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Relationship between virulence and repellency of entomopathogenic isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the termite *Macrotermes michaelseni*

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ABSTRACT

Termites encounter a diverse array of potentially useful and harmful fungi in their subterranean habitats. These vary from symbiotic to harmful species with varying levels of virulence. How these hemiedaphic insects survive in habitats with infective fungi is not well understood. Possible mediation of olfactory signals in avoiding contact with entomopathogenic fungi has been explored by a number of workers. In the present study, we initially found that *Macrotermes michaelseni* detected a virulent isolate of *Metarhizium anisopliae* from some distance and avoided direct physical contact. We hypothesized that there may be a relationship between virulence and repellency of different isolates of *M. anisopliae* and *Beauveria bassiana* to the termite. We compared these for selected isolates of the two fungi. Positive correlations between the two parameters for both sets of isolates of the fungi were obtained. The results show an interesting co-evolutionary phenomenon in which the termite's response to either *M. anisopliae* or *B. bassiana* is directly related to potential harm these fungi can inflict on the insect and that the virulent strains are more likely to be recognized from some distance and avoided.

1. Introduction

Foraging insects encounter a diverse array of natural enemies that include predators, parasitoids, parasites and pathogens. The ability to recognize and respond defensively to such dangers is an important survival trait for any species. Detection of enemy-specific semiochemicals is often a key part of the defensive behavioural repertoire of many insects (Dicke and Grostal, 2001). Although entomopathogenic fungi can cause significant mortality in susceptible insect populations (Rath, 2000), few studies have been reported on the olfactory responses of these insects to such fungi (Meyling and Pell, 2006). The generalist insect predator *Anthocoris nemorum* L. (Heteroptera: Anthocoridae) has been shown to detect and avoid pathogenic *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycetes: Hypocreales) when it forages on host plants to which it is adapted (Meyling and Pell, 2006). On the other hand, the ectoparasitoid *Cephalonomia tartsalis* (Ashmead) (Hymenoptera: Bethylidae) is apparently unable to similarly detect conidia of this fungus or living hosts infected by the fungus, resulting in mortality of infected parasitoids or their offspring (Lord, 2001).

The ability of edaphic and hemiedaphic insects, and particularly eusocial species, to survive in microbial-rich environments has been recognized (Rosengaus et al., 1999a, 2003; Traniello et al., 2002). Earlier studies on the termite *Nasutitermes exitiosus* (Hill) (Isoptera: Termitidae) exposed to a highly virulent strain of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycetes: Hypocreales) failed to cause epizootics in the nests of the termite, which led Hänel and Watson (1983) to conclude that 'unknown factors' limited the efficiency of the pathogen. Chouvenc et al. (2008) treated individual termites, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) with conidial suspensions of *M. anisopliae* and exposed them to untreated conspecifics in a foraging arena. After 5 days, 90% of the treated individuals died in the arena but the untreated termites did not exhibit a significant

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increase in mortality after 90 days of contact with the infected member suggesting that conidia were dispersed in the termite environment but did not survive. Several behavioural and physiological mechanisms have been identified in different termite species, which may counter pathogen transmission through social exchanges and pathogen-related mortality of infected individuals within a termite community (Cremer et al., 2007). Behavioural mechanisms include the use of vibratory displays to warn nestmates about the presence of lethal fungal concentrations to which they respond by absconding (Rosengaus et al., 1999b; Myles, 2002), or increasing rates of mutual grooming when exposed to pathogens (Rosengaus et al., 1998, 2000), and walling off of infected areas of a colony (Milner et al., 1998; Staples and Milner, 2000). Physiological mechanisms include fungistatic secretions associated with exocrine glands and faecal pellets of some termites that reduce microbial growth within nest chambers (Rosengaus et al., 1998, 2004), enhancement of resistance to infection through cellular and humoral immune responses (Rosengaus et al., 1999a; Traniello et al., 2002), and salivary gland secretions containing antibiotic peptides (Lamberty et al., 2001). These defensive mechanisms demand cooperation between members of social groups or altruistic behaviours of some colony members for the benefit of the whole colony, which result in avoidance, control or elimination of parasitic infections (Müller and Schmid-Hempel, 1993; Cremer et al., 2007).

Possible avoidance of contact with entomopathogenic fungi by termites through the mediation of olfactory signals has been explored in a number of laboratories. For example, Kramm et al. (1982) found that healthy members of *Reticulitermes virginicus* (Banks) (Isoptera: Rhinotermitidae) did not contact cadavers infected with *M. anisopliae* suggesting mediation of a volatile signal. Staples and Milner (2000) tested the effect of conidia of 24 isolates of this fungus on the degree of tunneling by *Coptotermes lacteus* (Froggatt) (Isoptera: Rhinotermitidae) when incorporated into sand and placed at the base of a 50-ml agar tube. They found that, in the presence of several highly virulent isolates of the fungus, tunnels reached only a very short distance into the sand–agar substrate compared to less virulent isolates. In addition, termites retreated from the substrate after initial contact with the infected sand and sealed off the tunnels preventing further contact. However, in the presence of other virulent strains termites failed to demonstrate these defensive responses and were eventually killed by the fungus. The authors interpreted the degree of tunneling by the termite as a measure of 'repellence' of the fungal isolates. However, it is doubtful if olfactory detection of fungi would be optimal in such aqueous agar environment with minimum gaseous space into which the repellent blend can diffuse. This may explain failure by the termites to avoid contact with some virulent isolates of the fungus used by the authors. In the present study, we used a specially designed choice olfactometer to measure the responses of *Macrotermes michaelseni* Sjölstedt (Isoptera: Macrotermes) to volatile signals from different isolates of *M. anisopliae* and *B. bassiana*. Our hypotheses were that (i) the termite is able to detect virulent fungi by olfaction and is thus repelled from a distance and (ii) there is a relationship between any repellency demonstrated and virulence of different isolates against the termite. Herein we describe the results we obtained.

2. Materials and methods

2.1. Termites

Worker castes of the termite, *M. michaelseni*, needed for virulence and repellency bioassays were trapped overnight in the field using a modified method described by Tamashiro et al. (1973) from three mounds located at (1) 1612 m asl, S01°13.366′ E036°53.766′, (2) 1610 m asl, S01°13.068′, 036°53.823′, and (3) 1618 m asl, S01°13.144′ E036°53.717′ at Kasarani, Nairobi, Kenya. The traps were covered, transported to the laboratory and placed on dark polyethylene plastic sheets. Termites were individually collected using a pair of soft forceps and placed in Petri dishes (9 cm diameter) lined with wet filter papers (Whatman No. 1, 9 cm in diameter), which were then transferred into an incubator (26 ± 2 °C and 90 ± 5% RH in the dark) where they were kept for 20 min for acclimatisation.

2.2. Fungal isolates

2.2.1. Preparation of conidial suspension for mortality responses

Fungal isolates were cultured on Sabouraud Dextrose Agar (SDA) medium (Oxoid, Basingstoke, Hampshire, England). Conidia were harvested from 2 to 3-week-old surface cultures by scraping and suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 (Fluka, Sigma–Aldrich, UK) and 3 mm glass beads in universal bottles. The suspensions were filtered through cheesecloth and homogenized in a Vortex (Genie 2 Scientific Industries, Bohemia, New York) for 5 min. Different concentrations were obtained through serial dilutions with the help of a haemocytometer (Hausser, Scientific Horsham, USA).

2.2.2. Conidial germination tests

The germination tests were performed on all isolates used in the mortality and repellency responses and these varied between 85.8 and 99.4%. The viability of conidia was determined for each isolate by spread-plating 0.1 ml of conidial suspension at 3×10^6 conidia ml^{-1} on Petri dishes containing SDA. Four sterile microscopic cover slips (22 mm × 22 mm) were placed on each plate. The plates ($n = 6$ per replicate) were sealed with parafilm and incubated at 26 ± 2 °C and 90 ± 5% RH and examined between 15 and 18 h under phase contrast microscope. The percentage germination of conidia was determined from 100 spore counts under cover slips at 400× magnification. A conidium was designated as having germinated if the length of its germ-tube was twice the diameter of the conidial propagule in question.

2.2.3. Initial screening of isolates

15 isolates of *M. anisopliae* and three of *B. bassiana*, which were initially isolated from different substrates (Table 1), were obtained from the *icipe*'s culture collection. For each treatment and control groups, six Petri dishes each containing 20 worker termites were used. The control samples were sprayed with sterile distilled water containing 0.05% Triton X-100 (Fluka, Sigma–Aldrich, UK) before inoculating conidia onto the termite using a spray tower (Burgerjon, 1956). For treatment groups, 10 ml of a standard concentration of 10^7 conidia ml^{-1} was equally sprayed among six Petri dishes containing the termite. The spray tower was cleaned with 90% alcohol and sterile distilled water between treatments. The experiment was repeated on three different occasions under similar laboratory conditions.

To maintain social cohesion within the group (Sun et al., 2003), two soldier termites were also added into each Petri dish after conidial inoculation. A piece of wet cotton wool was used to maintain high humidity in each Petri dish throughout the experiment. The lids of the Petri dishes had five aeration holes (2 mm in diameter) to ensure free flow of air. Two pieces of sterile cypress wood, *Cupressus lusilanica* Dallimore, (approximately 50 mm × 30 mm × 1.5 mm) and 0.5 g of fungal garden [*Termitomyces* sp. (Basidiomycetes: Agaricatidae)] from the termite mounds were provided as shelter and for food, respectively, after applications of conidia. The fungal gardens were from respective termite mounds. The groups of termites were maintained at 26 ± 2 °C and 90 ± 5% RH in darkness. The relative humidity in the

Table 1
Viability of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* used against *Macrotermes michaelseni*. These isolates of fungi were isolated from various substrates from different localities.

Species/isolates	Origin of isolates, locality and country	% germination of isolates	Year of isolation
Metarhizium anisopliae			
ICIPE 51	Soil, Kitui, Kenya	99.4 ± 0.4	2005
ICIPE 30	Lepidoptera (*Busseola fusca*), Migori, Kenya	98.6 ± 0.5	1989
ICIPE 18	Soil, Mbita, Kenya	97.8 ± 0.1	1989
ICIPE 56	Tree, Nairobi, Kenya	97.8 ± 1.1	1990
ICIPE 47	Soil, Kitui, Kenya	96.9 ± 1.2	1990
ICIPE 62	Soil, Kinshasa, DRC	96.7 ± 1.3	1990
ICIPE 7	Soil, Matete, DRC	94.4 ± 1.4	
ICIPE 95	Sandfly (*Lutzomyia* sp.) Baringo, Kenya	93.1 ± 1.5	1996
ICIPE 44	Forest soil, Meru, Kenya	91.7 ± 1.1	1990
ICIPE 49	Soil, Mt. Kenya, Kenya	91.7 ± 1.7	
ICIPE60	Soil, Kakelo-Seme, Kenya	90.8 ± 1.2	1990
ICIPE 20	Soil, Migori, Kenya	90.8 ± 1.6	1989
ICIPE 21	*Schistocerca gregaria*, Port Sudan, Sudan	90.3 ± 1.2	1999
ICIPE 41	Soil, Kitui, Kenya	90.5 ± 1.5	1990
ICIPE 69	Soil, Matete, DRC Congo	90.5 ± 1.5	1990
Beauveria bassiana			
ICIPE 276	Soil, Mbita, Kenya	89.7 ± 1.6	2004
ICIPE79	Tick (*Rhipicephalus appendiculatus*), Rusinga Island, Kenya	88.6 ± 1.3	1996
ICIPE 278	Soil, Kericho, Kenya	85.8 ± 1.4	2005

DRC: Democratic republic of Congo. ICIPE: International Centre of Insect Physiology and Ecology.

incubator was controlled using stable saturated solution of K_2SO_4 (Supelco, Sigma–Aldrich, UK). Mortalities were recorded daily for calculation of LT_{50} values (time needed to give 50% mortality during screening) of each of the fungal isolates.

2.2.4. Dose–response mortality experiments

After the initial screening, nine isolates of *M. anisopliae* and three of *B. bassiana* were used for dose–response studies. They were selected on the basis of their LT_{50} achieved after the initial screening and ranged from most virulent (ICIPE 51, ICIPE 30, and ICIPE 18), moderately virulent (ICIPE 49, ICIPE60, and ICIPE 20), to least virulent (ICIPE 21, ICIPE 41 and, ICIPE 69). For *B. bassiana*, all the three isolates (ICIPE 276, ICIPE 79 and ICIPE 278) were used. Each treatment and control Petri dish contained 20 worker termites. Termite control groups (six replicates) were sprayed before the treatment groups using Burgerjon spray tower as described above. For treatment groups, six conidial concentrations (10^5, 3×10^5, 10^6, 3×10^6, 10^7 and 3×10^7 conidia ml^{-1}) were used for each isolate. The Burgerjon spray tower was cleaned between treatments as described earlier. 10 ml of each conidial concentration was sprayed equally among the different sets of six replicate Petri dishes. The treatment and control experiments were repeated on three different occasions under similar conditions as described for time-mortality response experiments. Experimental procedure remained similar as described for time-mortality responses. Accumulative data for 4 days were used for analyses of the dose–mortality responses for both the treatment and control replicates.

2.2.5. Mycoses tests

To confirm that mortality was due to fungal infection, the cadavers were removed from treatments, surface-sterilized in 1% sodium hypochlorite solution and then in 70% alcohol (Supelco, Sigma–Aldrich, UK) for 3 s in each solution and rinsed for 3 min in sterile distilled water. They were then placed onto Petri dishes lined with filter papers (Whatman, 9 cm in diameter), which were then moistened with sterile distilled water. High humidity (>80%) was maintained throughout the experimental tests. The Petri dishes were then covered with their lids, the edges of which were sealed with Parafilm. This procedure was also followed for control replicates. Mycoses were confirmed by daily microscopic examination of hyphae and spores at a magnification of 400×. The tests for time–mortality and dose–mortality were terminated after 14 days of post-infection.

2.2.6. Scaled-up production of dry conidia for dose–repellency experiments

Dose–repellency bioassays required substantial numbers of dry conidia. This was achieved by growing conidia on long white rice substrates following the technique described by Maniania et al. (2003). Two kilograms of the rice (Pishori) were soaked in sterilized distilled water for 10 min, rinsed three times and transferred to steel trays (33 cm × 25 cm × 13 cm od). The trays were wrapped with polyethylene autoclave bags and sterilized for 1 h at 121 °C. The substrates were left to cool at room temperature after which they were inoculated with 3-day-old cultures of blastospores (50 ml) and thoroughly mixed for complete coverage of the rice with the inocula. The cultures were incubated in a controlled temperature room (26 ± 2 °C and 60–70% RH). After 21 days, the conidial substrates were allowed to dry overnight at room temperature (22–25 °C). The conidia were harvested by sifting the substrate through a sieve (295 μm mesh size) into hazard polyethylene bags, which were then sealed. Conidia were stored in a refrigerator (4–6 °C) before use and only 1-month-old dry conidia were used in the repellency bioassays.

2.2.7. Repellency of dry conidia at different doses

Repellency of the selected isolates was evaluated in dual-choice Y-tube olfactometers constructed from glass, each consisting of three compartments, "A", "B" and "C" (Fig. 1). "A" served as the release site of the termites, and "B" and "C" served as source of test odor or control. Nylon gauze (40 mesh size) was attached with an adhesive to the floor of each olfactometer to facilitate easy movement of the insects on the floor of the device. A tygon tube (5.3 mm id, 6.35 mm od) (Supelco, North Harrison Road, Bellefonte, USA) connected to a vent (6 mm in diameter) at the junction facilitated airflow from each compartment to the Y-junction and then to an aspirator. The setup ensured that the air from the three arms did not mix until at the Y-junction. A flowmeter (Cole Parmer, Chicago, USA) connected to the tygon tube helped to regulate the airflow at 5 ml s^{-1}. Compartment "A" was illuminated with two florescent bulbs (220 V, 13 A, AC). The rest of the olfactometer, including compartments "B and "C", were shielded with a dark cotton cloth (shaded part in Fig. 1). The combination of brightness

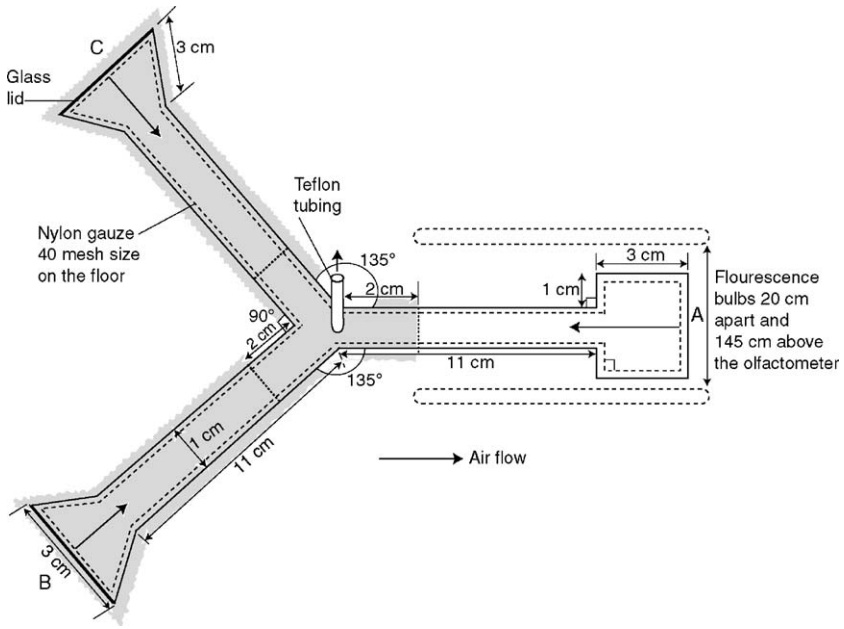

Fig. 1. Diagram of the olfactometer used in testing repellency of isolates of fungi. A–C are the compartments of the specially designed Y-olfactometer. The unshaded (push) and the shaded (pull) parts show the illuminated and unilluminated areas, respectively. The edge next to compartments B and C was briefly (<30 s) lifted to score the data.

and darkness acted as a 'push–pull' set of visual stimuli to induce the termites to move away from the release area toward the treated and control compartments. Bioassays were undertaken in a fume-hood.

In a preliminary experiment, a highly virulent isolate of *M. anisopliae* (ICIPE 30) was used to test for repellency against the termite. Steam sterilized sawdust (0.5 g) from cypress wood, *Cupressus lusilanica* dried at 85 ± 1 °C for 1 h was mixed with varying amounts of dry conidia (0.005, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g) held in folded aluminium foil and placed in either "B" or "C" compartment. The other compartment functioned as the control with aluminium foil containing only sawdust (also 0.5 g). Folded foils served to avoid direct physical contact of test materials by the test insects but allowed diffusion of volatiles through the folds into the compartment. At the flow rate of air (5 ml min^{-1}) deployed in the olfactometer, there was no evidence of conidia being drawn from the folded foils into the treatment arm upwind. This was confirmed by determining the weights of the foils with conidia before and after the assays (which showed no loss in weight) and close microscopic inspection (400× magnification) of the test termites (which showed no evidence of contamination with conidia). Glass lids were loosely placed at the openings of all the three compartments of the olfactometer to avoid escape of test insects, while facilitating airflow into the olfactometer. Groups of worker termites (20) were put in compartment "A". Most of the insects moved away from light into the Y-junction within a few minutes. The number of termites in the treated and control compartments together with those in their respective arms were recorded at an interval of 10 min for 90 min to give nine readings for each replicate. During the readings the edge of the black cloth at the two compartments was briefly (<30 s) lifted to avoid extended exposure of the termites to light. Termites that did not move beyond the Y-junction were treated as non-responders. After each replicate test, the olfactometer was sterilized with 70% alcohol to kill the conidia. Pure acetone was used to rinse the olfactometers to eliminate any possible conidial odours. The device was then thoroughly washed with residue-free liquid soap detergent in sterilized distilled water and dried in an oven (45 °C). The assay was replicated 10 times. The treatment and control arms were swapped after every replicate to eliminate any asymmetric bias of either the olfactometer or the surroundings. The above procedure was followed to determine the repellency–dose (RD_{50}) values of different amounts of dry conidia (0.0125, 0.025, 0.05, 0.1 and 0.2 g) of all nine and three isolates of *M. anisopliae* and *B. bassiana*, respectively. Each dose of each isolate was replicated 12 times. For the control assay, both arms of the olfactometer were untreated.

3. Statistical analysis

In all the tests, data for mortality and repellency were individually pooled before analyses. In virulence tests, mortality data were corrected for natural mortality in controls using Abbott's formula (Abbott, 1925) and arcsin transformed to normalise the data before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed of SAS version 9.1 (SAS Institute, 2003). Means were separated by Student–Newman–Keuls (SNK) test. Repellency to the termite was calculated using the formula:

$$\frac{P_{nc} - P_{nt}}{P_{nc} + P_{nt}} \times 100$$

where P_{nc} and P_{nt} represent the average percentage of worker termites in control and treatment arms, respectively (Wanzala et al., 2004).

Lethal time to 50% mortality (LT_{50}), lethal concentration to 50% mortality (LC_{50}) and the repellency–dose to 50% (RD_{50}) were estimated with repeated measures logistic regression via generalized estimating equations (GEE) (Throne et al., 1995; Stokes et al., 2000). These analyses were carried out using GENMOD procedure of SAS version 9.1 (SAS Institute, 2003). The relationship between virulence and repellency of the fungal isolates towards the termite was established through non-linear regression analysis. 95% confidence intervals were used to identify significant differences among the values of LT_{50}, LC_{50} and RD_{50} for the different isolates of the fungi. The level of significance was set at 5% for all analyses.

4. Results

4.1. Initial screening of isolates

Results from initial screening of isolates showed that there were no significant differences in mortalities among the termites

Table 2
The Lethal Time (LT_{50}) values of time-mortality responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni* at a constant conidial concentration (10^7 conidia ml^{-1}). CLs represent the confidence limits of the LT_{50} values.

Isolates of fungi	% mortality (mean ± SE)	% mycoses	LT_{50} values/CLs	Slope ± SE
Control	13.1 ± 0.8	0 ± 0		
M. anisopliae				
ICIPE 51	85.4 ± 2.3^{a}	99.4 ± 0.4^{a}	2.0^{a} (2–2.1)	7.1 ± 0.3
ICIPE 30	84.6 ± 2.3^{ab}	98.6 ± 0.5^{a}	2.1^{b} (2.1–2.2)	7.2 ± 0.4
ICIPE 18	83.1 ± 2.4^{bc}	97.8 ± 1^{ab}	2.3^{c} (2.2–2.4)	7.7 ± 0.2
ICIPE 56	82.6 ± 2.4^{bcd}	97.8 ± 1.1^{ab}	2.3^{c} (2.2–2.4	6.2 ± 0.2
ICIPE 47	81.7 ± 2.5^{cde}	96.7 ± 1.2^{ab}	2.4^{c} (2.3–2.5)	5.8 ± 0.3
ICIPE 62	80.8 ± 2.5^{def}	94.4 ± 1.3^{abc}	2.5^{c} (2.4–2.6)	6.9 ± 0.2
ICIPE 7	80 ± 2.5^{edf}	93.1 ± 1.4^{abcd}	2.5^{c} (2.4–2.7)	5.8 ± 0.3
ICIPE 95	79.7 ± 2.5^{ef}	91.7 ± 1.5^{bcd}	2.6^{cd} (2.5–2.7)	6.8 ± 0.1
ICIPE 44	78.8 ± 2.6^{ef}	91.7 ± 1.1^{cde}	2.6^{cd} (2.6–2.8)	6.7 ± 0.5
ICIPE 49	77.1 ± 2.7^{fg}	90.8 ± 1.7^{cde}	2.8^{cd} (2.7–3)	6.1 ± 0.4
ICIPE60	77 ± 2.7^{gh}	90.8 ± 1.2^{de}	2.9^{cd} (2.7–3.2)	5.6 ± 0.6
ICIPE 20	76.2 ± 2.7^{gh}	90.3 ± 1.6^{de}	2.9^{cd} (2.9–3)	7.5 ± 0.2
ICIPE 21	70.6 ± 3^{i}	90.3 ± 1.5^{de}	3.6^{e} (3.2–4)	9 ± 0.5
ICIPE 41	$70.2 \pm 2.^{8i}$	90.3 ± 1.2^{de}	4.1^{e} (3.9–4.3)	7.3 ± 0.4
ICIPE 69	65.5 ± 3.1^{j}	90 ± 1.6^{de}	4.4^{e} (4.6–4.6)	4.2 ± 0.9
B. bassiana				
ICIPE 276	60.5 ± 3^{k}	89.7 ± 1.6^{de}	4.5^{e} (4.1–4.9)	4.3 ± 0.3
ICIPE79	60.4 ± 2.9^{k}	88.6 ± 1.3^{de}	4.6^{f} (4.5–4.4.7)	5.8 ± 0.3
ICIPE 278	47.7 ± 2.3^{l}	85.8 ± 1.4^{e}	5.8^{g} (5.5–6.2)	4.4 ± 0.3

Percentage mortality (mean ± SE) and percentage mycoses (mean ± SE) followed with the same letters within a column are not significantly different ($\alpha = 0.05$, $P = 0.0001$, SNK test). LT_{50} values followed with the same letters within a column are not significantly different (Proc GENMOD, $P > 0.05$, $\alpha = 0.05$, $P = 0.0001$, SNK test,). 95% confidence limits of the LT_{50} values are shown in brackets in the column labeled LT_{50}. Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among LT_{50} values (Proc GENMOD) and the significance was also tested ($\alpha = 0.05$, $P = 0.0001$, SNK test,). ICIPE: International Centre of Insect Physiology and Ecology.

from the three mounds ($F_{df\ (2,\ 30)} = 0$, $P = 1$, $n = 33$, SNK test). In all tests, the mortality data were therefore pooled before analyses. Percentage mean mortalities of worker termites caused by isolates of *M. anisopliae* were significantly greater than those caused by isolates of *B. bassiana* at a concentration of 10^7 conidia ml^{-1} ($F_{df\ (17,\ 3366)} = 285.4$, $P < 0.05$, $n = 3564$, SNK test). ANOVA revealed significant time effects on mortalities among isolates of the two species of fungi ($F_{df\ (10,\ 3366)} = 5827.8$, $P < 0.05$, $n = 3564$, SNK test). There were significant interactions between the fungal treatments and time effects on mortalities of termite groups at a concentration of 10^7 conidia ml^{-1} ($F_{df\ (170,\ 3366)} = 39.6$, $P < 0.05$, $n = 3564$, SNK test). The variations in LT_{50} values of isolates of *M. anisopliae* (15) and *B. bassiana* (3) are shown in Table 2. There were significant differences in mycoses among isolates of the two fungi ($F_{df\ (17,\ 306)} = 9.3$, $P < 0.05$, $n = 324$). Mean percentage mortalities in the control groups were significantly less than in all the fungal treatments ($P < 0.05$, SNK test) (Table 2). Among the control replicates the mean percentage mortalities were not significantly different ($F_{df\ (2,\ 15)} = 3.6$, $P < 0.05$, $n = 18$, SNK test).

4.2. Mortality–dose responses

ANOVA showed significant differences in mortalities among the treatment groups ($F_{df\ (5,\ 1224)} = 540.3$, $P < 0.05$, $n = 1296$, SNK test) of isolates of the two fungi. The mean percentage mortalities among the termite groups were directly proportional to the conidial concentrations and differed significantly among fungal isolates ($F_{df\ (5,\ 1224)} = 646.9$, $P < 0.05$, $n = 1296$, SNK test). There were significant interactions between the treatment groups and the concentrations of conidia ($F_{df\ (55,\ 1224)} = 7.8$, $P < 0.05$, $n = 1296$, SNK test) on the mortalities of the termite groups. The variations among the LC_{50} values of isolates of *M. anisopliae* (9) and *B. bassiana* (3) are shown in Table 3. Mean percentage mortality in the control groups (13.1 ± 1.1, mean ± SE) were not significantly different among one another ($F_{df\ (2,\ 15)} = 4.3$, $n = 18$, $P > 0.05$, SNK test) but were significantly less than in all the fungal treated termite groups ($P < 0.05$, SNK test) (Table 3). No lethal mycoses were observed in all control groups. Moreover, mean percentage mortalities in these groups were not significantly different from one another ($F_{df\ (1,\ 34)} = 1.6$, $n = 36$, $P = 0.22$, SNK test).

4.3. Repellency–dose responses

In the push–pull olfactometer setup, worker termites showed a propensity to move towards the control arm (fungus-free) of the olfactometer. Those that entered into the arm of compartment that contained conidia of different isolates did not move close to the odour sources and with higher doses of the more virulent fungi

Table 3
The Lethal concentration (LC_{50}) values of mortality–dose responses of various isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni* when exposed to various concentrations of conidia (conidia ml^{-1}). The data used were for 4 days post-infection.

Isolates of fungi	LC_{50} values (conidia ml^{-1})	95% confidence limits	Slope ± SE
M. anisopliae			
ICIPE 51	1.5×10^{4a}	$(1.0–2.1) \times 10^4$	1.1 ± 0.06
ICIPE 30	1.9×10^{4a}	$(1.4–2.7) \times 10^4$	0.6 ± 0.03
ICIPE 18	3.9×10^{4b}	$(2.7–5.5) \times 10^4$	0.6 ± 0.05
ICIPE 49	7.9×10^{4c}	$(6.3–10) \times 10^4$	0.7 ± 0.03
ICIPE 60	11.8×10^{4c}	$(10.3–13.6) \times 10^4$	0.6 ± 0.03
ICIPE 20	12.7×10^{4cd}	$(10.5–16.6) \times 10^4$	0.5 ± 0.03
ICIPE 21	15.4×10^{4cd}	$(10.6–23.3) \times 10^4$	0.4 ± 0.04
ICIPE 41	46.4×10^{4e}	$(42–51.9) \times 10^4$	0.5 ± 0.02
ICIPE 69	149.3×10^{4f}	$(111.5–199.8) \times 10^4$	0.4 ± 0.03
B. bassiana			
ICIPE 276	240.0×10^{4g}	$(206.1–279.5) \times 10^4$	0.3 ± 0.01
ICIPE79	591.1×10^{4h}	$(451.2–774.5) \times 10^4$	0.3 ± 0.01
ICIPE 278	1140.9×10^{4i}	$(932.8–94.5) \times 10^4$	0.3 ± 0.01

LC_{50} values followed by the same letter within a column are not significantly different (Proc GENMOD, $P > 0.05$, $\alpha = 0.05$, $P = 0.0001$, SNK test,). Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among LC_{50} and were tested for significance ($\alpha = 0.05$, $P = 0.0001$, SNK test,). The dose–mortality response data were for 4 days after inoculation of the fungi. ICIPE-International Centre of Insect Physiology and Ecology.

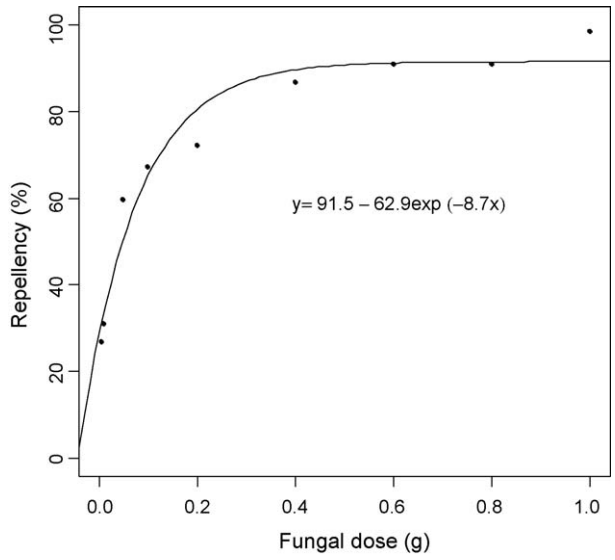

Fig. 2. Repellency of a highly pathogenic isolate of *Metarhizium anisopliae* (ICIPE 30) at different doses of dry conidia (g) against *Macrotermes michaelseni*.

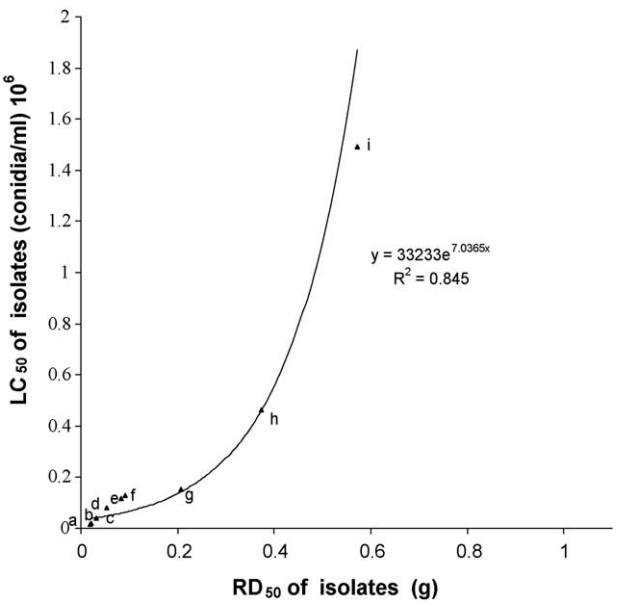

Fig. 3. Relationship between virulence (LC_{50}, conidia ml^{-1}) and repellency (RD_{50}, g) of *Metarhizium anisopliae*, towards termite, *Macroterms michaelseni* where a (ICIPE 51), b (ICIPE 30), c (ICIPE 18), d (ICIPE 49), e (ICIPE 60), f (ICIPE 20), g (ICIPE 21), h (ICIPE 41) and i (ICIPE 69) represent the different fungal isolates. The relationship shows positive correlation between virulence and repellency.

they rapidly retreated from the arm. The results of our initial assay with a highly virulent isolate (ICIPE 30) indicated a clear dose–response relationship ($R^2 = 0.8$, $F_{df\ (8,\ 81)} = 22$, $P < 0.05$, $n = 90$, SNK test) (Fig. 2). There were significance differences in repellency between isolates ($F_{df\ (11,\ 6420)} = 504.2$, $P < 0.05$, $n = 6480$, SNK test) and between the doses of conidia ($F_{df\ (4,\ 6420)} = 574.7$, $P < 0.05$, $n = 6480$, SNK test). ANOVA detected significant interaction effects between isolates and doses on repellency of the fungi against the termite groups ($F_{df\ (44,\ 6420)} = 6.6$, $P < 0.05$, $n = 6480$, SNK test). The repellency indices of *M. anisopliae* and *B. bassiana* as measured by RD_{50} values are shown in Table 4.

4.4. Correlation between virulence and repellency

There was a strong positive correlation between virulence and repellency of the nine isolates of *M. anisopliae* ($R^2 = 0.85$, $P < 0.05$) (Fig. 3). A similar relationship was found for isolates of *B. bassiana* ($R^2 = 0.99$, $P < 0.05$). Both virulence and repellency of isolates of *M. anisopliae* were significantly greater than those of *B. bassiana* ($P < 0.05$, SNK test).

Table 4
The RD_{50} values of repellency–dose responses of various strains of *Metarhizium anisopliae* and *Beauveria bassiana* against *Macrotermes michaelseni* when exposed to varying doses of dry conidia (conidia g^{-1}).

Isolates of fungi	Repellency dose (RD_{50}, g)	95% confidence limits	Slope ± SE
M. anisopliae			
ICIPE 51	1.9×10^{-2a}	$(1.7–2.1) \times 10^{-2}$	0.7 ± 0.04
ICIPE 30	2.2×10^{-2a}	$(2–2.4) \times 10^{-2}$	0.6 ± 0.03
ICIPE 18	3.2×10^{-2b}	$(2.9–3.7) \times 10^{-2}$	0.6 ± 0.03
ICIPE 49	5.2×10^{-2c}	$(4.9–5.6) \times 10^{-2}$	0.5 ± 0.04
ICIPE 60	8.2×10^{-2d}	$(7.4–9.2) \times 10^{-2}$	0.5 ± 0.02
ICIPE 20	9.3×10^{-2e}	$(8.8–9.8) \times 10^{-2}$	0.4 ± 0.01
ICIPE 21	20.7×10^{-2f}	$(16.9–25.3) \times 10^{-2}$	0.5 ± 0.03
ICIPE 41	37.4×10^{-2g}	$(31.1–45) \times 10^{-2}$	0.4 ± 0.04
ICIPE 69	57.3×10^{-2h}	$(48.–68.4) \times 10^{-2}$	0.4 ± 0.02
B. bassiana			
ICIPE 276	101.86×10^{-2i}	$(80.9–128.2) \times 10^{-2}$	0.5 ± 0.03
ICIPE 79	102.19×10^{-2i}	$(83.4–125.2) \times 10^{-2}$	0.6 ± 0.03
ICIPE 278	140.07×10^{-2j}	$(108.8–180.4) \times 10^{-2}$	0.6 ± 0.03

RD_{50} values followed by the same letter within a column are not significantly different (Proc GENMOD, $P > 0.05$, $\alpha = 0.05$, $P = 0.0001$, SNK test). Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among RD_{50} and were tested for significance ($\alpha = 0.05$, $P = 0.0001$, SNK test). The dose–mortality response data were for 4 days post-infection. ICIPE: International Centre of Insect Physiology and Ecology.

5. Discussion

Different fungal isolates of *M. anisopliae* and *B. bassiana* repelled the termite *M. michaelseni* in dose-related manner as illustrated by the results with one of the highly virulent isolates of *M. anisopliae* (Fig. 2). Of particular interest are clear positive correlations between virulence of the different isolates of the two species of fungi and their repellency to the insect (Fig. 3). In our assays termites that entered the arm of the olfactometer attached to the compartment with fungi did not move close to the odour sources. With higher doses of the more virulent fungi they came to a standstill some distance away from the odour source before retreating. This shows that the insect can detect potentially harmful concentrations of fungi by olfaction and avoid direct physical contact.

Previously, Staples and Milner (2000) tested the effect of conidia of different isolates of *M. anisopliae* incorporated into sand placed at the base of a 50-ml agar tube on the degree of tunneling by *Coptotermes lacteus* (Froggatt). In the presence of some highly virulent isolates of the fungus, the insect retreated from the substrate after initial contact with the infected sand and sealed off the tunnels, thus preventing further contact. However, their experimental set-up did not clearly differentiate between avoidance resulting from contact and that resulting from olfaction and may account for failure by termites in some experiments to demonstrate defensive behaviour. The olfactometer set-up in the present study with slow flow rate of air was designed to restrict the insect's contact with the fungi to olfactory signals. In our follow up study, we have found that volatiles collected from the fungi are repellent to the termite, particularly those associated with the more virulent isolates (Mburu et al., in preparation). This confirms that the termites are able to perceive the presence of fungi through their volatile emissions.

Olfactory detection and avoidance of contact with potentially infective fungi may have been an important evolutionary trait in *M.*

michaelseni and probably other termite species to counter the challenges of semi-edaphic habitats. When infection does occur to members of some termite species, several other behavioural mechanisms have been shown to limit possible social exchanges of infectious conidia within the community such as increased rate of mutual grooming (Rosengaus et al., 1998; Traniello et al., 2002), walling off infected areas of a colony (Milner et al., 1998) and removal of diseased individuals from their nests (Traniello et al., 2002). It would be interesting to see which, if any, of these mechanisms also operate in *M. michaelseni* as part of its defensive repertoire against fungal infections.

The differential virulence and repellency as depicted by LT_{50} and RD_{50} values of the fungi suggest qualitative and/or quantitative variations in the composition of volatile blends emitted by different strains. These variations may be used as signatures by termites to detect and avoid virulent strains and warn conspecifics of the presence of potent risks. We are currently studying the chemical composition of these blends. Olfactometric assays of the constituents individually and in mixtures will help to characterize those that contribute to the repellency of the natural repellent blends and to account for the differences in the repellency of the different fungal isolates. The results of this study will be reported elsewhere.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2009.04.015.

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