INTERACTIONS between FORMOSAN SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE) and WOOD DECAY FUNGI

Mary L. Cornelius, Donald J. Daigle, William J. Connick, Mario Tellez[1], Kelley S. Williams, and Mary P. Lovisa

United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124

[1]United States Department of Agriculture, Agricultural Research Service, Natural Products Center, P.O. Box 8048, Oxford, MS 38677

Abstract This study compares the responses of termites to the lignin-degrading basidiomycete, *Marasmiellus troyanus* (Murrill) Singer, grown on different substrates, and examines the tunneling behavior of termites in sand treated with extracts of fungus-infected pure lignin versus solvent-treated sand. Termites showed a significant preference for lignin infected with *M. troyanus* versus uninfected lignin and sawdust infected with *M. troyanus* versus uninfected sawdust, but not cellulose infected with *M. troyanus* versus uninfected cellulose. When termites were presented with a choice of lignin versus sawdust, there were no significant differences in the response of termites in the tests where both substrates were uninfected or where both substrates were infected with *M. troyanus*. However, in choice tests where only one of the substrates was infected, there were significantly more termites in the fungus-infected substrate. The number of termites was significantly greater in tips containing extract-treated sand compared with tips containing solvent-treated sand for tests with both the pentane and methanol extracts of lignin infected with *M. troyanus*. These results indicate that *M. troyanus* produces a chemical during the process of metabolizing lignin that elicits a positive response in termites.

Key Words *Coptotermes formosanus Marasmiellus troyanus* tunneling behavior foraging behavior lignin

INTRODUCTION

Both subterranean termites and wood decay fungi play an important role in forest ecosystems as decomposers of wood. Wood decay fungi may provide nutritional benefits to subterranean termites by increasing the availability of nitrogen and other nutrients, breaking down toxic compounds in the wood, and enhancing the ability of termites to metabolize cellulose by chemically modifying the wood (Waller and LaFage, 1987). However, in some cases, wood decay fungi may cause chemical modifications to the wood that negatively affect termite feeding behavior by metabolizing cellulose, producing toxic compounds, or by accelerating the process of decomposition of the wood to the point where the wood is no longer suitable as a food source for termites (Amburgey and Beal, 1977; Amburgey, 1979).

In order to increase our understanding of the ecological relationships between termites and fungi, this study examines how the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, responds to the lignin-degrading basidiomycete, *Marasmiellus troyanus* (Murrill) Singer, grown on different substrates. Previous studies determined that *C. formosanus* showed a strong preference for spruce sawdust infected with *M. troyanus* over uninfected sawdust, but that termites did not respond to *M. troyanus* when the fungus was grown on potato dextrose agar (PDA) (Cornelius et al., 2002). The current study compares the responses of termites to *M. troyanus* grown on spruce sawdust, pure cellulose, and pure lignin to determine how the fungus affects termite behavior when grown on these substrates. This study also examines the tunneling activity

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of termites in sand treated with extracts of fungus-infected pure lignin to determine whether termite behavior is influenced by chemicals produced by the fungus when cultured on pure lignin.

MATERIALS and METHODS

Collection and Maintenance of Termite Colonies and Fungus Cultures

Formosan subterranean termites were collected from field colonies in New Orleans, LA, using underground bucket traps (Su and Scheffrahn, 1986) baited with blocks of spruce wood (*Picea* spp). Termites were kept in the lab in 5.6-L covered plastic boxes containing moist sand and blocks of spruce wood until they were used in experiments. The litter rot fungus, *M. troyanus* (TF-1867), was isolated from leaf litter in an abandoned oil refinery in Darrow, LA. Stock cultures of fungi were maintained at 8° C on malt extract agar slants.

Inoculation of Fungus-Infected Material

The substrates used in this experiment were pure lignin (Westvaco, Charleston, SC); pure cellulose, Solka-Floc (F. S. & D., Urbana, OH); and spruce sawdust (*Picea* spp). Potato dextrose agar (PDA) plates were inoculated with *M. troyanus* and placed in incubators set at a temperature of 25° C with a photoperiod of 12:12 (L:D) h for 4 d. After 4 d, 15 (1-cm square) plugs were removed from the PDA plate and used to inoculate one liter of Sabouraud dextrose broth, using an orbital shaker at 120 rpm at room temperature for 7 d. After 7 d, the broth was filtered through a buchner funnel using a sterile Whatman #4 filter paper. The mycelium was rinsed with 650 ml of sterile Milli-Q™ filtered water. The mycelium was weighed and resuspended in 180 ml of sterile water, stomached on high for 60 seconds. The substrate (sawdust, lignin, or cellulose) was placed in an autoclavable polypropylene vent bag (36 x 61 cm) (Unicorn Imp. and Mfg. Corp., Commerce, TX) with a single 0.2 micron filter (7.6 cm x 25.4 cm). The bag was heat sealed, and a 2-cm slit, loosely covered by autoclave tape, was made in the bag. The bag was autoclaved, vent-side up, for 60 min on each of two consecutive days. After cooling to room temperature, the substrate was inoculated with 43.4 g of the mycelium. The vent bags were placed in an incubator set at a temperature of 25° C with a photoperiod of 12:12 (L:D) h for 14 d. For all three substrates (cellulose, lignin, or sawdust), bags of uninfected material were prepared using the same procedure to serve as controls.

Preparation of Extracts of Fungus-Infected Pure Lignin

Pure lignin, inoculated with 125.18 g of mycelium of *M. troyanus* and kept in an incubator as described previously, was extracted in a Waring commercial laboratory blender with pesticide grade pentane (300 ml X 3) at low speed for approximately 1 minute each time. Pentane extracts were separated from the insoluble lignin through vacuum filtration (Whatman paper # 1) and combined. The pentane was then removed under reduced pressure with the help of a rotary evaporator to ~5ml, transferred to a vial, and dried under nitrogen to afford 0.249 g of an oily yellow residue (pentane extract). The pentane-insoluble solids were further extracted with ACS grade dichloromethane in the same way to afford 0.306 g of a dark brown residue (dichloromethane extract). The pentane-insoluble and dichloromethane-insoluble solid material was further extracted overnight with HPLC grade MeOH (4 L) in a 4 L erlenmeyer flask fitted with a Lightning Lab Master mechanical stirrer at 250 rpm. Solids (43.619 g) were removed through vacuum filtration and the filtrate was placed in a freezer (-40°C) overnight. Precipitated solids (1.348 g) were removed through vacuum filtration. Solvent from the filtrate was removed under reduced pressure to afford 47.837 g of a dark brown residue (methanol extract).

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Termite Responses to Fungus-Infected Material

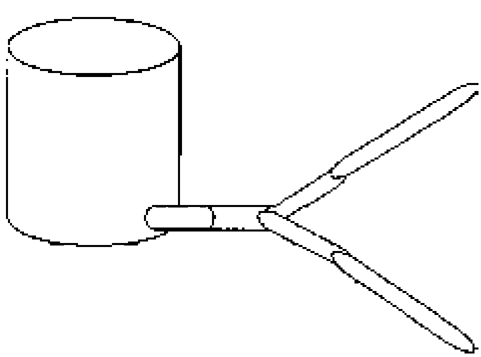
A series of choice tests were conducted to examine termite responses to *M. troyanus* cultured on pure cellulose, pure lignin, or spruce sawdust. Because termites were constructing tunnels in the pure lignin, choice tests were also conducted to compare tunneling behavior of termites in lignin versus untreated sand. The following choice tests were conducted: 1) lignin infected with *M. troyanus* versus uninfected lignin; 2) sawdust infected with *M. troyanus* versus uninfected sawdust; 3) cellulose infected with *M. troyanus* versus uninfected cellulose; 4) uninfected lignin versus uninfected sawdust; 5) lignin infected with *M. troyanus* versus sawdust infected with *M. troyanus*; 6) lignin infected with *M. troyanus* versus uninfected sawdust; 7) uninfected lignin versus sawdust infected with *M. troyanus*; 8) lignin infected with *M. troyanus* versus uninfected cellulose; 9) uninfected lignin versus uninfected cellulose; 10) lignin infected with *M. troyanus* versus sand; 11) uninfected lignin versus sand.

The testing device was comprised of a 9 cm high x 7 cm diameter, clear polystyrene, cylindrical screwtop container (Consolidated Plastics, Twinsburg, Ohio) with 5-cm length piece of Tygon tubing (0.8 cm I. D. diameter) inserted through a hole on the lower side of the container and sealed in place with a hot glue gun. A plastic Y-tube (stem: 3 cm; arms: 3 cm; diameter: 1 cm) was attached to the distal ends of the Tygon tube and another 5-cm length piece of Tygon tubing (0.8 cm I. D. diameter) was attached to each arm of the Y-tube. A 1-ml disposable pipette tip (length: 7 cm; diameter: 1 cm at wide end) was attached to the other end of the Tygon tubing (Figure 1). A thin layer of moist sand (Frey Scientific, Mansfield, OH) was placed on the bottom of the container. Termites were able to move freely from the container into the tubing. In each container, 200 *C. formosanus* workers were placed in the center of the container. For each experiment, two termite colonies were used, with five replicates from each colony.

For each replicate, the disposable pipette tips were filled with the material being tested (sawdust, lignin, cellulose, or sand). For each experiment, the position of the tips containing the different treatments was rotated on the arms of the Y-tube between replicates to preclude any positional effects. These tests were conducted in ambient conditions in the laboratory. For each experiment, data were recorded on the number of termites in each tip after 3 h.

Termite Responses to Extracts of Fungus-Infected Pure Lignin

Crude extracts of fungus-infected lignin were tested using the same testing apparatus as described previously (Figure 1). Crude extracts (dichloromethane, pentane, and methanol) were tested at different concentrations, proportional to extract recovery. For these tests, sand was treated



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with either an extract of fungus-infected lignin or the solvent control. Sand was allowed to air dry until the solvent completely evaporated, and then each pipette tip was filled with sand and moistened with 400 microliters of distilled water. For each replicate, one arm of the Y-tube was connected to a pipette tip filled with sand treated with an extract of fungus-infected lignin and the other arm of the Y-tube was connected to a pipette tip filled with sand treated with the solvent. The position of the treatment and control tips on the arms of the Y-tube was rotated between replicates to preclude any positional effects. Each pipette tip was marked on the outer surface with a permanent marker at a distance of 2 cm from the narrow end of the pipette tip. Tunneling activity was observed until termites reached this mark. As soon as termites reached this mark in one of the tips attached to each Y-tube, both tips were removed and the number of termites in each tip was counted. For any replicates in which termites did not reach this mark in either tip within 5 h, both tips were removed after 5 h, and the number of termites in each tip was counted. These tests were conducted in ambient conditions in the laboratory. These experiments were conducted using three colonies of termites with four replicates of each colony. For each experiment, data were recorded on the number of treatment and control tips where termites tunneled through sand until they reached the mark and the number of termites in treatment and control tips.

Data Analysis

Data on numbers of termites in pipette tips in paired choice tests were analyzed using a t-test for matched pairs. In order to compare the tunneling activity of termites in sand treated with extracts of fungus-infected lignin versus solvent controls, data on the number of treatments and control tips which termites tunneled through sand until they reached the mark within 5 h were analyzed using the binomial distribution (Sign test) (SYSTAT, 1998).

RESULTS

Termite Responses to Fungus-Infected Material

There were significantly more termites in tips containing lignin infected with *M. troyanus* versus uninfected lignin and sawdust infected with *M. troyanus* versus uninfected sawdust. However, there was no significant difference in the number of termites in tips containing cellulose infected with *M. troyanus* versus uninfected cellulose (Table 1).

Table 1. Tunneling behavior of ***C. formosanus*** in pipette tips attached to each arm of a plastic y-tube over a 3 h period

Experiment	Mean Number of Termites in Pipette Tips	
Tip A versus Tip B	Tip A	Tip B
Lignin (*M. troyanus*) vs Lignin (control)	74.1 ± 12.2	20.4 ± 3.8**
Sawdust (*M. troyanus*) vs Sawdust (control)	27.8 ± 3.3	16.1 ± 3.1**
Cellulose (*M. troyanus*) vs Cellulose (control)	18.2 ± 1.3	15.1 ± 1.8
Lignin (control) vs Sawdust (control)	17.9 ± 4.8	10.1 ± 2.0
Lignin (*M. troyanus*) vs Sawdust (*M. troyanus*)	30.1 ± 4.8	24.9 ± 3.5
Lignin (*M. troyanus*) vs Sawdust (control)	48.6 ± 8.2	16.1 ± 3.2**
Lignin (control) vs Sawdust (*M. troyanus*)	15.3 ± 2.2	30.0 ± 4.4**
Lignin (*M. troyanus*) vs Cellulose (control)	29.3 ± 4.6	16.8 ± 2.8*
Lignin (control) vs Cellulose (control)	16.9 ± 4.0	21.4 ± 2.2
Lignin (*M. troyanus*) vs Sand	37.6 ± 4.1	21.7 ± 4.1**
Lignin (control) vs Sand	27.3 ± 3.5	22.3 ± 2.5

*, **, indicate significant *t* values at $P \leq 0.05$, $P \leq 0.01$, respectively.

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When termites were presented with a choice of lignin versus sawdust, there were no significant differences in the number of termites in tips in the tests where both substrates were uninfected or where both substrates were infected with *M. troyanus.* However, in choice tests where only one of the substrates was infected, there were significantly more termites in the fungus-infected substrate. When lignin was paired with either untreated cellulose or untreated sand, there were significantly more termites in the tests where the lignin was infected with *M. troyanus*, but not in the tests where the lignin was uninfected.

Termite Responses to Extracts of Fungus-Infected Pure Lignin

For the dichloromethane extract, there was no significant difference in the number of termites in treated and control tips after 5 h (Table 2). However, within the 5-h exposure period, termites tunneled completely through sand (termites reached the mark) in nine of the tips filled with solvent-treated sand and none of the tips filled with extract-treated sand. In contrast, there were significantly more termites in tips filled with extract-treated sand than solvent-treated sand for tests with both the pentane and methanol extracts. Differences in the number of tips where termites tunneled completely through the sand were not significant for these two tests.

DISCUSSION

Termites showed a preference for lignin infected with *M. troyanus* over uninfected lignin, sawdust infected with *M. troyanus* over uninfected sawdust, but not pure cellulose infected with *M. troyanus* versus uninfected cellulose. These results were consistent with findings of a previous study in which termites preferred sawdust infected with three species of wood decay fungi, including *M. troyanus*, over uninfected sawdust, but did not show any preference for pure cellulose infected with the white rot fungus, *Phanerochaete chrysosporium* Burdsall over uninfected cellulose (Cornelius et al., 2002). These results suggest that *M. troyanus* produces a chemical that elicits a positive response in termites during the process of metabolizing lignin.

Although there were substantial textural differences in the materials tested (lignin, sawdust, cellulose, and sand) that could affect termite behavior, the number of termites in tips were significantly different when only one of the substances was infected with *M. troyanus*. In tests where either both substances were fungus-infected or both were uninfected, the number of termites in tips was similar. Despite the textural differences in the materials, termites were primarily responding to the presence of the fungus.

Termites responded to crude extracts of fungus-infected lignin. Although there was no difference in the number of termites in tips treated with the dichloromethane extract versus the control, termites tunneled through solvent-treated sand faster than through extract-treated sand,

Table 2. Tunneling behavior of *C. formosanus* in pipette tips filled with sand treated with an extract of lignin infected with *M. troyanus* versus solvent-treated sand over a 5-h exposure period

Solvent	Extract Concentration[1] (in 20 g sand)	Number of Tips Termites Tunneled Through Completely: Treated	Number of Tips Termites Tunneled Through Completely: Control	Mean Number of Termites in Tips: Treatment	Mean Number of Termites in Tips: Control
Dichloromethane	40.6 mg	0	9*	26.8 ± 5.0	32.6 ± 2.5
Pentane	40.6 mg	8	3	24.9 ± 4.8	16.9 ± 2.9*
Methanol	200 mg	8	4	28.5 ± 3.9	17.9 ± 1.8*

* indicate significant *t* values at $P \leq 0.05$.
[1]Extracts were tested at different concentrations, proportional to extract recovery.

suggesting that a nonpolar chemical produced by the fungus may inhibit tunneling activity. Our previous research has demonstrated that termite responses to material infected with *M. troyanus* varies from a strong preference to a strong avoidance, depending on the substrate and the degree of degradation. For instance, when *M. troyanus* was cultured on blocks of spruce in a 12 h light: 12 h dark cycle, termites showed a strong avoidance of fungus-infected blocks when the blocks were decayed for only 2 weeks, and showed a strong preference for fungus-infected blocks when they were decayed for 4, 8, or 12 weeks (Cornelius, unpublished). Although crude pentane and methanol extracts did not increase the tunneling activity of termites, there were significantly more termites in extract-treated sand versus solvent-treated sand for both of these extracts, suggesting that the preference of termites for fungus-infected lignin is due to chemicals produced by the fungus. It is possible that *M. troyanus* produces chemicals that elicit a negative response in termites, as well as chemicals that elicit a positive response and that termite behavior is influenced by changes in the ratio of these chemicals in fungus-infected material during the process of degradation. Further studies are necessary in order to isolate and identify specific chemicals produced by *M. troyanus* that affect termite behavior.

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