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Musty odor of entomopathogens enhances disease-prevention behaviors in the termite *Coptotermes formosanus* ☆

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ABSTRACT

Termites often eliminate pathogens directly through mutual grooming, and are thereby prevent infections from entomopathogenic fungi. Our previous study confirmed that the antennae of *Coptotermes formosanus* sensitively responded to the musty odor of entomopathogenic fungi. However, it is unclear if this odor has any effect on termite behavior. The purpose of this study was to clarify the effects of fungal odor on termite behavior, especially on conidia removal. The musty odor was prepared as an aqueous solution by immersing conidia in distilled water. When untreated termites were mixed with fungal-odor-treated termites at a ratio of 4:1, mutual grooming and attack of treated termites were frequently observed. This indicated that the fungal odor triggered these behavioral responses. While some components of the fungal odor were found in all of the entomopathogenic fungi tested, the odor profiles differed among the isolates.

1. Introduction

Hygiene behavior plays a key role in insect prevention against pathogens (Oi and Pereira, 1993; Swanson et al., 2009). Mutual grooming behavior, which has been well studied in termites, is a typical hygiene behavior (Kramm and West, 1982; Boucias et al., 1996; Shimizu and Yamaji, 2003). Through mutual grooming, termite workers ingest fungal conidia on the cuticle of a nestmate with the glossae and dispose of them through their alimentary tract (Yanagawa and Shimizu, 2007; Chouvenc et al., 2009). When together with nestmates, mutual grooming reduces the chances of termites getting infected by entomopathogenic fungi. This aspect of termite behavior is one of the key reasons that control of termite populations with entomopathogenic fungi had so far only limited if any effect. Since such biological approaches are an environment-friendly alternative to the current chemical control (Verma et al., 2009), it is important to identify the cues that induce termite hygiene behavior. Although microbes vary greatly with regard to competitive strength, attachment pattern, germination ability, environmental adaptability, and so on (Clarkson and Charnley, 1996), it is not yet clear what cues lead termites to notice the presence of entomopathogenic fungi on the cuticle of their nestmates.

Termite hygiene behaviors are most likely triggered by chemical information, since most termites are blind. Recent studies have revealed that termite antennae sensitively respond to the musty odors of entomopathogenic fungi (Yanagawa et al., 2009, 2010). To understand the role played by chemical perception in *Coptotermes formosanus* behavior, we investigated whether odor from entomopathogenic fungi may be the cue that induces termite hygiene behavior. We report here the results from a laboratory study.

2. Materials and methods

2.1. Insects

Matured workers of *C. formosanus* were obtained from a laboratory colony maintained since 2002 (Okayama, Japan) in the dark at 28 °C and more than 85% R.H. at Kyoto University, Japan. Termites were separated into two groups, A and B, and each group was placed in a Petri dish (90 × 15 mm). At the center of the dishes was a filter paper (about 90 mm in diam., Whatman No.1) that was impregnated with distilled water (group A) or an aqueous

☆ Fungal odor enhances termite disease-prevention behavior.

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solution of 0.05% (wt/wt) Nile blue A (group B). They were then kept at 25 °C for 1–2 weeks before use in the bioassay. This treatment stained all of the termites in group B blue.

2.2. Preparation for collecting fungal odor

Three isolates of highly virulent entomopathogenic fungi, *M. anisopliae* 455, *I. fumosorosae* K3 and *B. brongniartii* 782, and three low-virulence isolates, *M. anisopliae* UZ, *I. fumosorosae* 8555 and *B. bassiana* F1214 were selected. Termites show 90–100% mortality on highly virulent fungi and 10–50% mortality on low virulent fungi at 7 days after treatment, and there are 10- to 100-fold difference in LC_{50} between lower- and higher-virulence fungi when five termites are kept in a dish (for further information see Yanagawa and Shimizu, 2005).

All of the *Metarhizium*, *Isaria* and *Beauveria* fungi were maintained on L-broth agar (1% polypeptone, 0.3% yeast extract, 2.0% sucrose, 0.5% NaCl, 2.0% agar) at 25 °C. Entomopathogenic fungal conidia were harvested with a brush from 10- to 15-day-old cultures and suspended in distilled water. About 3 ml of conidia suspensions, which contained 1.59×10^7–3.94×10^8 conidia/ml, could be collected from one culture. The conidial suspensions were left overnight at 25 °C and the conidia were then removed through a 0.2 μm filter unit (Dismic-13CP, Advantec, Japan). Volatiles trapped in these filtered solutions were used for fungal odor.

One milliliter of distilled water was prepared as control solution W. Another distilled water solution was prepared by gently washing the surface of solid L-broth agar that had not been inoculated with entomopathogenic fungi as control solution L. These two control solutions were left overnight and filtered as described above.

2.3. Comparison of grooming behavior among the 6 isolates

In this assay, groups of four workers treated with control solution and one worker treated with fungus odor were kept in a Petri dish (35 × 15 mm) and their touching frequency was monitored. The odor-treated termites were taken from the blue-stained termite group B, all termites treated with control solution originated from the unstained group A. As a control, one termite in group B was treated with control solution W or L and added to four termites from group A treated with control solution W, and their touching frequency was estimated.

For treatment, termites were collected from the Petri dishes and put in 1 ml microcentrifuge tubes containing a fungus-odor solution. The termites were submerged in the solution with gentle swirling for 5 s and allowed to dry on Whatman filter paper. The treated termite groups were then partitioned into the dishes. After treatment, five termites were placed in Petri dishes and covered with a cardboard box during the experiment to reduce the effect of room light on termite movements. The termites were then left for 15 min to reduce the impact of the artificial treatment. Since it was impractical to observe and estimate the level of grooming behavior for the entire duration of termite activity, the frequency at which the termites touched each other was counted on photographs taken every 30 s for 15 min. Only termites for which the mouth parts touched their nestmates were counted. A total of 30 photographs were taken per dish to clarify differences in grooming behavior among the six isolates in addition to the two control solutions. Data were obtained from 20 replicates, thus 800 termites were used.

2.4. Comparison of disease-preventive behaviors and other responses

Daily observation of other hygiene behaviors was conducted using the same assay model as described above; four termites treated with control solution from group A were allowed to contact a single fungus odor-treated nestmate from group B, and their behaviors were observed. Dead individuals were not removed and the responses of other termites to the dead individual were also observed. Attack, cannibalism, burial and death caused by contact with the odor-treated termite in a dish were observed for a week, and the number of dead individuals and the duration until the first dead individual was found were estimated. The death rate was calculated both for all of the individuals in a dish and for only the odor-treated individual in a dish. Attacks were determined by the loss of body parts, and therefore cannibalism only included the eating of a dead body. As a control, one termite in group B treated with control solution W (distilled water solution) or L (distilled water solution treated with L-broth medium) and four termites in group A treated with distilled water solution were placed in a dish. Data were obtained from 20 replicates.

2.5. Identification of fungal volatiles

Volatiles from entomopathogenic fungal conidia collected in water were extracted with SPME fiber.

SPME fiber coated with 100 μm polydimethylsiloxane (Red: 100 m; Supelco, Bellefonte, PA, USA) was used to sample the volatiles in the solution. A SPME fiber was immersed in 3 ml of each of the solutions containing odor substances for 30 min at room temperature.

The gas chromatography apparatus was a GC-14A equipped with a polar capillary column, DB-WAX (30 m length, 0.25 mm diameter, 0.25 μm film thickness; J & W Scientific. Inc.) and a flame ionization detector. Helium was used as the carrier gas. The SPME fiber was inserted into the GC injection port kept at 200 °C for 1 min in splitless mode with a detector temperature of 220 °C. The column oven was programmed to hold at 40 °C for 5 min, to increase at 10 °C/min to 180 °C and then 20 °C/min to 220 °C, and finally to hold for 10 min at 220 °C. The substances collected from the solutions were also analyzed by a Shimadzu QP5000 GC–MS system (Shimadzu, Japan) equipped with a polar capillary column (DB-WAX polar column). Helium was used as a carrier gas at a flow rate of 50 ml/min. The 70 eV EI spectra were recorded at a rate of 0.5 s per scan. Volatile compounds were tentatively identified by comparison to the mass spectra and retention times of authentic compounds, which were purchased from Nacalai Tesque (Kyoto, Japan).

2.6. Statistical analysis

To compare the differences in the grooming behavior of termites, a Poisson regression (Proc GENMOD, SAS institute, 1999) was applied. For the analysis of behavioral differences and the number of dead individuals, a logistic regression was applied and the survival time of the first individual to die, i.e. the duration until the first dead individual was found, was analyzed by a Cox regression model using JMP 6.0 software (SAS). The differences between control groups, which were treated with distilled water and broth solution, and the odor-treated groups were described in terms of fungal odor parameters, and the differences among the six isolates were examined with respect to genera, isolates and virulence.

3. Results

3.1. Comparison of grooming behavior among the 6 isolates

Grooming behavior was estimated in terms of the touching frequency among five termites in a dish consisting of one odor-treated and four untreated termites. The frequency of mutual touching in a group is presented in Fig. 1A and that toward one