Effect of Orange Oil Extract on the Formosan Subterranean Termite (Isoptera: Rhinotermitidae)

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ABSTRACT The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), accidentally brought into the United States, has become a major urban pest, causing damage to structures and live trees. Because of increasing restrictions on the use of conventional termiticides, attention is focused on finding safer alternative methods for termite management. Oil from citrus peel, referred to here as orange oil extract (OOE), contains ≈92% *d*-limonene, and it is generally known to be toxic to insects. In laboratory experiments, 96 and 68% termites were killed in 5 d when OOE at 5 ppm (vol:vol) was dispensed from the top or bottom, respectively, with termites held at the opposite end of a tight-fitting plastic container. Apart from high mortality, workers exposed to vapor consumed significantly less filter paper than controls. However, when termites were exposed to OOE vapor, even at 10 ppm, in the void of a model wall, there was very little mortality. Termites did not tunnel through glass tubes filled with sand treated with 0.2 or 0.4% OOE. Sand treated with OOE was extracted each week for 8 wk to determine the remaining amount of *d*-limonene. Results indicated that there was a sharp decline in the quantity of *d*-limonene during the first 3 wk to a residual level that gradually decreased over the remaining period. With a suitable method of application and in combination with other control practices, OOE can be effectively used for the control of subterranean termites.

KEY WORDS *Coptotermes formosanus*, orange oil extract, *d*-limonene, fumigation, tunneling behavior

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), was first detected in the continental United States in 1965 in Houston, TX, and it has since spread rapidly throughout the southeastern United States, mainly by anthropogenic means such as transport of infested railroad ties, pallets, utility poles, and boats (Woodson et al. 2001). As a subterranean termite, *C. formosanus*, builds carton nests in dark spaces such as within walls, beneath houses, and in the hollow of trees. Direct spraying of an entire colony with pesticides is very difficult, if not impossible. With greater restrictions being placed on the use of conventional termiticides (Isman 2006), there is a growing need to find alternate and environmentally safer methods for the management of this urban pest.

Several studies have been carried out to evaluate the effect of natural products, in particular plant essential oils and their components on termites. Whereas, Wilkins (1992) reviewed naturally occurring antitermite compounds, Tellez et al. (2003) selectively reviewed natural products for the control of the Formosan subterranean termite. Most of the previous studies have looked at mortality after contact exposure and/or tunneling through sand treated with a test product (Grace and Yates 1992; Zhu et al. 2001, 2003; Chang and Cheng 2002; Ibrahim et al. 2004a, 2004b; Chauhan and Raina 2006; Fokialakis et al. 2006; Meepagala et al. 2006). Shaaya et al. (1991) tested essential oils and associated compounds from several plants as fumigants against several stored-product insects. Bläske and Hertel (2001) determined the effect of indirect exposure of four plant extracts on four species of termites, including *C. formosanus.* Park and Shin (2005) reported on the fumigant activity of essential oils against the termite *Reticulitermes speratus* (Kolbe).

Oils from citrus peel contain a diverse group of compounds (Wilson and Shaw 1971). Among these compounds, *d*-limonene [(*R*)-4-isopropenyl-1-methylcyclohexene] is the major component and can account for up to ≈98% (Hink and Fee 1986). *d*-Limonene is used in a variety of foods and beverages and in this context is classified by the U.S. Food and Drug Administration as a "Generally Recognized as Safe" compound. Limonene is also frequently used as an ingredient in cleaning products. Orange peel oil was reported to be toxic to seven species of insects (Sheppard 1984). The oil was effective by contact

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application as well as fumigation against house flies, *Musca domestica* L., and red imported fire ants, *Solenopsis invicta* Buren. Hollingsworth (2005) reported the use of citrus extract for mealybug and scale insect control. Hink and Fee (1986) noted that the vapors of *d*-limonene were the main cause of toxicity to all life stages of the cat flea, *Ctenocephalides felis* (Bouché). High concentrations of the vapors also caused mortality in the German cockroach, *Blattella germanica* (L.), and rice weevils, *Sitophilus oryzae* (L.) (Karr and Coats 1988). *d*-limonene also has proved very effective as a contact toxicant against drywood termites (R. Scheffrahn: personal communication).

The purpose of this study was to determine the effect of orange oil extract (OOE) on the Formosan subterranean termite, particularly if used as a fumigant. We also investigated the potential of orange oil-treated sand as a termite barrier and conducted residue analysis to determine its longevity in treated sand.

Materials and Methods

Termites and Chemicals. Formosan subterranean termites were collected from bucket traps (Su and Scheffrahn 1986) placed in New Orleans' City Park (colonies designated as 1753 and 1776) and the Lakefront campus of the University of New Orleans (colony UNO) in New Orleans, LA. After bringing the collection into the laboratory, all the debris was removed, and termites (workers and soldiers) were kept in plastic boxes with moist spruce slats. The boxes were stored in incubators maintained at 28°C, 65% RH, and constant darkness. The OOE used in this study was a product marketed under the name of XT-2000 (XT-2000 Inc., San Diego, CA) and contains purportedly 92% *d*-limonene.

Vapor Action of OOE. Tall rectangular plastic (polystyrene) containers (10 by 10 by 21 cm, inside volume 1,895 cm^3, Pioneer Plastics, Dixon, KY) were used for the first experiment (Fig. 1A). A 90-mm-diameter filter paper (Whatman no. 1, Whatman, Clifton, NJ) was oven-dried, weighed, and placed in a glass petri dish at the bottom of each container. The filter paper was moistened with 1.2 ml of distilled water, and 50 workers (average third instar) and five soldiers placed on it. A 4.25-cm-diameter filter paper was treated either with 4.75 or 9.5 μl OOE in 100 μl of hexane, and after allowing the solvent to evaporate, the filter paper was suspended from the top of the container to produce assumed (based on volume calculations) OOE concentration of 2.5 ppm or 5 ppm (vol:vol), respectively. For control, the filter paper was treated with hexane. Termites were checked for mortality, and dead termites removed every day for 5 d after which the filter paper that served as food source was removed, carefully cleaned of all debris, oven-dried, and weighed to determine consumption. Termites from three colonies were used in the test, and the test was replicated five times.

A second experiment was carried out in a glass cylinder (11.25 cm in diameter by 52.5 cm in height; volume 5.2 liters). Six 30-ml plastic cups, each with 10 g of moist sand, were suspended from the rim of the cylinder so that these were either 5 or 25 cm from the top (Fig. 1B). A 4.25-cm-diameter filter paper treated with 26 μl of OOE in 100 μl of hexane was placed in a glass petri dish at the bottom of the cylinder to produce an assumed OOE concentration of 5 ppm (vol:vol). Fifty termite workers from each of the three colonies were released into one of the cups at each level. The top of the cylinder was covered with aluminum foil tightly held in place with rubber bands. For control, the filter paper was treated with hexane. Cups were removed, and termites were examined daily for mortality over a period of 5 d. The experiment was replicated four times.

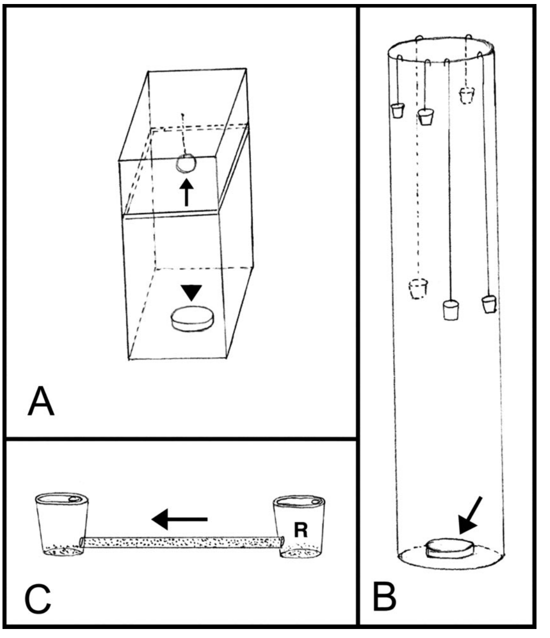

Fig. 1. Experimental setup to test the effect of OOE on *C. formosanus*. Effect of OOE vapor dispensed from top (A) arrow points to the filter paper with OOE, arrowhead indicates the petri dish with termites; vapor dispensed from bottom (arrow), with termites in cups kept at two different levels (B); and tunneling through a tube filled with sand mixed with OOE (C), R indicates the release cup and arrow the direction of termite tunneling.

A third test involved a model wall (82.5 by 32.5 by 10 cm) of 2 by 4 pine frame with the sides made of 9-mm-thick plywood. The inside hollow area of this wall was 0.0164 m^3. A 12.5-mm-diameter opening in the pine frame provided for the introduction of termites and monitoring of their activity with a VideoProbe XL (Everest Imaging, Austin, TX). The wall was placed over a layer of wet sand, and 500 ml of water was poured inside. After 2 d, allowing for the wood to absorb the water, 5,000 workers and 500 soldiers collected from a new colony (1559) from City Park, New Orleans were introduced into the hollow of the wall. After two additional days, a filter paper strip treated with 164 μl of OOE (10 ppm, vol:vol) was suspended from a rubber stopper from another hole at the top. Termite activity was periodically checked, and the wall was opened after 72 h to determine mortality.

Tunneling Assay. The procedure was previously described by Chauhan and Raina (2006). Briefly, medium grain sand was mixed thoroughly with 0.0, 0.2 or 0.4% (wt:wt) OOE in hexane. After allowing the hexane to evaporate, 2.8 g of the treated sand was filled into a glass tube (15 cm long, 4 mm ID), connected at either end to two 30 ml plastic cups with lids (Bio-Serv, Frenchtown, NJ) each containing 10 g of moist sand (Fig. 1C). Fifty workers were released into one of the cups and tunneling activity (distance tunneled) was monitored at 4, 20, 24, 28, 44, 52, 68 and 72 h. The experiment was conducted with workers from three colonies and replicated three times.

Residue Analysis of OOE in Sand. Medium grain dry sand (418.5 g) was mixed with OOE to obtain a concentration of 5,000 ppm (wt:wt) of *d*-limonine. The treated sand was filled into 13- by 100-mm glass tubes (15.5 g/tube) and placed uncovered in a rack at room ambience (21 ± 2°C and 50 ± 8% RH). OOE was immediately extracted from the sand in three tubes by using hexane. Thereafter, every 7 d for the next 8 wk, three tubes were extracted. The extraction was accomplished by gravity filtration of 4 by 4 ml of hexane through the sand contained in a sintered glass funnel. The final volume of the collected solution was adjusted to 12 ml. Samples were analyzed by gas chromatography-mass spectrometry on an HP 6890/5973 (Agilent Technologies, Wilmington, DE) by using a Zebron ZB-5 (30 m by 0.25 mm; 0.25-μm dp) column (Phenomenex, Torrance, CA). A 2-μl sample was injected using the following conditions: pulsed splitless injection (150°C, pulse pressure = 25 psi for 1.1 min); oven temperature programmed from 45°C with a 1-min hold to 180°C at 7°C/min; He carrier gas at 1.5 ml/min; detection based on single ion monitoring at m/z 68, 79, 93, 107, 121, and 136 with 20 ms dwell times; and 70 eV EI source.

Statistical Analysis of Data. Two-way analyses of variance were performed using treatment, colony, replicate, and/or day as possible class variables with mortality, consumption, or distance tunneled as the response variable. Least-squares means were calculated for main effects and their interaction. In the second experiment with the glass cylinder, the experimental design used was a split plot where factorial arrangements consisted of three colonies and four treatments, and subunit was a repeated measure over 5 d. Repeated measure error structure was modeled using a first-order autoregressive covariance structure. Statistical analyses were performed using SAS version 8.02 software (SAS Institute, Cary, NC).

Results

Vapor Action. When exposed to vapors of OOE from a filter paper suspended in a tightly sealed plastic container, 96 and 49% of the termites died in 5 d at the high and low doses respectively (Fig. 2). The UNO colony was most susceptible at the higher dose, showing 100% mortality in 4 d (data not shown). Mortality in the control group ranged from 4 to 17% with an average of 8.6% for all three colonies. There was a significant effect of both treatment ($P < 0.0001$) and colony ($P < 0.0001$) on the observed mortality; however, there was no significant effect of interaction of the variables ($P = 0.1777$). Consumption of the filter paper was significantly reduced ($P < 0.0001$) in treatments involving both high and low doses of OOE (Fig. 3). There was no significant effect of colony ($P = 0.6856$) or the interaction of colony and treatment ($P = 0.2966$). When analyzed as consumption per live termite, consumption was still significantly lower in the treatment groups, indicating that although some of the termites were alive, they were apparently not feeding.

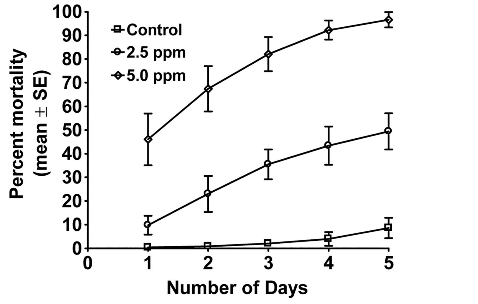

Fig. 2. Mortality of *C. formosanus* exposed to vapors of OOE dispensed from the top. Means are mortalities of workers from three colonies.

In the experiment where the treated filter paper was placed at the bottom of the chamber, the overall mortality was lower compared with the previous experiment in which OOE was dispensed from above the termites. At the end of 5 d, there was a significant effect of the treatment on mortality ($P < 0.0001$) as well as a slightly significant effect of colony ($P = 0.0148$), but no significant effect of the interaction of the two. Mean mortality among the control termites was 4.32 and 4.67% at 5 and 25 cm, respectively, over the 5-d test period (Fig. 4). From the data, it seemed that there may be significant differences between the 5- and 25-cm OOE-treated cups over the 5-d period. Because of the nonhomogenous variances between the control and noncontrol groups, treatments were

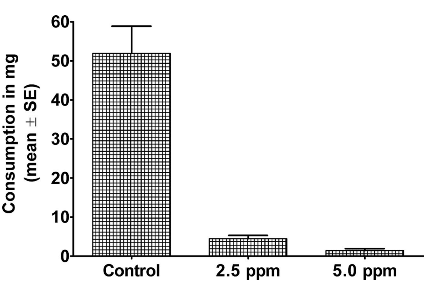

Fig. 3. Consumption of filter paper by termites exposed to vapors of OOE. Mean consumption is for workers from three colonies.

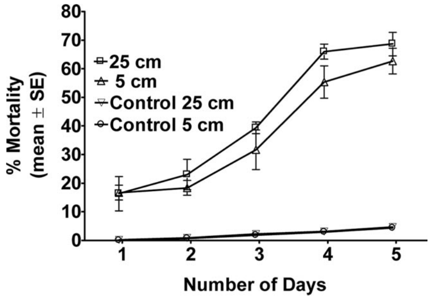

Fig. 4. Mortality of *C. formosanus* workers kept at five and 25 cm from the top and exposed to vapors of OOE dispensed at the bottom of the container.

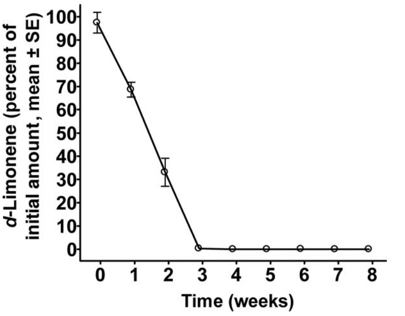

Fig. 6. Analysis of *d*-limonene residue in OOE treated sand over a period of 8 wk. Values indicate percent of original amount averaged for three samples each week.

split and control and noncontrol groups were analyzed separately. There were no significant differences in mortality between the 5- and 25-cm OOE-treated groups ($P = 0.07$). The results indicated that the only significant effect was day ($P < 0.0001$), and there were no significant effect of colony, treatment (cup height in this case), or any interactions between colony, day, and/or treatment. Application of OOE vapor inside the hollow of a wall, even at twice the higher concentration used in the earlier two experiments, did not cause desired mortality among the termites. Periodic observations revealed that the termites were actively moving and had even started tunneling through the wood. At the end of 3-d exposure, only 15.4% of the termites released in the wall were dead.

Tunneling Experiment. In control tubes filled with solvent-treated sand, the termites tunneled through the entire length (15 cm) within 24 h, and more than half way in the first 4 h (Fig. 5). However, when the sand was treated at either 0.2 or 0.4% wt:wt with OOE, no termites of any of the three test colonies were able to tunnel through the entire tube, even after 72 h. At the higher concentration, the termites did not make it beyond 3 cm, and most of them died in the attempt. Tunneling behavior of termites from individual colonies was very similar.

Residue Analysis. There was a sharp decline in the amount of *d*-limonene in OOE-treated sand during the first 3 wk (Fig. 6). After the 3-wk period, the amount was barely detectable, even when using single ion monitoring of limonene-m/z values, and it stayed that way for the next 5 wk. However, throughout this latter period, a slight odor associated with OOE was detected in the sand.

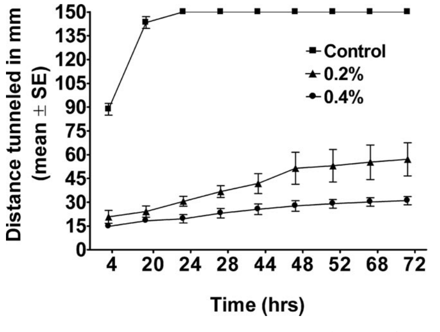

Fig. 5. Distance tunneled over time by *C. formosanus* workers through glass tubes filled with OOE-treated sand. Means are of three colonies.

Discussion

Whereas plant essential oils and compounds thereof have extensively been tested for their toxicity to insects, there are only few studies in which termites, including *C. formosanus*, were used as the target pest. In most of these studies, test compounds were evaluated for contact toxicity. There is only one previous report in which limonene as a component of the essential oil of *Calocedrus* sp. was tested against *C. formosanus* (Cheng et al. 2004). Although our results have confirmed previous reports of toxicity to insects of citrus peel oils with *d*-limonene as its major component, we have shown that in our experiments toxicity to *C. formosanus* was due to both fumigant (vapor action assays) and contact action (tunneling assay) of OOE. For the sake of calculations, we also assumed that all of the limonene applied to the filter papers vaporized and that there was no absorption or adsorption to the surface or diffusion from the test container.

When equivalent amounts of OOE were applied, there was a higher mortality (96%) when the OOE-treated filter paper was suspended from the top of the container, compared with the placement of the treated paper at the bottom (68 and 63% in cups situated at the middle and top of the cylinder, respectively). It is possible that some of the OOE vapor, particularly near the top, was lost when the container was opened for observations. Looking at the consumption of the filter paper, we observed that even when cumulative mortality was no >49% (at 2.5 ppm, vol:vol), the consumption was very low. Therefore, even though some of the termites were alive, they were presumably not feeding, and they may eventually die. When tested in an artificial wall, application of OOE vapor even at 10 ppm vol:vol resulted in very low mortality. There is no real explanation for this effect, except that the wood may have rapidly absorbed most of the *d*-limonene. Alternatively, some of the vapor may have escaped, resulting in lower total concentration. Moreover, this test was carried out for only 3 d and greater mortality may have resulted from longer exposure. Although it would be impractical to fumigate an entire house for a period of three or more days, voids in sections of a wall with termite infestation could be effectively treated with OOE.

In most previous studies involving vapor action of plant essential oils and their individual components (Sharma et al. 1994, Bläske and Hertel 2001, Park and Shin 2005), the actual volume of the container was not taken into consideration. Thus, application of eucalyptus oil at 5 mg/cm^2 of filter paper (Sharma et al. 1994) amounted to a very high concentration in a petri dish. Therefore, it is not surprising that they observed 100% mortality of the termite *Odontotermes brunneus* (Hagen) in 24 h. However, Shaaya et al. (1991), using actual volume of the treated space, reported 100% mortality in 24 h among stored-product insects, by essential oils of sage, lavender, and rosemary at 15 ppm.

Tunneling of termites through sand treated with plant essential oils and other natural compounds also has been extensively studied; the concept being that a barrier of treated sand placed around a structure could prevent subterranean termites from invading it. Mixed at either 0.2 or 0.4% wt:wt OOE, termites did not tunnel through the entire length of the tubes filled with treated sand. All the termites died before they crossed one third the length of the tube. Considering the low cost of OOE, this could be very promising except that the effectiveness of treated sand may not last very long. Residue analysis of sand treated with 0.5% OOE revealed that in 3 wk the quantity of *d*-limonene was almost negligible. Bläske et al. (2003) reported that there was no trace of isoborneol, 7 d after it was applied to sand at 0.02%. They further reported that loamy sand and sandy clay types of soil were somewhat better in retaining the compound during the same period.

d-Limonene is much less toxic to mammals than commonly used insecticides. The LD_{50} in rats is >5,000 mg/kg (Hall and Oser 1965). However, *d*-limonene has caused allergic contact dermatitis in humans and animals (Wakelin et al. 1998). Considering these results, OOE may not prove to be very effective as a barrier against the Formosan subterranean termite. However, with proper application technology and when used in combination with other control measures, OOE may prove to be effective against subterranean termites particularly as a fumigant for walls and other tight spaces.

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