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Behavioral and electrophysiological responses of *Coptotermes formosanus* Shiraki towards entomopathogenic fungal volatiles

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ABSTRACT

Termites adjust their response to entomopathogenic fungi according to the profile of fungal volatile organic compounds (VOCs). This study demonstrates the pathogenicity of *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) towards the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Using no-choice assays, *M. anisopliae* was found to be highly virulent (LT_{50} 3.10 d) when compared to *B. bassiana* (LT_{50} 6.62 d) and *I. fumosorosea* (LT_{50} 12.39 d). Also using choice assays, the foraging behavior of *C. formosanus* was determined in the presence of pathogenic fungi. The highly pathogenic fungi (*M. anisopliae*) elicited a repellent response, causing most of the termites to forage in a safe zone farthest from the fungal source. This repellency resulted in relatively low mortality similar to the controls. The repellency of *M. anisopliae* conidia can be used to protect human belongings and timber from termites. While *I. fumosorosea* cultures were not repellent to *C. formosanus* workers, the termites were highly susceptible to infection. Electroantennographic responses of workers showed approximately 47% and 78% lower level of response to conidia of *B. bassiana* and *I. fumosorosea*, respectively, as compared to *M. anisopliae*. The VOC profile of repellent cultures of *M. anisopliae* mainly consisted of paraffins (60.97%), while the major proportion of the *I. fumosorosea* profile consisted of branched and cyclic alkanes (84.41%). From the above findings, we conclude that the incorporation of *I. fumosorosea* may increase the control potential of bait.

1. Introduction

Termites are highly likely to be exposed to natural enemies that include predators, parasites and pathogens during foraging. Among these natural enemies, fungi are especially important pathogens of insects and many fungi thrive in the same environment as termites. Conditions in a termite nest, including moderate temperature and high humidity, are conducive to the growth of fungal species and are important factors in fungal survival and propagation (Kramm et al., 1982). In spite of this, few studies have been able to demonstrate consistent and effective control of termite colonies using fungi or fungal preparations in the field (Rath, 2000).

Reasons for this lack of success include both physiological and behavioral mechanisms that termites have evolved to resist infection by disease agents. Physiological mechanisms include cellular and humoral immune responses (Rosengaus et al., 2007; Chouvenc et al., 2009) and antifungal chemical production in termite nests by nest mates, which are primarily composed of fecal material (Rosengaus et al., 1998), sternal and salivary gland secretions (Rosengaus et al., 2004). Behavioral mechanisms include the use of allogrooming to remove fungal conidia from the surface of the cuticle of nest mates (Rosengaus et al., 1998; Shimizu and Yamaji, 2003), walling-off of infected areas of a colony (Milner et al., 1998; Staples and Milner, 2000), pathogen alarm behavior which warns nestmates about the presence of lethal fungi causing them to leave the area (Rosengaus et al., 1999; Myles, 2002) and removal of fungal-infected termites (Grace and Zoberi, 1992; Jones et al., 1996; Rath, 2000; Myles, 2002).

The work explored in many of the studies mentioned above was focused on the avoidance behavior of termites towards repellent entomopathogenic fungi. The authors Staples and Milner (2000); Myles (2002); Mburu et al. (2009) interpreted that repellency of termites towards virulent strains of entomopathogenic fungi served as a means to eliminate the chance of fungal epizootics in the nest. Furthermore, they suggested that repellency occurred mainly because of variations in the composition of volatile blends emitted by different strains. However, no attempt has been made to compare the volatile organic compounds (VOCs) of entomopathogenic fungi in the past. Recent studies by Mburu et al. (2009) on *Metarhizium anisopliae* and *Beauveria bassiana* suggested that

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termite responses towards entomopathogenic fungi are directly related to potential harm the fungi can inflict on the insect, and virulent strains are more likely to be detected from some distance and avoided. Furthermore, they suggested that termites are able to perceive the presence of fungi through their volatile emissions. But their findings did not give any information regarding VOCs of the entomopathogenic fungi. Thus, the objectives of this study were the following: (1) to compare the virulence of *M. anisopliae*, *B. bassiana* and *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) by no-choice assays for the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), (2) to study the behavioral responses of termites towards fungal cultures by choice assays, (3) to compare the electroantennographic response of *C. formosanus* workers towards entomopathogenic fungi, and (4) to qualitatively analyze the VOCs of entomopathogenic fungi by HS-SPME using GC-MS.

2. Materials and methods

2.1. Entomopathogenic fungi

M. anisopliae (EBCL 02049), *B. bassiana* (EBCL 03005) and *I. fumosorosea* (EBCL 03011) were isolated from the cadavers of *C. formosanus* from China during 2002–2003. The strains were deposited at the European Biological Control Laboratory, Montpellier, France. The fungal cultures were maintained on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, US) at 25 ± 2 °C in complete darkness.

2.2. Collection and preparation of termites

Formosan subterranean termites were obtained from South China Agricultural University, Guangzhou, China. Four different *C. formosanus* colonies were chosen to prevent colony vitality from biasing the data. Termites were maintained separately at 24–27 °C in plastic buckets containing pine wood stakes (*Pinus* sp.) placed over moist sterile soil in complete darkness.

2.3. Experiment 1. Virulence level determination by no-choice assays

Virulence of *M. anisopliae*, *B. bassiana* and *I. fumosorosea* was determined by inoculating filter paper (Whatman #1) with a 1 mL suspension of 24-day-old sporulating cultures in 0.05% Tween 80 (Sigma–Aldrich, St. Louis, MO, US) at a concentration of 1×10^7 conidia/mL. Both the concentration of the conidial suspension (1×10^7 conidia/mL) and viability (91–99%) were calculated as described by Hussain et al. (2009). In brief, conidia were harvested by flooding each plate with 10 mL 0.05% Tween 80 in sterile distilled water and dislodging the conidia into suspension with a glass rod. The conidial suspension of 1×10^7 conidia/mL was prepared by using a Neubauer haemocytometer (0.0625 m^2; Fuchs-Rosenthal Merck Euro Lab, Darmstadt, Germany). A conidium was considered to have germinated if the germ tube was more than half the diameter of the conidium. Control treatments were prepared by adding 1 mL 0.05% Tween 80 solution to the filter paper in Petri dishes (95 × 15 mm).

One hundred *C. formosanus* workers were added to each Petri dish containing treated filter paper. Each treatment was replicated four times, once for each of the four different termite colonies. The experimental units were placed at 26–28 °C in complete darkness. Mortality was recorded daily for 16 d post-treatment. Dead termites were removed and placed in dishes lined with wet filter paper and maintained at 25 °C in growth chamber for mummification and sporulation to prove that the insects died due to fungal infection. The entire study was conducted three times. Data were analyzed using analysis of variance (ANOVA) and means were compared by Student Neuman Keuls test (SAS Institute, 2000).

2.4. Experiment 2. Termite behavior related to fungal cultures in choice assays

The test arena consisted of two glass containers (A and B) same size (7 cm diameter × 9 cm high) that were connected with a 15 cm long (1.5 cm., ID) glass tube (Fig. 1). In addition, container A was attached to a culture tube containing 24-day-old fungal culture (*M. anisopliae*, *B. bassiana* and *I. fumosorosea*) grown on PDA. Both containers contained a block (2.3 × 2.3 × 2.3 cm^3) of pine wood along with 15 g of sterilized soil. The soil was moistened with sterilized distilled water. One hundred workers of *C. formosanus* were introduced into container A. A control for each colony contained only PDA in the culture tube attached to container A. Test arenas were kept in total darkness at 26–28 °C and 85 ± 5% RH for the duration of the study. The whole study was repeated three times. The location and mortality of the termites was monitored daily for 64 d. The data on mortality and termite's presence were angularly transformed before analysis that was back-transformed for presentation. Data were analyzed using analysis of variance (ANOVA), and means were compared by Student Neuman Keuls test (SAS Institute 2000).

2.5. Experiment 3. Electroantennographic (EAG) bioassays

The EAG experiments were designed to investigate the selectivity of antennal receptors of *C. formosanus* workers towards different entomopathogenic fungi. The experiments were performed using an excised antenna of a *C. formosanus* worker. An electrically conductive gel (Spectra 360® electrode gel; Parker, Orange, New Jersey) was applied to the metal electrodes fixed to the base and tip of the excised antenna. Fifty micro-liters of conidial suspensions of *M. anisopliae*, *B. bassiana* or *I. fumosorosea* at a concentration of 1×10^{12} conidia/mL, was applied onto a piece of filter paper and placed into a 15 cm Pasteur pipette. A Pasteur pipette containing a filter paper dipped in 0.05% Tween 80 was used as a control. Humidified air at a flow rate of 4 ml/min was passed through the pipette and directed onto the antenna. The antennal responses were amplified and recorded with a data acquisition controller and software EAG (Syntech). Ten antennae from ten different workers were used to observe the EAG response of *C. formosanus* toward entomopathogenic fungi.

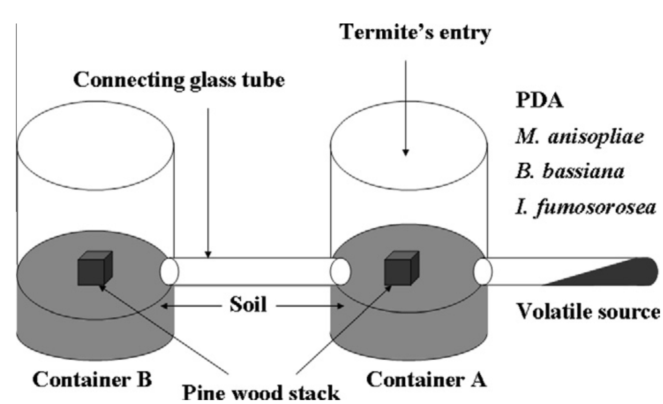

Fig. 1. Choice assay for testing behavioral response of *C. formosanus* to volatile organic compounds released by fungal pathogens.