Survival Rate, Food Consumption, and Tunneling of the Formosan Subterranean Termite (Isoptera: Rhinotermitidae) Feeding on Bt and non-Bt Maize

by

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ABSTRACT

Although several termite species were reported to be susceptible to some *Bacillus thuringiensis* (Bt) subspecies, no research has been conduced to evaluate the possible non-target effect of genetically modified (GM) Bt crops on termites. In this study, plant tissues of three commercial planted Bt maize (YieldGard* Corn Borer, Genuity* VT Triple PRO™ and Genuit[y]* SmartStax™) and two non-Bt maize hybrids were provided to Formosan subterranean termite, *Coptotermes formosanus*, as food. Five food sources including wood blocks and filter paper treated with maize leaf 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 differences in survival rate, food consumption and length of tunnels between termites feeding on Bt and non-Bt maize planting materials, indicating that Bt proteins expressed in the three Bt maize products did not negatively affect *C. formosanus*. However, compared to wood block and filter paper treatments, termites feeding on maize tissues showed different consumption pattern and tunneling behavior. Our study also suggests that maize stalk is a good candidate for termite bait matrices.

Key words: *Coptotermes formosanus,* GM Bt maize, non-target effect, consumption behavior, tunneling

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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 for 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 found targets to 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, Hernández-Rodríguez et al. 2009, Bravo et al. 2011). With the advance of modern molecular technology, some Cry and 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 for years (Zhang et al. 1995, Li et al. 2001, Henderson 2008, Zhang et al. 2009). In this study, Formosan subterranean termite, *Coptotermes formosanus*, 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.

MATERIALS AND METHODS

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.

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™ traits and DKC 61-21 possessing Genuity* SmartStax™ 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™ 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™ corn contains all Bt genes expressed in Genuity* VT Triple Pro™ plus Cry1F targeting lepidopteran species and Cry34Ab1/Cry35Ab1 targeting rootworms (Monsanto, 2012). Genuity* VT Triple PRO™ and SmartStax™ 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, 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 °C 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.

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.

Substrate and Bioassay Arena

Autoclaved (121°C 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.

Wood Block and Filter Paper

Wood blocks (1.9×1.9×0.9cm southern yellow pine) were autoclaved (121°C, 15min) and dried in an oven dryer (45 °C, 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.

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.

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 ° C) 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 °C, 1d), the weight of wood blocks and filter paper 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.

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 α=0.05.

RESULTS

Survival Rate

The mean survival rates of the two controls at 14 d were 89.6% (wood block) and 85.4% (filter paper) (Table 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 on
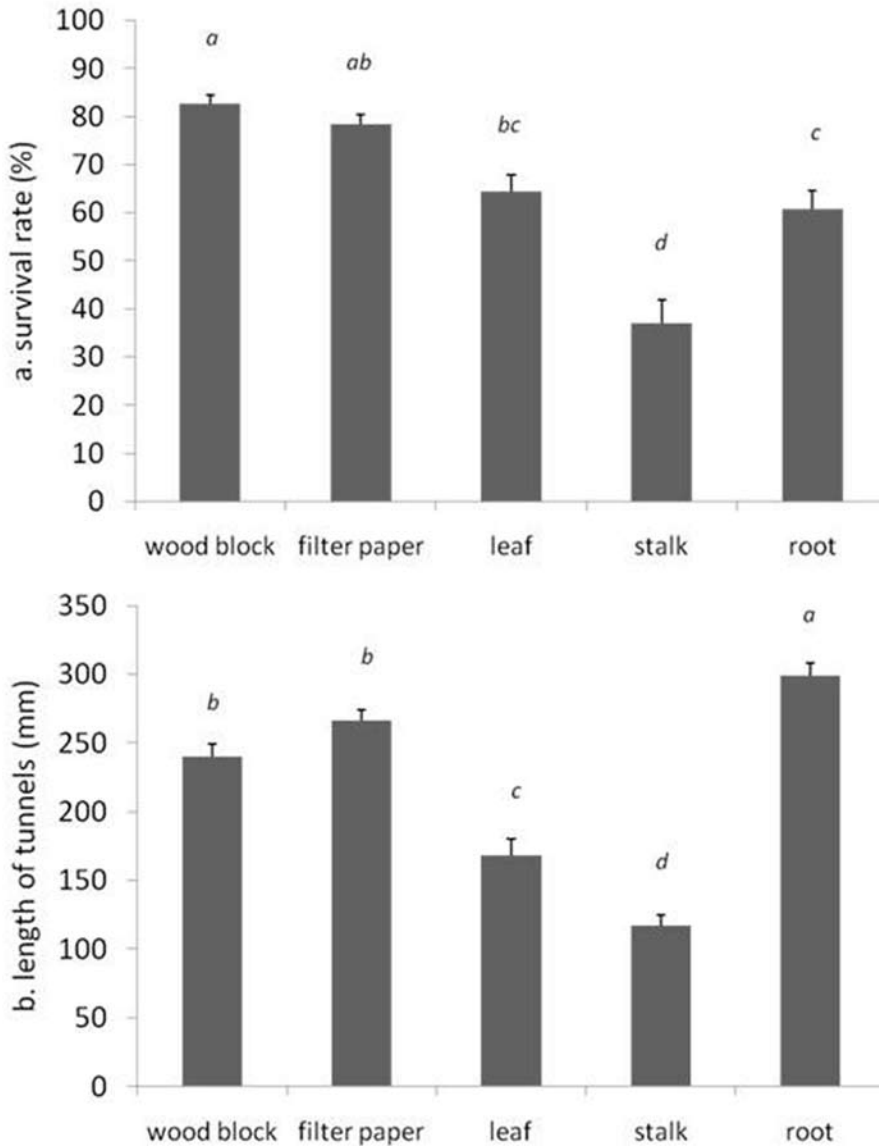
Fig.1 (A) Mean survival rate ND (B) mean tunnel length of Coptotermes formosanus on diferemnt food sources across Bt and nd non-Bt maize hybrids. Mean v alues followed by the same letter are notsignkficantly different (P>0.05)

Table 1: Mean survival rate (±SE), food consumption (±SE), and tunnel length (±SE) of *C. formosanus* 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	-

wood bock was 81.2%, which was significantly greater ($P < 0.05$) than that observed for those feeding on maize leaf tissue (64.3%) or root (60.7%), but it was not significantly different compared to the survivorship (77.1%) of the
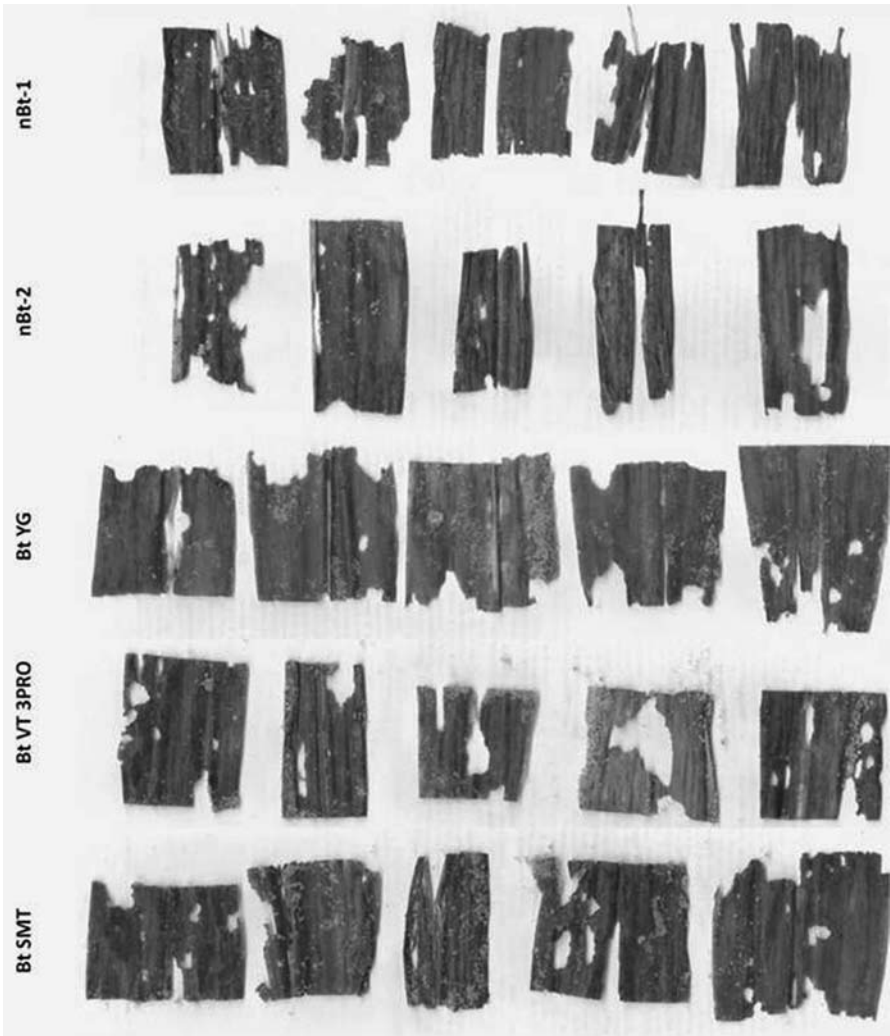

Fig. 2. Consumption pattern of *Coptotermes formosanus* feeding onnon-Bt (on nonBt-1, and Bt maize leaves.

termites 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 (Fig. 1a).

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 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 ($F = 0.42$;
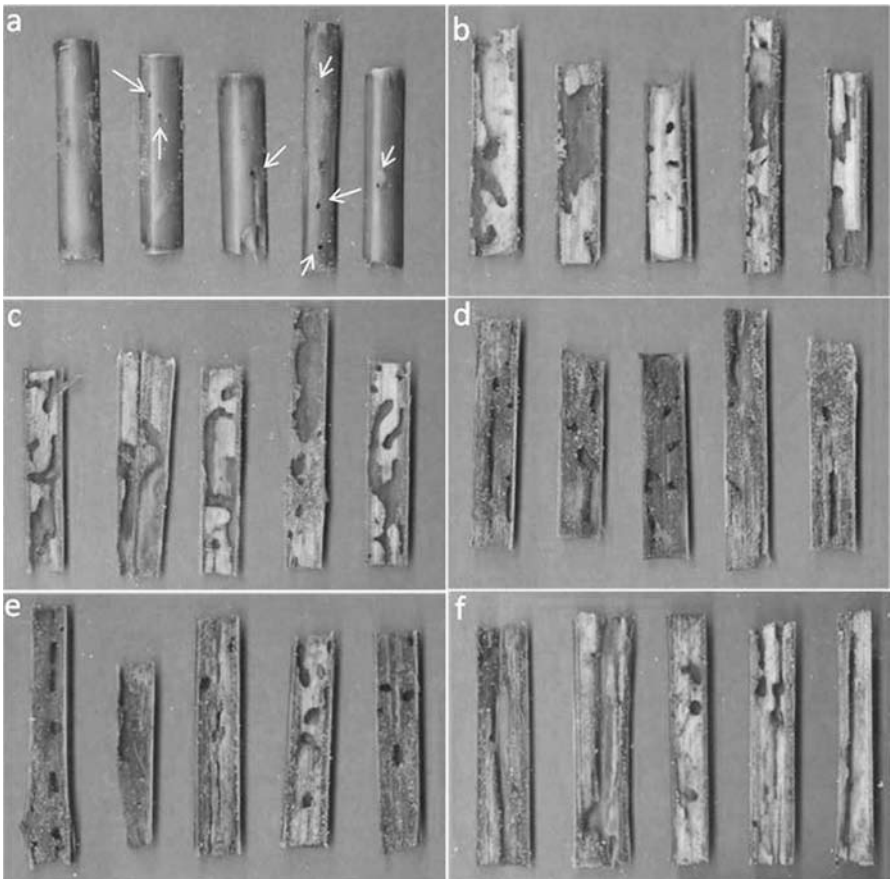

Fig. 3.(a) Holes made by *C, formosanus* pn the srface of split stalks at day 2-3 and the consumption pattern of termites feeding of (b) n-Bt-1, (c) nBt-2, (d) BtYG, (e) BtVT, (e) BtVTPROaand (f) BtSMT maize stalks.

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.

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 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 = 0.6511for

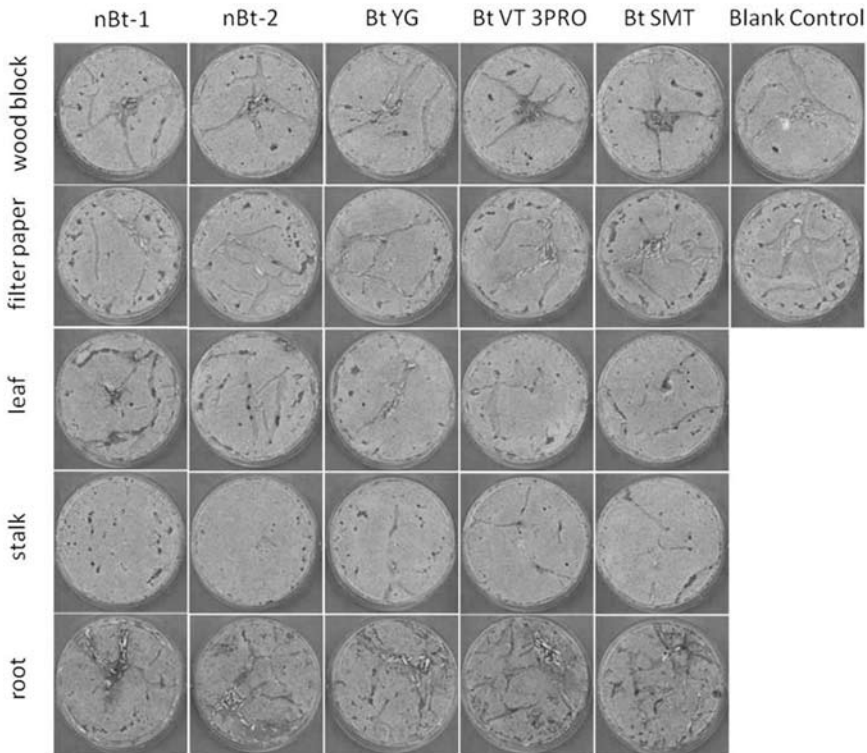

Fig. 4. Tunneling pattern of *C, formosanus* in the bottom sides of Petrie dishes containing food sources withBt and non-Bt plant tissue extracts.

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) (Fig. 1b).

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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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. 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 (Fig. 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.

ACKNOWLEDGMMENTS

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