Effects of Multiple Generations of *Metarhizium anisopliae* on Subterranean Termite Feeding and Mortality (Isoptera: Rhinotermitidae)

by

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ABSTRACT

This research evaluated the attractancy and mortality caused by *Metarhizium anisopliae* on two species of subterranean termites, *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki. This work was done by testing the mycelium mat matrix of *M. anisopliae* cultured on rice or corn in an olfactometer and a glass tube bioassay system. The tunneling distances of *R. flavipes* and *C. formosanus*, when exposed to aged strains of *M. anisopliae*, were measured along with the mortality caused by the fungus to populations of the termites. In addition, comparisons were made to determine if *R. flavipes* were attracted to ethanol extracts of the mycelium of *M. anisopliae* (X-5), or a commercial preferred feeding product (Summon®). The extracts and the Summon® disks were tested in the laboratory using glass plate bioassays, and in the field using commercial termite monitors containing each of the treatments individually.

The results with attractancy and mortality varied with age and generation of *M. anisopliae* mycelia, but all treatments were more attractive and caused more mortality than the controls. When presented with choices in the olfactometer, both *R. flavipes* and *C. formosanus* showed preference to both the mycelium and the extract forms of *M. anisopliae*. In the glassplates, the 1:1000 dilution of *M. anisopliae* extract (X-5) was strongly preferred over the other treatments, and all of the dilutions were preferred over the Summon® and ethanol (40%) treated disks in the laboratory. An analysis of the consumption of test cellulose matrix showed that Summon® did not attract termites, but it was a preferred food source. When the undiluted ethanol extract of *M. anisopliae* was tested in the field, there were more termite visits to the ethanol extract of *M. anisopliae* (X-5) treated monitor stations, and the fewest termite visits were observed in the monitors containing the untreated fiber pulp disks.

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INTRODUCTION

In the family Rhinotermitidae, there are at least two economically important subterranean termite pest species in the United States: the Eastern subterranean termite, *Reticulitermes flavipes* and the Formosan subterranean termite, *Coptotermes formosanus*. Both of these subterranean termite colonies provide a controlled microclimate, due in part to limited exchanges of air with the outside environment. The humid microclimate within the nest is ideal for fungal growth, so many strains of fungi normally occur within the nests. In fact, these subterranean termites generally prefer fungus-infested wood because the fungi partially decompose the cellulose and lignin into a more digestible form (Sands 1969).

The fungus, *Metarhizium anisopliae*, is a promising biological agent for controlling termites. It is a commonly found pathogen in nature and is in the Phylum Deuteromycota (imperfect fungi), which infects over 200 insect species, including termites at all life stages. Foraging termites usually become contaminated with fungal spores and subsequently transport the spores to the colony. Once the spores attach to the host, they may be deposited within the colony. Once the spores are in the colony, they can be spread by: 1) mutual exchange of nutrients between colony members, called trophallaxis; 2) grooming, which consists of termites licking the body surfaces of nest mates; and, 3) cannibalism, which regulates the number of individuals in each caste and recycles nitrogenous nutrients (Kramm *et al.* 1982). The infection process begins when fungal spores or conidia land on the cuticle of a susceptible insect (Gillespie *et al.* 2000). A complex of specific interactions, such as enzymes, and nonspecific interactions, such as hydrophobicity, are responsible for the spore-cuticle interaction that mediates attachment. The rodlet layer of the spores come into contact with the host's epicuticle, and the topography and chemical properties of the epicuticle enhance adhesion of the spore and help to orientate the germ tubes into the cuticle (Cole & Hoch 1991). Sporulation is followed by penetration of the host's cuticle, usually between the mouthparts, at inter-segmental folds, or through spiracles. These areas have a high level of moisture that promotes germination (Zimmerman 1993). The fungal spore uses a combination of mechanical pressure and cuticle degrading enzymes, such as lipases and proteases, to attack and dissolve the cuticle (Khetan 2001).

After the fungus penetrates through the cuticle, it invades into the hemocoel to form a dense mycelial growth (Zimmerman 1993). As the mycelium penetrates through the host, *M. anisopliae* produces toxins

known as destruxins and cytochalasins (Wright *et al.* 2000). Destruxins are produced as the mycelium grows inside the insect. Destruxin forms A and B depolarize muscle membranes by activating calcium ion channels, which leads to lifelessness and paralysis. Destruxins C and E are immunosuppressive and cytopathic (Boudas & Pendland 1998). The toxin cytochalasins acts to block actin filament elongation. The fungus forms green chains of cylindrical conidia that densely compact on the infected host, causing green muscardine disease (Zimmerman 1993).The life cycle of the fungus is completed when it sporulates on the cadaver of the host. The external hyphae produce conidia that ripen and are released into the environment. This allows horizontal transmission of the disease within the termite colony (Khetan 2001).

There are numerous advantages when using fungi as biocontrol agents, such as relative safety to humans and other non-target organisms, reduction of pesticide usage, and increased activity of natural enemies (Lacey *et al.* 2001). Fungal biocontrol agents can be improved by genetically altering the fungus' efficacy, stability and marketability (Milner 1991). Therefore, multiple generations of *M. anisopliae* need to be tested to select for traits that increase its effectiveness as a biocontrol agent.

MATERIALS AND METHODS

Olfactometer

An olfactometer was constructed (Williams 2001) and used to evaluate volatiles produced by fungal strains. This was done to quantitate the attractancy of these volatiles to the termites. The olfactometer (Fig. 5-6) consists of a main 5 cm diameter PVC pipe that was octagon in shape with 55.9 cm sides that were connected in eight places with 5.1 cm joints 10.2 cm in length. The main PVC pipe had a total diameter of 127 cm that was raised by 30.5 cm wooden boards, so the main PVC pipe was above all of the petri dishes positioned below. The purpose of the main PVC pipe was to serve as a reservoir for air used in all of the radiating petri dishes. A Cole-Parmer Model No. L-79200-00 pump was connected to the 5.1 cm PVC pipe with flexible 1.0 cm ID PVC tubing, which allowed 2.5 ml/min of outside air to enter into the main PVC ring. The pump was placed outside the treatment chamber in order to avoid excess vibration to the platform that could alter the natural behavior of the termites. The pump was run day and night for the length of the experiment. Each of the six test chambers consisted of a center Falcon 150 X 25 mm sterile petri dish with two 5.1 cm pieces of glass tubing attached to the upper end of the dish to serve as a release valve for excess air. At the lower end of the center dish, six radiating Fisher brand

100 X 15 mm sterile petri dishes were connected with 10.2 cm glass tubing to the center dish (Fig. 6). Each of these radiating dishes contained either a treatment of *M. anisopliae* mycelia that had been refrigerated (4° C) or ethanol extract of *M. anisopliae* (X-5), controls of sterile rice or corn, or untreated cardboard made from FC kraft pulp disks produced from 100% Douglas Fir in Samoa, California. The wooden platform for the olfactometer was 182.9x182.9 cm that accommodated, six center dishes and thirty-six radiating petri dishes. In order to allow equal airflow across the thirty-six treatments in the radiating dishes, a 55.9 cm (length) of 1.00 cm ID flexible PVC tubing was attached from the main PVC pipe by Fisher brand disconnects to a Cole-Parmer Model No. PMR 1-010265 aluminum flow controller. The flow controller allowed the appropriate volume of air to flow into each test chamber. The incoming air was regulated to 1.12 ml/min. The air flowed out of the flowmeter into 10.2 cm (length) of 1.00 cm ID flexible PVC tubing connected to a Gelman AquaPrep filter that removed particles 0.5 µm and larger. These filters prevent outside contaminants from entering the closed apparatus. The filtered air then flowed through a 35.6 cm (length) of 1.00 cm ID flexible PVC tubing and into each radiating dish.

The olfactometer evaluated the attractancy of both the mycelia mat matrix on corn or rice, and the fungal extract (X-5). When termites were placed in the apparatus, they orientate and move towards the most attractive odor source. The first generation of *M. anisopliae* cultured on rice and the second generation of *M. anisopliae* cultured on corn were tested in the olfactometer. Testing involved aging the strains of fungi cultured on the rice or corn for a maximum of 3 months in the refrigerator (4° C), and testing the attractancy of the fungi as it aged at 1, 2, 4, 8, and 12 weeks, respectfully. The mycelia treatments placed into the radiating dishes were eight corn kernels or sixteen pieces of rice, respectively. The control treatments involved the placement of eight corn kernels or sixteen pieces of rice into the radiating dishes, respectively.

R. flavipes also were tested in the olfactometer to evaluate the attractancy of ethanol extracted mycelium of *M. anisopliae* and Summon® (FMC, 2003). The treatments used in these tests were ethanol treated fiber cement kraft pulp disks, *M. anisopliae* extract treated disks, and Summon® disks. The disks were emersed into their appropriate testing treatment, and then allowed to dry for 8 hours. For the olfactometer experiments a total of 300 termites from each species, with one soldier for *R. flavipes* and two soldiers for *C. formosanus*, were placed separately in the center dish at the time of initiation. Tests were

conducted separately for each termite species. Termites were allowed to forage randomly into the radiating dishes. The apparatus was placed in a separate room to reduce the possible contamination by spores in the ambient air. Counts of termite preference were made hourly for the first 5 hrs and then daily for two days. The light in the room was kept on day and night (L:24 hrs.). The apparatus allows three replicates of each species to be evaluated in each trial.

Glass Tube Bioassays

Glass tube bioassays allow mortality and attractancy to be evaluated. The bioassay tubes are similar to those described by Su *et al.* 1993, Gold *et al.* 1994, Gold *et al.* 1996, Waite *et al.* 2004. The total length of the glass tubes was 85 mm. The treated tubes contained a 10 mm top layer of agar, followed successively by a 15 mm layer of soil, a 35 mm layer of *M. anisopliae* impregnated rice or corn, a 15 mm layer of soil, and a 10 mm bottom layer of agar. Two types of control tubes were used. The first contained a 10 mm top layer of agar, followed by a 15 mm layer of soil, a 35 mm layer of sterile rice or sterile corn, a 15 mm layer of soil, and a 10 mm bottom layer of agar. The second control type contained a 10 mm top layer of agar, 65 mm of soil, and a 10 mm bottom layer of agar. The bottom of all the tubes contained a 3 cm piece of wooden applicator stick, and both ends of the glass tube were enclosed by foil and a rubber band. The soil used in the bioassay tubes was comprised of 100 g of soil from College Station, Texas mixed with 10 ml of water.

The mycelia used in these bioassays were the first generation of *M. anisopliae* cultured on rice, and the second generations of *M. anisopliae* cultured on rice and corn. Groups of 30 termites from each species, including one soldier for *R. flavipes* and two soldiers for *C. formosanus*, were tested separately by placing them on top of the agar in each tube and allowed to tunnel vertically through the tube. The bioassay tubes were maintained in an environmental chamber to regulate temperature at 25 ± 2°C and humidity. The tunneling distance was observed after a period of six days. On the sixth day, the tubes were carefully disassembled and mortality of the termites was determined. Three replicates of each treatment in the bioassay tubes were made for each of the two termite species.

Glass Plate Bioassay Studies

Glass plate bioassays were set up in laboratory to determine *R. flavipes* recruitment and percent consumption of the cellulose matrix in each treatment. Recruitment is defined as one termite finding a preferred treatment that caused the redirection and movement of nest

mates to the preferred treatment. The experimental design followed similar designs used by N.-Y. Su & H. Puche (2003) and Robson *et al.* (1995). The nest was defined as the center petri dish on the upper glass plate. Two glass plates, each measuring 35.6 X 35.6 cm, were used in this experiment. The two glass plates were centered over each other, with a 0.3 cm gap between them. On all of the sides of the square, a 2.5 cm wide glass strip that was 35.6 cm long and 0.3 cm in height serves as a barrier to prevent termites from escaping from the treatment areas. In order to form a 0.3 cm amount of thickness between the two glassplates, two FC kraft pulp disks for each treatment were glued together with Elmer's All Multipurpose Glue. The glued disks were allowed to dry for 24 hrs, to avoid altering the termites' natural foraging behavior. In the glass plate study, five replicates were made for each treatment including: dried pre-weighed untreated fiber cement kraft pulp disks; ethanol extract of *M. anisopliae* (X-5) treated fiber cement kraft pulp disks; 40% ethyl alcohol treated FC kraft pulp disks; and Summon®. Each disk was approximately 3.5 cm in diameter. There were four different dilutions used for the ethanol extract of *M. anisopliae* (X-5) treated disks including, 1:0 (undiluted), 1:10, 1:100, and 1:1000. To prepare each dilution, the full strength 40% ethanol extract was diluted with ethanol, to make the desired concentrations. For instance, when making the 1:10 dilution, 10 ml of X-5 shipped from Fungi Perfecti was combined with 90 ml of 40% ethanol in a plastic 237 ml Fisherbrand wide mouth bottle. This solution was then inverted to ensure thorough mixing. All of the FC kraft paper disks were soaked in the test solution until they were completely saturated, and then the disks were dried in a fume hood for 8 hrs.

The upper glass plate had five holes drilled through the glass. A single center hole was drilled 17.8 cm from the edge of the glass plate. The other four holes were 7.6 cm from each of the four corners. A Falcon 150 X 25 mm sterile petri dish with a 0.3 cm hole burned in the middle of the dish was attached to the middle of the upper glassplate with silicone. The treatments were placed in all four corners within the glassplates, and sifted College Station soil, which was free of rocks and other debris, was placed in between the two glassplates. The soil was funneled into the glassplates, until the inner 30.5 X 30.5 cm square was full of soil. On all sides, clamps were always used to keep the plates together. The plates were then laid flat on a table and 4 ml of distilled water was added through each hole to moisten the soil.

Approximately five hundred termites (1.5 g) were placed in each of the petri dishes attached to the center of the upper glassplate. Termites were placed in the petri dish along with one tongue depressor cut in half

and placed on each side of the petri dish. Termites were allowed to tunnel normally from the center petri dish into the soil between the two glassplates to the treatments found in the four corners. Termite foraging was observed, so preference between the treatments could be determined in this experiment. Once the termites had tunneled to the treatments, daily counts of termites feeding on each treatment were taken daily for 12 days. Every other day, 4 ml of water was added to each of the four holes around each treatment to provide adequate moisture to the foraging termites.

Digital pictures were taken daily of each glassplate, to document the number of termites in each treatment. After 12 days, the glassplates were diassembled and the treatment disks were removed. The disks were placed in an oven and dried for 4 hrs. At the end of 4 hrs, the disks were allowed to cool, and then were reweighed. Comparisons were then made to the pre-testing weight to determine the amount of each disk that had been consumed by the termites. There were a total of 20 replications of the glassplate bioassays.

Field Evaluation

A field plot was established in Bryan, Texas to test the attractancy of *M. anisopliae* extract on the native subterranean population of *R. flavipes*. The field plot was 4.6 X 6.4 m. The field plot was monitored twice a week for 8 months, to determine the areas with active termite populations. The experimental design is similar to that used by Houseman *et al.* (2001). The Whitmire® monitoring system was used, with four monitors placed 30.5 cm from each other at each of the five active sites in the field plot.

The attractancy of the ethanol extract of *M. anisopliae* (X-5) treated fiber cement kraft pulp, and Summon® made by FMC Corporation were evaluated in this study. Four treatments were used that included untreated fiber cement kraft pulp, 40% ethyl alcohol treated fiber cement kraft pulp, ethanol extract of *M. anisopliae* (X-5) extract treated fiber cement kraft pulp, and a Summon®. The fiber cement kraft pulp sheets were cut into 3.5 cm circles that were the same size as the Summons®. The undiluted ethanol extract of *M. anisopliae* (X-5) and 40% ethyl alcohol fiber cement kraft pulp disks were immersed in their respective treatment and then left under the fumehood to completely dry. After the treatments dried, they were added individually to the bottom of each Whitmire® monitor.

The same treatments evaluated in the field plot were also tested around urban structures with active termite infestations. A total of 304 commercial Termitrol® monitoring stations were installed around 5

structures. The monitors were installed around the perimeter of the structures in clusters of four and each cluster was approximately 3 m apart. In the Termitrol® stations, the wooden monitor was replaced with a 12.7x20.3 cm sheet of fiber cement kraft pulp cardboard cellulose matrix. The treatments were: an untreated disk, ethanol treated disk, 1:0 (undiluted) ethanol extract of *M. anisopliae* treated disk, or Summon®. One of the treatments was randomly assigned to one of the four monitors in each cluster using a statistical calculator. Readings were taken twice a week for 17 weeks to determine the presence or absence of termites in the monitoring systems. Twice a week, 4 ml of undiluted ethanol extract of *M. anisopliae* (X-5) and 4 ml of 40% ethanol was added to the appropriate station on the FC kraft pulp disk located on the bottom of the station. Summon® was replaced through time as needed.

Statistical Analysis

SPSS (2001) was used to run an analysis of variance (ANOVA) of means where indicated. Levels of significance were $p < 0.05$.

RESULTS

Olfactometer

The results of the comparisons of *M. anisopliae* (C15) cultured on sterile corn and then aged for 1, 2, 4, 8, and 12 weeks, respectively before being tested in the olfactometer with three hundred workers of *R. flavipes* are shown in Fig. 1. The most attractive treatment was C15 aged for 1 week prior to testing in the olfactometer. There appeared to be a trend for recruitment of *R. flavipes* with C15 aged for 1 and 2 weeks through time, while remaining treatments had consistently low levels of attractancy during testing. Initially the termite responses to the *M. anisopliae* treatments were relatively low; however, the untreated sterile corn control was apparently less attractive than treatments with the fungal mycelium, regardless of the age of mycelia prior to testing.

The second generation of *M. anisopliae* (C16) cultured on sterile corn was tested in the olfactometer with 300 *R. flavipes* and the results are summarized in Fig. 2. There were no significant differences between the untreated sterile corn control and C16 that had been aged 4, 8, and 12 weeks in the early stages of the experiment (60, 120, and 300 minutes). However, for all of the remaining test intervals, the non-treated control was less attractive than any of the C16 mycelia treatments. In these trials, *M. anisopliae* (C16) aged for only 2 weeks prior to testing was significantly ($p \leq 0.05$) more attractive than the other treatments through 2880 hours post-testing. It was also apparent from these trials that all

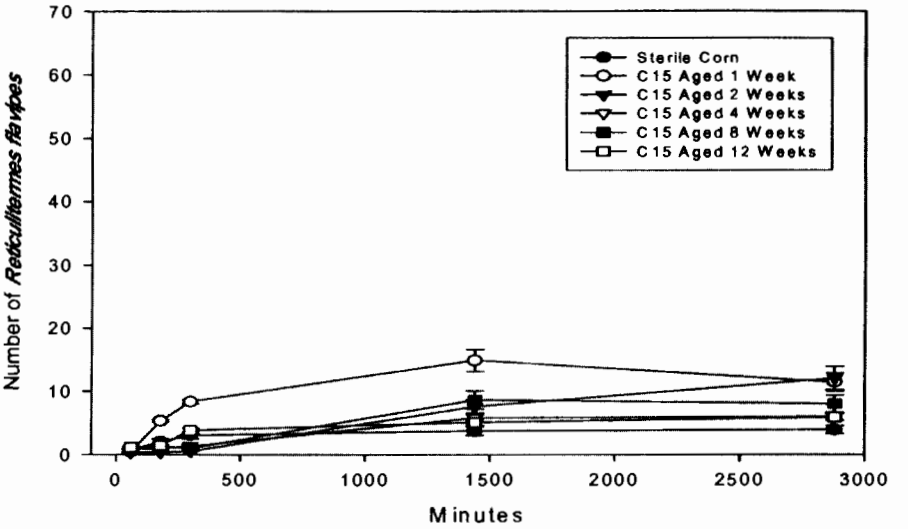

Fig. 1. Mean number of *Reticulitermes flavipes* observed in the first generation of *M. anisopliae* cultured on corn (C15) or the control through time in an olfactometer.

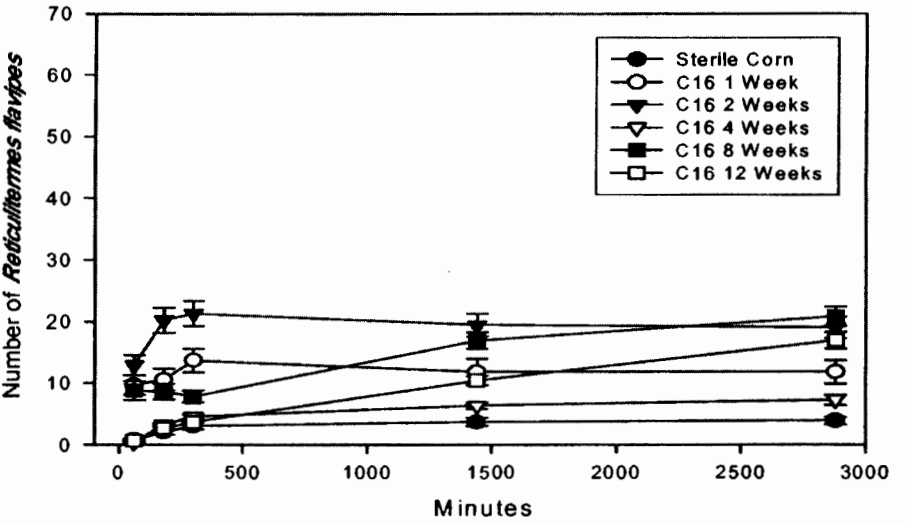

Fig. 2. Mean number of *Reticulitermes flavipes* observed in the second generation of *M. anisopliae* cultured on corn (C16) or the control through time in an olfactometer.

treatments with C16 retained a measurable level of attractiveness through 12 weeks. The C16 treatments were consistently more attractive than the C15 treatments. *R. flavipes* in these tests and noted in a comparison of Fig. 1 & 2.

When *M. anisopliae* was cultured on sterile rice (FF1000) and tested with 300 *R. flavipes* in an olfactometer, there were no significant differences in attractiveness during the first three test periods of 60, 120 and 300 minutes, with the exception of FF1000 that had been aged for 12 weeks. This treatment was significantly different ($p \leq 0.05$) than all of the other treatments through time and showed some evidence of recruitment of the other termites.

Another difference was that two of the treatments (FF1000 aged 4 and 8 weeks, respectively) were significantly less attractive than the untreated controls. The mean number of *R. flavipes* observed at each time period for the different treatments are shown in Fig. 3.

Olfactometer tests were conducted with 300 *C. formosanus* workers using *M. anisopliae* cultured on sterile corn (C15 (Fig. 4). Mycelia aged for 1, 4, 8, and 12 weeks were approximately equal in attractiveness; however, these treatments were more attractive to *C. formosanus* (Fig. 4) than to *R. flavipes* (Fig. 1). There was limited recruitment of termites to treatment chambers in the olfactometer after 300 minutes adjustment period.

The second generation of *M. anisopliae* cultured on sterile corn (C16) and tested with *C. formosanus* was significantly ($p \leq 0.05$) more attractive than the untreated control (Fig. 5). The most attractive cultures were those aged 4 and 12 weeks prior to testing in the olfactometer, and were significantly ($p \leq 0.05$) more attractive than either the sterile corn control or the other aged (C16) mycelia tested. There was a general trend for *C. formosanus* (Fig. 5) to be more attracted to C16 than were *R. flavipes* (Fig. 2).

When *M. anisopliae* was cultured on sterile rice (FF1000) and tested in the olfactometer with 300 *C. formosanus*, there were significant ($p \leq 0.05$) preferences for the mycelia that had been aged for 8 weeks prior to testing. After the initial adjustment period, the first generation of mycelium cultured on rice (FF1000) was significantly ($p \leq 0.05$) more attractive than the sterile rice control at 1440 minutes. The mean number of *C. formosanus* observed at each time period for the different treatments are shown in Fig. 6.

Ethanol (40%) extracts were prepared from *M. anisopliae* mycelium cultured on sterile rice (X-5). Comparisons that included dilutions of X-5 at 1:0, 1:10, 1:100, and 1:1000, an ethanol control, and Summon®, were made in an olfactometer with 300 *R. flavipes*. The most preferred treatments were the 1:1000 dilution of X-5 and Summon®, both of which were significantly different ($p \leq 0.05$) from each other as well as from the other treatments (Fig. 7). The least preferred treatment was the 1:10 dilution of X-5 extract, which was significantly different ($p \leq 0.05$)

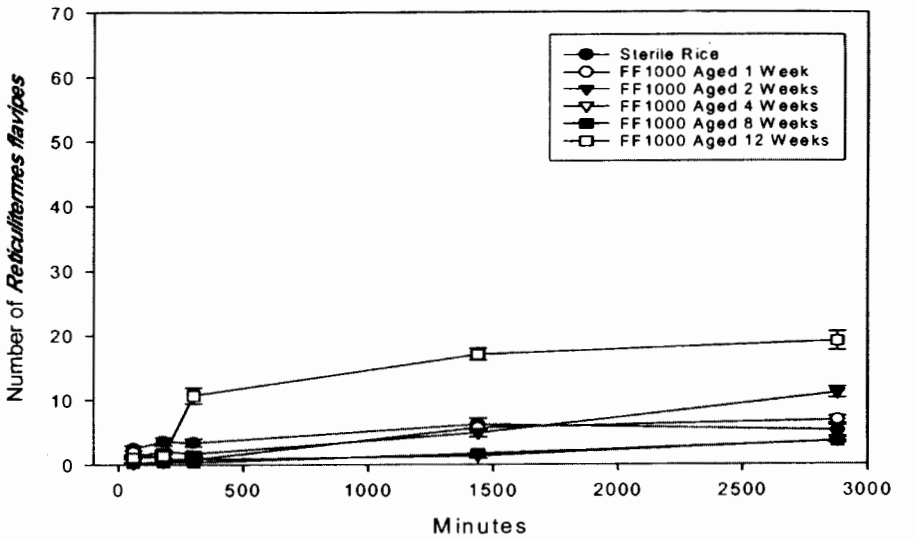

Fig. 3. Mean number of *Reticulitermes flavipes* observed in the first generation of *M. anisopliae* cultured on rice (FF1000) or the control through time in an olfactometer.

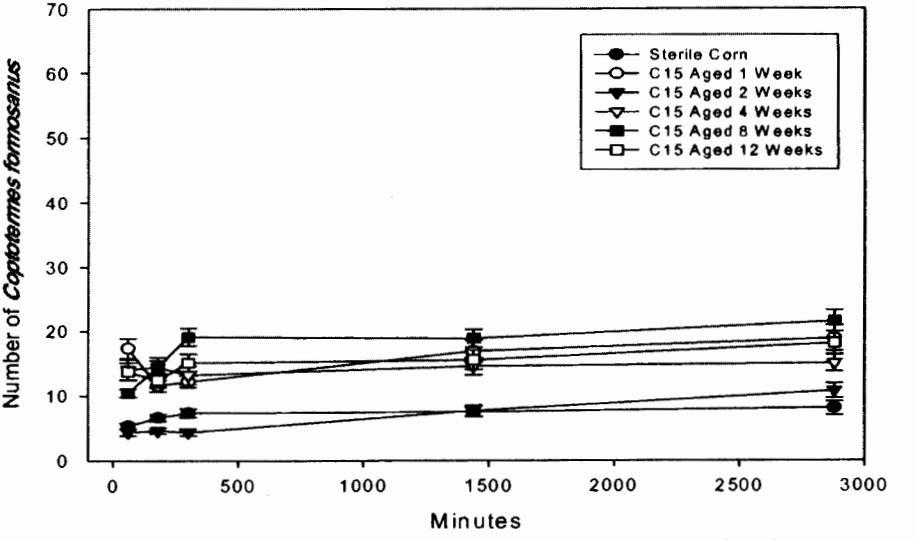

Fig. 4. Mean number of *Coptotermes formosanus* observed in the first generation of *M. anisopliae* cultured on corn (C15) or the corn control through time in an olfactometer.

from all other treatments. Of the six treatments, the only one that showed potential recruitment by foraging workers of *R. flavipes* was the 1:1000 dilution of X-5 extract (Fig. 7).

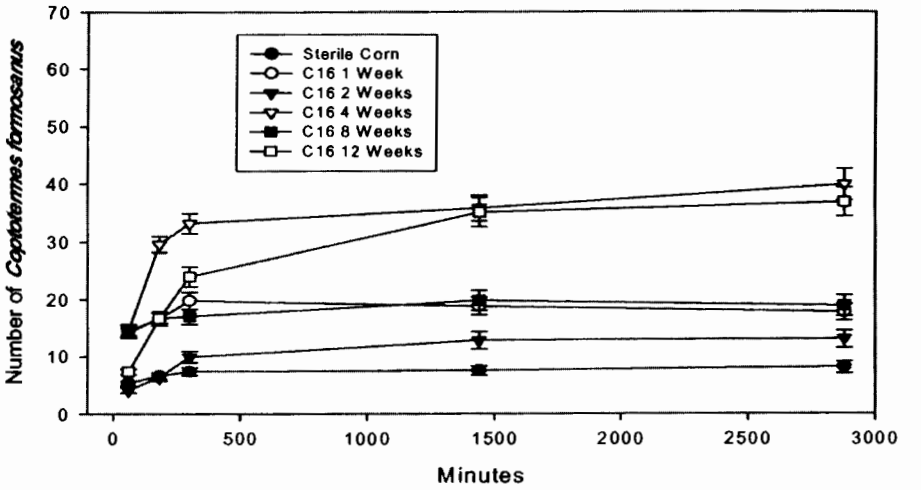

Fig. 5. Mean number of *Coptotermes formosanus* observed in the second generation of *M. anisopliae* cultured on corn (C16) or the corn control through time in an olfactometer.

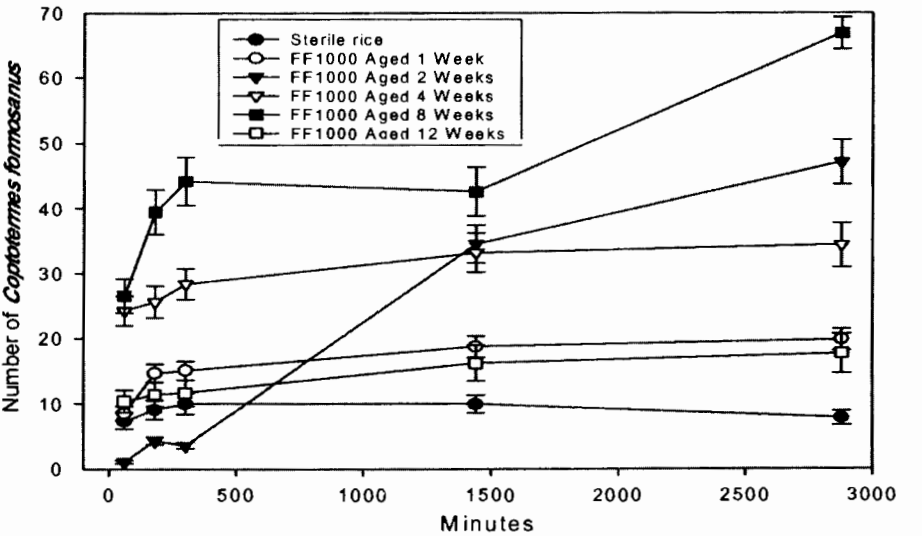

Fig. 6. Mean number of *Coptotermes formosanus* observed in the first generation of *M. anisopliae* cultured on rice (FF1000) or the rice control through time in an olfactometer.

Glass Tube Bioassays

When 30 *R. flavipes* were provided access to aged mycelium of *M. anisopliae* cultured on sterile corn (C15), there were no significant differences between treatments (Fig. 8). The termite workers were able

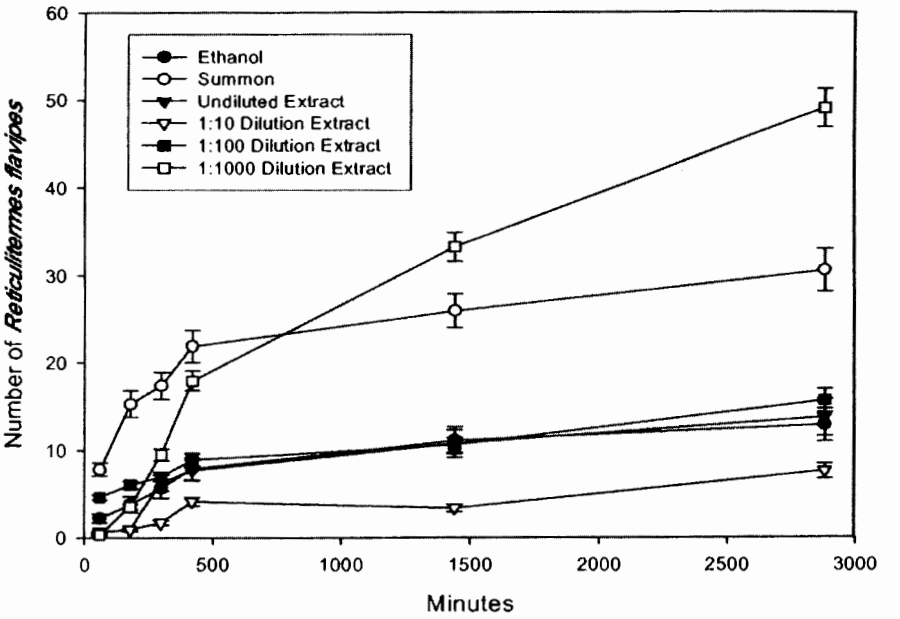

Fig. 7. The mean number of *Reticulitermes flavipes* found in each treatment when testing the ethanol extract of *M. anisopliae* (X-5) cultured on rice and Summon® disk through time in an olfactometer.

to penetrate the bioassay tubes within the 6 day test period. Whereas there was less tunneling in the untreated soil and sterile corn controls, these differences were not significant. The presence of mycelium in the treatments apparently did not inhibit tunneling in these tests.

In the bioassay tubes that included the second generation of *M. anisopliae* cultured on corn (C16) and *R. flavipes*, there was a disruption of tunneling with the mycelium that had been aged for 2 weeks at 4° C and C16 aged for 12 weeks at 24° C (Fig. 9). These treatments were not significantly different ($p \geq 0.05$) from each other or from the untreated controls of soil and sterile corn, but they were significantly different than other C16 mycelia treatments.

When *M. anisopliae* was cultured on sterile rice (FF1000) and tested with thirty *R. flavipes* in the bioassay tubes, there was a strong correlation between the aged mycelium and inhibition of tunneling (Fig. 10). The termites were able to tunnel completely through the non-treated sterile rice controls, which indicated that it was the mycelium that was inhibiting tunneling. In general, there were significant differences between the treatments with aged FF1000, compared to the untreated controls.

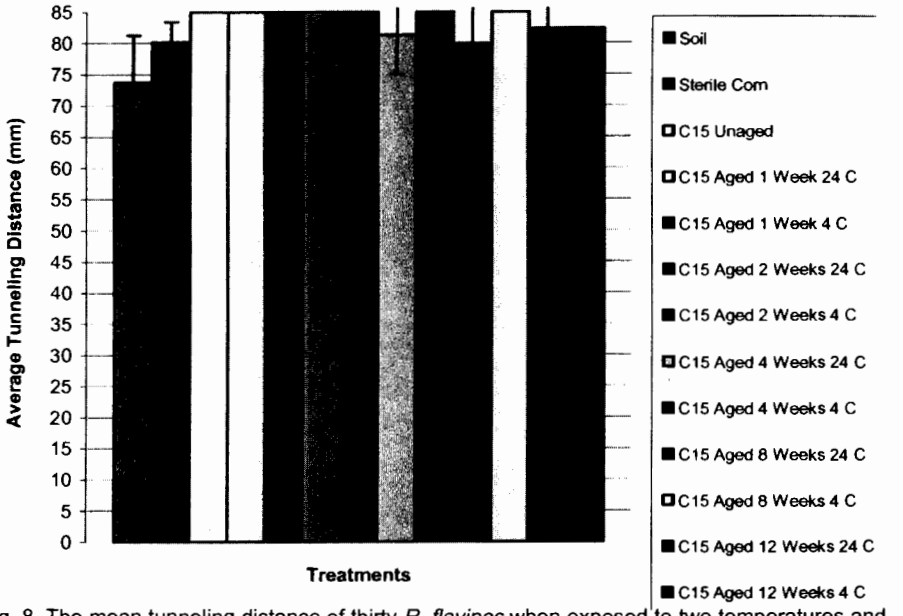

Fig. 8. The mean tunneling distance of thirty *R. flavipes* when exposed to two temperatures and five different ages of the first generation *M. anisopliae* cultured on corn (C15) for 6 days in bioassay tubes held at 25° C.

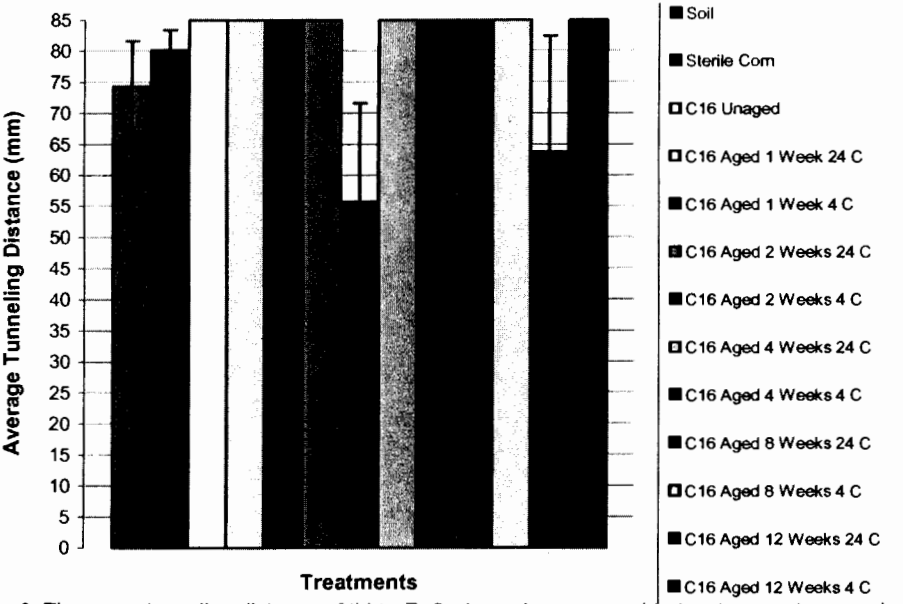

Fig. 9. The mean tunneling distance of thirty *R. flavipes* when exposed to two temperatures and five different ages of the second generation of *M. anisopliae* cultured on corn (C16) for 6 days in bioassay tubes held at 25° C.

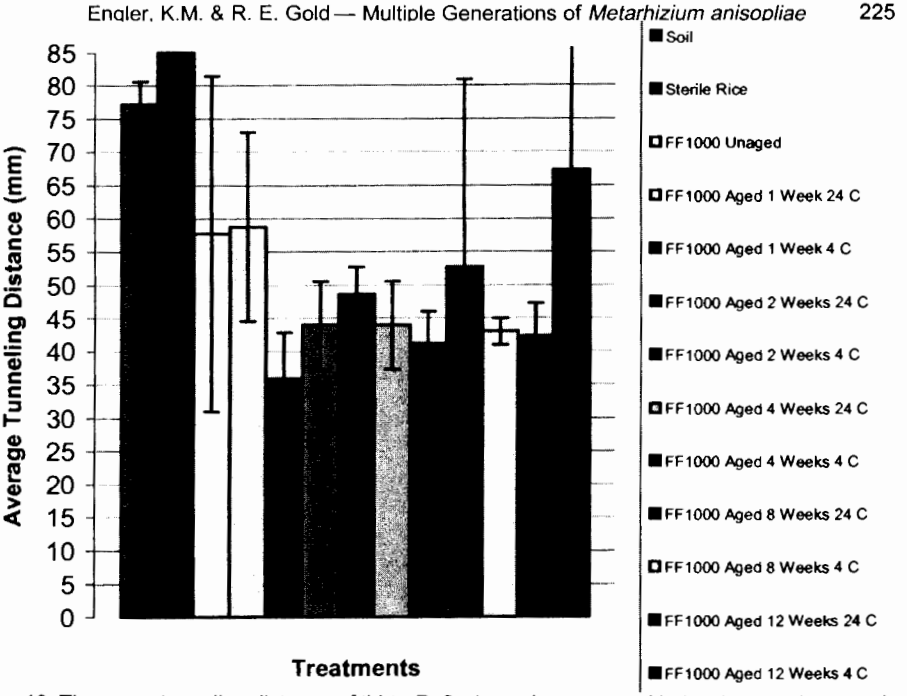

Fig. 10. The mean tunneling distance of thirty *R. flavipes* when exposed to two temperatures and five different ages of the first generation of *M. anisopliae* cultured on rice (FF1000) for 6 days in bioassay tubes held at 25° C.

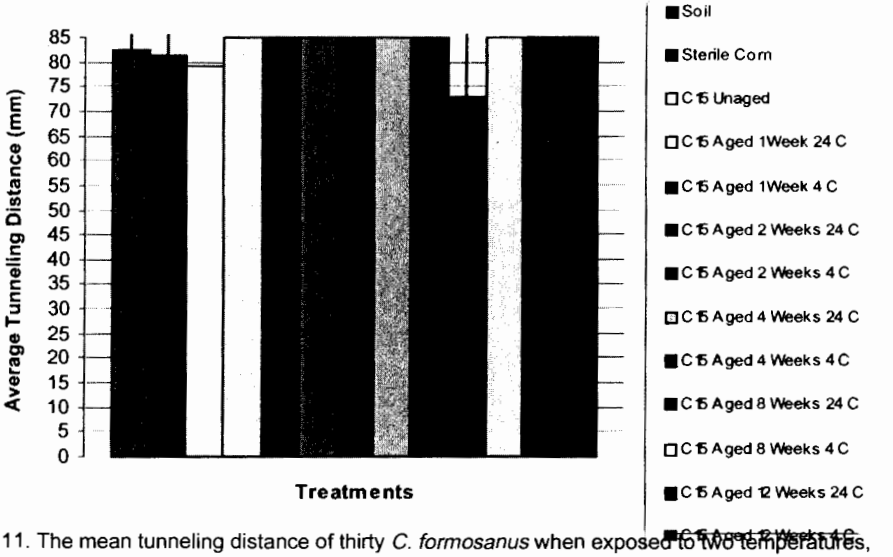

Fig.11. The mean tunneling distance of thirty *C. formosanus* when exposed to two temperatures, and five different ages of the first generation of *M. anisopliae* cultured on corn (C15) for 6 days in bioassay tubes held at 25° C.

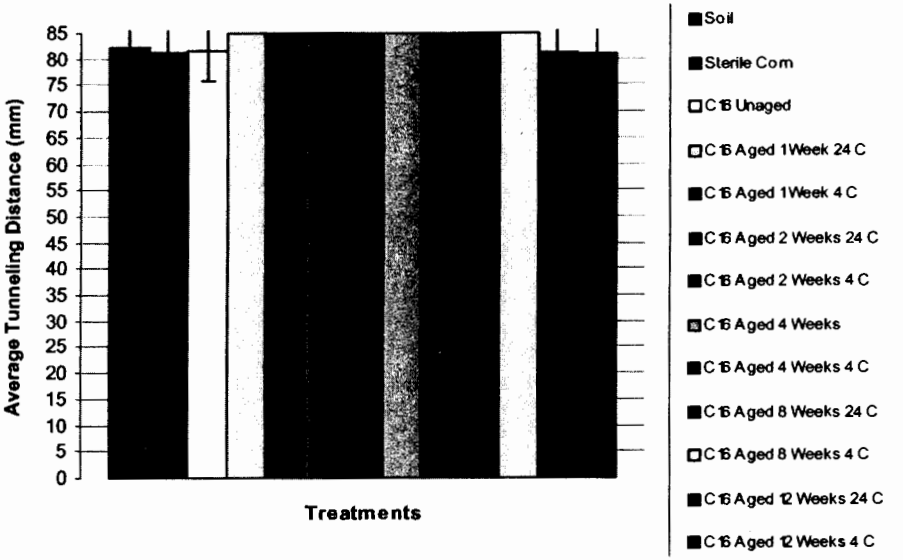

Fig. 12. The mean tunneling distance of thirty *C. formosanus* when exposed to two temperatures and five different ages of the second generation of *M. anisopliae* cultured on corn (C16) for 6 days in bioassay tubes held at 25° C.

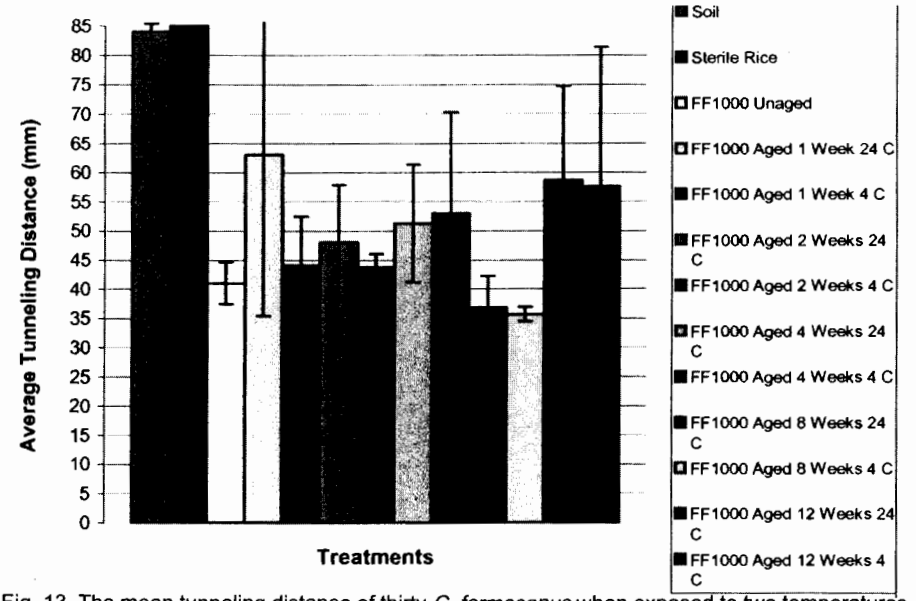

Fig. 13. The mean tunneling distance of thirty *C. formosanus* when exposed to two temperatures and five different ages of the first generation of *M. anisopliae* cultured on rice (FF1000) for 6 days in bioassay tubes held at 25° C.

The results of bioassay tube experiments conducted on thirty workers of *C. formosanus* allowed access to mycelia of *M. anisopliae* are found in Fig. 11-13. When the first generation of *M. anisopliae* was cultured on sterile corn (C15) and aged from 1 to 12 weeks at 24 or 4° C, there were no significant differences between the soil and sterile corn controls and aged mycelium (Fig. 11). Apparently, mycelium did not inhibit tunneling by *C. formosanus* in these tests.

When the second generation of *M. anisopliae* was cultured on sterile corn (C16) and exposed to foraging *C. formosanus* in bioassay tubes, there were no significant differences between the treatments (Fig. 12).

When *M. anisopliae* was cultured on sterile rice (FF1000) and provided to 30 *C. formosanus* workers in bioassay tubes, there were significant differences ($p \leq 0.05$) between all of the FF1000 treatments (Fig. 13), except FF1000 aged for 1 week at 24° C, and the soil and sterile rice controls. It was apparent that foraging termites tunneled to the area containing mycelium, and remained there for the 6 day testing period. These effects were due to the presence of aged mycelium and not to sterile rice. These results are similar to those determined for *R. flavipes* exposed to FF1000 (Fig. 13).

At the end of 6 days, the bioassay tubes were carefully disassembled and live termites were counted. Mortality rates were then determined for each treatment, and the results are provided in Tables 1-6.

Table 1. The mean percent mortality and the standard deviation of thirty *R. flavipes* when exposed to the first generation of *M. anisopliae* cultured on corn (C15) at various different temperatures and ages for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks[2]	4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M. anisopliae cultured on corn (C15)	24° C	11.0±1.5[a]	11.0±1.3[a]	15.6±2.1[a]	6.7±2.0[a]	10.0 ± 2.7[a]	23.4±2.1[a]
M. anisopliae cultured on corn (C15)	4° C	[1]	1.0±1.0[b]	3.3±1.0[a]	7.7±0.6[a]	4.3±2.3[a]	20.0±2.6[a]
Soil	24° C	1.1±0.6[b]	0.0±0.0[b]	1.1±0.6[b]	0.0±0.0[b]	1.1±0.6[a]	0.0±0.0[b]
Sterile Corn	4° C	2.2±0.6[b]	1.1±0.6[b]	1.1±0.6[b]	2.2±0.6[a]	0.0±0.0[a]	3.3±0.0[b]

[1]Could not be tested

[2]Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

For *R. flavipes*, the mortality found in the bioassay tubes containing the unaged treatment of the first generation of *M. anisopliae* cultured on corn (C15) was significantly different ($p \leq 0.05$) from the soil and sterile corn controls. The lowest mortality was observed in the soil and sterile corn treatments. The mortality found in the mycelia treatments at the end of 6 days in this experiment were significantly different

Table 2. The mean percent mortality and the standard deviation of thirty *R. flavipes* when exposed to the second generation of *M. anisopliae* cultured on corn (C16) at two different temperatures and 6 ages for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks[2]	4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M. anisopliae cultured on corn (C16)	24° C	11.0±3.5[a]	1.0±0.6[a]	3.3±1.7[a]	15.6±3.5[a]	24.3±1.2[a]	36.7±2.6[a]
M. anisopliae cultured on corn (C16)	4° C	[1]	2.2±1.2[a]	20.0±2.6[b]	8.9±2.1[a]	12.7±2.3[b]	33.3±3.6[a]
Soil	24° C	1.1±0.6[b]	0.0±0.0[a]	1.1±0.6[a]	0.0±0.0[b]	1.1±0.6[c]	0.0±0.0[b]
Sterile Corn	4° C	2.2±0.6[b]	0.0±0.0[a]	1.1±0.6[a]	2.2±0.6[b]	0.0±0.0[c]	3.3±0.0[b]

[1]Could not be tested

[2] Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

Table 3. The mean percent mortality and the standard deviation of thirty *R. flavipes* when exposed to the first generation of *M. anisopliae* cultured on rice (FF1000) at two different temperatures and aged for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks[2]	4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M. anisopliae cultured on rice (FF1000)	24° C	2.2±0.6[a]	36.7±4.0[a]	11.0±0.6[a]	4.3 1.5[a]	6.7±2.9[a]	11.0±2.1[a]
M..anisopliae cultured on rice (FF1000)	**4° C**	[1]	31.0±2.5[a]	7.7±0.6[a]	9.2±1.3[a]	13.3±1.8[b]	15.9±2.4[a]
Soil	24° C	0.0±0.0[a]	1.1±0.6[b]	1.1±0.6[a]	1.1±0.6[a]	0.0±0.0[a]	0.0±0.0[b]
Sterile Rice	4° C	2.2±0.6[a]	1.3±0.6[b]	1.3±0.6[a]	1.1±0.6[a]	3.3±0.0[a]	2.2±0.6[b]

[1]Could not be tested

[2] Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

($p \leq 0.05$) compared to the soil controls. The first generation of mycelium cultured on corn (C15) that was aged for 12 weeks produced the highest mortality both at 4° C and 24° C, and were significantly different ($p \leq 0.05$) from the soil and sterile corn controls. The mean percent mortality and the standard deviation observed in each treatment are shown in Table 1.

For *R. flavipes*, mortality associated with unaged C16 was significantly different ($p \leq 0.05$) from soil and sterile corn controls. The lowest mortality was observed in soil and sterile corn treatments. The second generation of mycelium cultured on corn (C16) that was aged for 12 weeks showed the highest mortality both at 4° C and 24° C, and were significantly different (pd•0.05) from the soil and sterile corn controls. The mean percent mortality and the standard deviation observed in each treatment are shown in Table 2.

For *R. flavipes*, mortality associated with FF1000 aged for 1 week at 24 and 4° C were significantly different ($p \leq 0.05$) from that for soil and sterile rice controls. The lowest mortality was observed in the soil and sterile rice treatments. The first generation of mycelium cultured on rice (FF1000) that was aged for 1 week showed the highest mortality both at 4 and 24° C and these mortalities were significantly different ($p \leq 0.05$) from these for soil and sterile rice controls. The mortality effects of exposure of *R. flavipes* to *M. anisopliae* were maintained for up to 12 weeks. The mean percent mortality and the standard deviation observed in each treatment are shown in Table 3.

For *C. formosanus*, the most significant effects of the first generation of *M. anisopliae* grown on corn were observed in the 8 and 12 week aged trials (Table 4). The lowest mortality was observed in soil and sterile corn treatments. The first generation of mycelium cultured on corn (C15) and aged for 8 weeks showed the highest mortality at 24° C, and this mortality was significantly different ($p \leq 0.05$) from that for mycelia aged for 8 weeks at 4° C, and the soil and sterile corn controls. The mycelium aged for 12 weeks at 4° C showed the highest mortality. The mycelium aged for 12 weeks at 4° C was significantly different ($p \leq 0.05$) from the soil and sterile corn controls. The mean mortality and standard deviation observed in each treatment for *C. formosanus* are shown in Table 4.

The results of testing mortality with aged mycelium of the second generation of *M. anisopliae* grown on corn with *C. formosanus* are presented in Table 5. The lowest mortality was observed in the soil and sterile corn treatments. The second generation of mycelium cultured on corn (C16) and aged for 4 weeks showed the highest mortality at 24° C, and the mortality was significantly different ($p \leq 0.05$) from that for

Table 4. The mean percent mortality and the standard deviation of thirty *C. formosanus* when exposed to the first generation of *M. anisopliae* cultured on corn (C15) at two different temperatures and 6 ages for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks[2]	4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M. anisopliae cultured on corn (C15)	24° C	7.7±2.5[a]	5.7±1.5[a]	16.7±3.6[a]	14.3±2.3[a]	31.0±7.1[a]	20.0±5.0[a]
M. anisopliae cultured on corn (C15)	4° C	[1]	4.3±1.5[a]	3.3±1.0[b]	9.2±0.6[a]	13.3±3.8[b]	15.9±1.2[a]
Soil	24° C	1.1±0.6[a]	0.0±0.0[a]	1.1±0.6[b]	3.3±0.0[b]	3.3±0.6[c]	0.0±0.0[b]
Sterile Corn	4° C	2.2±0.6[a]	1.1±0.6[a]	0.0±0.0[b]	1.1±0.6[b]	1.1±0.6[c]	0.0±0.0[b]

[1]Could not be tested
[2] Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

Table 5. The mean percent mortality and the standard deviation of thirty *C. formosanus* when exposed to the second generation of *M. anisopliae* cultured on corn (C16) at two different temperatures and 6 ages for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks	[2]4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M.. anisopliae cultured on corn (C16)	24° C	5.3 ± 1.5[a]	3.5 ± 1.3[a]	4.3 ± 1.5[a]	11.0 ± 1.5[a]	2.2 ± 1.2[a]	8.9 ± 2.1[a]
M. anisopliae cultured on corn (C16)	4° C	[1]	4.3 ± 0.6[a]	2.2 ± 1.2[a]	1.7 ± 1.8[b]	1.0 ± 0.6[a]	14.3 ± 1.2[a]
Soil	24° C	0.0 ± 0.0[a]	0.0 ± 0.0[a]	1.1 ± 0.6[a]	3.3 ± 0.0[b]	3.3 ± 0.0[a]	0.0 ± 0.0[b]
Sterile Corn	4° C	0.0 ± 0.0[a]	1.1 ± 0.6[a]	0.0 ± 0.0[a]	1.1 ± 0.6[b]	1.1 ± 0.6[a]	0.0 ± 0.0[b]

[1]Could not be tested
[2]Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

mycelia aged for 4 weeks at 4° C, and the soil and sterile corn controls. The mycelium aged for 12 weeks at 4° C showed the highest mortality and was significantly different (pd•0.05) from the soil and sterile corn controls.

The results of testing with aged mycelium of *M. anisopliae* cultured on rice (FF1000) on the mortality of *C. formosanus* are summarized in

Table 6. The mean percent mortality and the standard deviation of thirty *C. formosanus* when exposed to the first generation of *M. anisopliae* cultured on rice (FF1000) at two different temperatures and 6 ages for 6 days in bioassay tubes.

Treatment	Exposed Temperature	Unaged[2]	1 Week[2]	2 Weeks[2]	4 Weeks[2]	8 Weeks[2]	12 Weeks[2]
M. anisopliae cultured on rice (FF1000)	24° C	2.2±1.2[a]	21.0±1.2[a]	16.7±3.0[a]	15.5±1.2[a]	13.3±2.6[a]	15.6±1.2[a]
M. anisopliae cultured on rice (FF1000)	4° C	[1]	22.2±1.5[a]	5.6±0.6[b]	11.4±1.2[a]	13.3±3.6[a]	20.0±2.6[a]
Soil	24° C	0.0 ± 0.0[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	1.1 ± 0.6[b]	0.0 ± 0.0[b]
Sterile Rice	4° C	2.2 ± 0.6[a]	3.3 ± 0.0[b]	2.2 ± 0.6[b]	2.2 ± 0.6[b]	2.2 ± 0.6[b]	2.2 ± 0.6[b]

[1]Could not be tested

[2] Means followed by the same letter within the same column were not significantly different ($p \geq 0.05$) from each other using Univariate Analysis of Variance (SPSS 2001).

Table 6. The lowest mortality resulting from exposure to FF1000 was observed in the soil and sterile rice treatments. The first generation of mycelium cultured on rice (FF1000) that was aged for 1 week showed the highest mortality both at 4 and 24° C and were significantly different ($p \leq 0.05$) from the soil and sterile rice controls. There was a low but significant level of mortality, associated with mycelia aged from 1 to 12 weeks. The mean mortality and standard deviation observed in each treatment for *C. formosanus* are shown in Table 6. There was a significant difference ($p \leq 0.05$) in mortality in *C. formosanus* exposed to FF1000 cultured on rice rather than corn (C15 and C16).

Glass Plate Bioassays

The results of *R. flavipes* observed at each of the seven treatments are shown in Fig. 14. The results indicated that Summon®, untreated disks, and ethanol treated disks, although acceptable to foraging *R. flavipes*, did not recruit over time. There was evidence of recruitment by *R. flavipes* to all dilutions of the *M. anisopliae* ethanol extract (X-5) cultured on sterile rice through time. *R. flavipes* preferences, from the least to the greatest, occur in the following order: Summon®, ethanol, untreated, 1:0, 1:10, 1:100, and 1:1000 dilution of X-5 extract, respectfully. Clearly, *M. anisopliae* extract dilutions were preferred over other treatments; however, there was an inverse relationship, in which more recruitment was observed for 1:1000 dilutions and the least

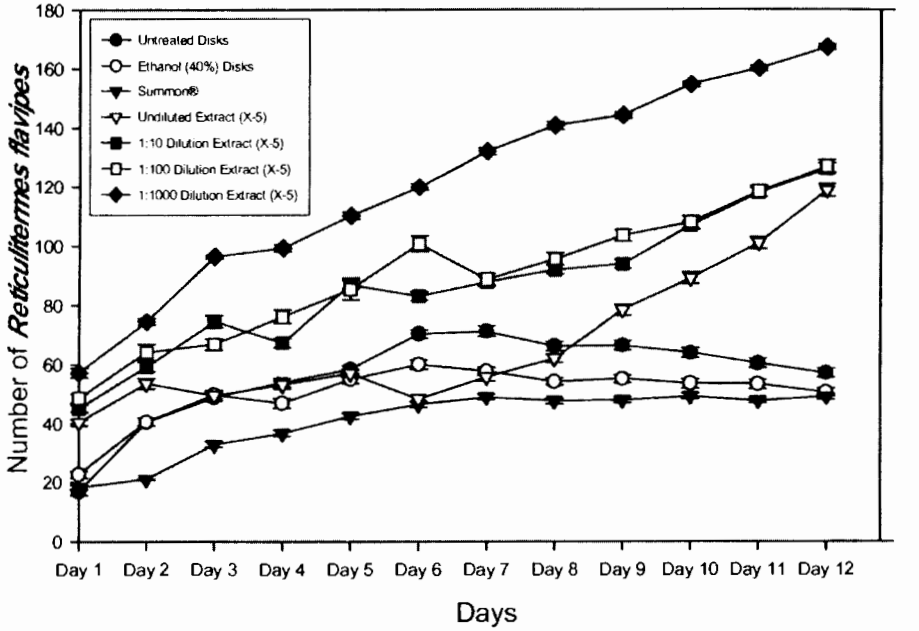

Fig. 14. Mean number and standard error of *Reticulitermes flavipes* observed around each treatment for 12 days in the glass plate bioassays.

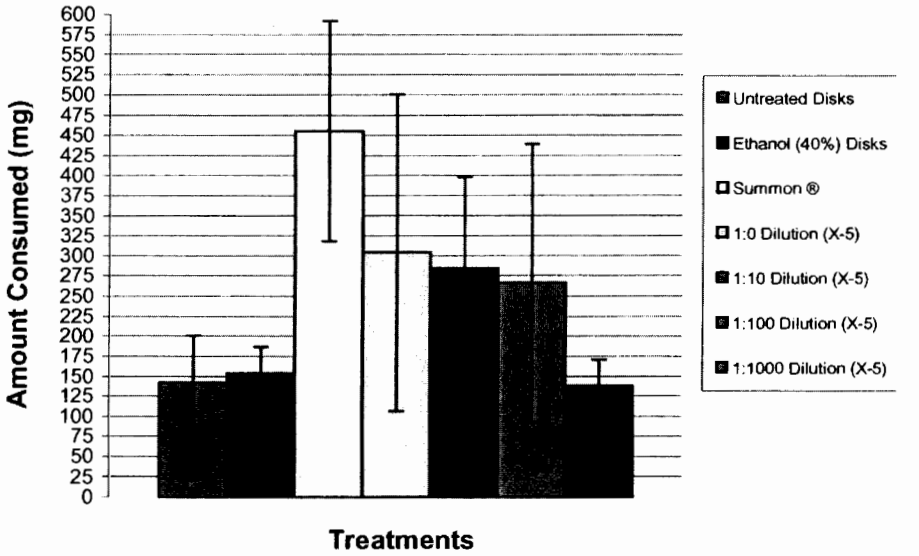

Fig. 15. The amount (mg) of each treatment consumed by *Reticulitermes flavipes* after 12 days in the glass plate bioassay study.

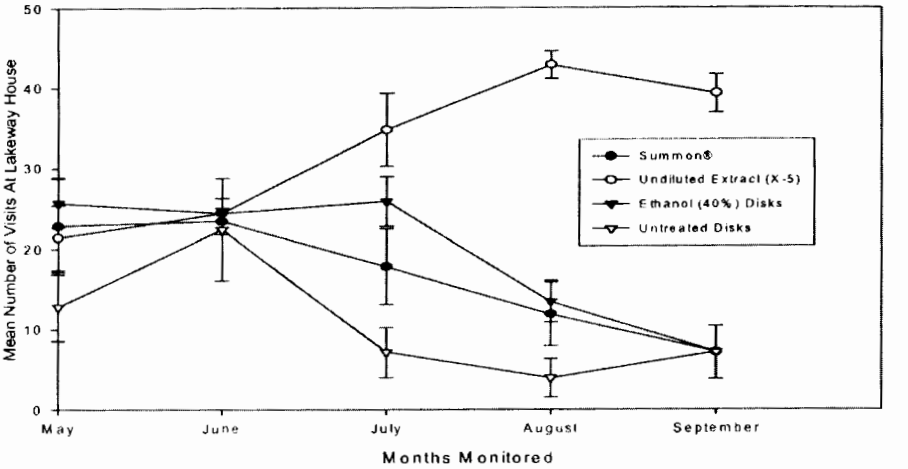

Fig. 16. Mean number of visits per month by *R. flavipes* discovered at the various treatments around the Lakeway House at thirty-one different time periods from May 16th through September 18, 2003.

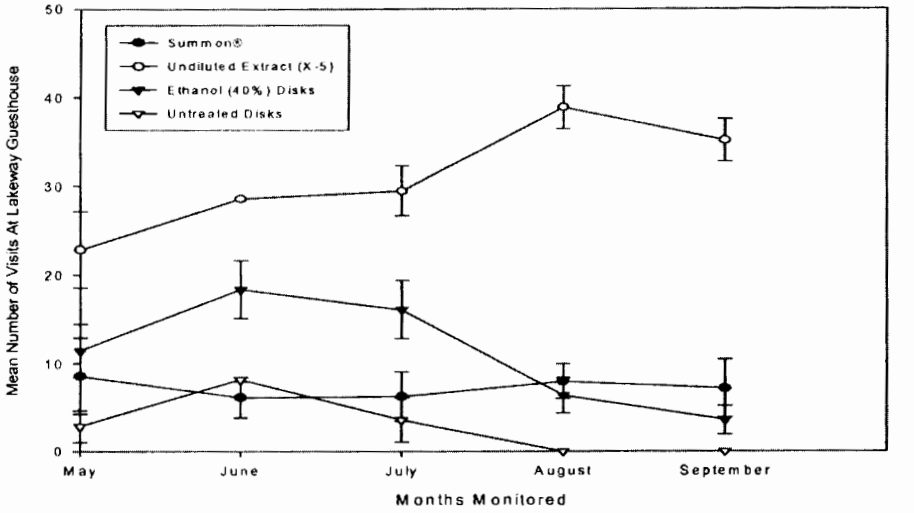

Fig. 17. Mean number of visits per month by *R. flavipes* discovered at the various treatments around the Lakeway Guesthouse at thirty-one different time periods from May 16th through September 18, 2003.

recruitment was with undiluted treatments. The 1:1000 dilution of *M. anisopliae* ethanol extract (X-5) was significantly different ($p \leq 0.05$) from the other treatments throughout the study. The mean number of termites observed at each treatment for each of the 12 days is shown in Fig. 14.

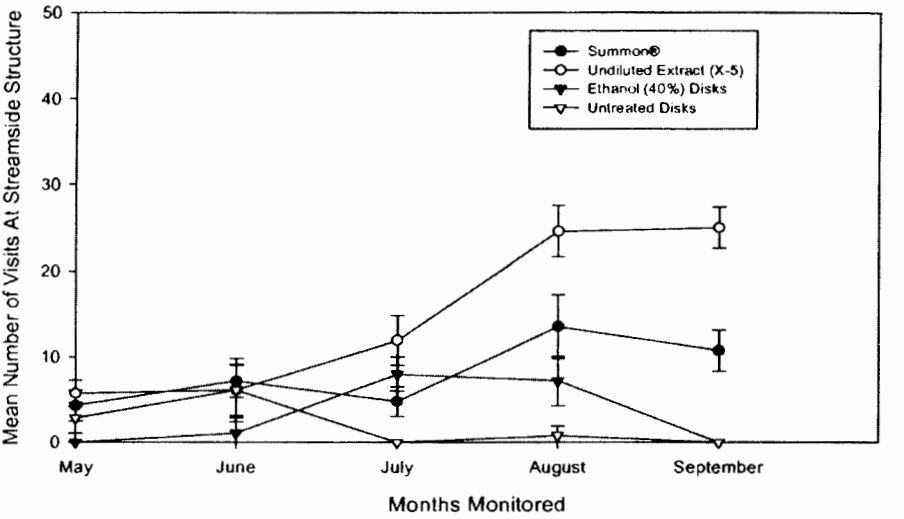

Fig. 18. Mean number of visits per month by *R. flavipes* discovered at the various treatments around the Streamside House at thirty-two different time periods from May 16th through September 18, 2003.

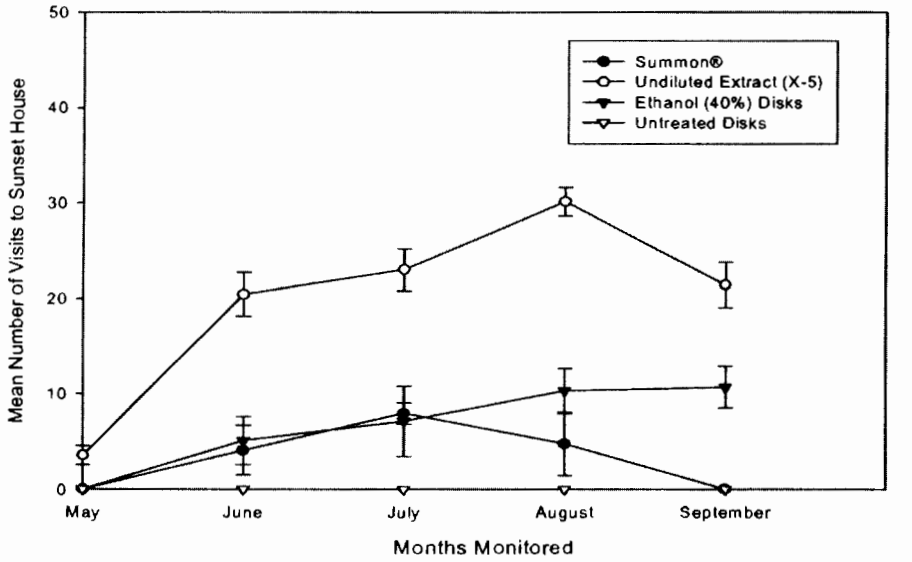

Fig.19. Mean number of visits per month by *R. flavipes* discovered at the various treatments around the Sunset House at thirty-one different time periods from May 21st through September 18, 2003.

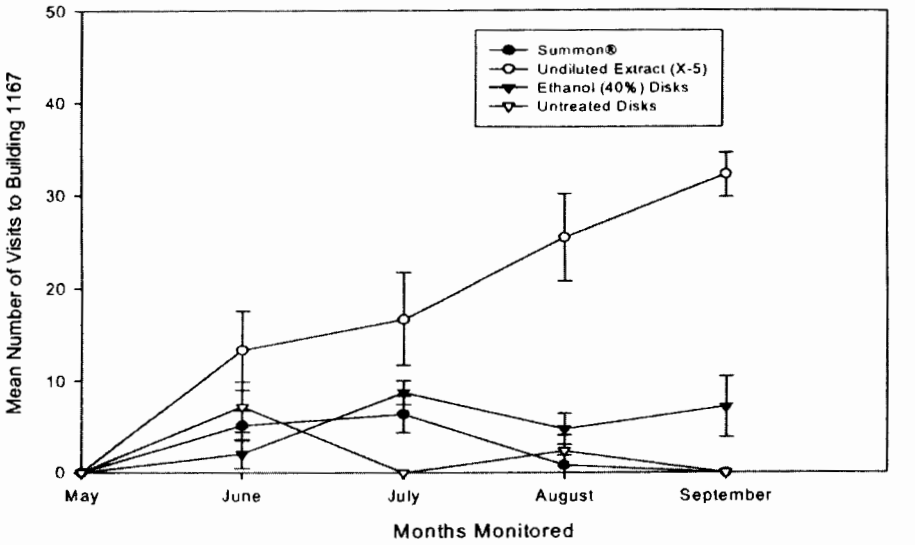

Fig.20. Mean number of visits per month by *R. flavipes* discovered at the various treatments around Building 1167 at twenty-seven different time periods from May 30th through September 18, 2003.

The results of the tests that compared consumption of the cellulose matrix associated with each of the treatments are summarized in Fig. 15. The largest difference between the initial and the final weight of the treatment disks after 12 days was observed for Summon® followed, in order of decreasing consumption levels, by 1:0 dilution of X-5 extract, 1:10 dilution of X-5 extract, 1:100 dilution of X-5 extract, ethanol treated disks, and untreated disks, respectively. The smallest difference in weight was associated with the 1:1000 dilution of *M. anisopliae* ethanol extract (X-5) treated disks.

Field Evaluation

The monitoring stations around each of the five urban structures were observed between 27 and 32 times, depending upon the date of installation and weather conditions. For all urban structures, the highest numbers of visits by termites were observed for *M. anisopliae* ethanol extract (X-5) treated monitors, which were significantly different ($p \leq 0.05$) from other treated monitors for July, August, and September 2003. The lowest numbers of visits were observed for untreated monitors, and these numbers were significantly different ($p \leq 0.05$) from those for other monitors and identical time periods. There was a decline in termite activity in the Summon®, undiluted extract, and ethanol control. The mean numbers of visits observed at each structure for each treatment at each time interval are shown in Figures 16-20.

The monitoring stations in the field plot were monitored thirty-one times. In the field plot, the most termite visits were observed in the *M. anisopliae* ethanol extract (X-5) treated monitors, which differed significantly ($p \leq 0.05$) from other treatments through time. The Summon® treated monitors were significantly different from untreated controls, and showed slight recruitment during the summer months, but declining recruitment in the fall. Both the ethanol treated monitors and the untreated monitors were the least preferred treatments. The mean number of visits observed in the field plot for each treatment and for each time period monitored is shown in Fig. 21.

DISCUSSION

The use of an olfactometer focused on determining if the fungus emitted volatiles to which other termites would respond. *M. anisopliae* was attractive to both *R. flavipes* and *C. formosanus* when compared to untreated matrix controls. Both species of termites showed different levels of attractiveness depending on the generation and age of *M. anisopliae*, to which they were exposed in the olfactometer. Overall, *C. formosanus* showed more movement towards the treatments in the olfactometer than did *R. flavipes*.

The effect of an ethanol extract of *M. anisopliae* (X-5) was also observed in the olfactometer to test preference by *R. flavipes* for different dilutions of the fungal extract on treated disks, compared to

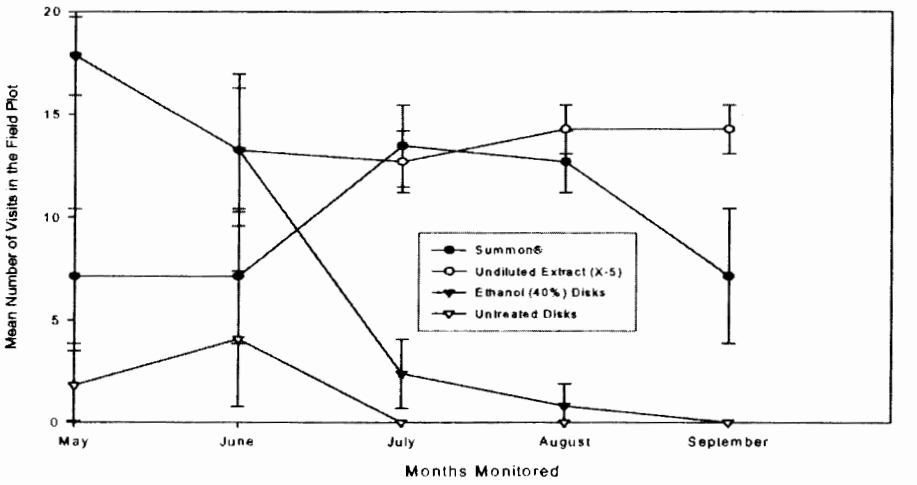

Fig.21. Mean number of visits per month discovered at the various treatments within the Field Plot at thirty-one different time periods from May 21st through September 18, 2003.

an ethanol (40%) treated disk and Summon®. The 1:1000 dilution of *M. anisopliae* extract and Summon ® was strongly preferred over the other treatments, and the 1:10 dilution was the least attractive treatment.

When *R. flavipes* and *C. formosanus* were exposed to different generations of *M. anisopliae* mycelia, they showed varying degrees of attractancy and mortality. There was variation among the generations of *M. anisopliae* as well as among different statues and ages of mycelia. When the tunneling distances of both species of termites were analyzed, greater tunneling was displayed in the first (C15) and second (C16) generations of *M. anisopliae* cultured on corn compared to the first generation of *M. anisopliae* cultured on rice (FF1000). Neither species was inhibited by the presence of the first and second generations of *M. anisopliae* cultured on corn, permitting them to tunnel all the way through the bioassay tubes. However, the first generation of *M. anisopliae* cultured on rice caused both species of termites to stop tunneling and live within the fungal matrix, rather than tunnel farther in the bioassay tubes.

By observing the mortality of *R. flavipes* and *C. formosanus* after being exposed to the fungal matrix in the bioassay tubes for six days, allowed us to conclude that some strains of the fungi adversely affected termite survival. The lowest mortality was observed in the soil, sterile rice and sterile corn controls for both species. There was a mortality effect on the termites when the fungi were aged. As the three strains of *M. anisopliae* were aged for 12 weeks, the lowest amount of mortality for *R. flavipes* was observed in the first generation of *M. anisopliae* cultured on rice (FF1000), and the highest mortality was observed in the second (C16) and first (C15) generations of *M. anisopliae* cultured on corn, respectively. For *C. formosanus*, the lowest mortality was observed in the second generation of *M. anisopliae* cultured on corn (C16) and the highest mortality was observed in the first generation of *M. anisopliae* cultured on rice (FF1000) and the first generation of *M. anisopliae* cultured on corn (C15), respectively.

The ethanol extract of *M. anisopliae* (X-5) was tested using glass plates bioassays to determine preference and amount of consumption by *R. flavipes*, when given a choice of untreated fiber kraft pulp disks, an ethanol (40%) treated disks, Summon®, and different dilutions (1:0, 1:10, 1:100, 1:1000) of *M. anisopliae* ethanol extract (X-5) treated disks. The termites showed a strong preference to the 1:1000 dilution of the fungal extract. Summon® was the least preferred treatment and did not elicit recruitment. There was a trend for increased recruitment to all ethanol extract (X-5) treatments. One possibility for this observation was the dilution of the extract became more acceptable to the termites,

as degradation of the chemical compounds occurred over time. The lower concentrations were most attractive, and there was an inverse relationship based on concentration through time.

An analysis of the consumption of test cellulose matrix showed that while Summon® was not attractive, it was very palatable. The ethanol extract (X-5) of *M. anisopliae* was attractive, but was not a phagostimulant. Summon® is composed of a matrix of ground cellulose materials that is formed into a cookie. There are no adhesive products holding the cookie together, so it is possible that the cookie could have disintegrated when 4 ml of water was added every two days. Also, Summon® appeared to be readily consumed by the termites as compared to fiber kraft pulp treatments. Another possibility in regards to a greater number of termites found around the extract treatments, but not consuming large amounts of these treatments, is that extract treatments elicit visitation but not consumption. It was apparent that attractancy and consumption are not necessarily related to each other.

The undiluted ethanol extract of *M. anisopliae* (X-5) was placed in termite monitors around existing urban structures and in a field plot. There appeared to be recruitment by other termites to the extract treated monitors through time. Overall, the most termite visits occurred in the ethanol extract of *M. anisopliae* (X-5) treated monitors, and the fewest in monitors containing untreated fiber kraft pulp disks. The most termite visits found for the undiluted extract were around the Lakeway Manor and Guest House, respectfully. This could be due to a frequent watering routine that would dilute the extract and make it more attractive to *R. flavipes*.

Summon® were also placed in bait stations to determine the effect of this commercial product in natural urban environments. FMC claims that subterranean termites preferred Summon® when they forage normally, but are not attracted to the product from far distances. This correlates with the findings in this study, since Summon® did not recruit other termites but was a palatable food source to the termites.

Since the undiluted extract emits volatiles in the soil, it can be viewed as a possible termite attractant. The most effective and time efficient treatment for termite control would be to use Summon® in conjunction with the ethanol extract of *M. anisopliae* (X-5). Since the extract is an attractant and the Summon® is a preferred food source, the termites would be attracted to the termite monitor and continue to feed within the monitor. This would allow the termites to feed on the cellulose and distribute it to the rest of the population. This research has yet to be done.

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