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Research in Biological Control of the Formosan Subterranean Termite

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RESEARCH IN BIOLOGICAL CONTROL OF THE FORMOSAN
SUBTERRANEAN TERMITE

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ABSTRACT

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a worldwide distributed pest of wooden structures and living plants that causes huge economic losses. Compared to chemical pesticides, biological control may provide a more environmentally friendly and persistent method for the control of *C. formosanus*. In this research, a series of studies were conducted to understand the termite-pathogen interaction and to develop a feasible biological control strategy.

In the first part of the research, the toxicity of Bt toxins expressed by genetically modified maize to termites was tested. Plant tissues or extracts of three commercially planted Bt maize and two non-Bt maize were provided to termites as food. The results revealed no significant difference in survival rate, food consumption or length of tunnels among termites feeding on Bt and non-Bt maize. The following experiments show that maize cob can be used as a termite bait matrix.

In the second part of the research, the susceptibility of *C. formosanus* to MosquitoDunks®, which contains about 10% of the entomopathogenic bacterium, *Bacillus thuringiensis* subspecies *israelensis*, was tested. No-choice and choice bioassays did not show a promising lethal effect of MosquitoDunks® on termites. Furthermore it was shown that *C. formosanus* can suppress the growth of *B. thuringiensis*. Also, clay was tested for its potential to be a termite bait matrix that can be used to encapsulate biological control agents. Choice tests showed that significantly more termites aggregated in chambers where clay was provided, indicating the possibility of clay to be used as a termite attractant.

In the third part of the research, the potential to combine a biological control agent and a chemical pesticide against termites was investigated. The effect of low concentrations of lufenuron, a chitin synthesis inhibitor, on termite physiology and behavior was tested. Results showed that lufenuron significantly reduced vigor and disease resistance of termites. In the following experiments, termite mortality was significantly higher and synergistic in the combination of lufenuron and *Pseudomonas aeruginosa* (Schroeter) compared to treatment of lufenuron or *P. aeruginosa* alone. To combine lufenuron and a termite pathogen may bring a successful IPM strategy for the control of termites.

CHAPTER 1. INTRODUCTION

1.1 Formosan Subterranean Termite

Termites feed on various types of cellulose. In the natural environment, termites play an important role in cellulose degradation and carbon recycling. Verma et al. (2009) reported that termites consume and recycle one third of all wood and plant material each year in tropical and subtropical areas. However, some termite species can be serious pests of structural wood when their habitats overlap with humans. The annual economic loss caused by termite pests was estimated to be 40 billion dollars worldwide (Rust and Su 2012). In the United States, the most destructive termites are subterranean termites belong to the family Rhinotermitidae. The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is an invasive species originating from China and Pacific areas. Since first collected from Charleston, South California in 1957 (Chambers 1988), *C. formosanus* colonies has been reported established in 11 states in the continental United States (Woodson et al. 2001). *C. formosanus* is called "super termite" because of its wide distribution, huge colony size, extensive foraging areas, and great damage to structural wood and living plants. Suszkiw (2000) estimated that the repair and control cost of *C. formosanus* is about 1 billion dollars in the United States each year. A more recent study stated that *C. formosanus* caused an annual economic loss of 300 million dollar in New Orleans alone (Lax and Osbrink 2003). In addition, Henderson (2008) reported that the massive foraging activity of *C. formosanus* may have caused serious damage to the levee system in New Orleans.

1.2 Control of Termites with Chemical Pesticides

Currently, two types of treatment, liquid termiticides and baiting systems, are widely used for the control of subterranean termites. Soil treated with liquid termiticides, such as fipronil, imidacloprid and chlorantraniliprole, places chemical barriers between termites and wooden structures (Ibrahim et al. 2003, Osbrink et al. 2005, 2011, Parman and Vargo 2010). According to a 2002 survey, liquid termiticides account for three fourths of the market share of termite control (Rust and Su 2012). Although the effectiveness of liquid termiticides has been proven by laboratory and field studies (Hu 2005, Mao et al. 2011, Vargo and Parman 2012, Gautam et al. 2014), they are not free from shortcomings. Soil treatments with liquid termiticides requires the use of a large amount of chemical, which not only increase the cost to homeowners, but also exert non-target effects to soil and aquatic organisms (Mostert 2002, Clasen et al. 2012, Hayasaka et al. 2012). Baiting systems provide another option for long-term control of subterranean termites (Henderson and Forschler 1997, Henderson 2001, Rust and Su 2012). A baiting system will deliver slow-acting pesticides, such as hexaflumuron and noviflumuron, to the whole colony of termites through direct feeding and secondary transfer (Su 1994, 2005, Su et al. 1997, Sajap et al. 2000, Getty et al. 2000, 2007, Husseneder et al. 2007, Osbrink and Cornelius 2013). Baiting systems decrease environmental exposure of pesticides. However, the present bait stations are very labor intensive for checking and replacement, and thus limits their application for the termite control.

1.3 Overview of Biological Control of Termites

Compared to the use of chemical pesticides, biological control may provide an environmental friendly method for termite control. Many potential biological control agents, including nematodes, bacteria, and fungi, have been tested against termites under laboratory conditions. According to a review by Chouvenc et al. (2011), a total of 227 scientific reports related to termite biological control were published between 1960 and 2011. Many of them reported high termite mortality caused by pathogens, such as *Bacillus thuringiensis*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Beauveria bassiana*, *Paecilomyces fumosoroseus*, and *Metarhizium anisopliae*, in laboratory bioassays (Connick et al. 2001, Castilhos-fortes et al. 2002, Sun et al. 2003, Wang and Powell 2004, Meikle et al. 2005, Wright et al. 2005, 2008, Dong et al. 2007, Devi and Kothamasi 2009, Singha et al. 2010, Wright and Cornelius 2012). However, in spite of the success of laboratory studies, few termite pathogens show positive results to suppress termite colonies in the field. Therefore, Chouvenc et al. (2011) concluded that "the fifty years of attempted biological control of termites" is a "failure". However, the "failure" of biological control is a result of ignoring factors such as termite disease resistance and efficient method to deliver a termite pathogen. Further study of these aspects may bring insights to develop a feasible biological control method against termites.

1.4 Termite Disease Resistance

It is known that termites have strong resistance in response to infection of microbial pathogens. Compared to other insects, termites benefit from their social living as it strengthens their resistance to disease. Traniello et al. (2002) reported that, when exposed to

the entomopathogenic fungus *M. anisopliae*, groups of dampwood termites, *Zootermopsis angusticollis*, had significantly lower mortality than did isolated individuals. Interestingly, they also found that the resistance to *M. anisopliae* can be transferred from immunized members to unimmunized nestmates. The mechanism for this social transfer of immunity has not been fully understood. However, a study on the carpenter ant, *Camponotus pennsylvanicus*, showed that trophallaxis may play an important role in this process (Hamilton et al. 2011). In addition, the grooming behavior between individuals can efficiently remove pathogens attached on the cuticle of termites and decrease disease risk (Yanagawa and Shimizu 2007, Yanagawa et al. 2008, Chouvenc et al. 2009). Some studies also showed that termite will bury infected and dead termites to reduce the contact between healthy termites and pathogens (Chouvenc and Su 2012). Termites are also known to produce antimicrobial components, such as naphthalene, butylated hydroxytoluene, dioctyl phthalate, fenchone and adipic dioctyl ester, that suppress the growth of pathogens in their habitats (Wiltz et al. 1998, Wright et al. 2000).

1.5 Delivering of Termite Pathogens

For a successful biological control strategy, it is essential to maintain the viability of pathogens delivered to the target insect. Unfortunately, very few studies have focused on how to deliver pathogens to termite colonies. Wang and Powell (2004) reported that, under laboratory conditions, incorporating *M. anisopliae* into a cellulose bait increase fungal transmission in two subterranean termite species, *Reticulitermes flavipes* and *C. formosanus*. However, this study did not test how long *M. anisopliae* conidia could survive in cellulose

baits to maintain an efficient concentration that kill termites. Dunlap et al. (2007) reported

that keratin hydrolysate can be used as a foam forming compound to increase the

bioavailability of *Paecilomyces fumosoroseus* for the control of *C. formosanus*.

1.6 Prospect for the Biological Control of Termites

Because of the strong disease resistance of termites, traditional biological control

alone may not ensure effective termite control under natural conditions. Inhibition of the

immune or behavioral response may be necessary to increase the effectiveness of a termite

pathogen in the field. Connick et al. (2001) reported that some immune inhibitors such as

ibuprofen and ibuprofen sodium salt significantly increased the mortality of C. *formosanus*

caused by *S. marcescens* infection. Hamilton and Bulmer (2012) also reported that feeding

dsRNA knockdown immune-related genes to *Reticulitermes flavipes* and increase their

susceptibility to the fungal pathogen *M. anisopliae*. Combining these technologies with

traditional biological control may bring a feasible termite control strategy that decreases the

use of chemical pesticides. Meanwhile, for future studies of termite biological control, it

would be valuable to pay more attention on methods to deliver a pathogen to termite colonies

and ensure the viability of pathogens in the long-term.

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CHAPTER 2. SURVIVAL RATE, FOOD CONSUMPTION AND TUNNELING OF THE FORMOSAN SUBTERRANEAN TERMITE (ISOPTERA: RHINOTERMITIDAE) FEEDING ON BT AND NON-BT MAIZE[1]

2.1 Introduction

Bacillus thuringiensis (Bt) is a group of gram-positive, spore forming bacteria that have great agricultural importance. Since it was first isolated in 1901 by Japanese biologist, Ishiwata Shigetane, a considerable number of studies have been conducted on its application as a biological pesticide (Schnepf et al. 1998, Roh et al. 2007). Based on their flagellar antigens, phage susceptibility and plasmid profiles, approximately 100 Bt subspecies have been identified and have have been found to target a variety of insect hosts and nematodes (Mohan et al. 2009, Sanahuja et al. 2011). Although not considered as typical pathogens of termites, some Bt subspecies were reported to be toxic to some termite species, such as *Reticulitermes flavipes*, *Nasutitermes ehrhardti*, *Heterotermes indicola, Microcerotermes championi, Bifiditermes beesoni*, *Microcerotermes beesoni* and *Microtermes obesi* (Smythe and Coppel 1965, Khan 1981, Castilhos-fortes et al. 2002, Khan et al. 1977, 1978, 1985, 2004, Singha et al. 2010).

The pathogenic mechanism of Bt on its target insects depends on two types of crystal proteins, Cry and Cyt toxin (also known as δ-endotoxins), and other toxins such as Vips (vegetative insecticidal proteins) (Frankenhuyzen 2009, Hern ández- Rodr ǵuez et al. 2009, Bravo et al. 2011). With the advance of modern molecular technology, some Cry and

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[1] This chapter previously appeared as Cai Wang, Gregg Henderson, Fangneng Huang, Bal K. Gautam, and Chenguang Zhu. Survival rate, food consumption, and tunneling of the Formosan subterranean termite (Isoptera: Rhinotermitidae) feeding on Bt and non-Bt maize. *Sociobiology* 59 (2012): 1335-1350. It is reprinted by permission of *Sociobiology*.

Vip genes have been cloned and transformed to maize and cotton against a variety of pests (Koziel et al. 1993, Vincent 2010). Presently, GM Bt crops are critically important for modern agriculture. By 2011, Bt crops (maize and cotton) were planted on 65 million hectares worldwide (James 2011).

Our interest in the relationship between GM Bt maize and termites was based on two academic facts: (1) very few studies have focused on the non-target effect of GM Bt maize on termites; and (2) maize stalks and other agricultural waste have already been used as a termite bait matrix in China (Zhang et al. 1995, Li et al. 2001, Henderson 2008, Zhang et al. 2009). In this study, the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, an important economic pest in the southern United States, was fed with materials of three Bt maize hybrids, YieldGard® Corn Borer (Bt YG), Genuity® VT Triple PRO™ (Bt VT 3PRO) and Genuity® SmartStax™ (Bt SMT) , and two non-Bt maize hybrids (nBt-1 and nBt-2). YieldGard® Corn Borer maize expressing the Cry1Ab protein was the most commonly planted Bt maize for controlling stalk borers in the world before 2010. Genuity®VT Triple Pro™ and SmartStax™ are two new Bt maize technologies which contain multiple Bt genes. The objectives of this study were to determine if *C. formosanus* was susceptible to toxins expressed by Bt maize hybrids and to study the consumption behavior of *C. formosanus* feeding on maize materials.

2.2 Materials and Mathods

2.2.1 Termites

C. formosanus was collected from Brechtel Park, New Orleans on March 17, 2011, using milk crate traps as described in Gautam and Henderson (2011a). Termites were maintained in trash cans (140L) with wet wood under high relative humidity conditions for less than one month.

2.2.2 Bt and Non-Bt Maize Hybrids

Plants of three Bt maize and two non-Bt maize (Monsanto Company, St. Louis. MO) were collected from a green house located at Louisiana State University Agricultural Center, in Baton Rouge, LA. The three Bt corn hybrids were DKC 67-23 RR2 containing YieldGard[®] Corn Borer trait, DKC 67-88 expressing Genuity[®] VT Triple Pro[TM] traits and DKC 61-21 possessing Genuity[®] SmartStax[TM] traits. YieldGard[®] Corn Borer contains a single Bt gene, Cry1Ab, which was the most commonly planted Bt maize for controlling stalk borers worldwide before 2010. Genuity[®] VT Triple PRO[TM] is a stacked/pyramided Bt corn that expresses three Bt genes including Cry1A.105 and Cry2Ab2 for controlling above-ground lepidopteran species and Cry3Bb1 for managing underground rootworms, *Diabrotica* spp. Genuity[®] SmartStax[TM] corn contains all Bt genes expressed in Genuity[®] VT Triple Pro[TM] plus Cry1F targeting lepidopteran species and Cry34Ab1/Cry35Ab1 targeting rootworms. Genuity[®] VT Triple PRO[TM] and SmartStax[TM] maize were among the first stacked/pyramided Bt maize technologies that were commercialized in 2010 in the United States and Canada. The two non-Bt maize hybrids were DKC 61-22 and DKC 67-86. The hybrid, DKC 61-22,

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was genetically closely related to the Bt maize hybrid, DKC 61-21, while DKC 67-86 was closely related to the Bt corn hybrids DKC 67-23 and DKC 67-88. Expression of Cry proteins in the corn hybrids was confirmed using an ELISA-based technique (EnviroLogix, Quantiplate™ kits, Portland, ME). Leaves, stalks and roots of each maize hybrid were put in separate Ziploc® bags with a small amount of water and stored in 4℃ for less than one week. Before use, the plant tissues were carefully washed with distilled water to clean the pollen and dust off the surface.

2.2.3 Experimental Design

A two-way completely random design was used in the study with corn hybrid and food source as the two main factors. The experiment contained five corn hybrids mentioned above. For each corn hybrid, tests were conducted in five different ways as food sources for the termite: (1) wood block containing maize leaf extract, (2) filter paper containing maize leaf extract, (3) maize leaf tissue, (4) maize stalks, and (5) maize root. In addition, wood block and filter paper treated with distilled water only were also included in the tests as blank controls. There were five replications in each treatment combination. Therefore, a total of 27 treatment combinations and 135 experimental units were tested in this experiment.

2.2.4 Substrate and Bioassay Arena

Autoclaved (121℃, 45min) sand was weighed and mixed uniformly with distilled water in a Ziploc® bag to make the 15% moisture sand by weight. Thirty grams wet sand was placed in each Petri dish (100×15mm) and pressed by bottom side of a smaller Petri dish (60×15mm) to form a thin layer as the substrate for termites.

2.2.5 Wood Block and Filter Paper

Wood blocks ($1.9 \times 1.9 \times 0.9$cm southern yellow pine) were autoclaved (121℃, 15min) and dried in an oven dryer (45℃, 1d). Dry weight of wood blocks and filter paper (4.25 cm diameter, Whatman®) was recorded. Maize leaves (25 g) were cut into small pieces and extracted with 20 ml distilled water. Approximately 10 ml of extract was collected from each hybrid. One ml of extract was added to the surface of wood block and filter paper and air-dried at room temperature. Wood blocks and filter paper treated with 1 ml of distilled water only were used as blank control.

2.2.6 Maize Leaves, Stalks and Roots

Maize stalks were straight-cut to check infection of stalk bores, which could make tunnels inside the stalk. Leaves and non-infected stalks were cross-cut into small segments (4-5 cm). Roots of maize (5 g) were weighted and cut into 3 cm segments and mixed with 30 g sand containing 15% moisture in each replicate of the root treatment.

2.2.7 Bioassays and Data Recording

Based on the colony structure, 50 termite workers and 2 nymphs (wing budded individuals) were introduced into each experimental unit. The bioassays were maintained at room temperature (23±1℃) for two weeks. Dead termites were removed daily and distilled water was added when necessary. After two weeks, live termites of each experimental unit were counted. Wood blocks, filter paper, leaves and stalks were carefully brushed clean of sand. The bottom side was scanned to observe the consumption areas and patterns. After completely drying in an oven dryer (45℃, 1d), the weight of wood blocks and filter paper

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were recorded to determine consumption. Because maize leaf, stalk and root used in this test were fresh, the consumption calculated by difference of dry weight was not available. The bottom side of each Petri dish was scanned to record the tunneling behavior and length of tunnels.

2.2.8 Statistical Analysis

The assumptions of independent and normal distribution were verified by the diagnostics plots in SAS 9.3 (SAS Institute, 2011), A two-way analysis of variance (ANOVA) was performed using PROC MIXED procedure to compare the survival rate, consumption, and tunnel length of termites feeding on different maize hybrids and different food sources. Post ANOVA comparisons were performed using Tukey's HSD test. Significant levels were determined at $\alpha=0.05$.

2.3 Results

2.3.1 Survival Rate

The mean survival rates of the two controls at 14 d were 89.6% (wood block) and 85.4% (filter paper) (Table 2.1). The main effect of food source on survival rate of termites at 14 d was significant ($F = 24.57$; df = 4,99; $P < 0.0001$), but the effect of maize hybrid and the interaction of food source and maize hybrid was not significant ($F = 1.41$; df = 4,99; $P = 0.2348$ for maize hybrid and $F = 0.91$; df = 16,99; $P = 0.5652$ for interaction). An average of 36.9% of termites feeding on maize stalks survived after 14 days across the five maize hybrids (both Bt and non-Bt), which was significantly less ($P < 0.05$) than that observed for any other food source. Survival rate of termites feeding wood bock was 81.2%, which was

significantly greater ($P < 0.05$) than that observed for those feeding maize leaf tissue (64.3%) or root (60.7%), but it was not significantly different compared to the survivorship (77.1%) of the termites feeding on filter paper. A difference was also significant ($P < 0.05$) between the filter paper and maize root, but not significant between filter paper and maize leaf tissue or between leaf tissue and root (Figure 2.1a).

2.3.2 Amount of Food Consumption

The mean consumption of the two controls at 14 d was 0.120 g (wood block) and 0.048 g (filter paper) (Table 2.1). As observed in the survival rate, the main effect of food source on food consumption after 14 days was significant ($F = 69.90$; df = 1,40; $P < 0.0001$), but the effect of maize hybrid and the interaction of food source and maize hybrid was not significant ($F = 0.42$; df = 4,40; $P = 0.7908$ for maize hybrid and $F = 1.21$; df = 4,40; $P = 0.3204$ for interaction). An average of 0.098 g wood block was consumed after 14 days across the five maize hybrids, which was significantly greater than that (0.051 g) recorded for the termites feeding on filter paper.

2.3.3 Tunnel Length

The mean tunnel length of the two controls after 14 days was 258.8 mm (wood block) and 292.1 mm (filter paper) (Table 2.1). Similarly as observed in the termite survival and food consumption, the main effect of food source on survival rate of termites at 14 d was significant ($F = 58.98$; df = 4,100; $P < 0.0001$), but the effect of maize hybrid and the interaction of food source and maize hybrid was not significant ($F = 0.62$; df = 4,100; $P = 0.6511$ for maize hybrid and $F = 1.30$; df = 16,100; $P = 0.2092$ for interaction). The length of

tunnels among different food sources from the highest to the lowest was: root (298.6 mm) >

filter paper (261.2 mm) = wood block (236.3 mm) > leaf (168.2 mm) > stalk (117.0 mm)

(Figure 2.1b).
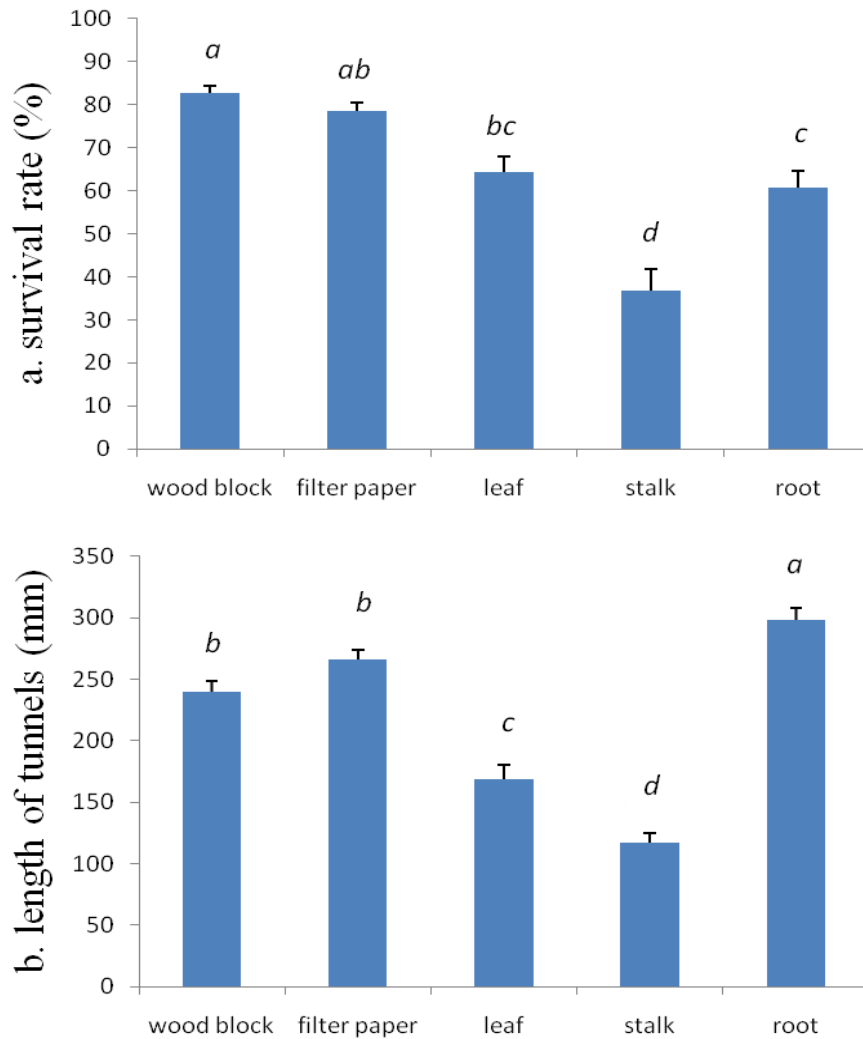
Figure 2.1 (a) Survival rate (mean $\pm$ SEM) and (b) tunnel length (mean $\pm$ SEM) of termites on different food sources across five Bt and non-Bt maize hybrids. Mean values followed by the same letter are not significantly different ($P > 0.05$).

Table 2.1. Survival rate (mean ± SEM), food consumption (mean ± SEM), and tunnel length (mean ± SEM) of termites feeding on different food sources containing Bt and non-Bt maize plant tissue or extract.

		nBt-1	nBt-2	Bt YG	Bt VT 3PRO	Bt SMT	Blank control
Survival rate (%)	wood block	78.8±3.7	83.1±3.5	76.6±7.9	83.1±4.7	84.6±3.7	89.6±2.1
	filter paper	73.8±2.9	81.5±4.1	76.1±8.9	80.0±2.3	73.8±5.3	85.4±2.4
	leaf	71.2±5.5	72.7±5.2	72.7±5.2	46.9±8.2	58.1±10.7	-
	stalk	43.5±13.1	52.3±9.6	32.3±13.8	27.7±10.4	28.8±8.8	-
	root	61.8±7.9	59.5±14.4	65.1±7.8	67.3±4.7	49.8±10.2	-
Consumption (g)	wood block	0.096±0.013	0.092±0.011	0.098±0.011	0.100±0.003	0.102±0.006	0.120±0.013
	filter paper	0.046±0.009	0.066±0.008	0.058±0.009	0.040±0.004	0.046±0.007	0.048±0.006
Tunnel length (mm)	wood block	253.1±13.7	226.8±36.2	241.2±25.7	241.2±7.2	219.3±11.3	258.8±29.2
	filter paper	236.2±23.4	290.1±13.2	278.4±18.6	239.5±8.5	261.6±24.3	292.1±15.8
	leaf	181.6±26.3	211.6±18.5	158.8±21.4	145.3±30.4	143.6±37.0	-
	stalk	121.6±17.1	78.4±20.5	120.2±17.2	126.5±9.4	138.4±17.1	-
	root	287.8±26.6	331.4±9.9	277.0±24.1	280.8±16.5	316.0±20.5	-

2.3.4 Consumption Pattern and Tunneling Behavior of Termites Feeding on Maize Materials

In the leaf treatments, both the daily observations and scanned pictures of leaf tissue (Figure 2.2) showed that termites prefer to eat primarily the vein. A tunnel inside the vein was regularly observed. Observations also revealed that termites prefer to stay on the surface of sand and leaf tissue. In the stalk treatments, termites made tunnels inside the stalks (Figure 2.3b-f). Within the second or third day after release of termites, 1 to 3 holes were made on the surface of split stalks by termites (Figure 2.3a). Observations and scanned pictures also showed that termites stay inside the split stalks, rather than making tunnels in the sand, resulting in fewest tunnels in sand substrate when compared to other food source treatments (Figure 2.4). In the root treatments, the termites consumed a large quantity of roots and broke them down into small pieces and pellets. Extensive tunneling was found in the root treatments (Figure 2.4).
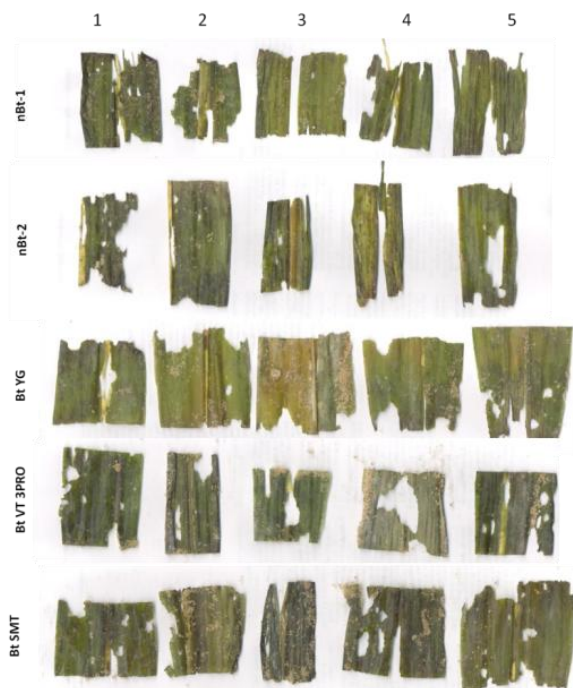
Figure 2.2 Consumption pattern of termites feeding on non-Bt (nBt-1, nBt-2) and Bt (Bt YG, Bt VT 3PRO and Bt SMT) maize leaves.

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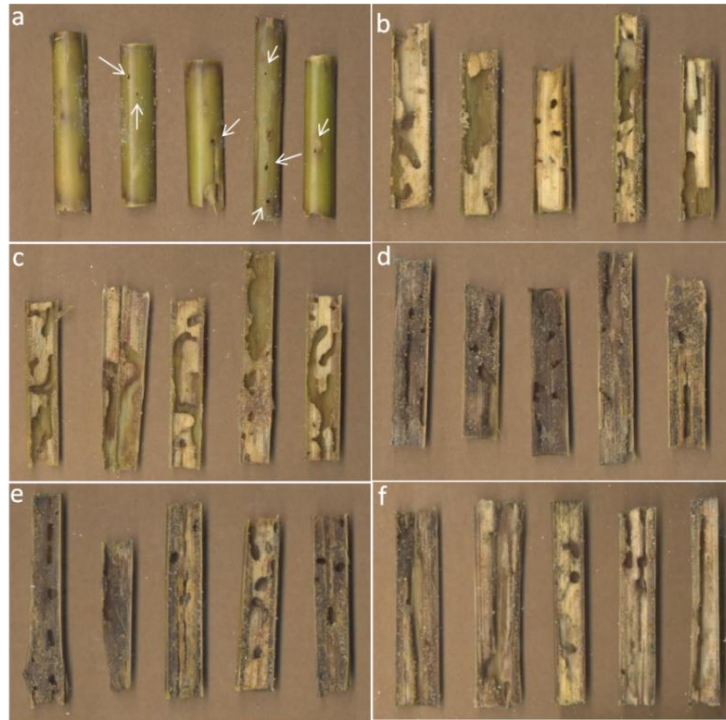
Figure 2.3 (a) Holes made by termites on the surface of split stalks at day 2-3 and the consumption pattern of termites feeding on (b) nBt-1, (c) nBt-2, (d) Bt YG, (e) Bt VT 3PRO and (f) Bt SMT maize stalks.
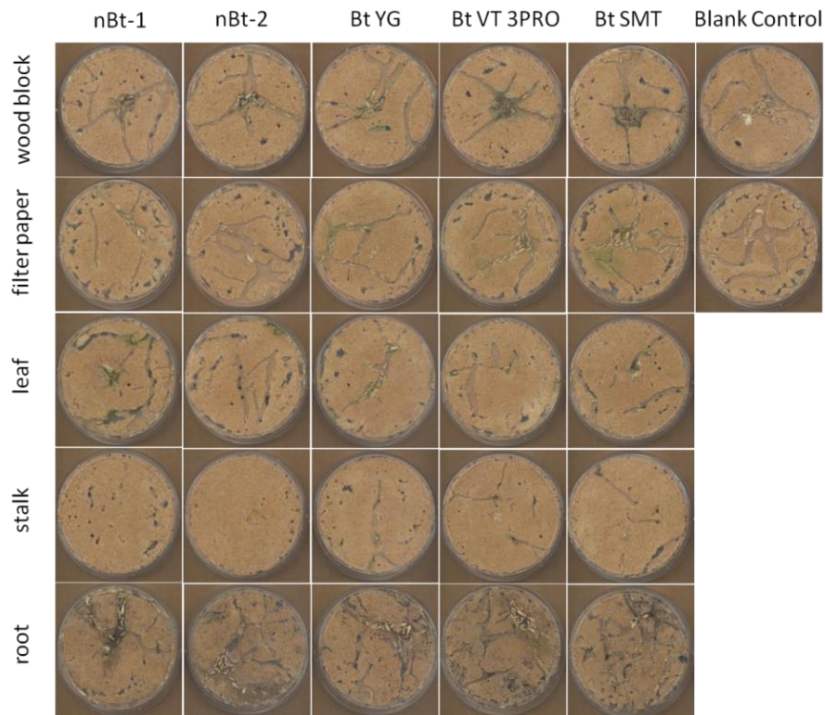
Figure 2.4 Tunneling pattern of termites in the bottom side of Petri dish containing different food sources with Bt and non-Bt plant tissue or extract.

2.4 Discussion

Despite their great value in modern agriculture, non-target effects of GM Bt crops have been of major concern. Meta-analysis showed that, by 2008, more than 360 original papers focusing on the non-target effect of GM Bt crops had been published (Naranjo 2009). However, among those papers, few studies related to termite species were included. In nature, termites could interact with GM Bt crops in various ways. For example, more than 10 termite species, such as *Ancistrotermes latinosus*, *Macrotermes falciger*, *Pseudacanthotermes spiniger*, *Cornitermes cumulans*, *Procornitermes triacifer*, *Ancistrotermes latinosus*, attack maize directly; some of them even cause 20 to 50% loss in corn yield (Mill 1992, Nkunika 1994, Rouland-Lefèvre 2011). In addition, Bt toxins produced by GM Bt crops can be released into soil by residue decomposition and root exudates (Tapp and Stttzky 1998, Muchaonyerwa and Waladde 2007, Saxena 2010, Helassa et al. 2011, Das and Chaudhary 2011). Subterranean termite species such as *C. formosanus* are likely to be exposed to Bt toxins remaining in soil (Muchaonyerwa and Waladde 2007). Our results suggest that three GM Bt maize involved in our study have no effect on *C. formosanus*.

Husseneder and Grace (2005) developed a method to deliver foreign genes to termite colonies through genetically modified gut bacteria, which indicates a potential application of Bt toxin(s) in termite control. However, despite studies on susceptibility of termites to Bt subspecies, no termite-targeted toxin have been identified. Our study showed that seven Bt toxins expressed in three GM Bt crops, including Cry1Ab, Cry1A.105, Cry2Ab2, Cry3Bb1, Cry1F, Cry34AB1 and Cry35AB1, do not negatively affect *C.*

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formosanus. This result will provide valuable information for the future screening work of termite-sensitive Bt toxins.

Significant difference in survival rate of termites was found among different food sources (Figure 2. 1a). The lowest survival rate observed in termites feeding on maize stalk could be caused by fungi growing on the surface of stalks observed from day 5 of the experiment. Gautam and Henderson (2011b) showed that, in laboratory conditions, attack of pathogenic fungi may lead to high mortality of termites. However, in nature, various strategies are used by termites to control fungi. For example, Chouvenc and Su (2012) reported that *C. formosanus* avoid the entomopathogenic fungus *Metarhizium anisopliae* by employing several behavioral patterns. Some anti-fungal chemicals associated with termites also inhibit the growth of fungi in natural conditions (Chen et al. 1998, Bulmer and Crozier 2004, Rosengaus et al. 2007).

Although *C. formosanus* is not considered an agricultural pest, some studies showed that they consume herbaceous crops such as sugarcane and bamboo (Dai and Luo 1980, Su and Scheffrahn 1986, Chen and Henderson 1996, Hapukotuwa 2011). Chen and Henderson (1996) stated that the feeding preference of *C. formosanus* for sugarcane may be caused by glutamic acid and aspartic acid in sugarcane juice. Li et al. (2000) reported that sugarcane powders were significantly preferred by *C. formosanus* over pine wood powders or starch. Our study reveals that, leaves, stalks and roots of maize also can be alternative food sources for *C. formosanus*. Moreover, termites showed special consumption and tunneling behaviors when feeding on maize tissues.

One possible application of this maize consumption behavior is to develop a stalk bait for use against *C. formosanus*. In China, maize stalks have already been used as a termite bait matrix to control subterranean termites such as *Reticulitermes chinensis* (Zhang et al 1995, Zhang et al. 2009). Compared to traditional bait matrices such as pinewood and cardboard, stalk bait shows some obvious advantages. Firstly, as an agricultural waste, maize stalk is a quite abundant resource for bait production, thus reducing the cost and the over utilization of forestry resources. Moreover, since termites made tunnels inside the stalks, more contact area can be attained between termites and the stalk bait, which may enhance toxicant contact and transfer.

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CHAPTER 3. EVALUATION OF THREE BAIT MATERIALS AND THEIR FOOD TRANSFER EFFICIENCY IN THE FORMOSAN SUBTERRANEAN TERMITE (ISOPTERA: RHINOTERMITIDAE)[1]

3.1 Introduction

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is one of the most economically important termite pests with a widespread global distribution (Henderson 2008, Rust and Su 2012). Two main methods for subterranean termite control, based on bait technologies and liquid termiticides, have been widely used (Henderson 2001, Gautam and Henderson 2012, Rust and Su 2012). In the United States, termite bait products account for about one third of the market share according to a 2002 survey (Rust and Su 2012). However, the labor costs associated with bait placement and inspection have hampered sales of baits compared to liquids. Meanwhile, termite baits have some obvious advantages in long-term termite control and structural protection for its low chemical expense and environmental friendliness (Pawson and Gold 1996, Su and Scheffrahn 1998, Grace and Su 2000, Thoms et al. 2009, Liu et al. 2011, Gautam and Henderson 2012).

A successful baiting system depends on understanding the foraging behavior of subterranean termites. A toxicant-laced cellulosic food material can be introduced to the whole colony through direct feeding, trophallaxis, cannibalism, and mutual grooming (Su and Scheffrahn 1998, Valles and Woodson 2002, Bagnères et al. 2009, Dhang 2011, Gautam and Henderson 2012). Two cellulosic food materials, wood and cardboard, have been

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commercially used in baiting systems. Maize cob is another material that contains abundant cellulose (Azubuike et al. 2011, Shogren et al. 2011). In some regions of China, maize cob is used non-commercially as a bait material for termite control and led to this investigation (Zhang et al. 1995, Zhang et al. 2009, Liu et al. 2011).

Although many studies focused on wood and cardboard baiting systems, very few have paid attention on comparing the effectiveness between different bait materials (Haverty et al. 2010, Lenz et al. 2011). Formosan subterranean termite consumption of maize stalks and cobs has been previously observed by us (unpublished data). Here, we compared three bait materials, wood, cardboard and maize cob, in three aspects: (1) no-choice tests and choice tests were conducted to study the consumption and preference of Formosan subterranean termites on different bait materials; (2) a Nile blue A transfer bioassay was employed to evaluate the efficiency of dyed food transfer from the bait material to termites; and (3) a donor and recipient bioassay was employed to evaluate the efficiency of dyed food transfer between treated termites (dyed) and untreated termites (undyed), when both (a) food was provided and (b) food was not provided.

3.2 Materials and Methods

3.2.1 Termites

Worker and soldier *C. formosanus* were collected from Brechtel Park, New Orleans, LA, on 14 October 2011 using milk crate traps as described in Gautam and Henderson (2011a). Termites were maintained in trash cans (140L) with wet southern yellow pine (*Pinus* sp.) wood under constant conditions (70-80% RH, 26-28℃) for 1-2 months before they were

used in the bioassays, as described in Mao and Henderson (2010).

3.2.2 Maize Cobs, Wood Blocks and Cardboard

Ears of a non-Bt corn hybrid were collected from Macon Ridge Research Station, LSU AgCenter, Winnsboro, LA, on 19 October 2011. Before the experiment, mature corn ears were selected and washed carefully and kernels were completely removed. Fresh dekerneled cobs were then crosscut into 8mm segments (dry weight: 1.097 ±0.049g). Southern yellow pine wood was purchased in Baton Rouge, LA in 2011 and cardboard roll was in the lab for the last few years. Wood was cut into small blocks (size: 8×20×20mm, dry weight: 1.442 ±0.036g). Cardboard was cut into round shaped pieces according to the size of the cross section of cobs (diameter: 20mm, dry weight: 0.119 ±0.003g). Cob segments, wood blocks and cardboard were autoclaved (121℃ for 15min) and dried in an oven dryer (70℃ for 48h).

3.2.3 Consumption Bioassay

The bioassays were conducted in Petri dishes (100×15mm) containing 30g sand (12% moisture). The bioassays included three no-choice tests, three two-choice tests and one three-choice test. In the no-choice test, cobs, wood blocks or cardboard were placed on top of the sand substrate in the Petri dish. In the two-choice test and three-choice test, the combinations of wood block and cardboard, wood block and cob, cardboard and cob, or wood block, cardboard and cob were provided. Cobs, cardboard and wood blocks were put on the substrate such that they were not touching and equally distanced to each other. Each treatment had 6 replicates.

For the two-choice tests and the three-choice test, red, green and blue lines were drawn under the bottom side of Petri dishes to indicate the location of each food choice during scanning. In each experimental unit, 50 workers and 5 soldiers were released before sealing with Parafilm® (Structure Probe, Inc., West Chester, PA). The bioassays were maintained in a 28℃ incubator (3710, Forma Scientific, Inc., Marietta, OH) in total darkness for two weeks. Moisture was monitored every day and distilled water was added to sand whenever required.

Before and after the experiment, the dry weight of wood blocks, cardboard and cobs was measured. The bottom side of these materials was scanned to observe the consumption patterns. At the end of the experiment, the survival rate of termites in each experimental unit was recorded. The bottom side of each Petri dish was scanned and tunneling was quantified by measuring the length of tunnels with the help of a string overlaid on tunnels as described in Gautam (2011). For two choice and three-choice tests, only length of tunnels that intersected with the scanned colored lines under each food source was measured. In all tests, tunnels created by termites circling the Petri dishes (edge effect) were not measured.

3.2.4 Nile Blue A Transfer Bioassay

A suitable persistent stain, Nile blue A (Sigma-Aldrich Co. LLC., St. Louis., MO), was used to evaluate the food transfer from bait to termites. Southern yellow pine wood blocks, cardboard and cobs were weighed and the required amount of Nile blue A to make 0.1% dye (w: w) was dissolved in acetone and pipetted on the food sources as described in Gautam

and Henderson (2011b). The bioassay arena and substrate was the same as in the consumption and preference bioassay. The dyed wood blocks, cardboard and cobs were put in the center of the Petri dishes. Each treatment had 21 experimental units. Fifty workers and 2 soldiers were released in each Petri dish, which was sealed with Parafilm® and maintained in a 28℃ incubator in total darkness. Three of the Petri dishes from each treatment were randomly chosen and checked at 6h, 12h, 1d, 2d, 3d, 4d, 5d after set up of the experiment. At each time point, the number of living termites and the number of termites turning blue were recorded to compare the efficiency of Nile blue A transfer from the three bait materials to termites. At the end of 5 days, blue termites feeding on different food sources were carefully collected using soft brushes and put in separate Petri dishes with wet sand in preparing for the donor and recipient bioassay.

3.2.5 Donor and Recipient Bioassay

A donor and recipient assay was employed to study the efficiency of Nile blue A transfer between termites. Termites feeding on different dyed food sources were used as Nile blue A donors and untreated termites were used as recipients. Two experimental groups, a starvation group and a fed group, were included in the bioassay. For the starvation group, no food was provided; whereas for the fed group, a piece of untreated southern yellow pine wood block ($8 \times 20 \times 20$mm) was provided in each experimental unit. Both starvation and fed groups had 3 treatments, in each of which, 50 blue workers previously feeding on one of three dyed bait materials were combined with 50 undyed workers and 2 soldiers. Each treatment (starvation and fed groups) contained 6 replicates. Petri dishes were sealed with

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Parafilm® and maintained in a 28℃ incubator in total darkness for 19 days. At the end of the experiment, the number of surviving dark blue termites (donor termites), light blue termites (untreated termites turned blue) and white termites (untreated termites that did not turn blue) were counted.

3.2.6 Data Analyses

SAS PROC UNIVARIATE was used to test the normality of data (survival rate, food consumption and length of tunnels). If the data was normally distributed, the means of treatments were compared by ANOVA and Tukey's test (F-value (F), degree of freedom (df), and P-value (P) were provided) or t-test (t-value (t), df and P were provided). For the data that were not normally distributed, a rank was assigned and compared by ANOVA and the Kruskal-Wallis test (Chi-square (χ^2), df and P were provided). All significant levels were determined at $\alpha < 0.05$.

3.3 Results

3.3.1 Survival Rate of Termites in Consumption Bioassays

In the no-choice test, the survival rate of termites feeding on cobs was significantly lower than that on cardboard ($\chi^2 = 7.0936$, df=2, $P = 0.0288$); neither were significantly different from wood blocks (Figure 3. 1a). In the two-choice test, the survival rate of the wood and cob treatment was significantly lower than that of the wood and cardboard treatment ($\chi^2 = 6.7765$, df=2, $P = 0.0338$); there was no significant difference between them and the treatment of wood and cob (Figure 3.1b).
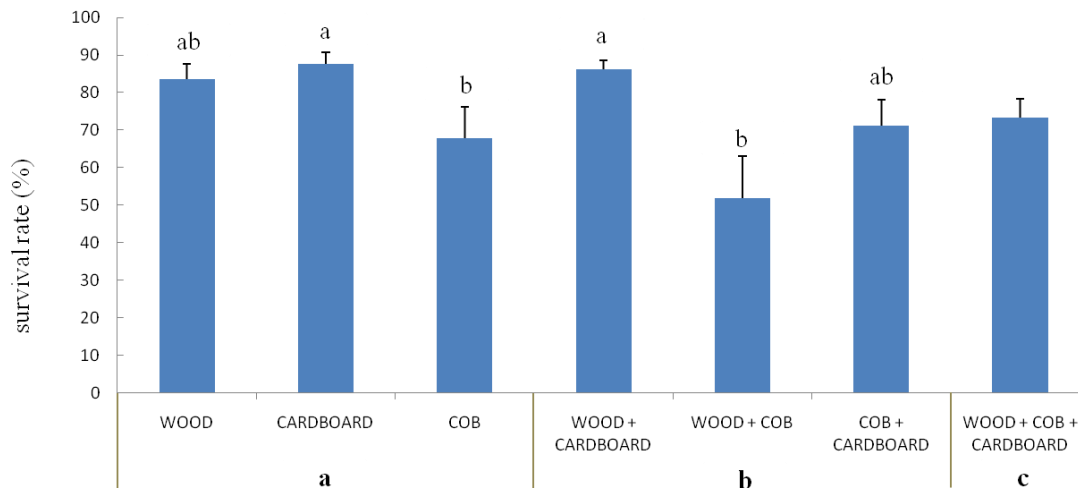
Figure 3.1 Survival rate (mean ± SEM) of termites of (a) no-choice tests, (b) two-choice tests and, (c) three-choice test. Different letters represent significant difference within each group (*P*<0.05).

3.3.2 Consumption and Tunneling in No-choice Test

The consumption of cardboard was significantly lower than that of wood blocks or cobs (χ^2=12.3158; df=2; *P*=0.0021); no significant difference was found between wood blocks and cobs (Figure 3. 2a). Termites feeding on cobs primarily consumed the centers and made a large consumption hole inside the cob (Figure 3.2a). No significant difference in length of tunnels was found among the three treatments (*F*=0.39; df=2,15; *P*=0.6830; Figure 3.3a).

3.3.3 Consumption and Tunneling in Two-choice Tests

The consumption between different food sources in the three treatments was: cobs significantly higher than wood blocks (*t*=3.67; df=10; *P*=0.0043); wood blocks significantly higher than cardboard (*t*=3.19; df=10; *P*=0.0096); cob and cardboard were similar (*t*=1.30; df=10; *P*=0.2225; Figure 3. 2b). Termites made consumption holes inside the center part of cobs in 8 of 12 combinations containing cobs (Figure 3.2b). More tunnels were built under

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and through the cobs than in the case of cardboard (t=2.50; df=10; P=0.0315), and there was

no significant difference between wood blocks and cardboard (χ^2=2.1818; df=2, P=0.1396) or

wood blocks and cob (t=0.48; df=10; P=0.6430; Figure 3. 3b).
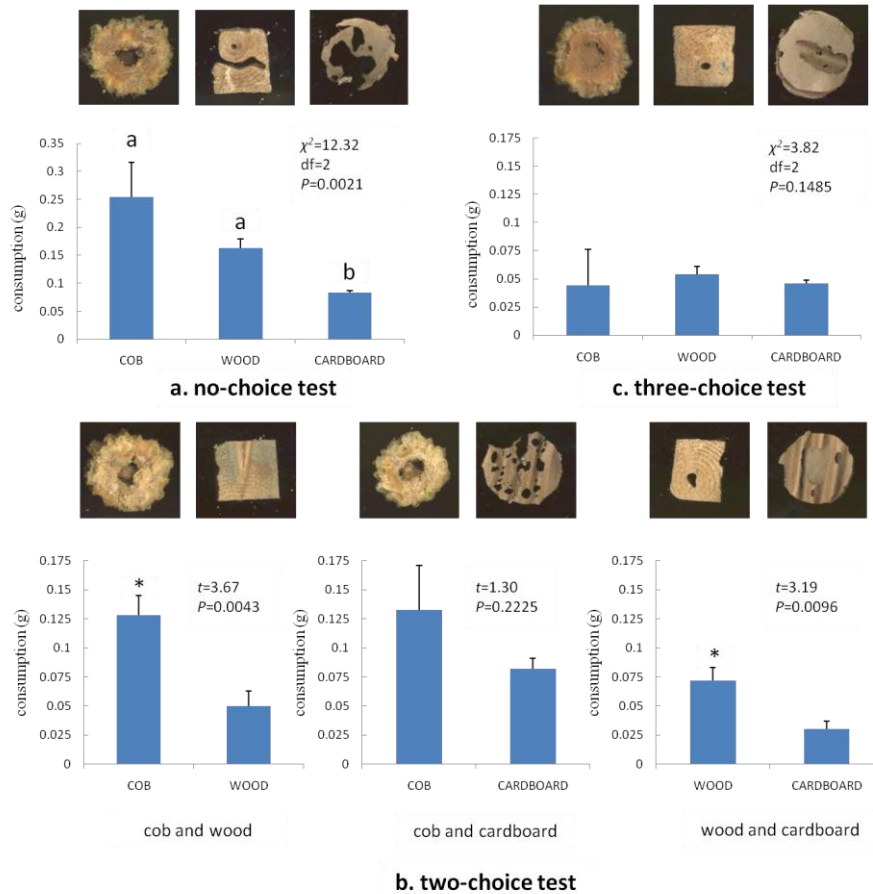
Figure 3.2. Consumption patterns and mean of consumption (±SE) of *C. formosanus* of (a) no-choice tests, (b) two-choice tests, and (c) three-choice test. *Bars and different letters represent significant difference (P<0.05).

3.3.4 Consumption and Tunneling in Three-choice Test

No significant difference in consumption was found between the three bait

materials (χ^2=3.8189; df=2; P=0.1485; Figure 3.2c). Unlike the no-choice tests and two-

choice tests, no obvious consumption holes were found in the center area of cobs in the three-

choice test (Figure 3.2c). However, tunnels under and through cobs were found in four of six

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replicates, whereas no obvious tunnels under wood blocks and cardboard were observed (Figure 3.3c). The length of tunnels made under and through the cobs was significantly longer than that under wood blocks and cardboard (χ^2=9.5253; df=2; P=0.0085).

3.3.5 Efficiency of Food Transfer from Bait Materials to Termites

At 6h, workers feeding on cobs, wood blocks and cardboard began to turn blue. However, the percentage of blue workers in the wood block treatment was significantly higher than that in cobs or cardboard treatments (F=29.31; df=2,6; P=0.0008); the percentage of blue workers in the cobs and cardboard treatments was similar. After 12h, no significant difference in the percentage of blue workers was detected between the three treatments (Figure 3.4). Blue soldiers were observed both in the cob and wood block treatments at day 1, while soldiers in the cardboard treatment began to turn blue at day 3.

3.3.6 Efficiency of Food Transfer between Termites

The survival rate of donor termites previously feeding on dyed wood blocks in fed group was significantly higher than that of the cob treatment in fed group (χ^2=15.1531; df=5; P=0.0097), but both of them were not significantly different from cardboard (Figure 3.5a). The survival rate of recipient termites in the wood treatment in fed group was significantly higher than that of the cob treatment in fed group or the cardboard treatment in starvation group (χ^2=13.7096; df=5; P=0.0176); the survival rate of recipient termites between the cob treatment in fed group and the cardboard treatment in starvation group was similar (Figure 3.5b).
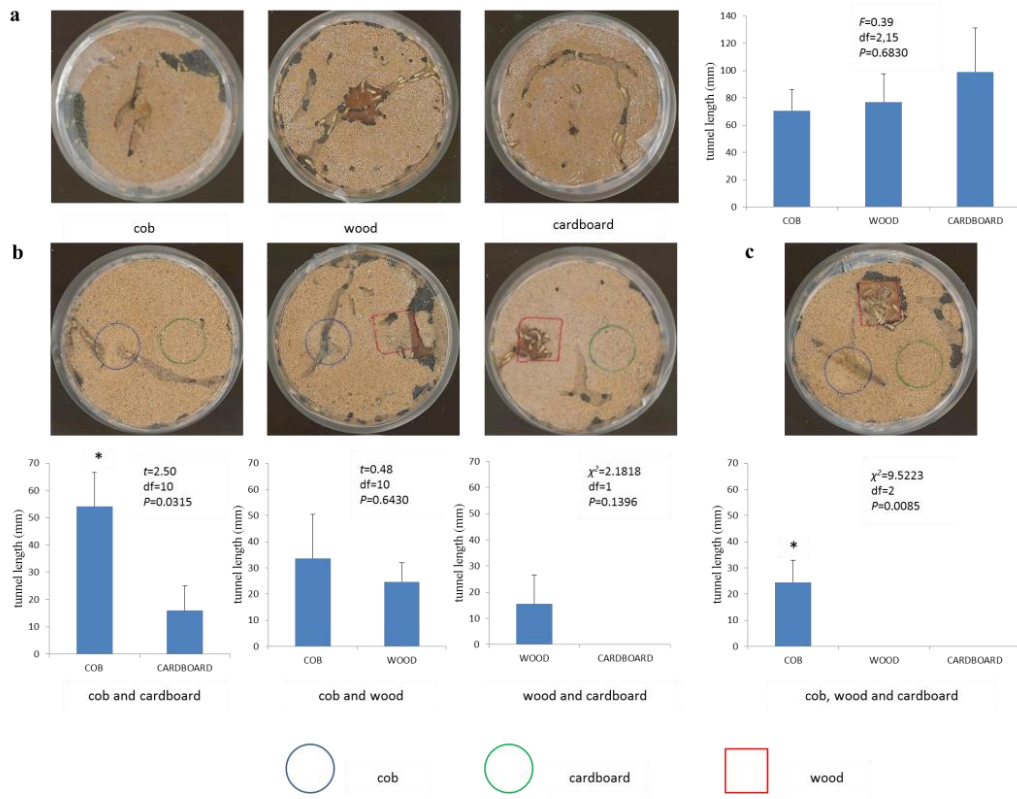
Figure 3.3. Tunneling conditions and tunnel length (mean ±SEM) under or through food materials made by termites in the (a) no-choice tests, (b) two-choice tests and, (c) three-choice test. *Bars represent significant difference (*P*<0.05).
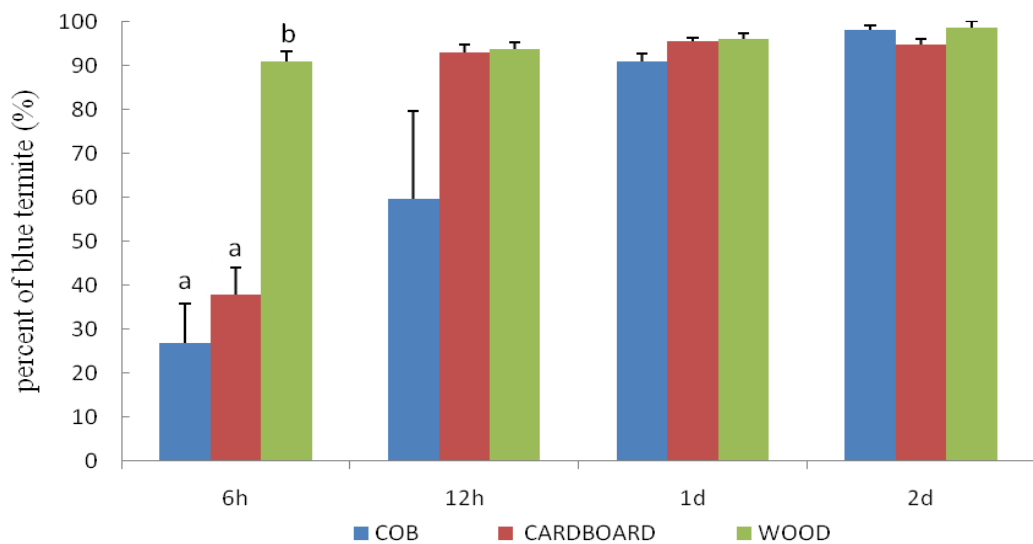
Figure 3.4. Percentage of termites turned blue (mean ±SEM) after 6h, 12h, 1d and 2d of feeding on three dyed bait materials. Different letters represent significant difference within each time period (*P*<0.05).

No significant difference in percentage of blue recipient termites was detected in the three treatments of fed group. However, the percentage of blue recipient termites in all treatments of fed group was significantly lower than that of starvation group (χ^2=28.7266; df=5; P<0.0001; Figure 3.5c). For the starvation group, in the wood treatment, the percentage of recipient termites turning blue was significantly higher than that in cardboard treatment (χ^2=28.7266; df=5; P<0.0001); both of them were not significantly different from the cob treatment.
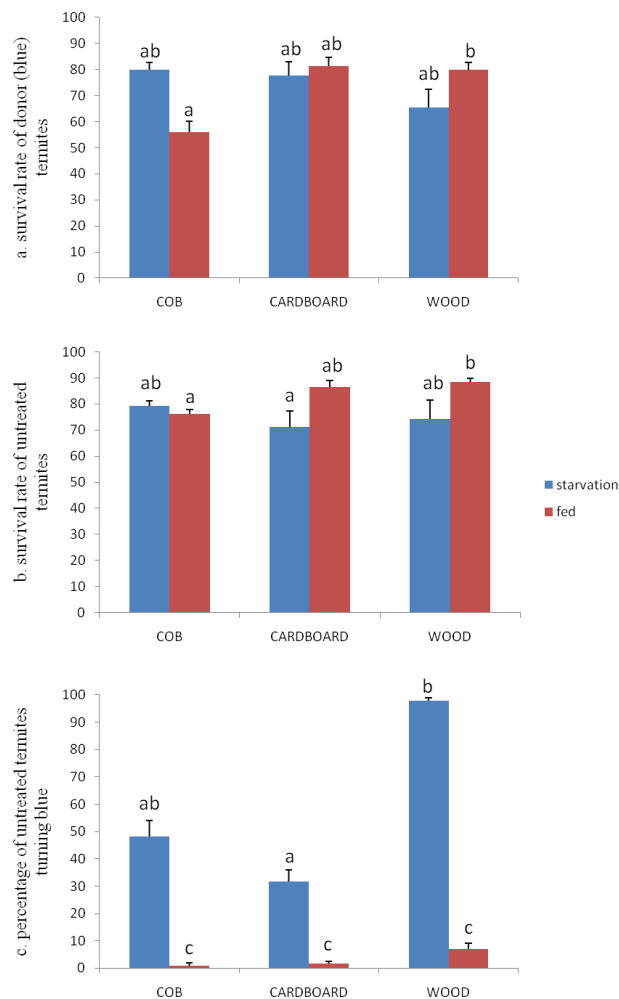
Figure 3.5. (a) Survival rate of donor termites (mean ±SEM); (b) Survival rate of recipient termites (mean ±SEM); (c) Percentage of recipient termites turned blue (mean ±SEM) in three treatments of starvation and fed groups after 19 days. Different letters represent significant difference (P<0.05).

3.4 Discussion

Based on our results, the mean consumption of cobs was the highest in the no-choice test and in all two-choice tests. For the two-choice test where cob and cardboard were present, this difference in consumption was not significant, probably due to a large variance in cob consumption resulting from one replicate where cob was not consumed at all. Removing the outlier, the consumption of cobs was significantly higher than that of cardboard (P=0.0506). Lenz and Evans (2002) stated that three components should be considered in developing a baiting system: active ingredient (toxicants), matrix (bait materials) and termite biology. Many studies focused on discovering and evaluating a variety of toxicants used in termite baits (Su 1994, Henderson and Forschler 1997, Haverty et al. 2010, Neoh et al. 2011). However, the development and evaluation of bait materials and related termite biology were not given as much priority. One of the most important factors determining the success of bait materials is the composition, evaluated by termite preference (Lenz and Evans 2002). With a consumption bioassay, Lenz et al. (2011) compared the consumption of *C. formosanus* and *Reticulitermes speratus* on 10 different types of paper and showed that cardboard was the most preferred. Li et al. (2001) compared the consumption of three bait matrices, pinewood powder, sugarcane powder and starch and found that sugarcane powder was preferred most by *C. formosanus* and *Reticulitermes flaviceps*. In our study, one possible reason for the preference of cobs by termites is that cobs absorb more water from the substrate. Bal and Henderson (2011c) reported that the highest wood consumption by termites always was found in wood blocks with high moisture content. Cobs also contain

more sugars and amino acids, which are known to increase bait attractiveness to termites (Chen and Henderson 1996, Lenz and Evans 2002). Moreover, Hedlund and Henderson (1999) found that an increase in surface area of food increased the consumption by termites. Cobs are full of nooks and crannies and surface area is large relative to dimensions.

Subterranean termites rely on tunnels for their high efficiency to search and transport food resources (Lee et al. 2007, Henderson 2008). The relationship between tunnel abundance, food searching and consumption behavior of *C. formosanus* has been shown in lab and field studies (Hedlund and Henderson 1999, Su 2005). According to the wood-consumption hypothesis developed by Li and Su (2008), a gain of tunnel space results from food removed from the space. In our study, though no significant difference in consumption was detected between cobs and cardboard in the two-choice test, or cobs, cardboard and wood blocks in the three-choice test, the mean length of tunnels under cobs was significantly more extensive than that under wood blocks or cardboard, indicating a preference of cobs.

The higher mortality in two treatments containing cobs may be the result of fungus that developed on the surface of the cobs. The attack of pathogenic fungi is one reason that high mortality occurs in the lab studies (Jayasimha and Henderson 2007, Gautam and Henderson 2011d). However, the relationship between fungi and termites is complex. In nature, some chemicals associated with termites inhibit the growth of fungi (Chen et al. 1998, Bulmer and Crozier 2004). On the other hand, some wood decaying fungi are attractive to *C. formosanus* and have been used as auxiliary compounds in termite baits in China and the United States (Esenther and Beal 1974, Su 2005, Liu et al 2011). Li et al (2001) reported that

the sugarcane powder infected by *Gloeophyllum trabeum* was significantly preferred by *C. formosanus* and *R. flaviceps* over undecayed powders. The possible role of cob fungi in termite mortality or attractiveness has not been determined yet. However, the issue of possibly greater susceptibility to fungal colonization has to be considered.

The Nile blue A transfer bioassay was used to evaluate the food transfer from bait materials to termites. Nile blue A is a persistent dye that can be accumulated in the fatty body of termites (Su et al. 1991). It has been widely used to estimate the population size, foraging distance, and toxicant transfer between termites (Evans et al. 1999, Stansly et al. 2001, Rust and Saran 2006, Bagnères et al. 2009, Gautam and Henderson 2011b, Eger et al. 2012). We found that Nile blue A provides a direct and obvious opportunity to estimate the amount and rate of transfer of bait materials to termites, by calculating the percentage of blue termites and the darkness of blue termites. The result showed that the percentage of blue workers feeding on wood blocks was significantly more than cobs or cardboard at 6h. The difference might be caused by the physical character of the three materials. Because of the low permeability, Nile blue A is at a higher concentration on the surface of wood blocks; thus, termites would initially take in higher amounts of dye.

Nile blue A also was used to evaluate the efficiency of food transfer from termite to termite. Trophallaxis, cannibalism and mutual grooming are main ways to transfer bait toxicants between termites (Valles and Woodson 2002, Bagnères et al. 2009, Dhang 2011). Nile blue A can transfer little between termites by trophallaxis and grooming (Su et al. 1991, Haagsma and Rust 1995, Baker et al. 2009). However, the role of cannibalism in bait

chemical transfer between individuals can be studied separately from other factors by using

Nile blue A. In starvation conditions, wood blocks treatments showed a stronger effect of

cannibalism via Nile blue A transfer than did cobs and cardboard treatments. Song et al.

(2006) reported that starved termites have higher non-caste-specific cannibalism levels than

fed termites during the first 30 days. The difference between starvation treatments and fed

treatments in Nile blue transfer is suggested to be caused by different cannibalism levels.

Since cannibalism can be an important factor to transfer bait chemicals between termites

under starvation conditions, in termite control practices, removing competing food sources

from termites may increases the effectiveness of bait toxicants.

Our study showed that cobs are as efficient as wood blocks and cardboard. As an

agricultural waste product, cobs are quite abundant and available (El-Geundi 2003, Varvel

and Wilhelm 2008, Wilaipon 2008). The use of cob can reduce the cost of a bait matrix and

help preserve forest resources.

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CHAPTER 4. SUSCEPTIBILITY OF FORMOSAN SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE) TO MOSQUITO DUNKS

4.1 Introduction

Termite is a diverse group of insects of the order Isoptera. There are at least four different families of termites found in the United States. Subterranean termites belong to the family Rhinotermitae. The main genera of subterranean termites that are economically important are *Coptotermes, Heterotermes,* and *Reticulitermes*. The genus *Coptotermes* contains the largest number of termite pests (28 species) among the 2,500 termite species worldwide (Rust and Su 2012). Of these, one of the most important is the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a global distributed pest that attacks structural wood and living trees (Henderson 2001).

Using *Bacillus thuringiensis* (Bt) as a biological control agent against termites was reviewed by Grace (1997, 2003), Culliney and Grace (2000), and Chouvenc et al. (2011). The conclusions were from positive to negative. The main concern focused on the effectiveness of Bt against termites in the field. Although Bt worked quite well in the lab that caused high termite mortalities, no successful result involved with field studies was reported. The main reason may be the short duration of Bt pesticide products in the field. The duration of viable Bt spores and toxins are determined by abiotic factors such as solar radiation and temperature (Leong et al. 1980). Dankle and Shasha (1989) reported that *B. thuringiensis* subspecies *thuringiensis* lost all spore viability and insecticidal activity within 4 days without protecting of ultraviolet screens. Pruett et al. (1980) reported that, after 135 days in the soil, the viable

spores of *B. thuringiensis* subspecies *galleriae* fell to 24%, while the pathogenicity fell rapidly to less than 1%. However, for termite control, bait products are placed in the ground and away from many of the degrading sources. As a result, Bt products with long duration of viability in the field would be a good candidate against termites.

One such product is MosquitoDunks®, a product which contains clay and about 10% *B. thuringiensis* subspecies *israelensis* (Bti) solids, spores, and insecticidal toxins. MosquitoDunks® have an active lifetime in water of 30 days. This product is advertised to be placed in fish ponds or other standing water to kill the mosquito larvae (Branton and Kase 1986, Ashton et al. 2005). Bti is known to be highly toxic to different *Aedes*, *Culex*, and *Anopheles* mosquito species that are main vectors of human diseases (Bravo et al. 2007). In this study, no-choice and choice tests were conducted to test the susceptibility of *C. formosanus* to mosquito dunks.

4.2 Methods and Materials

4.2.1 Termites and MosquitoDunks

Four colonies of *C. formosanus* were collected from Brechtel Park, New Orleans, LA, using the method descried with Gautam and Henderson (2011). Colony group 1 and 2 were collected on 14 February 2012, and colony group 3 and 4 were collected on 26 March 2013. Termites were maintained in the laboratory in 140L trash cans with wet wood for less than three mo. before testing. MosquitoDunks® (Summit Chemical Company, Baltimore, MD), which contain 10.31% Bti, were purchased in Baton Rouge in 2011. The concentration of Bti was previously tested by dilution-plate method. Each gram of mosquito dunk contains

about 2.7×10^9 colony forming unit (cfu). After autoclaving (121℃, 4h), the concentration of Bti decreased to 100 cfu/g.

4.2.2 No-choice Test

The bioassay arenas were plastic containers (90 mm in diameter and 38 mm in height) with 30 grams 15% moisture sand (autoclaved) as substrate. Five pieces of filter paper (4.25 cm in diameter, Whatman®) was placed on the substrate. The no-choice test contained three treatments: (1) mosquito dunk treatment, an mosquito dunk was placed on the filter paper and 10 ml distilled water was added; (2) autoclaved dunk treatment, an autoclaved mosquito dunk (121℃, 4h) was placed on the filter paper and 10 ml distilled water was added; and (3) control treatment, no mosquito dunk was placed and 1 ml distilled water was added on the filter paper. Each treatment contained 10 replicates (5 for each colony group). Two colonies of termites (colony group 1 and 2) were tested in the experiment. One hundred termites (colony 1: 100 workers, colony 2: 90 workers and 10 soldiers) were released to each experimental unit. The experiment was maintained in a 27℃ incubator for 31 days under darkness.

Before and after the experiment, the dry weight of filter paper was weighed to determine the consumption. At the end of the experiment, the mortality of termites was recorded. The bottom side of the container was scanned to determine the tunneling condition. The mortality, length of tunnels, and filter paper consumption of each treatment were analyzed by UNIVARIATE statement of SAS 9.3 (2011 SAS In statute Inc., Cary, NC). If the data was normally distributed, ANOVA and Tukey test was used to compare the mean of each

treatment. If the data was not normally distributed, a rank was assigned and compared by ANOVA and the Kruskal-Wallis test. All significant levels were determined at $\alpha < 0.05$.

4.2.3 Choice Test

The bioassay arenas were three-chambered transparent polyacrylic containers (18.0 cm $\times$ 8.0 cm $\times$ 4.5 cm each box, and 6.0 cm $\times$ 8.0 cm $\times$ 4.5 cm each chamber) with small holes (diameter $\approx$ 1.5 cm) at the bottom of both inner walls of the chamber. Thirty gram sand was put in each chamber as substrate and moistened with 3 ml distilled water. Before the experiment, a hole with same size to mosquito dunks was made in the center of each wood block. There were three types of 2-choice tests: (1) wood block (clay) vs. wood block (sand), the center hole of wood block was filled with either clay or sand and was placed in right or left side chamber; (2) wood block vs. mosquito dunk, a wood block and an mosquito dunk was placed in right or left side chamber; and (3) wood block vs. autoclaved dunk, a wood block and an autoclaved mosquito dunk was placed in right or left side chamber. The required amount of distilled water was added to wood blocks and mosquito dunks (untreated or autoclaved) to make to same moisture. Two colonies of termites (colony group 3 and 4) were tested in the experiment. One hundred termites (90 workers + 10 soldiers) were released to the center chamber. Each type of 2-choice test contained 12 replicates (6 for each colony group). The containers were covered by lids and sealed with clear plastic wrap to prevent water loss. The bioassays were maintained in a 27℃ incubator for 35 days under darkness.

At the end of the experiment, the survivorship of termites in each type of the two-choice test was recorded. For each type of two-choice test, the percentage of termites, area of

tunnels, and wood consumption in each side chamber were recorded. The data was compared

with 2-way ANOVA with colony and chamber as fixed factors. All significant levels were

determined at α<0.05.

4.3 Results

4.3.1 No-choice Test

Termites from both colony groups were found aggregated inside the mosquito

dunks (either untreated or autoclaved) and the abdomen was shrinking as compared to

termites in the controls (Figure 4.1). For the colony group 1, there was no significant

difference in mortality among the three treatments ($P = 0.0665$). However, significantly

longer tunnels were made in the controls, as compared to the mosquito dunk treatment ($P =$

0.0431, Figure 4.2). For the colony group 2, the mean mortality of termites were significantly

higher in the mosquito dunk treatment than that in the autoclaved dunk treatment ($P =$

0.0404), but the length of tunnels were similar among the three treatments ($P = 0.0976$,

Figure 4.2). For both colonies, termites in the controls had significantly higher filter paper

consumption ($P = 0.0092$ for colony group 1, $P < 0.0001$ for colony group 2, Figure 4.2).

Figure 4.1. Termites in (a) mosquito dunk treatment, (b) autoclaved mosquito dunk treatment and, and (c) control.
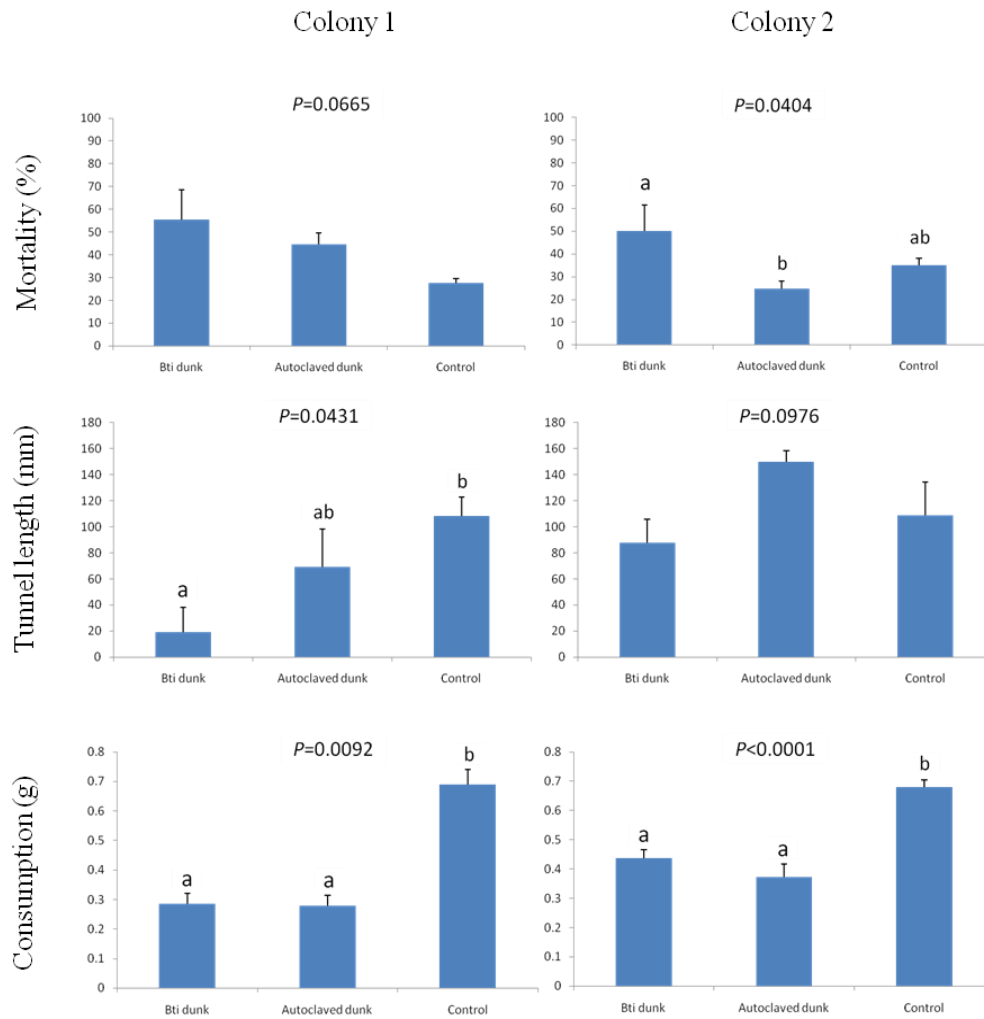
Figure 4.2. Mortality (mean ±SEM), tunnel length (mean ±SEM), and consumption (mean ± SEM) of termites in no-choice tests. Different letters present significant difference ($P < 0.05$).

4.3.2 Choice Test

The main effect of colony was significant in survivorship ($P = 0.0012$). However, there was no significant difference in survivorship among the three 2-choice tests ($P = 0.1592$, Figure 4.3). For the 2-choice test wood (clay) vs. wood (sand), there was no significant difference in number of termites (Figure 4.4), area of tunnels (Figure 4.5), and wood consumption (Figure 4.6) among the three chambers. For the 2-choice test wood vs. mosquito dunk, there were significantly more termites aggregated in the chambers containing wood, as

compared to center or mosquito dunk side chambers (Figure 4.4). The area of tunnels made in

the three chambers was similar (Figure 4.5). Significantly more wood than mosquito dunk

was consumed in the colony 2, but was not in the colony 1(Figure 4.6). For the 2-choice test

wood vs. autoclaved dunk, there were significantly more termites in the wood side chamber

than the center or autoclaved side chamber (Figure 4.4). The area of tunnels in the wood side

chamber was significantly more than that in the autoclaved dunk side chamber, but neither

was different from the center chamber (Figure 4.5). Significantly more wood than autoclaved

dunk was consumed in the colony 2, whereas no difference was found in the colony 1 (Figure

4.6).
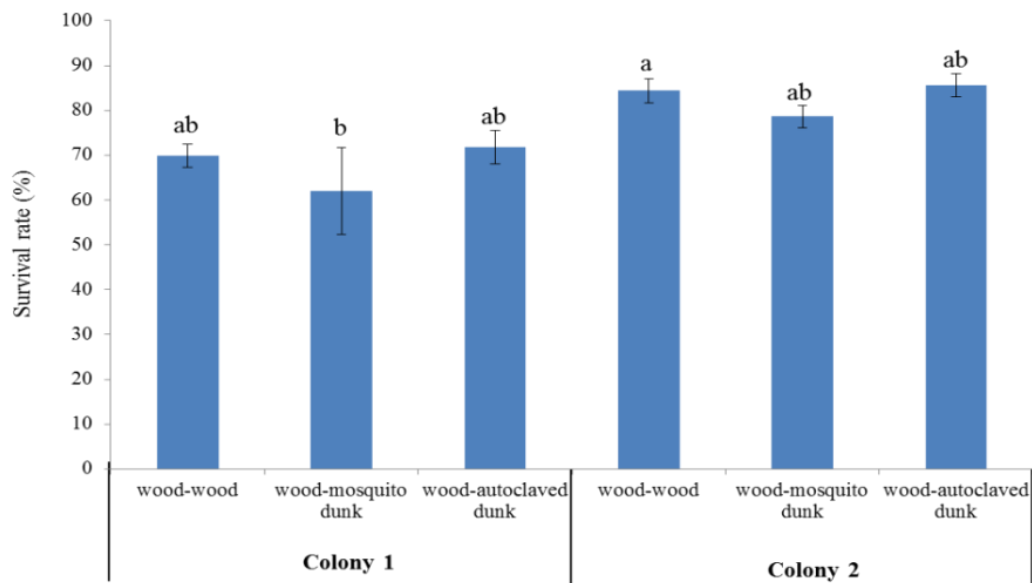
Figure 4.3. Survivorship of termites (mean ± SEM) in the three 2-choice tests (wood [clay] vs. wood [sand], wood vs. mosquito dunk, and wood vs. autoclaved dunk). Different letters present significant difference ($P < 0.05$).

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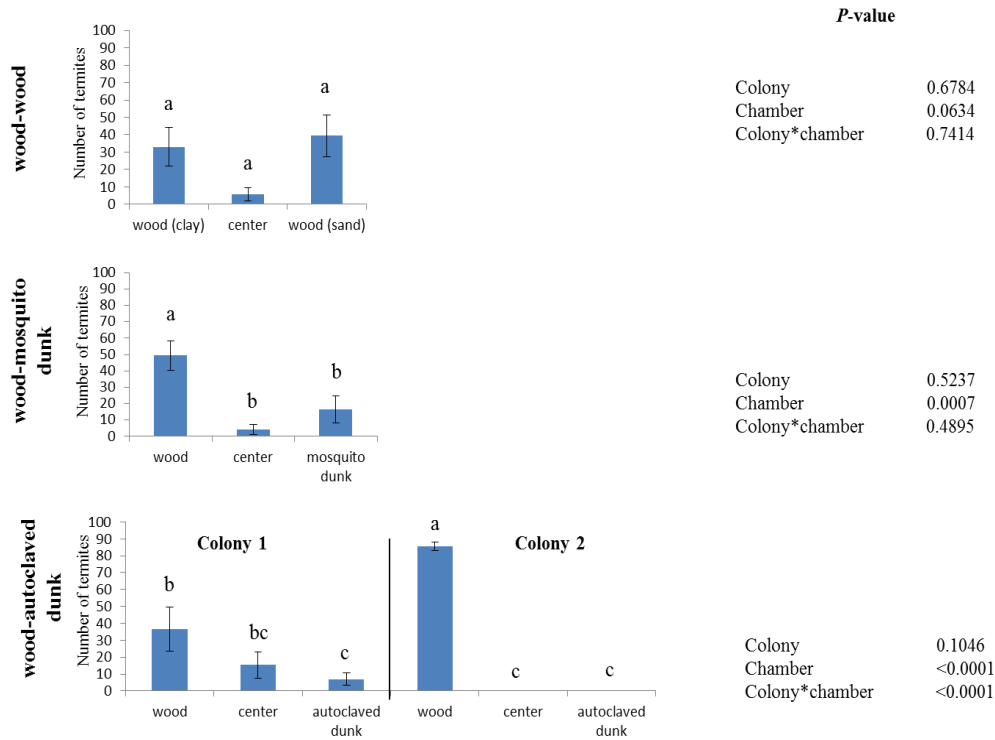
Figure 4.4. Number of living termites (mean ±SEM) aggregated in the three chambers in each 2-choice test. Different letters present significant difference ($P < 0.05$).
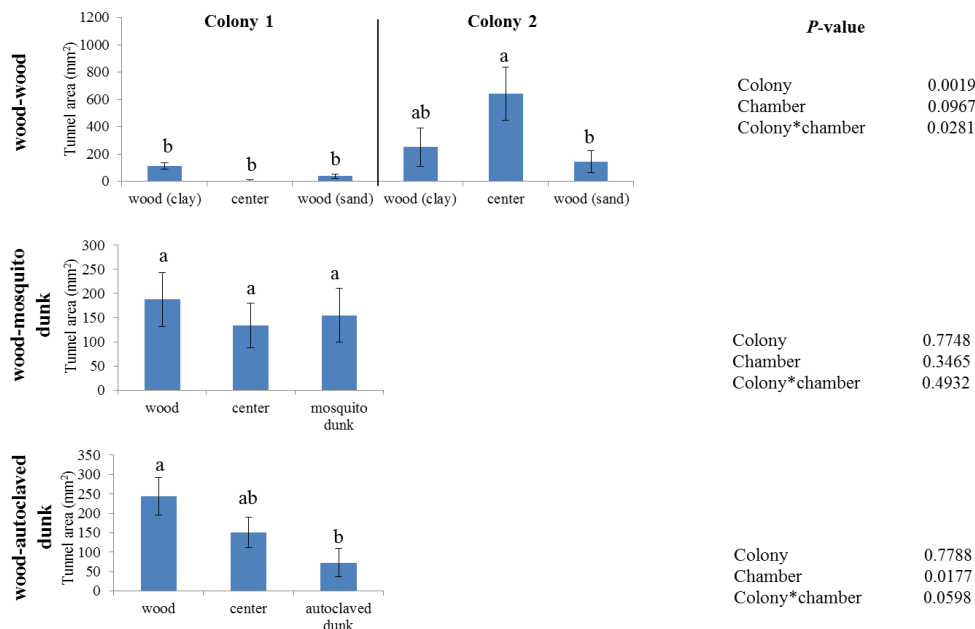
Figure 4.5. Area of tunnels (mm^2, mean ±SEM) in the three chambers of each 2-choice test. Different letters present significant difference ($P < 0.05$).
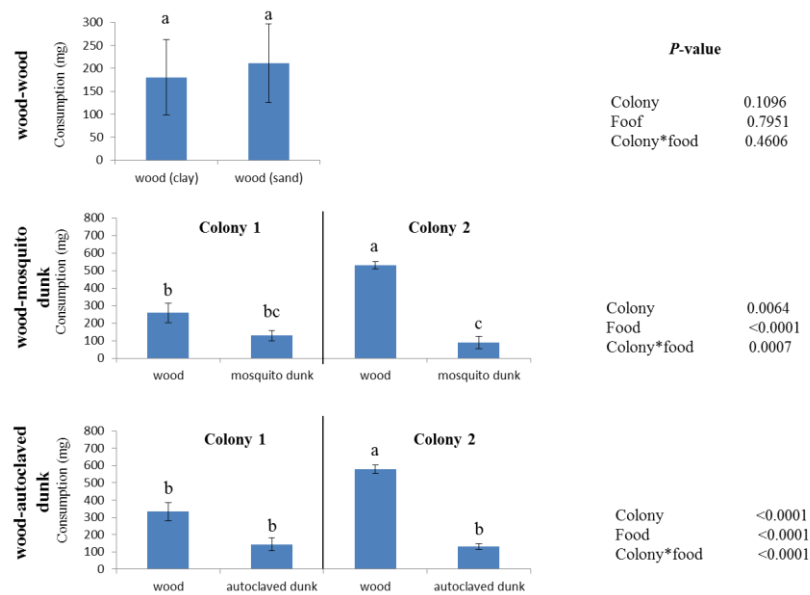
Figure 4.6. Consumption (dry weight loss, mg, mean ± SEM) of termites in the two side chamber of each 2-choice test. Different letters present significant difference ($P < 0.05$).

4.4 Discussion

In previous studies, Bti and other Bt subspecies caused high mortalities in *Reticulitermes flavipes*, *Bifiditermes beesoni*, *Heterotermes indicola, Nasutitermes ehrhardti*, *Microcerotermes championi, M. beesoni* and *M. obesi* (Smythe and Coppel 1965, Khan 1981, Castilhos-fortes et al. 2002, Khan et al. 1977, 1978, 1985, 2004, Singha et al. 2010). However, in the present study, mosquito dunks with high concentration of Bti only caused slightly but significant higher mortality in one of the colonies in the no-choice test. No obvious difference in mortality was found among the three 2-choice tests.

The results showed that the use of mosquito dunks itself cannot bring a promise control of *C. formosanus.* Wright and Lax (2013) combined Bt with another termite pathogen, *Isaria fumosorosea*, and observed a significantly higher mortality of *C. formosanus* as

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compared to Bt or *I. fumosorosea* treatment alone. It would be valuable to test the potential to combine mosquito dunks or Bti with a chemical pesticide or termite pathogen for the control of *C. formosanus.*

In the no-choice test, an aggregation of termites in dunks was observed, and significantly less filter paper consumption was recorded when dunks were provided. However, in the choice test, no preference or consumption effect of dunk was found. This may be due to the differences in moisture levels of mosquito dunks in the two experimental settings. In the no-choice test, 10 ml distilled water was added to dunks, which is saturated. Whereas in the choice test, only 2-3 ml distilled water was added to dunks to make the same moisture of wood blocks. It is possible that dunks in the choice tests were too dry to show an attraction of termites. In the third two-choice test, the hole of wood blocks was filled with clay or sand. Clay is a compound of mosquito dunks that may be used to encapsulate Bti. Under natural conditions, large amount of clay has been found in the tree cavities created by termite consumption (Henderson 2008), indicating a possible attraction effect of clay on termites. Although no preference of aggregation, tunneling and consumption was detected in the 2-choice test wood (clay) vs. wood (sand), it is important to note that in the present study, a small amount of clay was placed in a big piece of wood block. More experiments to test the effect of clay on termite aggregation and behavior are needed.

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CHAPTER 5. EVIDENCE OF FORMOSAN SUBTERRANEAN TERMITES GROUP SIZE AND ASSOCIATED BACTERIA IN THE SUPPRESSION OF ENTOMOPATHOGENIC BACTERIA[1]

5.1 Introduction

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is one of the most destructive pests in the world. It has been estimated that, in 11 U.S. states, the combined damage repair and control cost of *C. formosanus* was more than 1 billion US dollars each year (Suszkiw 2000). Currently, two principle methods are widely used for control: (1) slow-acting toxic baits to eliminate termite colonies, and (2) liquid insecticide treated soil as chemical barriers to prevent termites from attacking wood (Henderson 2001, Rust and Su 2012). However, insecticides may pose unintended threats to non-target organisms and water resources (Jayasimha and Henderson 2007a).

Biological control may provide long-lasting and environmental friendly technologies for the control of *C. formosanus*, but termites are resistant to microbial pathogens. Currently, most research is focused on entomopathogenic fungi. Three mechanisms account for fungal resistance by termites include: (1) immune responses to infection, (2) synthesis of antifungal molecules, and (3) behavioral responses that prevent fungal epizootics (Chen et al. 1998, Bulmer and Crozier 2004, Rosengaus et al. 2007, Chouvenc et al. 2009, Yanagawa et al. 2008, 2009, 2010, Chouvenc and Su 2012). Moreover,

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[1] This chapter previously appeared as Cai Wang, and Gregg Henderson. Evidence of Formosan subterranean termite group size and associated bacteria in the suppression of entompathogenic bacteria, *Bacillus thuringiensis* subspecies *israelensis* and *thuringiensis*. *Annals of the Entomological Society of America* 106 (2013): 454-462. It is reprinted by permission of Entomology Society of America.

Jayasimha and Henderson (2007b, 2007c) found that fungi isolated from *C. formosanus* show

antagonistic effects against brown rot fungus, *Gleophyllum trabeum* (Pers.) Murrill. Although

G. trabeum is not pathogenic to termites, it does compete for cellulose.

Few studies have focused on the interaction between termites and

entomopathogenic bacteria. *Bacillus thuringiensis* Berliner, a well-known biological control

agent, has been evaluated against termites (Smythe and Coppel 1965, Grace 1997, Khan et al.

2004, Singha et al. 2010). Laboratory studies show that *B. thuringiensis* subspecies

thuringiensis (*Btt*) was toxic to *Reticulitermes flavipes* Kollar, *R. virginicus* Banks, *R.

hesperus* Banks, and *Zootermopsis angusticollis* Hagen (Smythe and Coppel 1965, Khan et al.

2004). Singha and Dutta (2010) reported that *B. thuringiensis* subspecies *israelensis* (*Bti*) was

effective against *Microtermes obesi* Holmgren and *Microcerotermes beesoni* Snyder. Our

previous research also showed that *C. formosanus* exposed to Summit® mosquito dunks

(Summit Chemical Company, Baltimore, MD), which contains 10.31% *Bti*, had higher

mortality and lower tunneling activity when compared to termites exposed to autoclaved

dunks, indicating that *C. formosanus* was negatively affected by this bacterium (CW and GH,

unpublished data).

In this study, *Bti* and *Btt* were used as models to study the ability of *C. formosanus*

to suppress entomopathogenic bacteria and how termite associated bacteria are involved in

this process. Termite exposure bioassays were conducted to study: (1) whether *C. formosanus*

can suppress the growth of *Bti* and *Btt*; and (2) whether the different group sizes of termites

influence the suppression ability. Moreover, antagonistic tests were used to study whether

bacteria associated with *C. formosanus* contribute to the suppression of *Bti* and *Btt*.

5.2 Materials and Methods

5.2.1 Termites

Two colony groups of *C. formosanus* were collected from Brechtel Park, New Orleans, LA, on 14 February 2012 using milk crate traps as described in Gautam and Henderson (2011a). Both colony groups were kept in 140L containers with wet pine wood (*Pinus* sp.) in the laboratory for ≈ 3 mo before testing.

5.2.2 *Bti* and *Btt*

B. thuringiensis subspecies *israelensis* strain HD735 (ATCC 10792^{T}) and *thuringiensis* strain HD522 (ATCC 35646) were provided by Daniel Zeigler (*Bacillus* Genetic Stock Center (BGSC), Department of Biochemistry, Ohio State University, Columbus, OH). Spores of two *B. thuringiensis* subspecies were stored on filter paper discs sealed in aluminum foil packets and kept at room temperature. Before testing, filter paper discs with *Bti* or *Btt* spores were placed on Luria-Bertani (LB) agar plate and incubated at 30℃ for 24 h. A single colony of *Bti* or *Btt* was picked from the incubated plates and transferred to a new tube containing 1 ml sterile LB broth. Then these tubes were incubated at 30℃ for 10 h with shacking (200 rpm) for inoculating the bioassay.

5.2.3 Bioassay Arena

Bioassay arenas were Petri dishes (100×15mm) containing 20 ml LB agar medium. Four colonies of *Bti* or *Btt* were inoculated on each agar plate using a sterile inoculating needle. Colonies were uniformly distributed on the agar plate (Figure 5.1A). The agar plates

were then incubated at 30℃ for 30 h and plates with *Bti* or *Btt* colonies of similar size were selected for the study.

5.2.4 Experimental Set Up and Maintenance.

Both of *Bti* and *Btt* experimental groups contained four treatments. Fifty, 25, 10 or no termites (control) were placed on each agar plate (colony group 1: 90% workers and 10% soldiers; colony group 2: 100% workers, according to their collected colony structures). There were 12 replicates in each treatment (six replicates for each colony group). The agar plates were covered with black poster paper to block light and maintained at room temperature (23.5 ± 0.5℃).

5.2.5 *Bti* and *Btt* Colony Growth Observations.

To compare the *Bti* or *Btt* growth between the four treatments, diameters of bacteria colonies were measured with a digital caliper (Carrera Precision®) once per day until the edge of *Bti* or *Btt* colonies became unclear (Figure 5.1B). For regularly shaped colonies, two measurements were acquired from each colony horizontally and vertically, then averaged (Figure 5.1C). For irregular colonies, diameters were recorded as the average of the longest and shortest axis (Figure 5.1D).

5.2.6 Viability Test.

To estimate the viability status, on days 1 and 5, six *Bti* or *Btt* colonies were randomly selected from the twelve agar plates of each treatment (three colonies from agar plates containing each termite colony group). A sterilized tooth pick was inserted into the center of *Bti* or *Btt* colonies. Then a straight line (≈ 6 cm long) was drawn on a new agar plate

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(test plate) with the inoculated tooth pick so that the dominant bacterium would grow. The

test plates were incubated at 30℃ for 24 h. The viability of *Bti* or *Btt* was confirmed if they

regenerated on the test plates, which was identified by colony growth characteristics and

Schaeffer-Fulton staining (Laridon 2006). Other bacteria species growing on the test plates

indicated substitution of dominance and loss of *Bti* or *Btt* viability. These new bacteria, which

brought into the agar plates by *C. formosanus*, were subcultured to obtain pure cultures. Five

bacteria strains with different colony color and shape characteristics (compared to *Bti* and *Btt*)

were obtained.
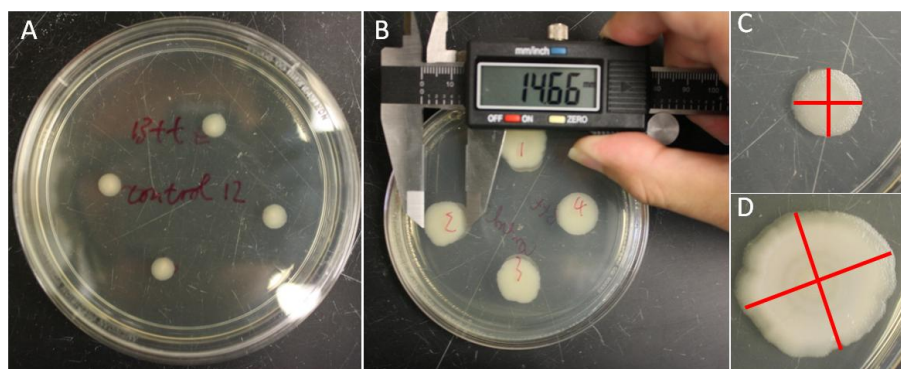
Figure 5.1. (A) bioassay arena- four *Bti* or *Btt* colonies were originally grown on LB agar plates before exposed to different group sizes of termites; (B) measurement of diameters of *Bti* or *Btt* colonies with a digital caliper; (C) measurement of diameters of regular shaped colonies; (D) measurement of diameters of irregular colonies.

5.2.7 Antagonistic Test

Additional tests were conducted to further study the possible antagonistic effect of

bacteria naturally associated with *C. formosanus* against *Bti* and *Btt.* A single colony of the

five bacteria strains isolated in the viability test as well as *Bti* and *Btt* were individually

picked, transferred to 1 ml sterile LB broth and incubated at 30℃ for 24 h with shaking (200

rpm). One hundred µl *Bti* or *Btt* broth was added onto each LB agar plate and spread evenly.

A piece of autoclaved (121℃ for 30 min) filter paper disc (7.5mm, excised using a 7.5-mm-

diameter hole puncher) was gently placed at the center of each agar plate with sterile forceps.

Ten µl broth of one of each five bacteria strains was pipetted, and added onto the filter paper

disc on the agar plate. For controls, 10 µl sterile LB broth was added onto the filter paper disc.

The agar plates were then incubated at 30℃ for 24 h. Antagonistic effects against *Bti* or *Btt*

was determined if an inhibitory zone was observed around the filter paper disc. Diameters of

inhibitory zones were measured with a digital caliper. Each inhibitory zone was measured

twice (horizontally and vertically for circular inhibitory zones or largest and smallest for

irregular shaped inhibitory zones) and measurements were averaged. The antagonistic test of

each bacterium against *Bti* or *Btt* was repeated three times.

5.2.8 Identification of Antagonistic Bacteria

Bacteria that inhibited the growth of *Bti* or *Btt* were identified by 16S rRNA gene

sequencing. Template DNA of antagonistic bacteria strains for polymerase chain reaction

(PCR) was prepared using DNeasy® blood & tissue kit (Qiagen, Valencia, CA). A pair of

universal bacterial 16S rRNA gene primers, 5'- AGA GTT TGA TCC TGG CTC AG -3'

(27F), and 5'- TAC GGC TAC CTT GTT ACG ACT T -3' (1492R), were used for

amplification of 16S rRNA gene fragments (Lane 1991). PCR was performed in a 20 µl

volume of AccuPower® PCR PreMix (Bioneer, Chungwon, Korea) with 1 µl of template

DNA and 10 pmol of each primer as follows: initial denaturation at 95 ℃ for 5 min, followed

by 35 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 1.5 min, and a final extension

step at 72 ℃ for 10 min. The amplified 16S rRNA gene fragments were sent to Eurofins

MWG Operon (Huntsville, AL) for bidirectional sequencing using primers 1492R and 27F.

Derived sequences were aligned and compared to the GenBank database through BLAST

(Basic Local Alignment Searching Tool, Altschul et al. 1990). The exact species match was

assumed if the sample showed more than 99% similarity to the published sequence of

identified species in the database (Lee et al. 2008, Husseneder et al. 2010a).

5.2.9 Data Analysis

Diagnostics plots in SAS 9.3 (SAS Institute, Cary, NC, 2011) were used to verify

the assumptions of independent and normal distribution of data. Then SAS PROC MIXED

was used to analyze the diameters of *Bti* or *Btt* colonies in each observation period if the

termite colony effect was not significant. Otherwise, the data of the two termite colonies was

analyzed separately using PROC GLM or PROC TTEST (two samples). Post ANOVA

comparisons were performed using Tukey's honestly significant difference (HSD) test. In all

tests, $\alpha = 0.05$.

5.3 Results

5.3.1 Suppression of *Bti* and *Btt* Growth by *C. formosanus*

In general, prior to placement of termites (day 0), there was no significant

differences in the diameters of *Bti* or *Btt* colonies between the four treatments (*Bti*: $F_{3, 92} =$

0.05; $P = 0.9854$ for termite colony 1; and $F_{3, 92} = 1.11$; $P = 0.3511$ for termite colony 2; Table

5.1; *Btt*: $F_{3, 92} = 1.59$; $P = 0.1965$ for termite colony 1; and $F_{3, 92} = 0.85$; $P = 0.4689$ for

termite colony 2; Table 5.2). Colony effect was significant on day 1 (*Bti*: $F_{1, 184} = 5.38$; $P =$

0.0215; and *Btt*: $F_{1, 183} = 4.22$; $P = 0.0414$) and day 2 (*Btt*: $F_{1, 92} = 5.04$; $P = 0.0271$), therefore

data of the two termite colony groups were analyzed separately. On day 1, for both the two

termite colony groups, mean diameters of *Bti* or *Btt* colonies from the smallest to the greatest

were: 50-termite < 25-termite < 10-termite < control (Table 5.1, Table 5.2). *Bti* or *Btt* colony

diameters in the treatments containing termites were significantly smaller than in the controls.

The diameters in the 50-termite treatment were significantly smaller than in the 10-termite

treatment, but neither of these were significantly different from the 25-termite treatment (*Bti*:

$F_{3, 92} = 48.29$; $P < 0.0001$ for termite colony 1; and $F_{3, 92} = 31.17$; $P < 0.0001$ for termite

colony 2; Table 5.1; *Btt*: $F_{3, 91} = 107.23$; $P < 0.0001$ for termite colony 1; and $F_{3, 92} = 28.81$; P

< 0.0001 for termite colony 2; Table 5.2). By day 2 the edge of *Bti* colonies became unclear

in the 50-termite treatment (Figure 5.2). The diameters of *Bti* colonies in the 25- and 10-

termite treatments were similar but significantly smaller than in the controls ($F_{2, 69} = 324.91$;

$P < 0.0001$ for termite colony 1; and $F_{2, 69} = 420.57$; $P < 0.0001$ for termite colony 2; Table

5.1). The *Btt* edge in 25 and 50 termite densities also became unclear on day 2 (Figure 5.2).

Btt colonies exposed to 10 termites were significantly smaller than the controls ($t_{46} = 29.69$; P

< 0.0001 for termite colony 1; and $t_{46} = 17.36$; $P < 0.0001$ for termite colony 2; Table 5.2).

5.3.2 Viability of *Bti* and *Btt* Colonies Exposed to *C. formosanus*

      All test plates showed regeneration on day 1 (Table 5.3). On day 5, inhibitory zones

were observed in treatments containing termites; unclear edges of these inhibitory zones were

observed in the 25- and 50-termite treatments (*Bti* and *Btt*) (Figure 5.2). The viability test

showed that both *Bti* and *Btt* regenerated on test plates after transfer from the 25- and10-

termite treatments as did controls, but all of six test plates in the 50-termite treatment showed

no regeneration of *Bti* or *Btt* (Table 5.3).
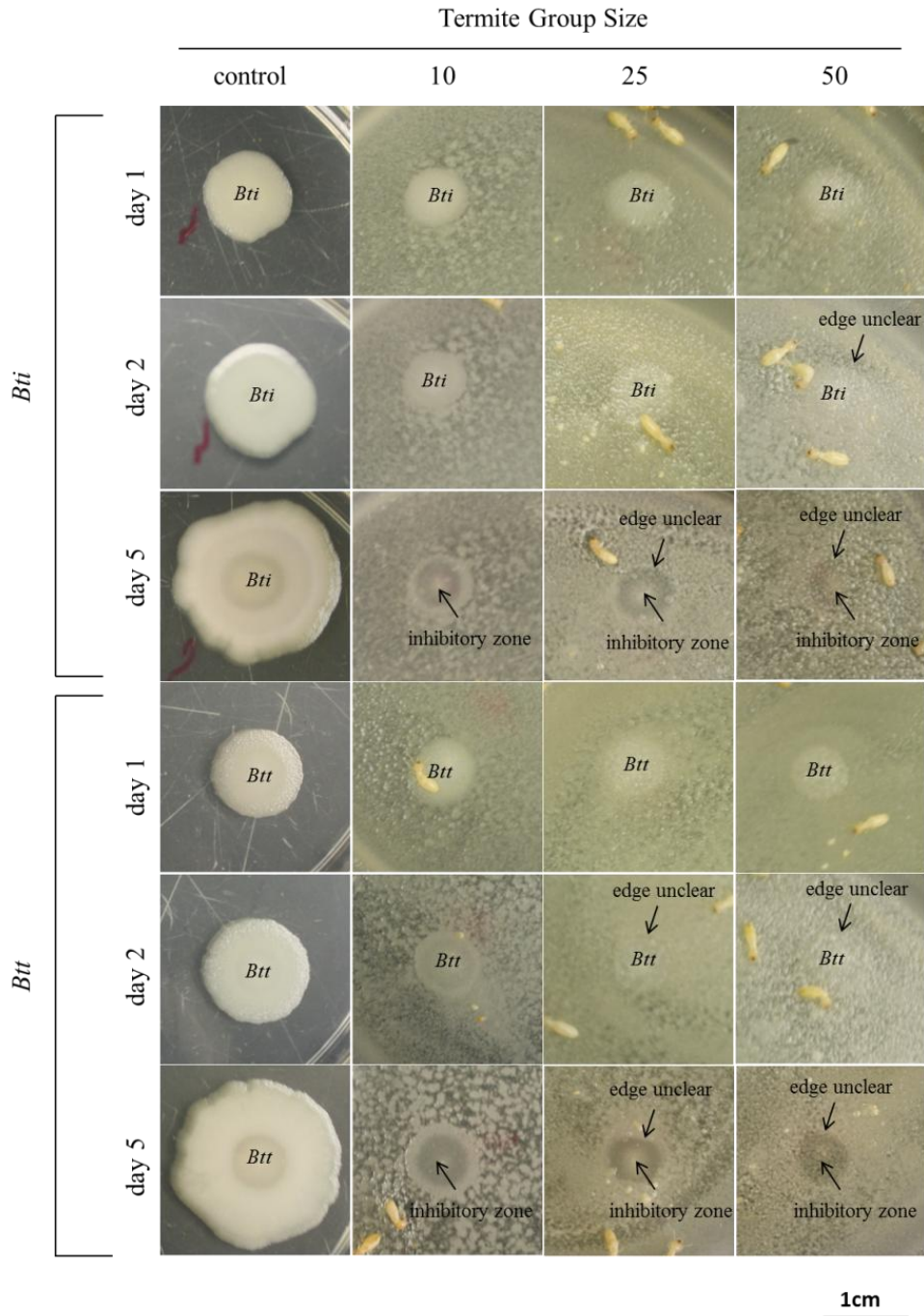
Figure 5.2. *Bti* or *Btt* colonies in the four treatments (control, 10-, 25-, or 50-termite) on day 1, 2 and 5.

Table 5.1. Diameter of *Bti* colonies (mm, mean $\pm$ SEM) exposed to termites with different group sizes (50-, 25-, 10-termite, and control) of the two termite colony groups on day 0, 1 and 2.

| Colony | Day | Termite Group Size | | | | *P* |
		50	25	10	control	
	0	5.68±0.08 (a*)	5.70±0.08 (a)	5.68±0.07 (a)	5.72±0.10 (a)	0.9854
1	1	6.79±0.17 (a)	7.35±0.17 (ab)	7.62±0.29 (b)	9.95±0.14 (c)	<0.0001
	2	-	7.40±0.16 (a)	7.80±0.31 (a)	14.61±0.17 (b)	<0.0001
	0	5.69±0.09 (a)	5.74±0.06 (a)	5.84±0.09 (a)	5.64±0.08 (a)	0.3511
2	1	7.30±0.24 (a)	7.74±0.13 (ab)	8.15±0.23 (b)	9.81±0.17 (c)	<0.0001
	2	-	7.70±0.10 (a)	8.20±0.19 (a)	14.65±0.25 (b)	<0.0001

*Different letters represent significant differences among group sizes within each time period ($P < 0.05$).

Table 5.2. Diameter of *Btt* colonies (mm, mean ±SEM) exposed to termites with different group sizes (50-, 25-, 10-termite, and control) of the two termite colonies on day 0, 1 and 2.

| Colony | Day | Termite Group Size | | | | *P* |
		50	25	10	control	
	0	6.41±0.11 (a*)	6.44±0.08 (a)	6.41±0.06 (a)	6.62±0.06 (a)	0.1965
1	1	7.63±0.14 (a)	7.95±0.16 (ab)	8.39±0.10 (b)	11.11±0.20 (c)	<0.0001
	2	-	-	8.70±0.11 (a)	14.86±0.17 (b)	<0.0001
	0	6.56±0.10 (a)	6.52±0.08 (a)	6.50±0.12 (a)	6.36±0.08 (a)	0.4689
2	1	8.16±0.16 (a)	8.65±0.13 (ab)	8.91±0.24 (b)	10.30±0.13 (c)	<0.0001
	2	-	-	9.88±0.21 (a)	14.42±0.15 (b)	<0.0001

*Different letters represent significant differences among group sizes within each time period ($P < 0.05$).

Table 5.3. Number of *Bti* or *Btt* regeneration on test plates after transfer from four treatments (50-, 25-, 10-termite, and control) on day 1 and 5.

| | Day | Termite Group Size | | | |
		50	25	10	control
Bti	1	6/6*	6/6	6/6	6/6
	5	0/6	5/6	4/6	6/6
Btt	1	6/6	6/6	6/6	6/6
	5	0/6	3/6	5/6	6/6

* Number of test plates showing positive result out of 6 tests in each treatment.

5.3.3 Antagonistic Effect of Termite Associated Bacteria to *Bti* and *Btt*

Four of five bacteria strains collected from treatments containing termite were found to be antagonistic to *Bti* and *Btt*. They were identified as *Lysinibacillus sphaericus* (Meyer and Neide) Ahmed et al., *Serratia marcescens* Bizio, *Cedecea davisae* Davis, and *Pseudomonas aeruginosa* (Schroeter) Migula (Table 5.4). *Lysinibacillus sphaericus* resulted in irregular shaped inhibitory zones, while the other three bacteria strains resulted in circular inhibitory zones. No inhibitory zone was observed in the controls (Figure 5.3A). For *Bti* smear plates, mean diameters of inhibitory zones were: *L. sphaericus* (21.56 mm) > *P. aeruginosa* (17.75 mm) > *S. marcescens* (14.29 mm) > *C. davisae* (9.48 mm) (Figure 5.3B). Mean diameters of inhibitory zones in *Btt* smear plates were: *P. aeruginosa* (15.99 mm) > *L. sphaericus* (14.94 mm) > *S. marcescens* (10.75 mm) > *C. davisae* (8.80 mm) (Figure 5.3C).

Table 5.4. BLAST identification of termite associated bacteria antagonistic to *Bti* and *Btt*.

Closest match in GenBank	Accession number of closest match*	% identity to closest match
Lysinibacillus sphaericus strain Ot4b.39	JQ744626.1	99%
Serratia marcescens strain SER1	JQ439899.1	100%
Cedecea davisae isolate PSB5	HQ242718.1	99%
Pseudomonas aeruginosa strain S7-628-2	JQ660238.1	100%
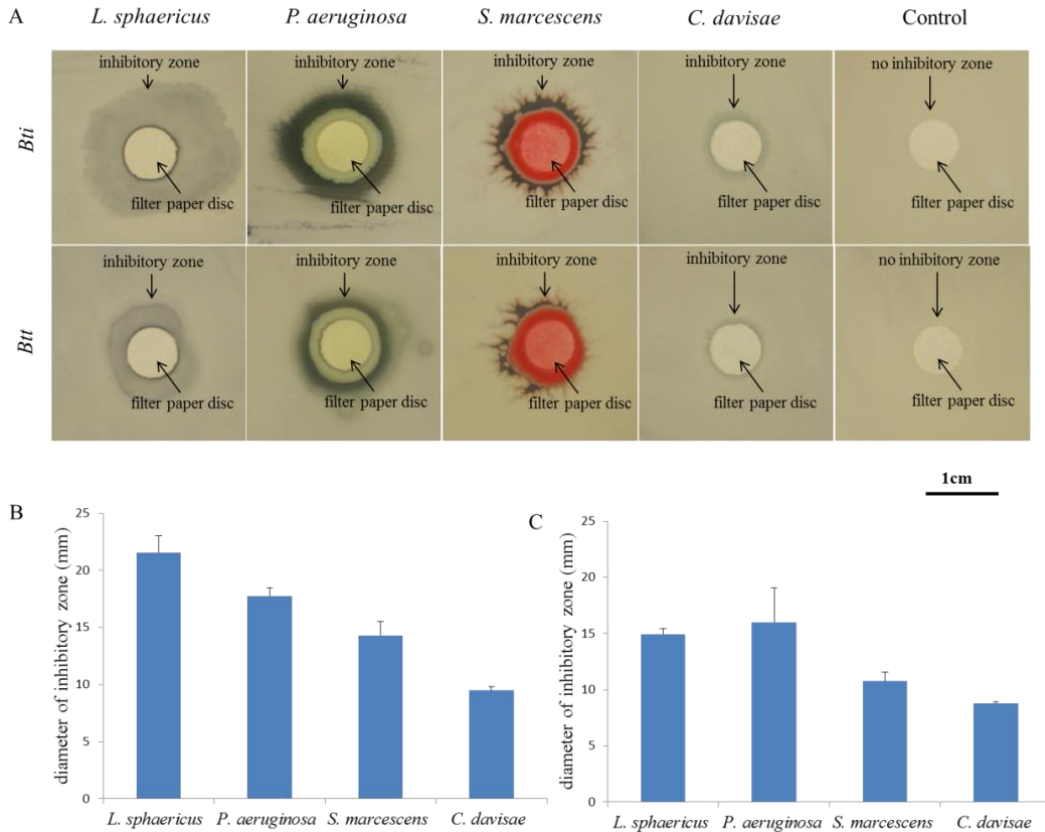
Figure 5.3. Antagonistic effect of four termite-associated bacteria strains on *Bti* and *Btt*: (A) inhibitory zone; (B) diameters of inhibitory zones (mean ± SEM) of *Bti* smear plates; (C) diameters of inhibitory zones (mean ± SEM) of *Btt* smear plates.

5.4 Discussion

Even the smallest termite group (n = 10) suppressed the growth of *Bti* and *Btt* on LB medium. Moreover, this suppression effect was enhanced with increased of group sizes of *C. formosanus*. Some researchers have shown that group living is essential for disease resistance in termites. For example, Traniello et al. (2002) reported that, when in groups, *Z. angusticollis* has higher survivorship after exposure to the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin and its ability to develop immunity was enhanced. Calleri et al. (2010) also found that compared to isolated termites, *Incisitermes schwarzi* Banks nesting in groups of 10 or 25 had significantly lower mortality when exposed to *M. anisopliae*.

Our study showed that group living not only contributes to the "passive" disease resistance of termites, but also to their "initiative" suppression of microbial pathogens. As a subterranean species, *C. formosanus* lives in humid and moist environments that favor growth of microorganisms; therefore, they are under high risk of exposure to various pathogens (Connick et al. 2001, Jayasimha and Henderson 2007a, Husseneder et al. 2010a, Gautam and Henderson 2011b). Also, *C. formosanus* is well known for its large colony size (Su and Scheffrahn 1988, Gautam and Henderson 2008, Henderson 2008, Rust and Su 2012). Su and Scheffrahn (1988) estimated that foraging population of *C. formosanus* can reach to several millions per colony. Based on our results, it is suggested that *C. formosanus* may benefit greatly from its large colony size by suppressing microbial pathogens in the natural environment, and reducing disease risk.

The importance of bacterial communities on pathogen resistance has been studied in many insects. For example, Dillon et al. (2005) found that gut bacteria of the desert locust *Schistocerca gregaria* Forskal can significantly decrease the density of the pathogenic bacterium *S. marcescens*. Likewise, Yoshiyama and Kimura (2009) reported that bacteria isolated from the digestive tract of the Japanese honeybee, *Apis cerana japonica* Takeuchi, suppressed *Paenibacillus larvae* (White), the causal agent of American foulbrood. Mattoso et al. (2011) found that bacteria on the cuticle of leafcutting ant, *Acromyrmex subterraneus* Forel, significantly reduced the susceptibility of worker ants to infection of *M. anisopliae.*

It is also well established that bacteria communities have complex coevolutionary history and are important for termites (Warnecke et al. 2007, Hongoh et al. 2008, Ohkuma 2008, Noda et al. 2009, Strassert et al. 2010, Rosengaus et al. 2011, Schauer et al. 2012). The bacterial load on the cuticle and in the gut of *C. formosanus* was investigated using molecular and culture-dependent methods by Shinzato et al. (2005), Adams and Boopathy (2005), Husseneder et al.

(2005, 2009, 2010a, 2010b), and Xiang et al. (2012). Although termite bacterial pathogens has not been fully studied, Matsui et al. (2012) found that one termite gut bacteria strain, CA1, which belong to the genera *Streptomyces*, can produce antibiotics that attack gram-positive bacteria including *Bacillus* spp.

Interestingly, the three antagonistic bacteria tested, *S. marcescens*, *P. aeruginosa*, and *L. sphaericus*, are known termite pathogens (Satdykov 1970, Khen et al. 1992, Culliney and Grace 2000, Osbrink et al. 2001, Connick et al. 2001, Devi et al. 2007, Sindhu et al. 2011). Adams and Boopathy (2005) reported that *S. marcescens* may contribute to creating anaerobic conditions in the gut of *C. formosanus*, thus benefiting digestion of cellulose. *L. sphaericus* also is involved with the degradation of lignin monomers and hemicellulose in the gut of many termites (Schäfer et al 1996, Kuhnigk and König 1997, König 2006). Another bacterium in our study, *C. davisae*, showed a weak antagonistic effect on *Bti* and *Btt*. Although this bacterium was found in cockroaches (Chaichanawongsaroj et al. 2004), its relationship with *C. formosanus* is unknown. Since only five dominant bacteria strains were isolated from inhibitory zones in this study, experiments for the further screening of antagonistic bacteria that are directly isolated from cuticle or gut of *C. formosanus* is needed. Also, a comparison between defaunated and untreated termites in suppressing entomopathogenic bacteria would likely provide more information for the antagonistic effect of termite symbionts under *in vivo* conditions.

It is possible that the immune responses and antimicrobial molecules synthesized by *C. formosanus* may also be responsible for the suppression for of *Bti* and *Btt*. Hussain and Wen (2012) reported that cell-free homogenates of *C. formosanus* previously exposed to *Staphylococcus aureus* Rosenbach, *Ralstonia solanacearum* Smith, or *B. thuringiensis* showed an inhibitory effect on the growth of each bacterium, indicating an induced immune defense by *C.*

formosanus. Antimicrobial peptides or enzymes have also been identified from termites. For example, Lee et al. (2003) identified a termite-derived antimicrobial peptide, spinigerin, which can work against both gram-negative and gram-positive bacteria. Termicin, another antimicrobial peptide, was identified in *Pseudacanthotermes spiniger* Sjostedt, *Odontotermes formosanus* Shiraki*, Macrotermes barneyi* Light*,* and *R. chinensis* Snyder (Da et al. 2003, Bulmer and Crozier 2004, Xu 2009). Matsuura et al. (2007) found that a 14.5 kDa protein, lysozyme, which is expressed in salivary glands and eggs of *R. speratus* Kolbe, protected eggs from gram-positive bacterial infection. Since genes encoding these peptides or enzymes are highly conserved among species, their homologous gene products in *C. formosanus* may also play a role in *Bti* and *Btt* suppression.

Although group sized dependent suppression of *Bti* and *Btt* was observed, it is premature to draw a conclusion that these bacteria cannot be used in termite control. Combining pesticides or biosynthesis inhibitors with pathogens may weaken termites and increase their susceptibility to pathogens (Connick et al. 2001, Grace 2003). Modern molecular technologies also give promise to extend the application of entomopathogenic bacteria. For example, Husseneder and Grace (2005), Zhao et al. (2008), and Zhang et al. (2010) developed methods to transform and express foreign genes in natural bacteria living in the gut of *C. formosanus.* Genes encoded bacterial toxins such as Cry and Vip proteins of *B. thuringiensis* can be an abundant source of candidate genes delivered to termite colonies.

Our bioassay provided a simple method to study the interaction among termites, their associated bacteria, and entomopathogens. In future, we suggest using this bioassay as a standard step to determine if termites and their associated bacteria have inhibitory effects on biological control agents.

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CHAPTER 6. CLAY PREFERENCE AND PARTICLE TRANSPORT BEHAVIOR OF THE FORMOSAN SUBTERRANEAN TERMITE (ISOPTERA: RHINOTERMITIDAE): A LABORATORY STUDY[1]

6.1 Introduction

Many higher termites (Isoptera: Termitidae) are associated with clay-rich habitats. For example, Tang (1983) reported that *Odontotermes formosanus* Shiraki and *Macrotermes barneyi* Light were more likely to attack fir forests in clay-loam areas as compared to sandy-loam areas. Hu et al. (2006) reported that in Guangdong Province, China, levees made of yellow clay (iron clay) were commonly inhabited by destructive higher termites, whereas sand levees were rarely infested. Likewise, in a laboratory study, Jouquet et al. (2007) reported that *Pseudacanthotermes spiniger* (Sjöstedt) significantly preferred belowground clay over sand to sheet the bridge-wood that connects the nest and foraging area. Some fungus-growing higher termites also use clay for nest construction. Edosomwan et al. (2012) reported that the clay and silt content in termite mounds in Ekpoma, Nigeria was 49.7% and 50.0% higher than adjacent soil, respectively. Abe et al. (2012) and Mujinya et al. (2013) also reported a significantly higher content of clay in *Macrotermes* mounds than in nearby soil. High clay content benefits mound-building termites by changing the physical properties, such as structure stability, infiltration rates and hydraulic conductivity, of the mound soil (Malaka 1977, Lys and Leuthold 1994, Jouquet 2004, Suzuki et al. 2007, Garba et al. 2011). Meanwhile, clay mounds accumulate high concentrations of inorganic ions and accelerate the recycling of organic nutrients, thus exerting a profound influence on the ecosystem (Ndiaye et al. 2004, Brossard et al. 2007, Abe et al. 2009, Jouquet et al. 2011, Joseph et al. 2012, 2013).

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Fewer studies note the relationship between clay and subterranean termites (Isoptera: Rhinotermitidae). Similar to higher mound-building termites, clay for nest construction has been observed in *Coptotermes* spp. For example, Rogers et al. (1999) reported that *C. lacteus* will often choose clay particles to build the outer-casing of mounds, thus enhancing the structural strength and reducing heat conductivity (Ewart 1988, French and Ahmed 2010). Henderson (2008) reported that *C. formosanus* can modify its habitat by using clay to fill tree cavities (often created after consumption of the xylem), and suggested that clay provides both water retaining and water shedding benefits. However, the full significance of clay to *C. formosanus* is not clear. We hypothesized that clay is attractive to *C. formosanus.* To test our hypothesis, two-choice and no-choice tests were conducted in the laboratory to study the preference of *C. formosanus* to clay under fed and starvation conditions. Aggregation, tunneling activity, and consumption by termites were recorded and compared between bioassay arenas where clay was present or not. In no-choice tests additional data were collected to compare survivorship, soldier proportions, and live and dry weight of workers to measure water content. The positive effect of clay on general health has been reported for many animals (Hladik and Gueguen 1974, Hunter 1993, Krishnamani and Machaney 2000, Mahaney et al. 2005, Ayotte et al. 2006, Ferrari 2008, Blake et al. 2010). Like many soil-dwelling and wood-feeding termites (Bignell et al. 1980, Boyer 1982), *C. formosanus* also may directly feed on organic matter contained in clay. Food absence was evaluated to encourage termites to consume the clay provided. Clay also may provide additional nutrition, especially minerals (Hladik and Gueguen 1974, Ayotte et al. 2006, Blake et al. 2010), which are low in concentration in cellulosic food of termites (La Fage and Nutting 1978, Kaspari et al. 2009, Botch et al. 2010).

6.2 Materials and Methods

6.2.1 Termites

Worker and soldier *C. formosanus* were collected from Brechtel Park, New Orleans, LA on 26 March 2013 using milk crate traps (colony group 1), as described in Gautam and Henderson (2011a). Another crate of termites (colony group 2) was collected from Bonnet Carre Spillway, La Place, LA on 27 March 2013. Termites were maintained in trash cans (140 L) with wet wood in the laboratory until the day of testing.

6.2.2 Clay and Sand Blocks

Clay was collected from the backyard of the Dr. Gregg Henderson (St. Gabriel, LA) in December 2012. Construction sand was purchased from Louisiana Cement Product (Baton Rouge, LA). A sample of the St. Gabriel collection and sand were analyzed by the Coastal Wetlands Soils Characterization Lab of LSU (Table 6.1). Before testing, clay and sand were autoclaved (121℃ for 1 h) and air dried. Eighty-five grams of dried clay or sand was put in a Ziploc® bag and completely mixed with 15 ml deionized water. Just prior to testing, 1.2 g of clay or sand (15% moisture) was weighed and kneaded into small blocks (≈ 1.5 cm $\times 1.5$ cm $\times 0.5$ cm [L $\times$ W $\times$ H]).

Table 6.1. Characteristics of clay and sand used in choice and no-choice tests.

Sample	pH	Organic Matter (%)	Particle Size Distribution (%)			Textural Class
			Sand	Silt	Clay	
Clay from St. Gabriel	7.09	11.58	7.6	48.9	43.5	Silty Clay
Construction sand	5.59	0.19	99.2	0.2	0.6	Sand

6.2.3 Choice Test

The preference of termites to clay was tested using two-choice bioassays. Bioassay arenas were three-chambered transparent polyacrylic boxes (18.0 cm × 8.0 cm × 4.5 cm [L × W × H] each box, 6.0 cm × 8.0 cm × 4.5 cm [L × W × H] each chamber, Pioneer Packaging Co., North Dixon, KY) with small holes (diameter ≈ 1.5 cm) at the bottom center of both inner walls of the chambers. Thirty grams of 15% moisture sand (moistened with deionized water) was placed in each chamber as substrate. To test termite preference under fed conditions, five filter paper discs (4.2 cm, Whatman®, Maidstone, UK) were moistened with 0.6 ml deionized water and placed on the substrate in both left and right chambers. A clay or sand formed block was placed on the filter paper in left or right chamber. To test termite preference under starvation condition, only clay or sand blocks were placed on the substrate of each side chamber. Bioassays were repeated 12 times (six replicates for each termite colony group). Fifty termites (45 workers and 5 soldiers) from each colony group were released to the center chamber. The containers were sealed with clear plastic wrap (polyethylene resins, 12.5 μm thick, Great Value ™) and kept in an incubator at 26℃ in total darkness.

On day 7, 14 and 21, the number of termites in each chamber (clay side, center and sand side) was counted. All termites were readily visible in the sand side or center chambers; whereas in clay side chambers termites were often hidden and difficult to count. As a result, termites in the clay side were determined by subtracting the number of termites in the center and sand side chamber from 50 (number released). On day 21, treatments were dismantled and the surviving termites in each chamber were counted. To compare tunneling activity of termites under clay or sand blocks, in each observation period, different colored pen inks were used to mark tunnels and the length of tunnels under each chamber as described in Gautam (2011), and

Wang and Henderson (2012). The percentage of termites and length of tunnels in each chamber were compared by Analysis of Variance (ANOVA) followed by Tukey's honestly significant difference (HSD) for means comparison (SAS 9.2, SAS Institute, Cary, NC, 2011). The consumption of clay also was evaluated in each observation period by observing color change in the digestive tract. Dry weight of filter paper (fed condition) in clay or sand side was weighed before and after the experiment to determine the consumption and compared by t-test (SAS). Significance levels were determined at $\alpha = 0.05$.

6.2.4 No-Choice Test

The bioassay arena was a Petri dish (100 by 15 mm, Fisherbrand®) with 30 g 15% moistened sand (moistened with deionized water) on the bottom as substrate. There were 4 treatments: (1) clay-fed: 5 filter paper discs (5.5 cm, Whatman®) were moistened with 1 ml deionized water and placed on the substrate, and a clay block on the filter paper; (2) sand-fed: 5 moistened filter paper discs were placed on the substrate and a sand block on the filter paper; (3) clay-starved: only a clay block was placed on the substrate; and (4) sand-starved: only a sand block was placed on the substrate. The Petri dishes were sealed with Parafilm® (American Can Co., Greenwich, CT, USA) and kept in an incubator set 26℃ in total darkness. Treatments contained 12 replicates and 50 termites (45 workers and 5 soldiers) as in choice tests.

At days 33 (colony 1) and 35 (colony 2), clay transport behavior was recorded. The bottom side of each Petri dish was scanned and the length of tunnels was quantified to determine tunneling activity and clay consumption was evaluated, using the same methods described in choice tests. The survivorship of termites, workers and soldiers, were recorded. Ten termites were randomly selected in each experimental unit and weighed with a 0.1 mg analytical balance (Mettler AE163, Mettler-Toledo Inc., Bradford, MA). These termites were then placed in a 55℃

oven dryer for 20 h and dry weight was measured. The water content of termites was calculated using the formula as described in Yuan and Hu (2011). The survivorship, soldier proportion, live and dry weight, water content and length of tunnel were compared among the four treatments by ANOVA followed by Tukey's HSD for means comparison (SAS). The dry weight of filter paper under fed conditions was weighed before and after the experiment. The consumption was compared by t-test (SAS). In all tests, $\alpha = 0.05$.

6.3 Results

6.3.1 Choice Test

In general, whether food was provided or not, there were significantly more termites aggregated in chambers containing clay blocks compared to center or sand side chambers, with an exception in termite colony 2 under fed conditions at day 21 (Figure 6.1 A, B; Table 6.2). Termite numbers in the center and sand side chambers were similar. There was no significant difference in length of tunnels made in the three chambers at each observation period (fed and starved; data not shown), but overall, there was more total tunneling on the clay side under both fed and starved conditions, as compared to center and sand side chambers (Table 6.3). No evidence of clay consumption was found at any observation period. On day 21, the survivorship of termites under fed conditions was significantly higher than under starved conditions (fed: 88.3 ± 1.5 %, starved: 76.0 ± 4.4 %, $t = 2.88$, df $= 9$, $P = 0.0183$ for colony group 1; and fed: 87.3 $\pm$ 1.4 %, starved: 73.3 ± 3.0 %, $t = 4.17$, df $= 10$, $P = 0.0019$ for colony group 2). Under fed conditions, the consumption of filter paper on clay and sand side was similar for colony group 1 (clay side: 108.1 ± 32.2 mg, sand side: 74.8 ± 11.9 mg, $t = 0.24$, df $= 10$, $P = 0.8183$) and colony group 2 (clay side: 64.3 ± 11.4 mg, sand side: 68.5 ± 7.5 mg, $t = 0.31$, df $= 10$, $P = 0.7613$). The transport of clay particles and sand was observed under both fed and starved conditions.

Termites spread clay particles on the substrate and attached them to the smooth surface of walls in clay side chambers (Figure 6.1 A1, B1), whereas sand was strewn on the wall of both side chambers (Figure 6.1 A1, A2, B1, B2). Upon fed conditions paper particles were also sometimes transported.

Table 6.2. Percentage of termites (mean $\pm$ SEM) aggregated in the three-chamber container under starved or fed conditions.

Colony	Nutritional condition	Day	Percentage of termites (%)			ANOVA results (F; df; P)
			Clay	Center	Sand	
1	Starved	7	70.7 $\pm$ 9.4a[†]	8.3 $\pm$ 1.3b	21.0 $\pm$ 8.7b	19.70; 2, 15; <0.0001
		14	83.7 $\pm$ 12.5a	5.0 $\pm$ 4.2b	11.3 $\pm$ 8.4b	23.57; 2, 15; <0.0001
		21	83.2 $\pm$ 16.1a	1.3 $\pm$ 1.3b	15.6 $\pm$ 14.9b	11.92; 2, 14; 0.0014
	Fed	7	65.0 $\pm$ 4.6a	7.3 $\pm$ 0.7c	27.7 $\pm$ 4.3b	62.97; 2, 15; <0.0001
		14	75.7 $\pm$ 10.7a	6.3 $\pm$ 2.7b	18.0 $\pm$ 8.2b	21.84; 2, 15; <0.0001
		21	79.5 $\pm$ 16.0a	0.8 $\pm$ 0.8b	19.7 $\pm$ 16.1b	9.83; 2, 15; 0.0019
2	Starved	7	91.3 $\pm$ 5.5a	1.3 $\pm$ 0.8b	7.3 $\pm$ 4.6b	145.33; 2, 15; <0.0001
		14	94.0 $\pm$ 3.4a	3.7 $\pm$ 3.3b	2.3 $\pm$ 2.0b	317.47; 2, 15; <0.0001
		21	100.0a	0.0b	0.0b	NA; 2, 15; <0.0001
	Fed	7	74.3 $\pm$ 4.7a	9.3 $\pm$ 2.3b	16.3 $\pm$ 3.1b	102.66; 2, 15; <0.0001
		14	81.0 $\pm$ 10.8a	4.0 $\pm$ 1.7b	15.0 $\pm$ 9.2b	25.39; 2, 15; <0.0001
		21	53.1 $\pm$ 18.4a	2.9 $\pm$ 2.9a	44.1 $\pm$ 18.4a	3.37; 2, 15; 0.0617

[†] Numbers followed by the different letters are significantly different at α=0.05 (Tukey's HSD). Comparisons are made within the same row.

Table 6.3. Additive length of tunnels (mm) made in clay side, center, or sand side chambers.

Nutrition condition	Day	Additive length of tunnels (mm)		
		Clay	Center	Sand
Starved	7	519	68	415
	14	533	68	481
	21	578	239	511
Fed	7	299	112	202
	14	546	162	320
	21	568	186	320
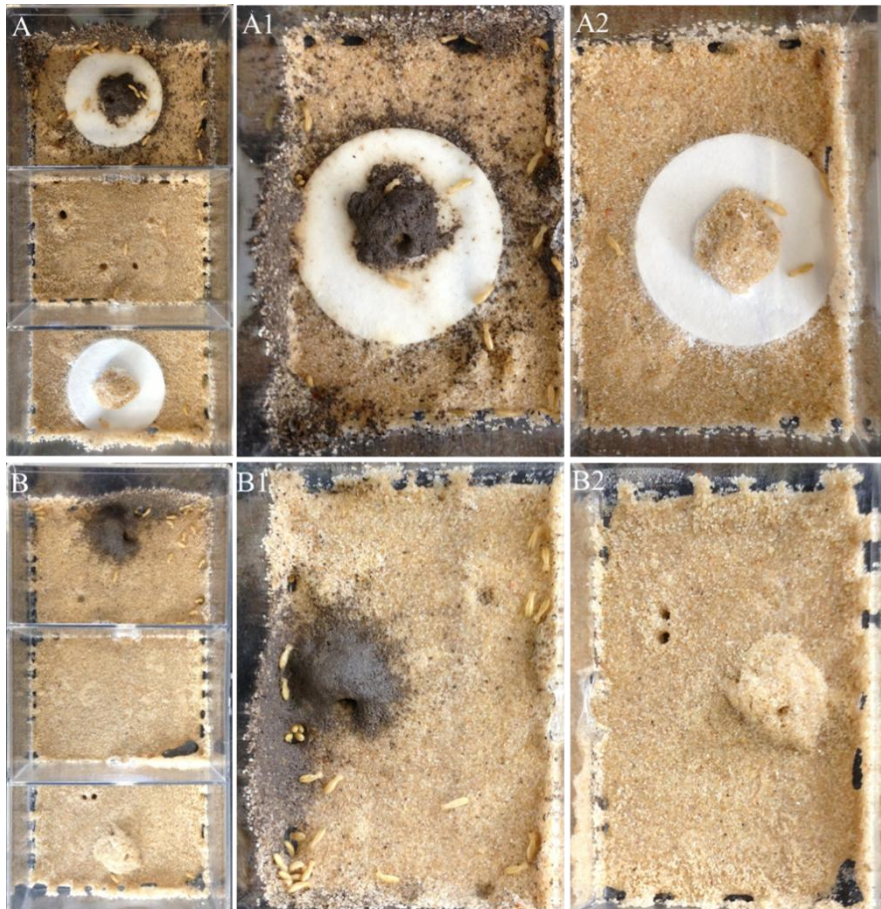
Figure 6.1. In two-choice tests, termites aggregated in clay side chambers under fed (A) or starved (B) conditions. Clay particles were spread on substrate and attached on the wall of clay side chambers. Sand was strewn on the wall of both side chambers. A1, B1: clay side chamber; A2, B2: sand side chamber.

6.3.2 No-Choice Test

Clay did not change survivorship, live and dry weight, water content, or tunnel length under fed or starved conditions (Table 6.4). It did not appear that clay had been consumed. For both colony groups, a significant decrease in filter paper consumption (dry weight loss) was found in the clay-fed treatment compared to the sand-fed treatment. Under fed conditions, colony group 1 had a significantly lower soldier proportion when clay was provided, but not in colony group 2 (Table 6.4).

Similar to the choice test, active particle transport behavior was observed. Clay particles were spread on the substrate (Figure 6.2 A1 [clay-fed], A3 [clay-starved]) and attached to the smooth surface of lids (Figure 6.2 B1 [clay-fed]). When clay was not provided, particles made from filter paper were spread on the sand blocks and substrate (Figure 6.2 A2 [sand-fed]); both sand and paper particles were attached to the smooth surface of lids (Figure 6.2 B2 [sand-fed]). In all treatments, the side of Petri dishes was strewn with sand (Figure 6.2 A1-A4). A clear tunneling pattern was observed in the clay-starved treatment. In 10 of 12 replicates, tunnels were made with a central point under clay blocks and lined with clay particles (Figure 6.2 C1 [clay-starved]), whereas in the sand-starved treatment, tunnels were made in only 4 of 12 replicates, without a clear pattern that centered under sand blocks (Figure 6.2 C2 [sand-starved]).

Table 6.4. Survivorship, soldier proportion, live and dry weight, water content, tunneling activity and consumption of termites (mean $\pm$ SEM) in no-choice tests.

Colony	Test	Clay-fed	Sand-fed	Clay-starved	Sand-starved	ANOVA results
1	survivorship (%)	74.6 $\pm$ 4.0a [d]	65.6 $\pm$ 3.2a	65.3 $\pm$ 4.2a	58.0 $\pm$ 6.1a	F=2.31; df=3,19; P=0.1085
	soldier proportion (%)	16.3 $\pm$ 1.3b	23.4 $\pm$ 2.1c	6.0 $\pm$ 1.0a	5.8 $\pm$ 0.7a	F=42.24; df=3,19; P<0.0001
	live weight [a] (mg)	51.2 $\pm$ 0.9a	50.5 $\pm$ 0.6a	50.5 $\pm$ 0.6a	49.6 $\pm$ 1.1a	F=0.62; df=3,19; P=0.6089
	dry weight [a] (mg)	19.0 $\pm$ 0.5a	17.7 $\pm$ 0.4a	18.4 $\pm$ 0.6a	17.8 $\pm$ 0.4a	F=1.56; df=3,19; P=0.2325
	water content [b] (%)	62.9 $\pm$ 0.7a	64.9 $\pm$ 0.8a	63.6 $\pm$ 0.8a	64.1 $\pm$ 0.9a	F=1.04; df=3,19; P=0.3956
	tunnel length (mm)	15.5 $\pm$ 9.8a	33.8 $\pm$ 14.3a	55.0 $\pm$ 19.6a	29.1 $\pm$ 18.7a	F=1.05; df=3,19; P=0.3933
	consumption [c] (mg)	167.7 $\pm$ 13.3a	331.1 $\pm$ 23.9b	----	----	t=6.25; df=9; P=0.0001
2	survivorship (%)	68.8 $\pm$ 2.0b	65.0 $\pm$ 5.5ab	46.0 $\pm$ 5.8a	50.4 $\pm$ 5.2ab	F=4.90; df=3,17; P=0.0124
	soldier proportion (%)	17.5 $\pm$ 0.5b	18.3 $\pm$ 2.3b	5.2 $\pm$ 1.8a	4.5 $\pm$ 1.4a	F=18.90; df=3,17; P<0.0001
	live weight [a] (mg)	41.3 $\pm$ 1.5a	42.6 $\pm$ 0.9a	38.9 $\pm$ 1.2a	39.5 $\pm$ 0.5a	F=2.69; df=3,17; P=0.0790
	dry weight [a] (mg)	12.4 $\pm$ 0.4b	12.9 $\pm$ 0.5b	10.2 $\pm$ 0.6a	10.5 $\pm$ 0.4a	F=5.56; df=3,17; P=0.0020
	water content [b] (%)	70.0 $\pm$ 0.6ab	69.8 $\pm$ 1.4a	73.8 $\pm$ 1.2b	73.4 $\pm$ 1.0ab	F=3.63; df=3,17; P=0.0344
	tunnel length (mm)	71.3 $\pm$ 22.5a	88.4 $\pm$ 32.1a	45.8 $\pm$ 13.0a	34.7 $\pm$ 22.7a	F=0.61; df=3,17; P=0.6160
	consumption [c] (mg)	268.5 $\pm$ 17.0a	388.9 $\pm$ 18.8b	----	----	t=4.66; df=9; P=0.0012

[a] Live and dry weight of ten termites were measured.

[b] Calculated by: (live weight – dry weight) / live weight.

[c] Measured by loss of dry weight of filter paper before and after the experiment.

[d] Numbers followed by the different letters are significantly different at α=0.05 (Tukey's HSD). Comparisons are made within the same row.
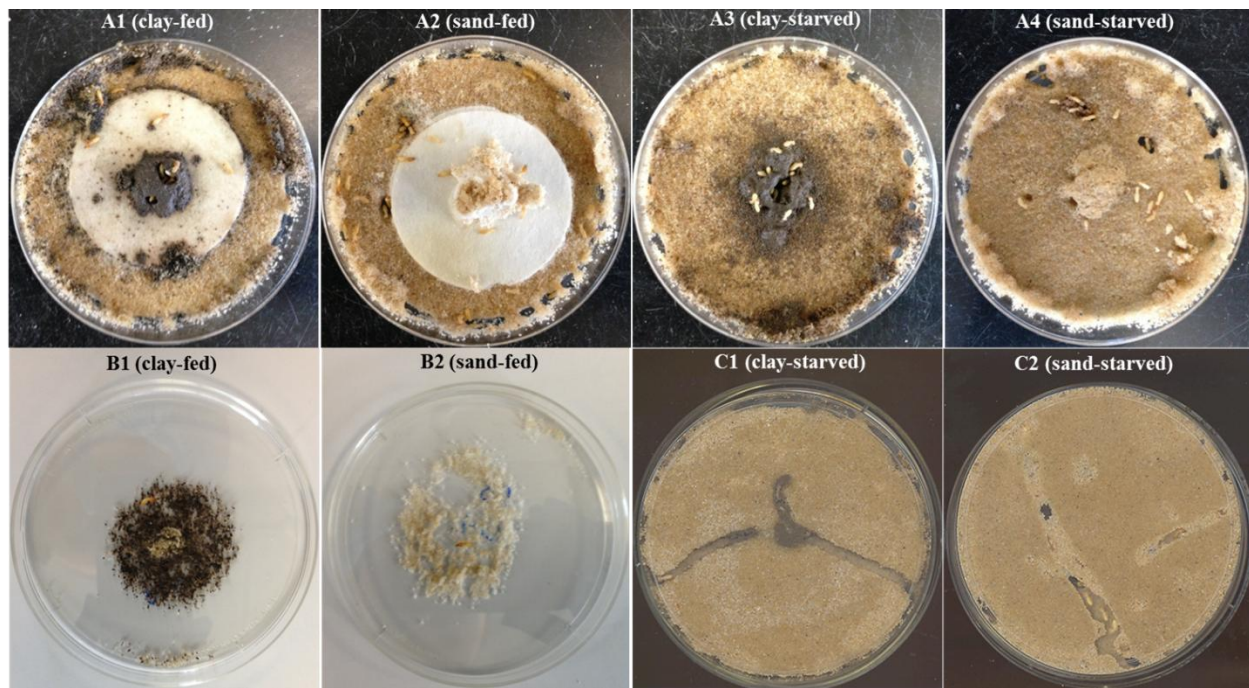
Figure 6.2. Particle transport behavior of termites in no-choice tests. A1-A4: up view of Petri dishes under fed or starved conditions; B1-B2: lids of Petri dishes under fed conditions; C1-C2: bottom view of Petri dishes under starved conditions.

6.4 Discussion

Clay caused termites to aggregate, supporting our clay attraction hypothesis. Many attractants to termites, such as amino acids, sugars and carbon dioxide, have been reported (Chen and Henderson 1996, Waller and Curtis 2003, Saran and Rust 2005, Bernklau et al. 2005, Broadbent et al. 2006, Wallace and Judd 2010, Castillo et al. 2013). A variety of wood decaying fungi, including the brown-rot fungi and blue-stain fungi, also can increase attractiveness of food sources to termites (Esenther et al. 1961, French 1978, Gao et al. 1987, Li et al. 2001, Cornelius et al. 2004, Little et al. 2012a, 2012b, 2013). In addition, some physical factors, such as surface area and size of food, are important for feeding and foraging preference of termites (Hedlund and Henderson 1999, Evans et al. 2005, 2007). This is the first report of an aggregation preference of *C. formosanus* to clay.

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The environmental cues that clay provides *C. formosanus* to cause aggregation are still not known. The inorganic ion content in the natural diet of termites is low (La Fage and Nutting 1978, Kaspari et al. 2009, Botch et al. 2010). In spite of this, Yoshimura et al. (2002) and Stewart et al. (2011) reported a high concentration of calcium, magnesium, zinc and manganese in the digestive tracts, Malpighian tubules, and mandibles of termites. Botch et al. (2010) reported that phosphate in artificial diets was attractive to *Reticulitermes flavipes* (Kollar). Likewise, Botch and Judd (2011) reported a foraging preference of *R. flavipes* to man-made potassium gradients in the soil. Clay can fix abundant inorganic ions because of its strong cation exchange capability (Reddy and Perkins 1974, 1976), and may help explain its attractiveness to *C. formosanus*. Feeding of organic matter in soil has been reported in many soil-dwelling termites (Bignell et al. 1980). The content of organic matter in our clay samples may also have contributed to the attractiveness of termites, especially under starvation conditions. In addition, clay and silt can hold a large amount of water (Brady and Weil 1999, Swoboda 2004), and the preference of *C. formosanus* to high moisture content of food and substrate has been well established (Delaplane and La Fage 1989, Gautam and Henderson 2011a, 2011b).

Attraction to clay also may be a result of a long association between termites and clay. In the present study, an active transport of clay particles to chamber walls and lids was observed. In the field, termites attach clay particles to vertical or inverted surfaces to construct bio-structures such as shelter tubes. Moreover, unlike other subterranean termite species, *C. formosanus* has a strong tendency to build above ground carton nests (Lai et al. 1983), and clay is found in the carton nest of *C. formosanus* (Henderson, unpublished data). Although various types of particles will be transported, materials with small particle sizes like clay may be essential for the construction behavior of *C. formosanus*. In a no-choice test, Cornelius and

Osbrink (2010) found that when *C. formosanus* use sand to construct shelter tubes, they are fragile and unlike shelter tubes made of clay or top soil. The unique properties of clay may be used for both water storage and waterproofing above ground (Henderson 2008). Gautam and Henderson (in preparation) observed that *C. formosanus* transferred more water to a food source (wood) when clay was provided as substrate, as compared to sand.

Inconsistent results of aggregation behavior and consumption by termites in choice tests have been reported previously by Green et al. (2005), Wong and Lee (2010), and Lima and Costa-Leonardo (2012). Likewise, in our choice tests, though termites significantly preferred to aggregate in the clay side, filter paper consumption between the two side chambers was similar. Boyer (1982) reported that wood-feeding termites may consume some clay constituents. Although we did not observe direct feeding of clay by *C. formosanus*, when termites transport moist clay particles with their mouthparts, it is possible that some is ingested. In the no-choice test, a significant increase in filter paper consumption was found when clay was not provided. However, we believe that this consumption is an artifact of the experimental design and an overestimation. This is due to paper particles being made when clay was unavailable. Similarly, Carter et al. (1983) reported that *C. formosanus* may use paper particles for carton-building. Chouvenc et al. (2011) observed that *C. formosanus* can deposit chewed "paper pellets" on the edge and lids of Petri dishes. Wang et al. (2013) also reported that *C. formosanus* made paper particles to bury dead individuals when filter paper was provided as food. As a pure source of cellulose, filter paper has been widely used as a diet in termite research. Traditionally, consumption was simply determined based on the dry weight loss of paper before and after the tests. Because of particle transport behavior, when careful weight measurement is needed, we

suggest the use of other food sources, such as wood blocks, to avoid overestimations of consumption.

The biological significance of clay to *C. formosanus* is now being understood. Our study may open the door for further research on the relationship of clay and *C. formosanus*. Four directions would be valuable for future investigation: (1) as a laboratory study, parameters such as moisture, temperature and ion concentration were strictly controlled and remained fairly constant in our bioassays. Though the extensive activity of *C. formosanus* in the clay-laden levees in New Orleans has been reported by Yang et al. (2009a, 2009b), a possible causal relationship between *C. formosanus* activity and clay-rich areas is not established. Field studies of clay preference and transport by *C. formosanus* would be of great value. (2) In the present study, only one type of field collected clay was tested. Jouquet et al. (2005) reported that the clay-particle size was more important for nest building than clay mineralogy by higher termites *Ancistrotermes cavithorax* (Sjöstedt) and *O.* nr. *pauperans* (Silvestri). The response of *C. formosanus* to clay minerals of different particle sizes and mineralogy should be investigated. (3) The preference of clay for bio-structure building was observed in many higher termites and *C. lacteus* (Rogers et al. 1999, Jouquet et al. 2007, Abe et al. 2012, Edosomwan et al. 2012, Mujinya et al. 2013). In the present study, we did not quantify the transported amount of the three materials (clay, sand and paper). However, when clay was available, less sand and paper were attached to the containers than when clay was not available. A well-designed choice test of clay utilization by *C. formosanus* in construction behavior would be helpful. (4) Though no significant effect of clay on general termite health was found, our no-choice test was of a short duration (33 or 35 d) with a small group of termites (n = 50) kept in Petri dishes and little if any clay was consuming. Clay may have long-term effects on termite health. For example, clay is

known for its detoxification function, which largely depends on negatively charged cation-exchange sites binding with small molecules (Diamond 1999, Houston et al. 2001, Voigt et al. 2008). Studies should be conducted to determine whether clay is regularly consumed in the field and exerts a detoxification effect on termites that would slow the action of termiticides and secondary plant metabolites.

The full understanding of the interaction between *C. formosanus* and clay may bring us new insights for control of this destructive pest. Baits placed in clay soils may have a greater probability of being infested by termites than baits placed in sandy soils. In locations where clay content is low, such as sandy areas, clay placed nearby or within baits may increase their attractiveness to *C. formosanus*. Moreover, clay may be useful as guidelines to help direct termites to in- and above-ground bait stations.

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CHAPTER 7. LETHAL AND SUBLETHAL EFFECT OF LUFENURON ON THE FORMOSAN SUBTERRANEAN TERMITE (ISOPTERA: RHINOTERMITIDAE)[1]

7.1 Introduction

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a worldwide distributed pest that attacks both living trees and structural wood (Henderson 2001, 2008, Rust and Su 2012). The annual economic loss caused by this pest was estimated about 1 billion dollars (USD) in the United States (Suszkiw 2000), and 0.8 billion yuan (RMB) in China (Zhong and Liu 2003). Baiting systems have been widely used for the control of *C. formosanus* and other subterranean termites (Henderson and Forschler 1997, Rust et al. 1998, Grace and Su 2001, Cornelius and Lax 2005). A baiting system will suppress termite populations with a slow-acting toxicant to be delivered to the whole colony through direct feeding and secondary transfer. Many bait toxicants, such as bistrifluron, chlorfluazuron, diflubenzuron, hexaflumuron, lufenuron, noviflumuron and sulfluramid, have been used or have potential to be used as active ingredients in termite baits (Su et al. 1995, Henderson and Forschler 1996, Su and Scheffrahn 1996, Grace et al. 2000, Peters and Fitzgerald 2003, Vargo 2003, Karr et al. 2004, Haagsma and Rust 2005, Kubota et al. 2006, Sukartana et al. 2009, Evans 2010, Neoh et al. 2011, Osbrink et al. 2011, Eger et al. 2012).

Lufenuron, a chitin synthesis inhibitor, has shown successfully to control cockroaches, cat fleas, and some agricultural pests (Kaakeh et al. 1997, Rust 2005, Sáenz-de-Cabezón et al. 2006, Moya et al. 2010, Correia et al. 2013). A lufenuron based termite bait was developed in 2003 (http://www.syngentaprofessionalproducts.com/news_releases/news.aspx?id=75353).

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Although this bait has not yet been commercially used, the effectiveness of lufenuron against termite was confirmed both in laboratory and field tests (Lewis and Power 2006, Lovelady et al. 2006, Vahabzadeh et al. 2007, Haverty et al. 2010, Lewis and Forschler 2010, Bowen and Kard 2012, Gautam and Henderson 2014). Most studies tested a concentration of 1500 ppm lufenuron in termite bait matrix. Few studies focused on the physiological and behavioral effect of lufenuron on *C. formosanus* at lower concentrations. Wang et al. (2013a) found that pre-fed 1000 ppm lufenuron significantly increased susceptibility of *C. formosanus* to the entomopathogenic bacterium *Pseudomonas aeruginosa* (Schroeter) Migula.

Clay affects many aspects of termite biology. For example, *C. formosanus* can transport clay to tree cavities or food sources, probably for water transfer and moisture maintenance (Henderson 2008, Gautam and Henderson, unpublished data). Wang and Henderson (2014) observed an aggregation preference of *C. formosanus* on clay, and suggested the use of clay as a bait attractant (Henderson et al. 2014). Here, we compared survivorship, running speed, body water content, food consumption, tunneling, microbial infection, and two behavioral patterns (carcass burying behavior and particle transport behavior) among termites fed the three concentrations of lufenuron-treated (250, 500, or 1000 ppm) or untreated (control) filter paper when clay or sand blocks were present.

7.2 Materials and Methods

7.2.1 Termites

Two colony groups of worker and soldier *C. formosanus* were tested. Colony group 1 was collected from Bonnet Carre Spillway, La Place, LA on 27 March 2013 using milk crate traps, as described in Gautam and Henderson (2011). Colony group 2 was collected from

Brechtel Park, New Orleans, LA on 23 April 2013 using the same method. Termites were maintained in trash cans (140L) with wet wood in the laboratory for < 2 month before testing.

7.2.2 Lufenuron Treated and Untreated Filter Paper

A stack of 50 filter paper discs (55mm, Whatman® No. 1, GE Healthcare, Maidstone, UK) was weighed and put in a Ziploc® bag (S.C. Johnson & Son, Racine, WI). The required amount of lufenuron (Sigma-Aldrich Co. LLC., St. Louis, MO) to make 250, 500 or 1000 ppm treated paper was dissolved in 25 ml acetone (HPLC grade, Mallinckrodt Specialty Chemicals Co., Paris, KY) and added into the Ziploc® bag. The untreated filter paper stack was added with 25 ml acetone only. Filter paper stacks were flipped several times to absorb the liquid. The open Ziploc® bags containing treated or untreated filter paper were placed under a fume hood for 48 h to evaporate the acetone.

7.2.3 Clay and Sand Block

Clay was collected from the backyard of Dr. Gregg Henderson (St. Gabriel, LA) in December 2012. A sample of clay was analyzed by the Coastal Wetlands Soils Characterization Lab of LSU and identified as silty clay (7.6% sand, 48.9% silt, and 43.5% clay). Construction sand was purchased from Louisiana Cement Product (Baton Rouge, LA). Clay and sand were autoclaved (121℃ for 1 h) and air dried before testing. Dried clay or sand (85 g) was put in a Ziploc® bag and completely mixed with 15 ml deionized water. Moistened clay or sand (1.2 g) was then kneaded by hand into small blocks (about 1.5 cm ×1.5 cm ×0.5 cm [L ×W ×H]).

7.2.4 Bioassay Arena.

To make a 15% moisture substrate, 25.5 g autoclaved sand (121℃ for 1 h) was put on the bottom of a Petri dish (100 by 15 mm, Fisherbrand®, Fisher Scientific, Pittsburgh, PA) and moistened with 4.5 ml sterile deionized water. Two filter paper discs (250, 500, 1000 ppm

lufenuron-treated or untreated) were weighed, moistened with 500 μl deionized water, and placed on one side of the bioassay arena. A clay or sand block was placed on the other side of the arena (Figure 7. 1 A, B).
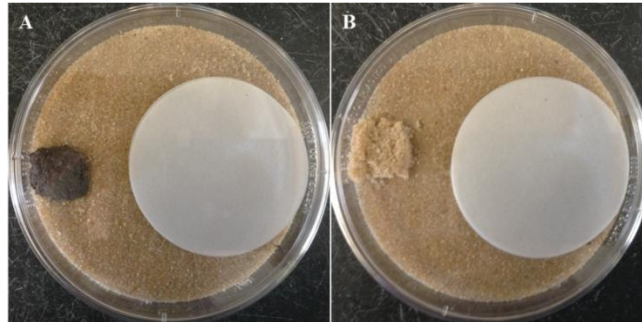
Figure 7.1. Bioassay arena. A clay block (A) or sand block (B) and two filter paper discs (lufenuron-treated or untreated) were placed on the substrate and not touched.

7.2.5 Bioassay Setting

The bioassay contained 8 treatments: 4 treatments (250, 500, or 1000 ppm lufenuron-treated and control filter paper) with clay blocks and 4 with sand blocks. Each treatment had 12 replicates (six replicates for each termite colony group). Fifty termites (45 workers and 5 soldiers) were released in each Petri dish, sealed with Parafilm® (Structure Probe, Inc., West Chester, PA) and kept in an incubator (3710, Forma Scientific, Inc., Marietta, OH) at 26℃ in total darkness for 30 d (colony group 1) and 32 d (colony group 2). All behavioral observations and data recordings were made at the end of the experiment.

7.2.6 Microbial Infection and Carcass Burying Behavior

A termite infected by microbial pathogens was determined when it assumed a color (red, yellow or black) or mycelium was growing around the body. The suppressed carcass-burying behavior of termites was determined if any infected and dead termites were left exposed

(unburied) on the substrate or filter paper. Any microbial infestation of filter paper itself was recorded based on color change or mycelium growth.

7.2.7 Particle Transport Behavior

Active particle transport behavior was recorded if termites attached particles (sand, paper and clay) onto the lid surface. The substrate area of lids was carefully marked with a red pen. The lids were then put on a paper printed with 0.5 cm $\times$ 0.5 cm gridlines, and the red ink areas were measured.

7.2.8 Survivorship and Body Water Content

The number of live termites was counted. Five termites were randomly selected in each experimental unit and weighed with a 0.1 mg analytical balance (Mettler AE163; Mettler-Toledo Inc., Bradford, MA). These termites were put in Petri dishes and placed in a 60℃ oven for 24 h for dry weight measure. The body water content of termites was calculated by the formula provided by Arquette and Forschler (2006).

7.2.9 Running Speed

A technique developed by Quarcoo et al. (2012) was modified to measure the running speed of termites. A circle (diameter: 95 mm; perimeter: 298 mm) was drawn on a piece of printing paper with a black ballpoint pen (BIC®-Crystal®, Société Bic, Clichy, France). To measure the running speed, a termite was randomly selected and put on the circular line. This termite was allowed to find and walk following the circular line for duration of 30 s. In the following 30 s, the distance the termites moved was recorded and running speed (cm/min) was calculated. If a termite did not move for 1 min after being placed on the circular line, the running

speed was counted as 0. Two termites from each experiment unit were tested. A new paper with a circle was used for each treatment and each colony group.

7.2.10 Tunneling and Consumption.

Red pen ink was used to mark the tunnels on the bottom side of Petri dish. The length of tunnels was measured with the help of a string overlaid on marked tunnels. The filter paper was air dried for 5 to 7 d and any particles on the paper were carefully removed with a soft brush. Dry weight loss of filter paper before and after the experiment was measured to calculate the consumption.

7.2.11 Data Analyses

A 3-way ANOVA (PROC MIXED, SAS Institute, Cary, NC) was used for the analysis of data (survivorship, running speed, water content, consumption, tunnel length, area of lids covered with particles) with colony, clay or sand block, and lufenuron concentration as fixed factors, followed by Tukey's HSD procedure for means comparison. If the colony effect was significant, the two termite colony groups were analyzed separately with 2-way ANOVA. Significance levels were determined at $\alpha = 0.05$.

7.3 Results

7.3.1 Survivorship

Colony had a significant effect on survivorship ($F_{1, 87} = 6.06$, $P = 0.0161$). For both colony groups, there was neither significant effect of clay or sand blocks nor the interaction effect between blocks and concentrations ($P > 0.05$, data not shown). However, lufenuron significantly decreased survivorship. For colony 1, survivorship in the controls was significantly higher than that in the treatments containing 250 or 1000 ppm lufenuron ($F_{3, 40} = 3.98$, $P = 0.0143$, Figure 7.2). For colony 2, all three concentrations caused significantly lower

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survivorship compared with the controls ($F_{3, 40} = 26.57$, $P < 0.0001$, Figure 7.2). Survivorship was not significantly different when compared among the three lufenuron concentrations.

7.3.2 Running Speed and Body Water Content

Colony had significant effect on running speed ($F_{1, 169} = 37.26$, $P < 0.0001$) and body water content ($F_{1, 81} = 154.65$, $P < 0.0001$). For both colony groups, the effect of clay or sand blocks and the interaction between blocks and concentrations were not significant in running speed or body water content ($P > 0.05$, data not shown). Lufenuron significantly decreased running speed of termites at all the concentrations when compared with the controls (colony 1: $F_{3, 78} = 21.19$, $P < 0.0001$; colony 2: $F_{3, 84} = 44.50$, $P < 0.0001$, Figure 7.2). However, lufenuron had no observed effect on body water content of termites (colony 1: $F_{3, 35} = 2.51$, $P = 0.0747$; colony 2: $F_{3, 39} = 0.94$, $P = 0.4287$, Figure 7.2).

7.3.3 Consumption

Colony had no significant effect on filter paper consumption ($F_{1, 87} = 0.03$, $P = 0.8664$). Clay or sand blocks had no significant effect on consumption ($F_{1, 87} = 2.03$, $P = 0.1574$), but the effect of concentrations and the interaction between blocks and concentrations were significant ($F_{3, 87} = 178.95$, $P < 0.0001$ for concentration, and $F_{3, 87} = 3.96$, $P = 0.0107$ for interaction). The filter paper consumption in the control-sand was significantly higher than in the control-clay. However, the consumption between clay and sand treatments was not significantly different for a given lufenuron concentration. Lufenuron at all three concentrations significantly decreased consumption compared with the controls (Figure 7.3).

7.3.4 Tunnel Length

Colony had no significant effect on tunnel length ($F_{1, 87} = 2.34$, $P = 0.1295$). Both blocks (clay or sand) and concentrations had significant effect on tunnel length ($F_{1, 87} = 4.61$, $P =$

0.0345 for block, and $F_{3, 87} = 6.05$, $P = 0.0009$ for concentration), but the interaction effect between blocks and concentrations was not significant ($F_{3, 87} = 1.27$, $P = 0.2914$). Length of tunnels in the control-clay treatment was significantly longer than in the 500 ppm-clay or 1000 ppm-clay treatments (Figure 7.3).
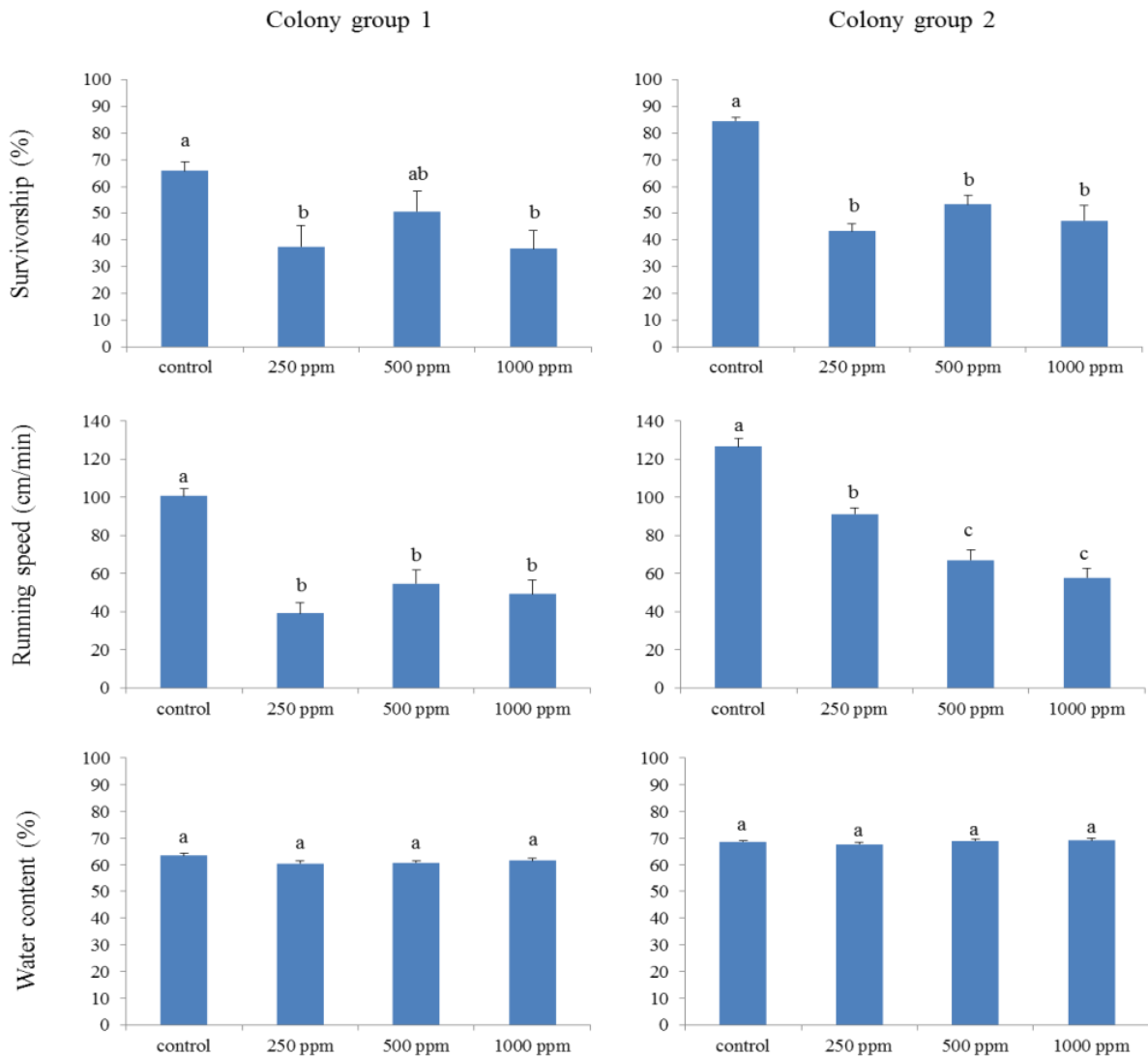
Figure 7.2. Survivorship, running speed, and body water content of termites (mean ± SEM) fed lufenuron-treated (250, 500 or 1000 ppm) or untreated (control) filter paper after 30-32 d. Different letters indicate significant difference ($P < 0.05$).
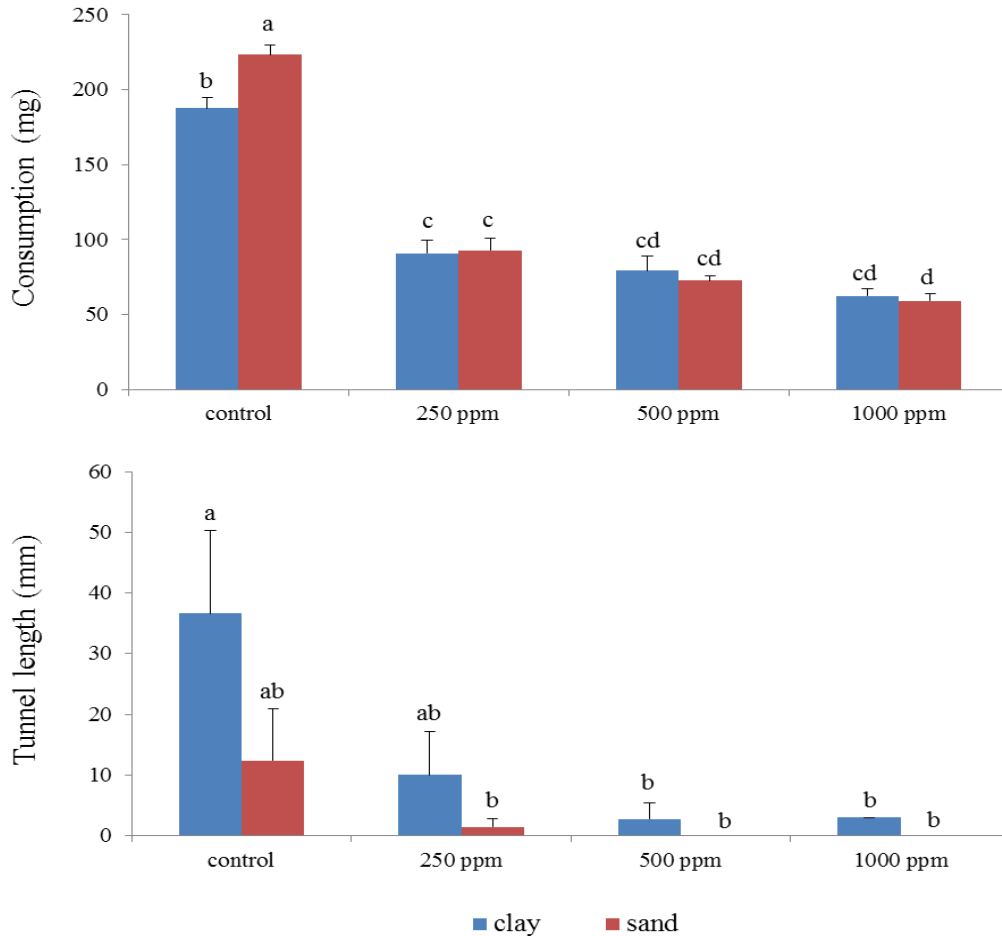
Figure 7.3. Consumption and tunnel length of termites (mean ±SEM) fed lufenuron-treated (250, 500 or 1000 ppm) or untreated (control) filter paper after 30-32 d. Different letters indicate significant difference ($P < 0.05$).

7.3.5 Microbial Infection and Carcass Burying Behavior

For both colony groups, no dead termites were found in the controls (Figure 7.4; Table 1). However, suppressed carcass-burying behavior was observed in 250, 500 and 1000 ppm lufenuron treatments (Table 7.1). Dead termites were colored (mostly red or black) or covered by mycelium, indicating the infection of microbial pathogens (Figure 7.4). Moreover, in all replicates containing lufenuron, filter paper discs were infested by various types of microbes

(assumed yellow and black colors or mycelium was growing) (Figure 7.4), whereas only 5 of 24

control filter paper discs were similarly infested.

Table 7.1. Replicates of each treatment showing normal ($+$) or inhibited ($-$) carcass burying behavior.

Colony group	Clay/sand	Lufenuron (ppm)			
		0	250	500	1000
1	Clay	+ + + + +	− − − − + +	− − − − − +	− − − − − −
	Sand	+ + + + +	− − − − + +	− − − − + +	− − − − − −
2	Clay	+ + + + +	− − + + + +	− − − + + +	− − − − − −
	Sand	+ + + + +	− − − − − +	− − − − − −	− − − − − −
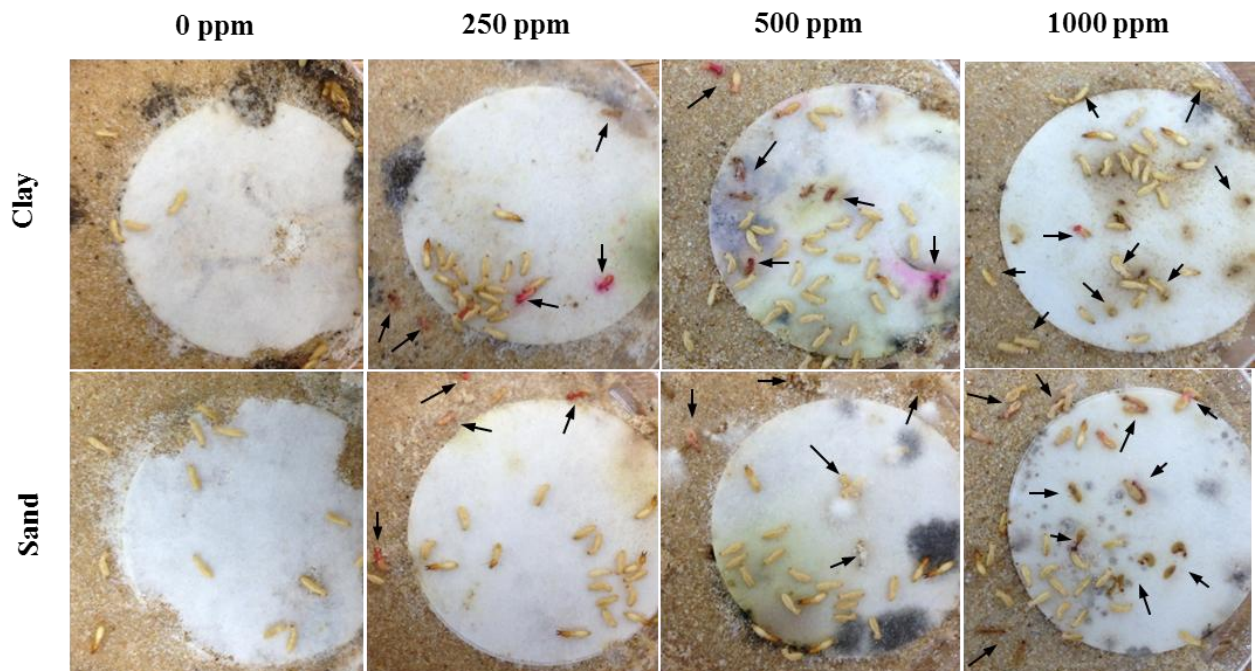
Figure 7.4. Termites fed lufenuron (250, 500, 1000 ppm) were infected by microbial pathogens and dead termites (indicated by arrows) were not buried. All lufenuron-treated filter paper discs were infested.

7.3.6 Particle Transport Behavior

For colony group 1, particles (sand, paper and clay) were transported in all controls and about half of the 250 ppm lufenuron replicates, whereas little or no particles were deposited to lids in 500 and 1000 ppm treatments (Figure 7.5A, Table 7.2). In colony group 2, active particle transport behavior was only observed in the controls (Table 7.2). Colony had a significant effect on the area of lids covered with particles ($F_{1, 87} = 7.78$, $P = 0.0065$). The effect of clay or sand blocks and the interaction effect between blocks and concentrations were not significant ($P > 0.05$, data not shown), however, lufenuron at all three concentrations significantly reduced the area covered with particles (colony 1: $F_{3, 40} = 62.68$, $P < 0.0001$; colony 2: $F_{3, 40} = 92.92$, $P < 0.0001$; Figure 7.5B).
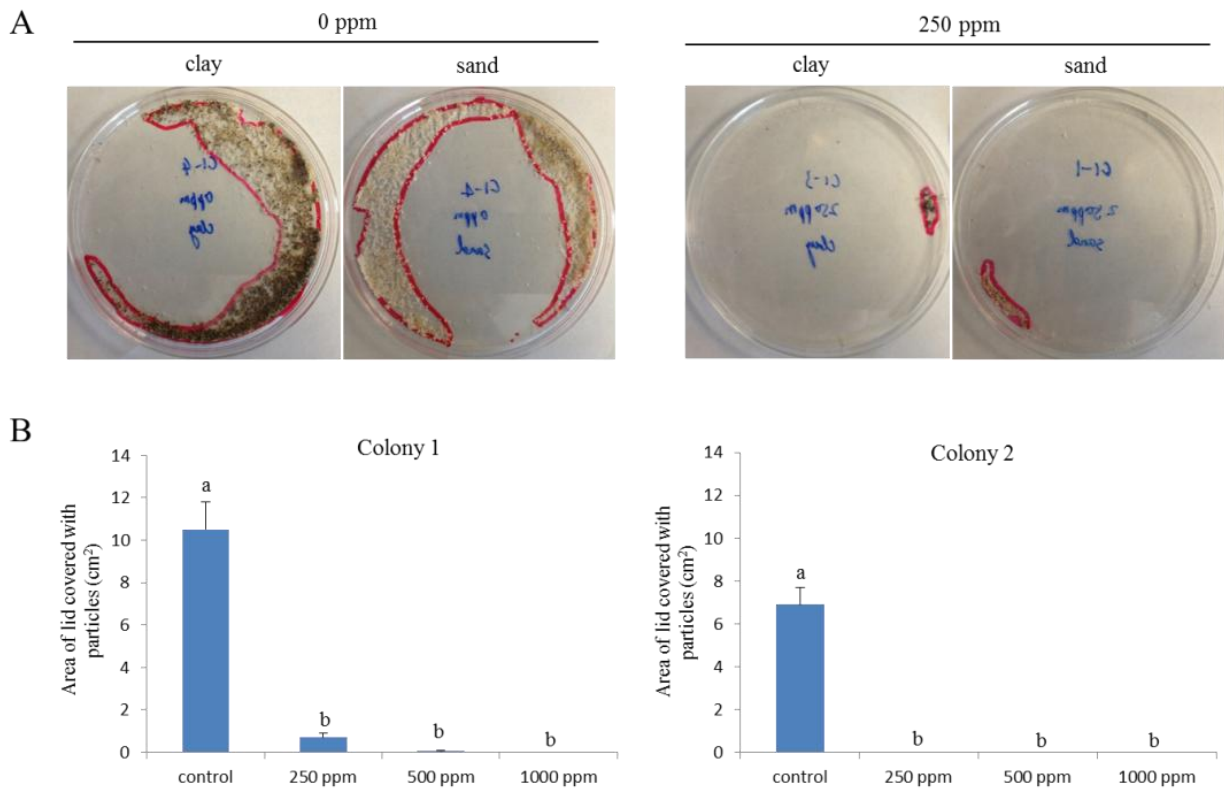
Figure 7.5. Particle transport behavior was suppressed in lufenuron treatments. A: example of lids that attached with particles (colony 1). B: area of lids covered with particles (mean ±SEM). Different letters indicate significant difference ($P < 0.05$).

Table 7.2. Replicates of each treatment that showing active ($+$) or inhibited ($-$) particle transport behavior.

Colony group	Clay/sand	Lufenuron (ppm)			
		0	250	500	1000
1	Clay	$+++++$	$--+++$	$------$	$------$
	Sand	$+++++$	$---++$	$-----+$	$------$
2	Clay	$+++++$	$------$	$------$	$------$
	Sand	$+++++$	$------$	$------$	$------$

7.4 Discussion

Although many studies focused on the termite mortality caused by chitin synthesis inhibitors, few paid attention on the physiological and behavioral impacts of these pesticides on termites. Rojas and Morales-Ramos (2004) compared the impact of three chitin synthesis inhibitors (10 ppm each), diflubenzuron, hexaflumuron, and lufenuron, on reproductive ability of paired *C. formosanus* alates. The results showed that the egg hatching was completely inhibited after 6 mo. treatment of the three chitin synthesis inhibitors, and lufenuron significantly decreased queen fecundity. Our study showed that lufenuron at the tested concentrations reduced termite survivorship, running speed, consumption and tunneling. Moreover, it induced microbial infection and suppressed carcass burying and particle transport behaviors.

Running speed is an important indicator of termite vigor (Arquette et al. 2006, Yuan and Hu 2011). Many termiticides, such as fipronil, indoxacarb and chlorfenapyr have been reported to reduce running speed of termites (Rust and Saran 2006, Saran and Rust 2007, Quarcoo et al. 2012). Wang et al. (2013a) reported that termite fed 1000 ppm lufenuron for 8 d became "sluggish". In the present study, lufenuron clearly reduced running speed of *C. formosanus*, indicating that the termite vigor was negatively affected.

Su and Scheffrahn (1996) reported a low concentration threshold of lufenuron to be between 1000- 2000 ppm for feeding deterrence of *C. formosanus*. Likewise, in our study, it appeared that lufenuron at all three concentrations reduced filter paper consumption. However, transport of paper particles was observed in the controls and some replicates of the 250 ppm lufenuron treatments. Wang and Henderson (2014) reported that the particle transport behavior of termites can lead to an overestimation of filter paper consumption. Gautam and Henderson (2014) reported that, in choice tests, consumption of bait matrix containing lufenuron (1500 ppm) was sinificantly higher than that containing noviflumuron (5000 ppm).

Wang et al. (2013a) reported that 1000 ppm lufenuron significantly increased susceptibility of *C. formosanus* to entomopathogenic bacterium *P. aeruginosa*. Interestingly, in the present study, we observed that termites feeding on low concentrations of lufenuron were infected by bacteria and fungus, though no microbes were purposely introduced. The microbes that attacked termites may be opportunistic pathogens. For example, dead termites turned red in many replicates treated with lufenuron, indicating an infection by *Serratia marcescens* Bizio, a bacterium naturally associated with termites (Adams and Boopathy 2005, Wang and Henderson 2013, Wang et al. 2013b). Studies have shown that subterranean termites can produce and secrete antimicrobial compounds to the cuticle and habitat (Wiltz et al. 1998, Bulmer et al. 2010, Hamilton et al. 2011). Filter paper discs in lufenuron treatments were infested by microbes, indicating a reduced ability of termites to suppress microbes in its surrounding environment.

Behavioral defense is important for disease resistance of termites. Carcass management will decrease the contact between carcasses and healthy individuals and reduce disease risks. Multiple behavioral patterns responding to carcasses, such as avoidance, cannibalism, removal and burial, were identified in termites (Chouvenc and Su 2012, Sun and

Zhou 2013, Sun et al. 2013). Chouvenc and Su (2012) reported that carcass burying behavior is dominant for *C. formosanus* when a high level of fungal infection and death occurred. Wang et al. (2013a) reported a suppression of carcass burying behavior of *C. formosanus* after feeding 1000 ppm lufenuron. In the present study, the carcass burying behavior was inhibited at all three concentrations, the most obvious being at 1000 ppm. The particle transport behavior also was suppressed at all lufenuron concentrations. Ulyshen and Shelton (2012) found that the particle transport behavior of *Reticulitermes virginicus* (Banks) was closely associated to the burial of carcass because termites moved significantly more sand to wood blocks that contained carcass. In addition, particle transport behavior is related to water transfer and bio-structure construction (Cornelius and Osbrink 2010, Mizumoto and Matsuura 2013, Wang and Henderson 2014, Gautam and Henderson, unpublished data).

The lufenuron-reduced disease resistance may provide new insight for the termite control industry. Subterranean termites live in moist environments that favor the growth of microbes (Husseneder et al. 2010). Many opportunistic pathogens, such as *P. aeruginosa, S. marcescens, Bacillus thuringiensis* Berliner, *B. cereus* Frankland and Frankland, *Aspergillus flavus* Link, and *A. nomius* Kurtzman, Horn and Hessltine, have been isolated from *C. formosanus* (Connick et al. 2001, Osbrink et al. 2001, Jayasimha and Henderson 2007a, 2007b, Husseneder et al. 2010, Chouvenc et al. 2012, Wang and Henderson 2013). Moreover, many biological control agents, including *P. fluorescens* Migula strain CHA0, *Beauveria bassiana* (Balsamo) Vuillemin, *Paecilomyces fumosoroseus* (Wize) Brown and Smith, and *Metarhizium anisopliae* (Metschinkoff) Sorokin, were quite effective against termites in the laboratory tests (Culliney and Grace 2000, Grace 2003, Sun et al. 2003, Wang and Powell 2004, Meikle et al. 2005, Wright et al. 2005, 2008, Dong et al. 2007, Devi and Kothamasi 2009, Wright and

Cornelius 2012). However, the strong immune and behavioral responses of termites limit the field application of these entomopathogenic microbes (Yanagawa and Shimizu 2007, Yanagawa et al. 2008, Chouvenc et al. 2009, 2011, Hamilton et al. 2011, Rosengaus et al. 2011, Chouvenc and Su 2012, Hussain et al. 2013). Therefore, the "best prospect" for the use of a termite pathogen is "as part of an integrated strategy" (Lenz and Evans 2002). Our study showed that lufenuron at low concentrations reduces the vigor of *C. formosanus* and suppresses its disease resistance. It would be valuable to further test the effectiveness of combinations of lufenuron and termite pathogens (Henderson et al. 2014). To achieve long-term viability, termite pathogens can be encapsulated in a clay-based matrix (Fravel et al. 1985), which we believe will not negatively interact with lufenuron as shown in this study.

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CHAPTER 8. LUFENURON SUPPRESSES THE RESISTANCES OF FORMOSAN SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE) TO ENTOMOPATHOGENIC BACTERIA[1]

8.1 Introduction

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, has become one of the most destructive termite species in the United States since its introduction from southern Asia (Haagsma et al. 1995, Henderson 2008, Rust and Su 2012). Currently, there are two widely applied methods for the control of this pest: termite baiting and liquid termiticides (Henderson 2001, Rust and Su 2012). Although chemical insecticides are popular in the termite control industry, they are not free from shortcomings, such as potential toxicity to non-target organisms and pollution to soil and water resources. Biological control methods may provide an environmentally friendly and sustainable option for the control of *C. formosanus* (Sun et al. 2002, Jayasimha and Henderson 2007, Sindhu et al. 2011). However, researchers have shown that many microbial pathogens cannot successfully infect *C. formosanus* due to its strong immune response and social behavior (Woodrow and Grace 2008, Yanagawa et al. 2009, 2010a, 2010b, Chouvenc and Su 2012, Wang and Henderson 2013). It is known that some pesticides can impact insect immunity, making insects more susceptible to pathogen infections (Azambuja et al. 1991, Sharma et al. 2003, 2008, George and Ambrose 2004, Büyükgüzel 2009, Wu et al. 2009, Alaux et al. 2010, Islam et al. 2010, James and Xu 2011, Shapiro-Ilan et al. 2011, Vidau et al. 2011, Aufauvre et al. 2012, Pettis et al. 2012, Zhu et al. 2012). The effect of pesticide on disease resistance of *C. formosanus*, however, is not well studied.

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We hypothesized that a low dose of lufenuron, a chitin synthesis inhibitor, will weaken *C. formosanus* and increase its susceptibility to the entomopathogenic bacteria, *Pseudomonas aeruginosa* (Schroeter) Migula, *Serratia marcescens* Bizio, and *Bacillus thuringiensis* Berliner subsp. *israelensis.* Lufenuron has been used for the control of cat fleas (Rust 2005a, 2005b, 2010). Its effectiveness as an active ingredient (toxicant) in termite baits has been confirmed by both laboratory and field studies (Rojas and Morales-Ramos 2004, Lovelady et al. 2006, Vahabzadeh et al. 2007, Lewis and Forschler 2010, Haverty et al. 2010, Bowen and Kard 2012, BKG and GH unpublished data). Lovelady et al. (2006), Haverty et al. (2010), and Bowen and Kard (2012) reported that 1500 ppm lufenuron baits can successfully suppress subterranean termite populations in the field. In the present study, we tested a lower concentration of lufenuron (1000 ppm), which did not effectively suppress *C. formosanus* under laboratory conditions in a previous study (Su and Scheffrahn 1996). Three entomopathogenic bacteria, *P. aeruginosa, S. marcescens,* and *B. thuringiensis* subsp. *Israelensis*, are known termite pathogens, as studied by Khan (1981), Khan et al. (1977, 1992), Osbrink et al. (2001), Connick et al. (2001), and Singha and Dutta (2010). Two of them, *P. aeruginosa* and *S. marcescens*, are naturally associated with termites and have been isolated from *C. formosanus* (Osbrink et al. 2001, Adams and Boopathy 2005, Wang and Henderson 2013).

To test our hypothesis, termites were first fed lufenuron or untreated filter paper and subjected to mortality bioassays involving the three entomopathogenic bacteria. The mortality and behavior of termites were recorded and compared.

8.2 Materials and Methods

8.2.1 Termites

Two colony groups of *C. formosanus* were collected > 100 m apart from Brechtel Park, New Orleans, LA, on 17 August 2012 using milk crate traps (Gautam and Henderson 2011). Termites were maintained in 140L trash cans provisioned with moist wood (*Pinus* sp.) for a maximum of 1 month before testing.

8.2.2 Termite Pre-Feeding Conditions

A stack of 12 filter paper discs (9 cm diam., Ahlstrom®, Grade 617) were weighed and treated with the calculated amount of lufenuron (Sigma-Aldrich Co. LLC., St. Louis., MO) dissolved in 14 ml of acetone to make 1000 ppm lufenuron treated filter paper. Control filter paper discs were added with an equal amount of acetone alone. The filter paper stacks were placed under a fume hood and flipped several times to absorb the liquid and left in place for 48 h until the acetone was completely evaporated. Six of the twelve lufenuron or untreated filter paper discs (2.80 ±0.01 g) were placed in a Petri dish (150 by 15 mm, Fisherbrand®) and moistened with 8 ml sterile distilled water. One thousand termites each from the 2 colonies (colony 1: 90% workers and 10% soldiers; colony 2: 100% workers, according to their collected colony structure) were released to each dish. Petri dishes were sealed with Parafilm® (Structure Probe, Inc., West Chester, PA) to maintain moisture and held in an incubator (3710, Forma Scientific, Inc., Marietta, OH) at 28 ℃ in total darkness for 8 days. At the end of day 8, the condition of termites (active or sluggish), filter paper consumption and survival were recorded.

8.2.3 Bacteria Strains, Growth Conditions and Bioassay Arena.

P. aeruginosa and *S. marcescens* were previously isolated from *C. formosanus* collected in New Orleans and identified by 16S rRNA sequencing (Wang and Henderson 2013).

B. thuringiensis subsp. *israelensis* was isolated from MosquitoDunk® (Summit Chemical Company, Baltimore, MD) and confirmed by colony growth characteristics and Schaeffer-Fulton staining. The three bacteria were streaked on Luria-Bertani (LB) agar plates and stored at 4℃ for less than one month before setting up the bioassay. Stored bacteria were streaked on Brain-Heart Infusion (BHI) agar plates and incubated at 30℃ for 24 h. A single colony of each bacterium was picked from the inoculated plates and transferred to 1 ml sterile BHI broth and incubated at 30℃ on a shaking platform set at 200 rpm for 12 h. Two hundred microliters of broth of each bacterium was then inoculated in 50 ml BHI broth and incubated at 30℃ for 34 h with shaking (200 rpm). Ten milliliters of broth of each bacterium was centrifuged at 4500 rpm for 20 min at 4 ℃. The concentrate was washed with 10 ml sterile distilled water and centrifuged again (4500 rpm, 20 min, 4℃). The concentrate was suspended and diluted using sterile distilled water until it reached an OD_{600} value of 1.0. One milliliter of bacteria suspension was added to a filter paper disc (7.5 cm, Ahlstrom, Grade 615) placed on a Petri dish (100 by 15 mm, Fisherbrand) of each treatment.

8.2.4 Mortality Bioassay.

The mortality bioassays contained 8 treatments. Termites pre-fed lufenuron or untreated filter paper were exposed to *P. aeruginosa*, *S. marcescens*, *B. thuringiensis* subsp. *israelensis*, or no bacteria. There were 12 replicates in each treatment (six replicates for each termite colony). From each colony, 25 termites pre-fed lufenuron or untreated filter paper (colony 1: 23 workers and 2 soldiers; colony 2: 25 workers) were released into each Petri dish. Petri dishes were then sealed with Parafilm® and kept in the incubator set at 28℃ in total darkness. The mortality of termites was recorded on day 2, 4, 6, 8, 10, and 12. The behavioral responses of termites to dead termites also were observed and recorded at each time period.

8.2.5 Data Analyses

For the test against each entomopathogenic bacterium, mortality was calculated and compared among termites: (1) pre-fed lufenuron and then exposed to bacteria, (2) only pre-fed lufenuron, (3) only exposed to bacteria, and (4) controls. Analysis of variance (ANOVA) SAS PROC MIXED model (SAS Institute, Cary, NC, 2011) was used to analyze the data and Tukey's honestly significant difference (HSD) test was performed for means comparison. All significant levels were determined at $\alpha = 0.05$.

8.3 Results

8.3.1 Termites Pre-Fed Lufenuron or Untreated Filter Paper

For both colonies, termites fed lufenuron moved slowly, appeared sluggish, and consumed less filter paper at the end of 8 d when compared with termites fed untreated filter paper. The survival rate within a colony between the treatments was similar, but a difference between colonies was evident (colony 1 ≈ 92%, colony 2 ≈ 79%). The colonies were similar in consumption of lufenuron treated paper, but the consumption of untreated filter paper of colony 1 was higher than that of colony 2 (Table 8.1). Because of the colony difference, data were analyzed separately.

Table 8.1. Conditions, consumption and survival rate of 1000 termites pre-fed lufenuron or untreated filter paper for 8 days.

Termite colony	Pre-treatment	Condition	Consumption (g)	Survival rate (%)
Colony 1	lufenuron	sluggish	0.55	91.1
	untreated	active	1.20	93.0
Colony 2	lufenuron	sluggish	0.56	76.1
	untreated	active	2.01	81.9

8.3.2 Termite Mortality in *P. aeruginosa* Test

The termites infected by *P. aeruginosa* assumed a yellow or green color after they succumbed to infection. In general, for both colonies, the mortality of termites pre-fed lufenuron and then exposed to *P. aeruginosa* (LU-PA) was significantly higher than that of termites only exposed to *P. aeruginosa* (PA), only pre-fed lufenuron (LU), or controls. There was no significant difference in mortality among the treatments PA, LU, and controls, with the exception of colony 2 on day 12 (Figure 8.1). The mean mortality of the treatment LU-PA was higher than the additive mortality of the treatments LU and PA for the majority of the observation period (from days 2 to 10), indicating that the combination had a synergistic effect on *C. formosanus* mortality.

8.3.3 Termite Mortality in *S. marcescens* Test

The termites infected by *S. marcescens* assumed a red color after they succumbed to infection. For colony 1, there was no significant difference in mortality among termites pre-fed lufenuron and then exposed to *S. marcescens* (LU-SM), only exposed to *S. marcescens* (SM), only pre-fed lufenuron (LU), and controls in any observation period (Figure 8.2). For colony 2, on day 2 and 4, mortality of the treatment LU-SM was significantly higher than that of the treatment SM or controls. From day 8 to 12, mortality of the treatment LU-SM was significantly higher than the controls, but neither were different from LU or SM. At the end of the experiment, the mean mortality of the treatment LU-SM was 1.5 times higher than that of the treatment SM or LU (Figure 8.2).

8.3.4 Termite Mortality in *B. thuringiensis* Test

For colony 1, no significant difference in mortality was detected among termites pre-fed lufenuron and then exposed to *B. thuringiensis* subsp. *israelensis* (LU-BTI), only exposed to

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B. thuringiensis subsp. *israelensis* (BTI), only pre-fed lufenuron (LU), and controls in any

observation period (Figure 8.3). For colony 2, on day 2 and 4, mortality of the treatment LU was

significantly higher than that of the treatment BTI. There was no significant difference in

mortality among the 4 treatments from day 6 to 10. On day 12, mortality of the treatment LU-

BTI was significantly higher than controls ($F_{3, 19} = 3.19$, $P = 0.0291$), but neither were different

from the treatment LU or BTI (Figure 8.3).

8.3.5 Carcass-Burying Behavior

For both colonies, in the treatments PA, SM, BTI and controls (termites not pre-fed

lufenuron), all dead termites were covered by paper pellets. In treatments LU-PA, LU-SM, LU-

BTI and LU (termites pre-fed lufenuron), no dead termites were covered by paper pellets.

8.4 Discussion

Pesticide effects on insect immunity have drawn much attention as a result of recent

investigations into colony collapse disorder (CCD) in honey bees. Exposed to sublethal doses of

fipronil, thiacloprid, or imidacloprid, honey bees were more susceptible to the gut pathogen

Nosema (Alaux et al. 2010, Vidau et al. 2011, Aufauvre et al. 2012, Pettis et al. 2012). In a study

aimed to determine the interaction of pesticide and microbial pathogens, Islam et al. (2010)

reported that the combination of a botanical pesticide, neem, and an insect pathogenic fungus,

Beauveria bassiana (Balsamo) Vuill, led to significantly higher mortality of the sweetpotato

whitefly, *Bemisia tabaci* Gennadius. Shapiro-Ilan et al. (2011) studied the interaction between

commercial pesticides (carbaryl or cypermethrin) and microbial agents (*Steinernema*

carpocapsae Weiser or *B. bassiana*) for the control of the pecan weevil, *Curculio caryae* Horn.

Their results showed that some combinations could be an effective control method. In the present

study, for both tested termite colonies, lufenuron significantly increased the virulence of *P.*

aeruginosa and the combination effect was synergistic. However, the interaction of lufenuron and *S. marcescens* or *B. thuringiensis* subsp. *israelensis* was not as strong as the interaction of lufenuron and *P. aeruginosa*, since higher mortality was only observed in one of the colonies.
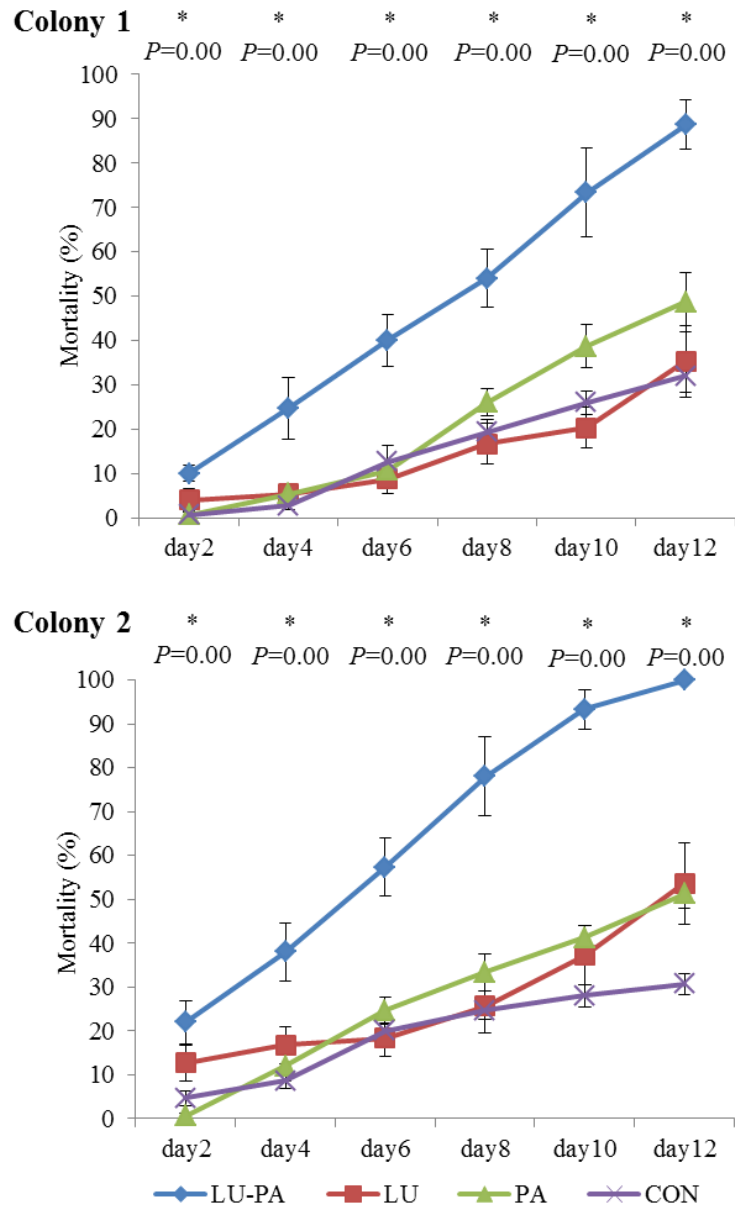
Figure 8.1. Daily mortality (mean ±SEM) of termites: (1) pre-fed lufenuron and then exposed to *P. aeruginosa* (LU-PA), (2) only pre-fed lufenuron (LU), (3) only exposed to *P. aeruginosa* (PA), and (4) controls. * represents significant difference among four treatments within each time period ($P < 0.05$).

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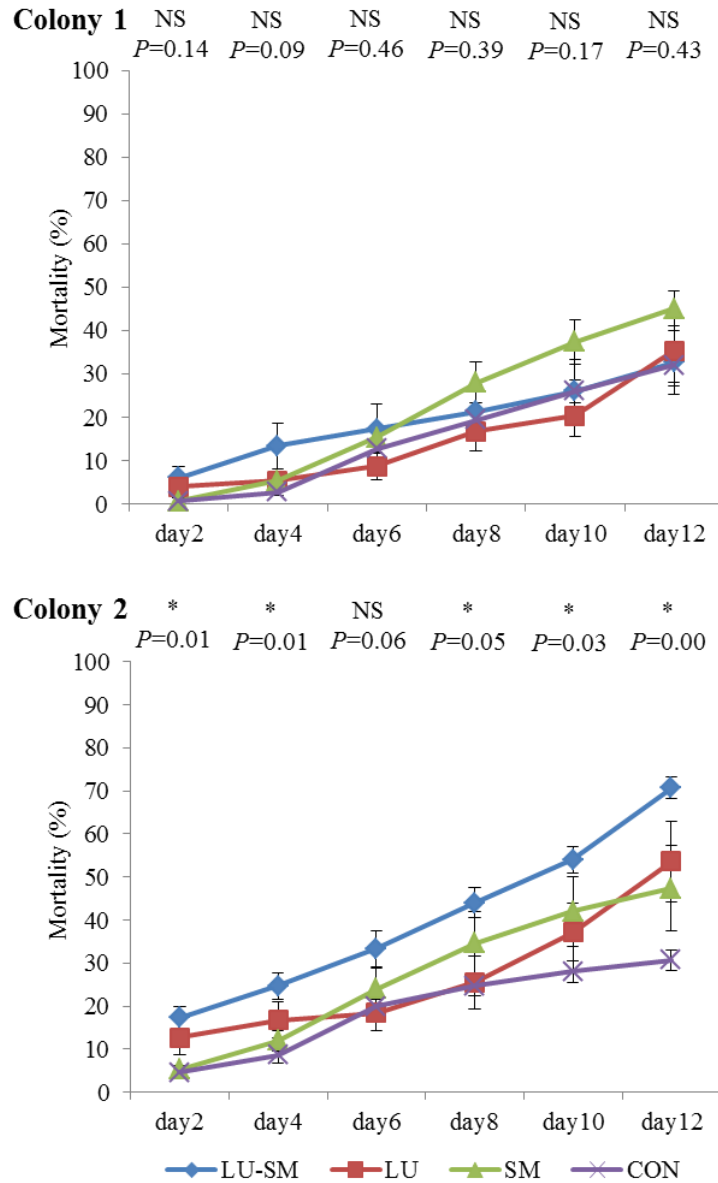
Figure 8.2. Daily mortality (mean ±SEM) of termites: (1) pre-fed lufenuron and then exposed to *S. marcescens* (LU-SM), (2) only pre-fed lufenuron (LU), (3) only exposed to *S. marcescens* (SM), and (4) controls. * represents significant difference among four treatments within each time period ($P < 0.05$); NS represents no significant difference among four treatments within each time period ($P > 0.05$).
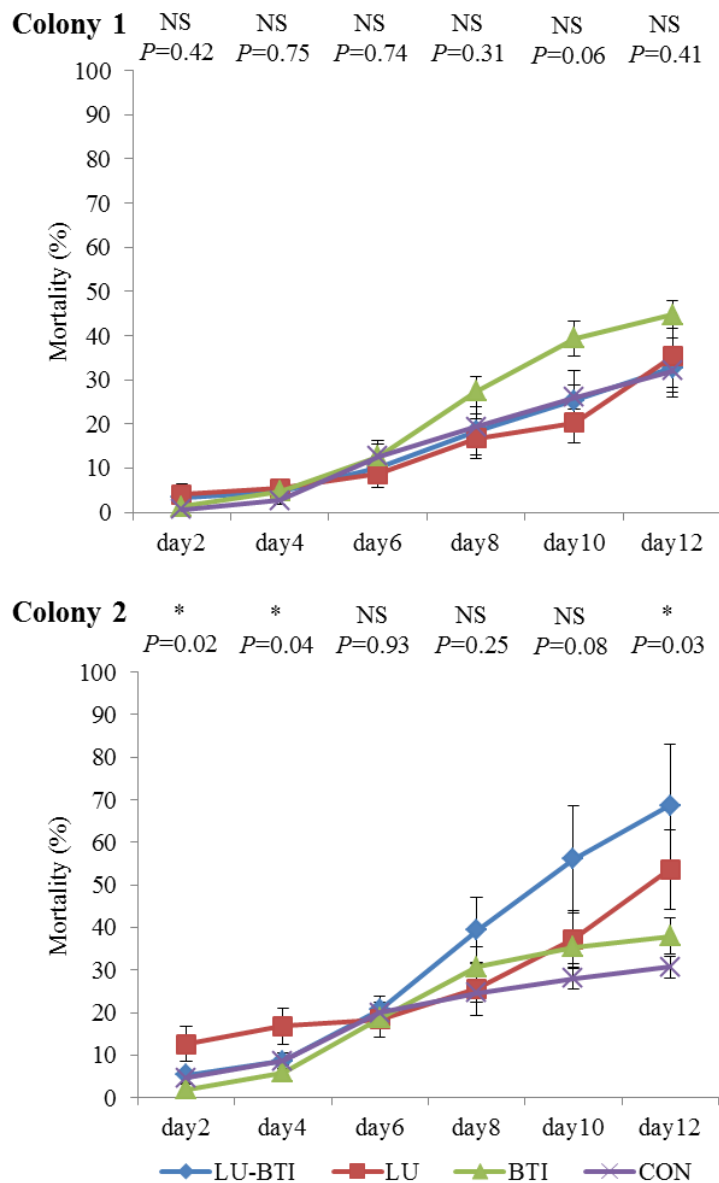
Figure 8.3. Daily mortality (mean ±SEM) of termites: (1) pre-fed lufenuron and then exposed to *B. thuringiensis* subsp. *israelensis* (LU-BTI), (2) only pre-fed lufenuron (LU), (3) only exposed to *B. thuringiensis* subsp. *israelensis* (BTI), and (4) controls. * represents significant difference among four treatments within each time period ($P < 0.05$); NS represents no significant difference among four treatments within each time period ($P > 0.05$).

Unlike vertebrates, insect immune systems lack an acquired immune response (Vilmos and Kurucz 1998, Schmidt et al. 2008). Cuticle, cellular responses, and humoral responses are major aspects of insect immunity (Vilmos and Kurucz 1998, James and Xu 2011). Some

enzymes, such as glutathione S-transferase (GST) and superoxide dismutase (SOD), which have multiple functions in metabolism, also play important roles in insect immune responses (Turrens 2003, Molina-Cruz et al. 2008, Huang et al. 2011, James and Xu 2011). It is known that organophospates, organochlorines and some botanical pesticides can suppress cellular responses by changing the viability and amount of hemocytes (Azambuja et al. 1991, Sharma et al. 2003, 2008, George and Ambrose 2004, James and Xu 2011, Koodalingam et al. 2013). A variety of synthetic pesticides also can reduce the activity of GST and SOD, thus increasing stresses on insect immunity (Büyükgüzel 2009, Wu et al. 2009, James and Xu 2011). Fewer studies on the effect of pesticides on cuticle and humoral responses exist (James and Xu 2011).

Lufenuron blocks the formation of the exoskeleton, thus may negatively affects the ability of termite cuticle to operate as a barrier against microbial pathogens. Merzendorfer (2013) stated that some chitin synthesis inhibitors can inhibit the formation of the peritrophic membrane of insects. Lufenuron may suppress disease resistance of termites by disrupting the function of peritrophic membrane that prevents microbial infection in the midgut. Zhu et al. (2012) reported that hexaflumuron, another chitin synthesis inhibitor, interferes with the balance of hemolymph compounds in the cutworm, *Spodoptera litura* Fabricius. Lufenuron may also influence cellular responses, humoral responses and metabolic enzymes associated with termite immunity, though the mechanisms of these processes are largely unknown.

Social behavior is important in effective disease defense of termites. To prevent infection, termites will often isolate dead cohorts into "quarantined barriers" made up of soil and fecal pellets (Logan et al. 1990, Rosengaus et al. 2011, Yu et al. 2012). Rosengaus et al. (1998), and Chouvenc et al. (2008, 2009) reported that chemical constituents of fecal pellets of *Zootermopsis angusticollis* Hagen and *Reticulitermes flavipes* (Kollar) play a role in inhibiting

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spore germination of the fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin. Neoh et al. (2012) closely studied the carcass-burying behavior of four termite species including *C. formosanus*. They found that, for *C. formosanus*, carcasses were actively detected, dragged and buried. In our study, when exposed to three microbial pathogens, termites pre-fed lufenuron did not show active carcass-burying behavior of dead cohorts. Other behavioral patterns that contribute to disease resistance of termites include self- and allogrooming. Chouvenc et al. (2009) found that, to prevent fungus infection, *R. flavipes* workers groom and ingest large amount of conidia of *M. anisopliae*.

The strong immune responses and pathogen defense behaviors of *C. formosanus* greatly decrease the risk of microbial infection, though it lives in humid environments that favor the growth of various pathogens (Husseneder et al. 2010). For the same reason, Chouvenc et al. (2011) believe it is improbable that biological control of termites will ever see the light of day. A few efforts have been taken to suppress the disease resistance of termites. For example, Bayer Corporation claims that one of its products, Premise Plus Nature ^{TM}, can make termites more susceptible to fungal infection by suppressing their grooming behavior (www.pctonline.com/Article.aspx?article_id=39807). Connick et al. (2001) reported that some immune inhibitors such as dexamethasone, ibuprofen, and ibuprofen sodium salt can significantly increase the mortality of *C. formosanus* exposed to *S. marcescens*. Bulmer et al. (2009) found that D-δ-gluconolactone, a nontoxic molecule derived from glucose, can block the β(1,3)-glucanase effector activity of termite Gram-negative bacteria binding protein-2 (tGNBP-2) and accelerate mortality of *Nasutitermes corniger* (Motschulsky) caused by the infection of *M. anisopliae*, *Serratia* sp. and *Pseudomonas* sp.. Hamilton and Bulmer (2012) used double-stranded RNA (dsRNA) to knockdown the expression of two antifungal defense genes of *R.*

flavipes, termicin and GNBP-2, and observed a decrease in cuticular antifungal activity. We believe that lufenuron has an advantage in suppressing termite resistance to microbial pathogens since its potential as a bait toxicant has been well studied.

Lufenuron also has a potential for the development of a combination bait with *P. aeruginosa.* The value to combine chemical termiticides and biological control agents has been discussed by Grace (2003), Lenz (2005), and Woodrow and Grace (2008). However, Chouvenc et al. (2011) expressed a concern about the "unrealistic optimism" of such potential based on data collected from "bioassays with poor biological relevancy". In our study, only one concentration each of lufenuron and bacteria was tested using small groups of termites. Therefore, before drawing a firm conclusion that integration of the two methods (chitin synthesis inhibitors and termite pathogens) can be a successful termite control strategy, more laboratory tests involving various concentration combinations and a bigger termite group size followed by multi-site field tests are needed.

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CHAPTER 9. SUMMARY AND CONCLUSION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a worldwide distributed pest of wooden structures and living plants that cause huge economic loss. Compared to chemical pesticides, biological control may provide a more environmental friendly and persistent method for the control of *C. formosanus*. Many pathogens have been tested previously for their ability against termites under laboratory conditions, and positive results were shown. However, recent studies showed that the strong immune and behavioral responses of termites may limit the application of these termite pathogens. In this Ph.D. research, a series of studies were conducted to understand the termite-pathogen interaction and to develop a feasible biological control strategy.

In the first part of the research, the toxicity of Bt toxins expressed by genetically modified maize to termites was tested. Plant tissues of three commercial planted Bt maize (YieldGard®, Genuity® VT Triple PRO™ and Genuity® SmartStax™) and two non-Bt maize were provided to termites as food. Five food sources including wood blocks and filter paper treated with plant extract as well as leaves, stalks, and roots of maize were tested in the laboratory. The experiment was maintained for two weeks and the survival rate of termites, food consumption, and tunneling behavior were recorded. The results revealed no significant difference in survival rate, food consumption and length of tunnels between termites feeding on Bt and non-Bt maize, indicating that Bt proteins expressed in the three Bt maize technologies did not negatively affect Formosan subterranean termites. However, comparing to wood block and filter paper treatments, termites feeding on maize tissues showed different consumption pattern and tunneling behavior, which suggests that maize stalk is a good candidate for termite bait matrices.

In the following experiments, the consumption and food transfer efficiency of two

commercially used termite bait materials, wood and cardboard, and one potential bait material, maize cob, were evaluated for use against termites in the lab. In the no-choice test, the consumption of wood and cob was similar and significantly more than cardboard. Tunneling under the food sources was similar. In the two-choice test, the consumption was: cob > wood, wood > cardboard, cob = cardboard, and tunneling under these choices was: cob = wood, wood = cardboard, cob > cardboard. In the three-choice test, no significant difference was detected in consumption, but tunnels made under the cob were significantly more than wood and cardboard. Nile blue A was used to study food transfer of bait material among termite cohorts. Dyed cardboard, cob or wood (0.1% Nile blue A) was provided to termites as food. Termites feeding on wood turned blue in significantly greater number at 6h compared to cardboard and cob, but there was no significant difference after 12h. Blue termites feeding on different bait materials were then collected and combined with undyed termites. When undyed (white) termites were placed with blue termites and food (wood block), termites turned blue in the same percentage regardless of original bait material fed on. However, when no food was provided (starvation group), the rate of white termites turning blue was dramatic; in dyed wood treatment, more termites turned blue than that of cardboard, though neither were significantly different from cob. Our study is the first to show that, cob, an otherwise waste product of the food and biofuel industry, is as efficient as wood and cardboard as a termite bait matrix.

In the second part of the research, the susceptibility of *C. formosanus* to MosquitoDunk®, which contain about 10% of the entomopathogenic bacteria, *Bacillus thuringiensis* subspecies *israelensis* (*Bti*), was tested. In the no-choice tests, mosquito dunks showed a weak but significant effect on the survival and tunneling of *C. formosanus* as compared to the controls. In choice tests, the mortality of *C. formosanus* was not significantly different between the treatments containing mosquito dunks and the controls.

To further study the *Bt* resistance, *C. formosanus* was tested for its ability to suppress the growth of *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) and *thuringiensis* (*Btt*). Different

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group sizes (50, 25, 10 and no termites [control]) of *C. formosanus* were placed on well-grown *Bti* or *Btt* agar plates. On day 1, the diameters of *Bti* and *Btt* colonies in the three treatments containing termites were significantly smaller than in the controls. The diameters of *Bti* and *Btt* colonies in the 50-termite treatment were significantly smaller than in the 10-termite treatment. However, neither was significantly different from the 25-termite treatment. This group size dependent suppression was even more distinct on day 2. On day 5, inhibitory zones were observed in all three treatments containing termites where *Bti* or *Btt* colonies originally grew. The *Bti* and *Btt* cells from these inhibitory zones regenerated on new plates after transfer from 25- and 10-termite treatments as did the controls, but no regeneration was observed after transfer from 50-termite treatment. Results show that the presence of *C. formosanus* can suppress the growth of *Bti* and *Btt* and the suppression effect enhanced with increased of group size. Moreover, antagonistic tests show that natural bacteria carried by termites play a role in the suppression of *Bti* and *Btt*.

Although the test of mosquito dunk did not show a promising lethal effect on *C. formosanus*, it brought us inspiration to pay attention to delivery method of a termite pathogen. Clay encapsulating of biological control agents has been well studied. In the present study, the biological significance of clay on *C. formosanus* was investigated. Choice tests showed that significantly more termites aggregated in chambers where clay blocks were provided, regardless of colony group, observation period or nutritional condition (fed or starved). No-choice tests showed that clay had no observable effect on survivorship, live or dry biomass, water content, and tunneling activity after 33-35 days. However, clay appeared to significantly decrease filter paper consumption (dry weight loss). Active particle (sand, paper and clay) transport behavior was observed in both choice and no-choice tests. When present, clay was preferentially spread on the substrate, attached to the smooth surfaces of the containers, and used to line sand tunnels.

Our study showed the potential to use clay for termite bait attraction and biological control agent encapsulation.

In the third part of the research, the potential to combine a biological control agent and a chemical pesticide was tested. A laboratory study was conducted to understand the effect of low concentrations of lufenuron, a chitin synthesis inhibitor, on termite physiology and behavior. Survivorship, running speed, body water content, food consumption, tunneling, microbial infection, and two behavioral patterns (carcass burying behavior and particle transport behavior) were compared among *C. formosanus* fed lufenuron-treated (250, 500 or 1000 ppm) or untreated (control) filter paper. In 30-32 days, all lufenuron treatments significantly reduced survivorship, running speed, consumption and tunneling, but had no substantial effect on body water content. In addition, termites fed the three concentrations of lufenuron became infected by opportunistic pathogens. Carcass burying and particle transport behaviors also were inhibited by lufenuron.

In the following experiments, *C. formosanus* previously fed lufenuron (1000 ppm) was exposed to each of the three entomopathogenic bacteria, *Pseudomonas aeruginosa* (Schroeter) Migula*, Serratia marcescens* Bizio*,* and *B. thuringiensis* subsp. *israelensis*. We found that termite mortality was significantly higher and synergistic in the combination of lufenuron and *P. aeruginosa* compared to treatment of lufenuron or *P. aeruginosa* alone. Other bacteria and lufenuron combinations were not quite as effective. Interestingly, only in treatments without lufenuron did termites show carcass-burying behavior. The results indicate that lufenuron, a chitin synthesis inhibitor, can suppress Formosan subterranean termite resistance to *P. aeruginosa*. To combine lufenuron and a termite pathogen may bring a successful IPM strategy for the control of termites.

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THE VITA

Cai Wang was born and raised in Wuhan, Hubei, P. R. China. Ever since he was a young boy, Cai had a deep love in observing animals and plants. Also, he had enriched collections in coins, mineral specimens and fossils. In 2002, he won first prize in a national Biology Olympiad Competition for high school students in China. In the next year, he was recommended for admission to the Department of Biology, Huazhong University of Science and Technology. From 2003 to 2007, he worked as an undergraduate research volunteer in a mosquito biological control program. Meanwhile, he took credit courses in Psychology at Huazhong Normal University. In 2007, he graduated with double bachelor's degrees in Biotechnology and Psychology. In the same year, he enrolled in the Institute of Hydrobiology, Chinese Academy of Science, under the supervision of Drs. Li Zhou and Jianfang Gui, and received a master's degree in Genetics in August, 2010. Soon after, he came to the United States and became a Ph.D. student in the Department of Entomology, Louisiana State University, under the supervision of Dr. Gregg Henderson. During his Ph.D. study, he worked on different aspects of Formosan subterranean termites, such as biological control, toxicology, behavior, ecology, and termite-microbe interaction.