies in Brazil. Neves and Alves (2000) found no significant difference in virulence between the most virulent *M. anisopliae* isolate and the most virulent *B. bassiana* isolate against a mound termite, *Cornitermes cumulans* (Kollar). Almeida *et al.* (1997) found that the most virulent *B. bassiana* isolate was more virulent than *M. anisopliae* against a termite, *Heterotermes tenuis* (Hagen). Based on these studies, further exploration is warranted for more virulent *B. bassiana* isolates for control of *R. flavipes* and *C. formosanus*.

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