Developmental and mortality responses of *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) following partial feeding on Bt-transgenic maize

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ABSTRACT

Objective: To determine i) the susceptibility of different instars of *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) to Bt maize (expressing *Cry1Ab*) and ii) the effects of transient feeding on Bt maize plants at the 3rd, 4th and 5th instars on the development of *C. partellus* and *S. calamistis*.

Methodology and Results: First to 5th instars of *C. partellus* and *S. calamistis* were reared on non-Bt vs. Bt plant material for five days and larval mortality assessed. For the development study, 3rd, 4th and 5th instars of *C. partellus* and *S. calamistis* were fed on Bt maize stems for 24h, then reared on non-Bt maize stems, and development monitored. Controls were fed on non-Bt maize only. The 1st instars were more susceptible to the Bt plants than the later instar stages. The duration of most stem borer instars were significantly prolonged at the instar at which feeding on Bt maize took place.

Conclusion and application of findings: Third to 5th stem borer instars, which are the preferred hosts of larval parasitoids, are less susceptible to Bt delta endotoxins. It is therefore likely that larval parasitoids would be exposed to Bt toxins via their hosts. The prolongation of development time of the insect pests as a result of feeding on Bt maize could result in increased attack rates by parasitoids due to increase in the 'window of vulnerability', the period during which the host is exposed to natural enemies. The combined effects of developmental delays may also result in temporal asynchrony of stem borer moths produced from Bt and non-Bt maize, a factor that would need to be considered in designing resistance management strategies.

Key words: *Bacillus thuringiensis*, *Cry1Ab*, environmental biosafety, natural enemies, GM maize, stem borers

INTRODUCTION

Crops modified to produce insecticidal proteins derived from *Bacillus thuringiensis* Berliner (Bt) are among the first genetically engineered crops (Duan *et al.*, 2002). Maize, *Zea mays* (L.), genetically modified to express the *Cry1Ab* gene from *B. thuringiensis* ssp. *kurstaki* strain HD-1, was the first transgenic crop to be commercialised in 1996 (EPA, 2000). Although transgenic maize was initially developed to control two crambid stem borers in North America, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae) (Archer *et al.*, 2001) and *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae) (Ostlie *et al.*, 1997), it has the potential to control other lepidopteran pests including *Pseudaletia unipunctata* Haworth (Lepidoptera: Noctuidae), *Helicoverpa zeae* Boddie (Lepidoptera: Noctuidae) (Sims *et al.*, 1996; Pilcher *et al.*, 1997) and *Papaipema nebris* Guenee (Lepidoptera: Noctuidae) (Binning & Rice, 2002). Bt maize is currently under limited commercial cultivation (in South Africa only, James, 2008) but under extensive research in other African countries, where the typical maize pests are different from those common in North America. Knowledge about the effect of Bt maize on such pest species is limited (Tende *et al.*, 2007).

The impact of Bt transgenic plants on non-target organisms, such as natural enemies (parasitoids and predators) is of concern (Wolfenbarger & Phifer, 2000; Lovei, 2001), especially in developing countries, where natural pest control is more important than in developed countries (Mertz *et al.*, 2007). Knowledge on the effect of Bt toxins on development of herbivorous insects is also important because this could have a direct effect on natural enemies by influencing the 'window of vulnerability', the period during which the host is exposed to natural enemies (Schuler *et al.*, 1999; Schoenmaker *et al.*, 2001). Furthermore, effects of Bt plants on host development could impact on the biology of a natural enemy developing in such host (Weseloh, 1984; Bourchier, 1991; Walker *et al.*, 2007).

Even though different larval instars of herbivores can have varying susceptibility to the same toxin (James *et al.*, 1993; Liu *et al.*, 1995; Huang *et al.*, 1999), studies on the efficacy of Bt maize have concentrated mostly on exposure of neonates to Bt plant material (Mugo *et al.*, 2004; Tende *et al.*, 2007). However, later instars could be less susceptible to Bt toxins (Huang *et al.*, 1999). Additionally, previous studies have mainly used sublethal Bt toxin concentrations with continuous, rather than partial exposure (Eizaguirre *et al.*, 2005). In reality, susceptible insects exposed continuously to Bt plants invariably suffer complete mortality; only those insects subjected to partial feeding on the Bt plants have high chances of survival (Obonyo *et al.*, unpublished data). Continuous exposure to sublethal Bt toxin concentrations therefore does not capture the actual situation in the field. Due to resistance management practices (Ives & Andow, 2002), patches of non-transgenic maize plants coexist alongside genetically modified (GM) plants. Several maize herbivores, such as stem borer larvae, especially later instars, can move between host plants (Ingram, 1958; Berger, 1992), for example from Bt to non-Bt maize plants (and vice versa).

Previous studies have primarily focused on the reduction of damage caused by pests on transgenic plants without much consideration of the effects on insect development. The few studies conducted on the effect of Bt crops on lepidopteran development (Pedersen *et al.*, 1997; Erb *et al.*, 2001; Huang *et al.*, 2005) have all used artificial diets. No study has reported using whole GM plants as food for the developing herbivores. In the studies with artificial diet, Bt was added as a microbial preparation, which contains protoxins and must be activated by midgut enzymes to become toxic to the target insects (Gill *et al.*, 1992; Bauer, 1995). This is a different exposure method from that provided by transgenic plants, which produce the active toxin (National Research Council, 2002). Direct exposure of lepidopteran pests to the transgenic plants would give more

accurate information regarding the effects of such plants on pest development.

The aim of this investigation was therefore to determine i) the susceptibility of different instars of *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) to Bt maize (expressing *Cry1Ab*) and ii) the effects of partial feeding on Bt maize plants at the 3rd, 4th and 5th instars on the development of *C. partellus* and *S. calamistis* stem borers.

MATERIALS AND METHODS

Insect species: *Chilo partellus* is a stem borer species of Asian origin (Zhou *et al.*, 2001), first reported in Africa from Malawi (Tams, 1932). Since then, it has spread to nearly all countries in eastern and southern Africa, with the first reports in Kenya in the early 1950's (Nye, 1960). It has spread throughout the maize growing areas of Kenya at elevations below 1500m (Overholt *et al.*, 1994; Zhou *et al.*, 2001; Songa *et al.*, 2002), and has become the most widely distributed, abundant and often the most damaging stem borer species (Songa, 1999). In some locations, it may even be displacing indigenous stem borer species (Ofomata *et al.*, 2003).

Sesamia calamistis is a native African stem borer species that occurs in low densities in all areas up to 2400m above sea level (Muhammad & Underwood, 2004). Little is known on the effects of transgenic maize on the development of either of these stem borer species.

Chilo partellus and *S. calamistis* eggs at the blackhead stage (the stage at which the eggs develop a black centre about one day before hatching) were obtained from the Animal Rearing and Quarantine Unit at the International Centre of Insect Physiology and Ecology (ICIPE), Kenya. These eggs originated from colonies maintained on artificial diet according to the procedure of Ochieng *et al.* (1985). The eggs were then incubated in jam jars in the laboratory at the National Agricultural Research Laboratories of the Kenya Agricultural Research Institute (KARI), Kabete, Nairobi, at 25±1°C and 12/12 h; light/dark photoperiod. Large numbers of larvae were reared in synchrony on an artificial diet according to the procedure of Ochieng *et al.* (1985) at 25±1°C and 12/12h; light/dark photoperiod, to provide adequate numbers of larvae for the tests. First instars were less than 12h old when the bioassay for this stage was conducted.

Plant material: Plant material was obtained from the Bt maize line, Event 216 (described in Obonyo *et al.*, 2008). The isogenic line, CML 216 (Mugo *et al.*, 2005), was used as a non-transgenic control in the experiments. Before use, pieces of plant material were washed in a 2% solution of commercially available bleach (0.05% sodium hypochlorite) to kill any microbial contaminants originating from the greenhouse, rinsed in distilled water and then dried.

Bioassays

Susceptibility of stem borer instars to Bt maize: Thirty (30) stem borer larvae of each instar (1st to 5th) for *C. partellus* and *S. calamistis* were fed on non-Bt or Bt plant material in moistened filter paper lined Petri dishes in the laboratory. First to 3rd instars were fed on leaf material while later instars were fed on stems. The larvae were transferred individually onto the plant material, using a camel-hair brush to handle sensitive 1st to 3rd instars and fine forceps for older ones, and covered with other leaf pieces, where leaf material was used. Leaf pieces were changed every 2 days, when the filter papers were also re-moistened with distilled water. Larval mortality was assessed on the 5th day after treatment. The experiment was replicated four times.

Effect of Bt maize on stem borer development: Depending on the availability of test insects, 20 - 30 each of 3rd, 4th and 5th instars of *C. partellus* and *S. calamistis* were starved for 24h before placing them individually on Bt maize stems for 24h, and on non-Bt maize for the control group. After this exposure, larvae were reared on non-Bt maize stems in moistened filter paper lined Petri dishes in the laboratory at 25±1°C and 12/12 h; light/dark photoperiod. Mortality of the larvae and stage specific larval development were observed and recorded daily until the emergence of adults. Larval development was monitored by checking for the presence of head capsules (which are indicative of moulting to the subsequent instar) in the Petri dishes.

Statistical analysis: To remove the effects of extraneous factors, mortality of the larvae exposed to Bt was corrected based on control mortality (Abbott, 1925). Analysis of variance was used to compare the

mortality of larvae as well as development time, for each of the stem borer species exposed to Bt maize and those reared exclusively on non-Bt maize. When significant differences were observed at P< 0.05, Student Newman Keul's (SNK) test was used to identify the means that were different from each other. Where significant differences in development times between the male and female stem borers were not observed,

the data were pooled for analysis. Instars with less than 5 larvae were not subjected to analysis for instar duration but were, however, considered in determining the treatment effects on overall larval development duration. Mortality data were arcsine transformed prior to analysis (Gomez & Gomez, 1984), in order to stabilise the variances.

RESULTS

Susceptibility of stem borer instars to Bt maize: For both insect pests, the 1st instars were more susceptible (F=3.2; df= 4, 8; P=0.042 for *C. partellus* and F=14.2; df= 4, 8; P=0.001 for *S. calamistis*) to the Bt plants compared to later instars. There were, however, no

significant differences in larval mortality between the 2nd to 5th instars (Table 1). *Chilo partellus* and *S. calamistis* background mortalities on non-Bt maize were 5.6 and 6.3%, respectively.

Table 1: Mortality (%)[1]of *Chilo partellus* and *Sesamia calamistis* instars after feeding on Bt maize for 5 days.

Stem borer instar	Mortality (%) of	
	Chilo partellus	*Sesamia calamistis*
1st	81.9 (67.4)a	87.7(70.8)a
2nd	25.6 (28.4)b	5.6 (12.8)b
3rd	28.0 (30.7)b	6.7 (14.0)b
4th	14.9 (21.8)b	21.3(26.7)b
5th	9.2 (17.1)b	15.7(19.9)b

[1]Corrected for control mortality; Means of arc sine transformed values in parenthesis. Means for each stem borer species followed by the same letter are not significantly different at P<0.05 using SNK test.

Table 2: Development time of various developmental stages of *Chilo partellus* and *Sesamia calamistis* fed on Bt maize or non-Bt maize for 24h at the 3rd larval instar.

Species and treatment	Duration of larval instars, days					Duration of pupal stage, days
	3rd	4th	5th	6th	Total	
Chilo partellus						
Control (non-Bt maize)	6.0±0.12a (17)	5.9±0.14a (17)	9.9±0.25a (17)	-	23.2±0.49a (17)	10.5±0.27a (17)
Bt maize	7.3±0.14b (12)	6.3±0.14a (12)	9.5±0.31a (11)	-	25.7±0.37a (11)	9.8±0.233a (11)
Sesamia calamistis						
Control (non-Bt maize)	7.5±0.24a (25)	7.4±0.24a (25)	8.7±0.35a (25)	9.4±0.38a (25)	33.4±0.83a (25)	12.7±0.40a (25)
Bt maize	8.8±0.25b (24)	7.5±0.27a (20)	8.5±0.39a (20)	9.8±0.42a (20)	34.3±0.92a (20)	12.6±0.45a (20)

Data are mean ±SE; Value in parenthesis indicates *n*. Means for each stem borer species in the same column followed by the same letter are not significantly different at P<0.05 using SNK test.

Effect of Bt maize on *Chilo partellus* development: Transient feeding of *C. partellus* on Bt maize at the 3rd and 4th instars significantly delayed their development at the instar at which exposure took place (F=50.6; df=1, 28; P=0.0005 and F=11.0; df=1, 30; P=0.002 for 3rd and 4th instar, respectively) but not on subsequent

developmental stages (Tables 2 and 3). Transient feeding on Bt maize at the 5th instar had no significant effect on the development time in either 5th or 6th larval instars (Table 4). However, the overall larval development time was significantly longer (F=20.9; df=1, 28; P=0.0005) for larvae subjected to transient

feeding on Bt maize as compared to those fed exclusively on non-Bt maize.

For the larvae subjected to transient feeding on Bt maize at the 3rd instar, 36% (4/11) of the larvae which pupated did so at the end of the 5th instar with the rest pupating at the 6th instar. In contrast, most (94%, 16/17) of the larvae reared exclusively on non-Bt maize which pupated did so at the end of their 5th instar.

For the larvae treated at the 4th instar, 37% (7/19) of the larvae exclusively fed on non-Bt maize which pupated did so at the 6th instar with the rest (63%) pupating at the 5th instar. In this treatment, 45% (5/11) of the pupated larvae subjected to transient

feeding on Bt maize did so at the 6th instar while 55% pupated at the 5th instar. For the larvae treated at 5th instar, 37% (7/19) of control larvae which pupated did so at the 6thinstar (with the rest having pupated at 5th instar), while 55% (6/11) of the Bt treated larvae pupated at their 6th instar. Only one of them pupated at their 5th instar, while four had an additional, 7th instar before pupation.

The duration of the pupal stage in larvae exposed to Bt maize at the 3rd and 4th instars was not significantly affected (Tables 2 and 3). Larvae exposed to Bt maize at the 5th instar had a significantly longer pupal duration compared to that of larvae reared on non-Bt maize (Table 4).

Table 3: Development time of various developmental stages of *Chilo partellus* and *Sesamia calamistis* fed on Bt maize or non-Bt maize for 24h at the 4th larval instar.

Species and treatment	Duration of larval instars, days				Duration of pupal stage, days
	4th	5th	6th	Total	
Chilo partellus					
Control (non-Bt maize)	5.8±0.18a (20)	8.5±0.41a (20)	-	20.3±0.82a (20)	10.4±0.24a (19)
Bt maize	6.7±0.24b (11)	7.5±0.55a (11)	-	21.5±1.09a (11)	10.7±0.32a (11)
Sesamia calamistis					
Control (non-Bt maize)	7.4±0.19a (30)	8.5±0.30a (30)	9.6±0.33a (29)	27.3±0.63a (29)	12.6±0.63a (29)
Bt maize	8.5±0.21b (26)	8.2±0.36a (22)	9.3±0.36a (20)	28.0±0.76a (20)	13.1±0.39a (20)

Data are mean ±SE; Value in parenthesis indicates *n.* Means for each stem borer species in the same column followed by the same letter are not significantly different at P<0.05 using SNK test.

Table 4: Development time of various developmental stages of *Chilo partellus* and *Sesamia calamistis* fed on Bt maize vs. non-Bt maize for 24h at the 5th larval instar.

Species & treatment	Duration of development stage, days			
	5th instar	6th instar	Total	Pupa
Chilo partellus				
Control (non-Bt maize)	9.9±0.36a (19)	6.7±0.45a (7)	12.4±0.87a (19)	9.6±0.26a (19)
Bt maize	10.2±0.48a (11)	6.7±0.38a (10)	18.9±1.14b (11)	11.6±0.34b (11)
Sesamia calamistis				
Control (non-Bt maize)	8.2±0.33a (29)	9.7±0.35a (29)	18.2±0.70a (29)	13.1±0.35a (29)
Bt maize	9.7±0.35b (26)	9.5±0.39a (24)	20.8±0.77b (24)	13.4±0.38a (24)

Data are mean ±SE; Value in parenthesis indicates *n.* Means for each stem borer species in the same column followed by the same letter are not significantly different at P<0.05 using SNK test.(Note: some of the *Chilo partellus* larvae pupated at fifth instar hence the lower number of sixth instar larvae compared to the number pupated).

Effect of Bt maize on *Sesamia calamistis* development*:* Transient feeding of *S. calamistis* on Bt maize at the 3rd, 4th and 5th instars significantly delayed their development at the instar in which exposure took place (F=15.2; df=1, 48; P=0.0005; F=15.4; df=1, 55; P=0.0005 and F=10.1, df=1, 55; P=0.003 for 3rd, 4th and 5th instars, respectively) but not on subsequent instars

DISCUSSION

In most lepidopteran species studied, the early instars were more susceptible to Bt toxins than later instars. This has been observed for *Ephestia cautella* Walker (Lepidoptera: Phycitidae), *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) (McGaughey, 1978), *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) (Fast & Regniere, 1984), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) (Hornby & Gardner, 1987), *Plutella xylostella* L. (Lepidoptera: Noctuidae) (Liu *et al.*, 1995) and *O. nubilalis* (Huang *et al.*, 1999). The susceptibility that we observed of different instars of *C. partellus* and *S. calamistis* to Bt maize is consistent with these previous results. This phenomenon could be related to size because later instars are larger and therefore able to physiologically tolerate more toxin (Huang *et al.*, 1999). Our experiments show that later stem borer instars, which are the suitable hosts of larval parasitoids, are less susceptible, as compared to neonates, to Bt delta endotoxins that are present in Bt maize. It is possible therefore that larval parasitoids could be exposed to Bt toxins via their hosts.

Bt toxins influence the development of a number of lepidopteran species (Binning & Rice, 2002; Horner *et al.*, 2003; Eizaguirre *et al.*, 2005), though most of these previous studies have used sublethal toxin concentrations over prolonged periods. However, larvae of the stalk borer *Papaipema nebris* Guenee (Lepidoptera: Noctuidae) are not sensitive to Bt maize (Pilcher *et al.*, 1997). Such inter-species differences could be due to variations in the processing, inactivation or binding affinity of the toxin in the insect midgut (Gill *et al.*, 1992).

In the present study, feeding the larvae on Bt plant tissue at the 3rd and 4th instars significantly lengthened the duration of the respective instar but not the subsequent ones while overall larval development time was not affected. Schoenmaker *et al.* (2001) suggested that the ingestion by lepidopteran larvae of sublethal doses of Bt toxin prolonged development time by temporarily inhibiting feeding. Continuous exposure

(Tables 2, 3 and 4). Regardless of the treatment, most of the *S. calamistis* larvae pupated at the end of the 6th instar with only 4% (2/49) of the larvae going into a 7th instar. However, both of these were larvae subjected to transient feeding on Bt maize. *Sesamia calamistis* pupal duration was not significantly affected by transient feeding on Bt maize (Table 2, 3 and 4).

to Bt toxin prolonged development of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) while exposure to toxin for shortened durations had no significant effects on larval development time (Dutton *et al.*, 2005). It therefore seems that larvae can recover from the effects of the Bt toxin, following transient exposure. Other lepidopteran larvae that ingest sublethal doses of Bt also resume normal development after a few days (Erb *et al.*, 2001; Siegfried *et al.*, 2001; Moreau & Bauce, 2003).

In our study (unlike previous studies), the larvae were exposed to Bt for a relatively short duration. Dutton *et al.* (2005) reported that when 3rd instars of *S. littoralis* larvae were exposed to Bt sprayed plants (such that the effect of the toxin does not persist for long, since the Bt spray is quickly degraded, Haddad *et al.*, 2005) there were no significant effects on overall larval development as compared to the significant effects noted when plants were reared for four days on Bt maize, such that the effect of the toxin was more persistent. Huang *et al.* (2006) observed larval development inhibition of *O. nubilalis*, *D. grandiosella* and *Diatraea saccharalis* F. (Lepidoptera: Pyralidae) fed on a diet prepared from *Cry1Ab* protein extracted from Bt corn leaves. Similarly, transgenic maize containing *Cry1Ab* delayed larval development of *H. zeae* (Stewart *et al.*, 2001; Horner *et al.*, 2003) and *Danaus plexippus* L. (Lepidoptera: Danainae) (Dively *et al.*, 2004).

Development time of the 5th instar for *C. partellus* larvae subjected to transient feeding on Bt maize at the same growth stage was not affected, possibly because pupation follows shortly after the 5th larval stage in this species at which time the larvae are relatively inactive and do not feed much as they clear up their guts in preparation for pupation (Tettamanti *et al.*, 2007). In addition, their large sizes enable them to tolerate more toxin (Huang *et al.*, 1999). Overall, larval development time in these larvae was significantly longer as a consequence of Bt exposure. This indicates

a disturbance to the "normal" development cycle, from which the larvae may eventually recover.

Sesamia calamistis generally went through more larval instars than *C. partellus* and (unlike *C. partellus*) was still actively feeding in its 5th instar, causing significant effects of transient feeding on Bt maize on development time of 5th instar *S. calamistis* larvae. Intraspecific variation in instar numbers was also noted in both species. Food quality can cause plasticity in instar number in many insect species, and under adverse conditions the number of instars tends to increase (Esperk *et al.*, 2007).

Pupal duration was not affected by transient feeding on Bt maize except for *C. partellus* exposed to Bt-maize at the 5th instar, which had longer pupal duration than the control larvae. This lack of effect on the pupal period was also previously observed on *H. zeae* (Horner *et al.*, 2003).

An extended developmental period could increase the 'window of vulnerability' during which larvae or pupae are exposed to parasitoids or predators. This was observed by Wallner *et al.* (1983) for gypsy moth larvae and Mascarenhas and Luttrell (1997) for *H. zeae*. Also, the combined effects of developmental delays may result in temporal asynchrony of moths emerging from Bt and non-Bt maize (Horner *et al.*, 2003). Current resistance management strategies using refuges are based on the assumption that susceptible individuals from the refuges mate randomly with the population under selection pressure by Bt toxin (Caprio, 1998).

Developmental delays caused by feeding on Bt maize and asynchrony of adult emergence could result in susceptible individuals mating before resistant adults emerge, thus potentially weakening this management option (Horner *et al.*, 2003). However, there are various alternative stem borer host plants (Khan *et al.*, 1997), which could be used to delay emergence of susceptible stem borer moths and hence enhance the chances of mating between resistant and susceptible individuals (Mulaa *et al.*, 2007). It would however be necessary to investigate the possible effects of Bt maize on the development of resistant larvae to assess if such an asynchrony occurs. Field assessments are also required to determine how strong the effect of Bt maize, reported here from laboratory experiments, would be on *C. partellus* and *S. calamistis* development under field conditions.

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