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Screening for development of resistance by the spotted stem borer, *Chilo Partellus* Swinhoe(Lepidoptera: Pyralidae) to Bt-maize delta-endotoxins

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Abstract Stem borers are one of the major limiting factors to maize *(Zea mays* L.) production in the world. In Kenya the damage caused by stem borers leads to 13.5% yield loss estimated to be 400,000 MT of maize annually. The spotted stem borer *Chilo partellus,* Swinhoe is one of the major species of stem borers in Kenya. Bt-maize has been proved to reduce losses due to stem borer damage. Development of insect resistance among stem borers is one of the concerns of using Bt-maize. A study was conducted at the KARI Biosafety Greenhouse level 11, to determine the development of stemborer resistance to two Bt cry proteins for over four generation cycles of selection. The cry proteins were cry1Ab and cry1Ba expressed from Bt-maize event 223 carrying Bt *cry1Ab* gene and event 10 carrying Bt *cry1Ba* gene. Three hundred neonates of *C. partellus* were infested into maize leaves and allowed to feed for 24 hours. The surviving larvae were reared in artificial diet up to adult stage. The performance of each protein was assessed over time by estimation of the number of surviving larvae over each generation. The results showed significantly fewer surviving larvae from the Bt-maize events compared to the non-transgenic CML 216 control. The means were70.4 for CML216, 13.3 and 7.4 for Event 10 and 223 respectively. There were highly significant differences between the control and the two Bt-maize events. The two Bt-maize events were statistically not different in controlling the pest over the studied generations, indicating that there was no development of resistance to cry proteins in the tested *C.partellus* colony.

Key words: Bt-maize, development, generations, Kenya, resistance, stem borers

Résumé Les foreurs de tiges constituent l'un des principaux facteurs limitant la production de maïs (*Zea mays*, L.) dans le monde. Au Kenya, les dommages causés par les foreurs de tiges ont conduit à une perte de 13.5% des récoltes, ce qui est estimé à 400 000 MT de maïs annuellement. Le foreur tacheté de tige, *Chilo partellus,* Swinhoe est l'une des principales espèces de foreurs de tige au Kenya. Il a été démontré que le Maïs-Bt réduit les pertes causées par les dommages du foreur de tige. Le développement de la résistance aux insectes forme l'une des préoccupations quant à l'utilisation de Maïs-Bt. En vue de pouvoir déterminer le développement de la résistance de foreurs de tige face à deux protéines 'cry' Bt pendant plus de quatre cycles de sélection de générations ; les protéines 'cry' étaient cry 1 Ab et cry 1Ba exprimées à partir de Maïs-Bt évènement 223 portant le gène Bt *cry 1 Ab* et évènement 10 portant le gène Bt *cry 1 Ba*. Trois cent neonates de *C. partellus* étaient infestés à travers les feuilles de maïs et abandonnées à développer pendant 24 heures. Les larves survivantes étaient nourries par régime artificiel jusqu'à l'âge adulte. L'action de chaque protéine était évaluée dans le temps par estimation du nombre de larves survivantes après chaque génération. Les résultats ont montré significativement peu de larves survivantes à partir des évènements avec Maïs-Bt par rapport au contrôle non-transgénique 216 CML. Les moyennes étaient 70.4 pour CML 216, 13.3 et 7.4 pour Evènement 10 et 223 respectivement. Il y avait des différences hautement significatives entre le contrôle et les évènements avec Maïs-Bt. Les deux évènements de Maïs-Bt n'étaient pas statistiquement différents dans le contrôle du parasite sur la période étudiée, indiquant qu'il n'y avait pas de développement de résistance aux protéines 'cry' dans les colonies testées de C. partellus.

Mots clés: Maïs-Bt, développement, génération, Kenya, résistance, foreurs de tige

Introduction

Maize is the principal staple food crop produced and consumed by most households in Eastern, Central and Southern Africa. Stem borers are a major constraint to maize production and food security for the majority of maize farmers in Kenya, and are estimated to cause yield losses of between 13-50% here in Kenya (Songa *et al.*, 2001a, Mugo *et al*., 2001). The major species of stem borers found in Kenya are the African stem borer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae), the spotted stem borer *Chilo partellus* Swinhoe (Lepidoptera: Crambidae), the pink stem borer, *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) the sugar cane stem borer *Eldana saccharina* Walker (Lepidoptera:Pyralidae) and the Coastal stem borer, *Chilo orichalcociliellus* Strand (Lepidoptera:Crambidae) (Songa *et al*., 2001a). However, two of these have been documented as being of greater economic importance; *B. fusca* and *C. partellus*.

Stem borers affect maize yields by reducing the photosynthetic area of the leaves. Crop losses also result from death of the growing point, early leaf senescence, reduced translocation, lodging and direct damage to ears. Secondary losses have ben documented due to infections by bacterial and fungal pathogens via entry points created by the stem borers within the plant tissues (Ndiritu, 1999). An up to 100% infestation level, has been recorded, and affects all stages of development of the plant. However infestations occurring at 4-8 leaf-stages have been found to cause greater damage because it can kill the growing point and lead to complete death of the plant.

The control measures employed mainly consist of chemical, biological, cultural and host plant resistance. Chemical control methods are most effective, but are expensive to the farmer, pause health risks to both the farmer and livestock, affect non-target organisms like bees and degrade the environment. Biological control methods are effective. However these methods are time consuming and the effects are felt in the long run, a time when most damage has already been done. Cultural methods of control methods reduce infestation levels but do not effectively control the pests. Host plant resistance is the preferred option because it is encapsulated in the seed. There are two types of host plant resistance, the conventional and the biotechnologically engineered. Scientists at CIMMYT have developed germplasm which has resistance to stem borers(Mulaa 1995)using conventional methods. Some of the lines developed include 1CS-cm (CIMMYT population 27) and 1CZ2-CM (CIMMYT population 22) among others. However developing insect resistance through conventional means is difficult and time consuming because of the quantitative nature of the inheritance, and the fact that breeding procedure involves two organisms, the pest and its host (Mugo *et al.*, 2001) .The deployment of Bt genes for the control of diverse insect pest is one of the most promising new technology for capturing increased potential yields from improved maize germplasm.

Introduction to Bt technology. *Bacillus thuringiensis* (Bt), is a soil bacterium that produces insecticidal proteins during its sporulation, and thousands of strains of Bt are known to exist. Each strain produces its own unique insecticidal proteins, or delta-endotoxins (McGaughey, 1985). The insecticidal activity of each toxin from Bt strain differ, affecting a variety of insects from different orders like Coleopterans (beetles), Lepidopterans (moths and butterflies) and Dipterans (flies and mosquitoes), (Gould and Keeton, 1996)Unlike many other pesticides, Bt toxins are very specific to harmful insects and are therefore safe to most beneficial insects and other animals. They are also biodegradable and do not persist in the environment (Van Frankenhuyzen, 1993).

Bt-maize. By using genetic engineering, modified novel genes from the soil dwelling bacteria, *Bacillus thuringiensis* (Bt) were introduced into maize, for control lepidopteran stem borers. The product is Bt-maize which has inbuilt resistance to stem borer due to the presence of crystal proteins produced by these genes. Two proteins were found effective against the local lepidopteran stem borers, *cry1Ab::Ubiquitin* and *cry1Ba::Ubiquitin*. Bt maize is planted in seven countries; USA, Canada, Argentina, South Africa, Spain, Honduras, and Germany (James, 2004). In the 2004, the global area planted with Bt-maize increased from 15.5 million hectares in 2003 to 19.3 million hectares. Use of Bt-maize may prove to be part of the solution in addressing hunger and food security along side poverty eradication.

Mode of action. Once the crystal (cry) proteins are ingested, they are dissolved in the insects' midgut, liberating protoxins called delta-endotoxins (Van Rie *et al*., 1992). They are then proteolytically processed into fragments, which bind to epithelial cells of the insects' midgut. Activated proteins disrupt the osmotic balance of these cells by forming pores in the cell membrane causing cells to lyse. The gut becomes perforated and the insect stops feeding and eventually dies ((Marrone and MacIntosh, 1993) However, binding does not always assure toxicity.

Resistance development. Insecticide resistance develops due to genetic variation in large populations, when a few individuals in the original insect population, remain unaffected by a given insecticide. This is due to either the nature of the insecticide's target molecules in the insect, or the method the insect uses to break down toxin molecules. When the insecticide is applied, individuals who are unaffected are the ones which survive to pass on their genes to the following generations. Over time, greater proportions of the insects populations is unaffected by the insecticide. Some of the factors related to development of resistance are: the rate of reproduction, shorter generation cycles, greater number of progeny, and larger, more genetically varied population. The only well-characterized mechanism of resistance to Bt is reduced binding of toxin to midgut membranes(Ferre *et al.*, 1991, 1995; Van Rie *et al*.,1990).The first evidence of resistance developing in the field against Bt-delta endotoxins was in *Plodia interpunctella*, the Indianmeal moth in storage bins of Bt-treated. In the open fields however, it is only *Plutella xylostella*, the diamondback moth, treated with sprays formulations of Bt toxins that was loosing control (McGaughey, 1985. This study showed continued susceptibility to Bt-delta endotoxins by *Chilo partellus* over generations. The two Bt-toxins used, cry1Ab and cry1Ba showed stability in controlling *Chilo partellus* throughout the study period even after the insects were exposed to sub-lethal doses of the delta-endotoxins.

Development of Bt maize in Kenya. Stem borers are distributed in all maize growing environments in Kenya. The country has increasing demand for maize due to a human population growing at 2.9% and due to increasing demand for quality products (Mugo *et al*., 2001). With increasing poverty and limited arable land, the country cannot afford to continue losing food to insects. Despite use existing control measures, losses have been ranging form 13-50% country wide (De Groote, 2002.).

Kenya Agricultural Research Institute (KARI) and CIMMYT (International Centre for research on Maize and Wheat) launched the Insect Resistant Maize for Africa (IRMA) Project in 1999 with the goal of increasing maize production and food security through the development and deployment of insect resistant maize to reduce losses due to stem borers.(Mugo *et al*.,2001)Both host plant resistance and genetically engineered maize (Bt maize) are mechanisms that will be use to ensure that African farmers realize maize yields by reducing stem borer damage.

So far appropriate laboratory and Biosafety Green house level 11 have been established at National Agricultural Research Laboratories (NARL), Kabete for development and evaluation of appropriate transgenic

germplasm. A confined field trial site, has already been set up at KARI-Kiboko, and field trials have begun. Bt-maize seeds have been imported and multiplication of the seeds is being done. However, one of the major concerns in the use of Bt-maize is the development of resistance to the toxins by the target pests. This study aims at finding out whether the maize stem borers will evolve resistance to the *Bt*-maize delta-endotoxins over time.

Materials and methods

Bt-maize Events containing different genes, *cry1Ab::Ubiquitin* and *cry1Ba::Ubiquitin*, were introduced from CIMMYT-Mexico into Kenya in May 2004. The parent line CML 216, non-transgenic was also imported (used as control). Stem borer species were collected from the various maize growing regions in Kenya. *C. partellus* mixed colony was established by mixing the insects collected from Eastern, Coast and Rift Valley provinces of Kenya. The insects were reared (mass rearing) and multiplied using artificial diet (Songa *et al.,* 2001) at KARI- Katumani, to make up to 500 individuals. This constitutes cycle zero (C0) of this colony. Transgenic maize seeds were planted in 10cmx10cm plastic pots at the Biosafety Greenhouse Level 11, located at NARL-Kabete. Twelve pots were used per event, each pot with one seed, were planted in synchrony to pupae stage of the stem borers such that at neonate stage the plants were at 6-8 leaf stage. Leaf bioassays were carried out at the 4-6 leaf stage to ascertain the presence of the genes. Event 223 (*cry1Ab*) and Event 10 (*cry1Ba*) leaves were harvested and infested with 300 neonates for 24 hours. Four replicates were used, for each event. A control was set using CML 216 (Non-transgenic). The larvae were transferred to artificial diet where they continued to die. The surviving larvae were counted and transferred to fresh diet and reared to the pupae stage. The pupae were then harvested and pupae weight determined, then transferred to cages to complete the life cycle. The entire life cycle took 35-42 days. The number of surviving larvae was recorded over the four generations, for each event and the control as well, and the protocol repeated for the subsequent generations.

Statistical analyses. The data for the mixed colony was analyzed by use of Analysis of variance (ANOVA) and the means were separated by use of LSD.

Results and discussion

There were highly significant differences between the non-transgenic control (CML216) and the transgenic events 10 and 223 in the number of surviving larvae in the tested colony of *C. partellus*. The means of surviving larvae over generation cycles were 70.4 for the control, 13.3 and 7.4 for events 10 and 223 respectively.

The mean for the non-transgenic control maize germplasm (70.4) was significantly different when compared with the transgenic Bt-maize germplasm. The results also show that the two Bt-maize cry genes, *cry1Ba* and *cry1Ab* means were not significantly different, indicating that they are both effective in controlling *C.partellus*. Event 10(cry1Ba) means remained stable from cycle 1 throughout to cycle 4 (Table1). This indicates that Event10 cycles were not significantly different; the subsequent generations maintained susceptibility as the dominant trait. Similar results were obtained for event 223(cry1Ab) from cycle 1 to cycle 4. The results of event223 cycles showed that cycle 4 was significantly different from cycle 1 at 5% level. However cycles 1, 2 and 3 were not significant at 5% level. A comparison of cycle 2, 3 and 4 at 5% level further revealed that they are not significantly different. Therefore the cycles for event 223 are not statistically different from each other and therefore did not influence the dependant variable. The non-transgenic control had a high CV (Table 1).

The replicates and the cycles were not significantly different and therefore the dependent variable (survivors) was not influenced by these treatments. A comparison of the cycles showed significant difference between cycle 1 and 2 the over the studied generations. Cycles 1, 3 and 4 were not significant at 5% level. Cycles 2, 3 and 4 were also not significant at 5%. There was environmental influence on cycle 2 Survivors. However the cycles were not statistically different at 1% level. A comparison of the different sources of variation within the experiment showed that, the events 1 and 2 are statistically different from 3 (control).

Recommendations

More generations cycles should be studied to see whether there is variation in the findings of this study. Studies should be done using other stem borer species like *Busseola fusca,* which already is known to have some level of resistance to Bt.

Conclusion

The low survivability of the stem borers which were exposed to Bt-maize delta endotoxins, indicates the effectiveness of the genes in controlling *C. partellus.* The decline of CML 216 survivors in the second generation can probably be attributed to fungal infection observed in some of the glass vials. *C. partellus* mixed colony survivors did not differ so much within the different events,

Table 1. Means of surviving larvae over generation cycles.

Cycle of selection	Maize germplasm		
	Event 10	Event 223	CML 216
1	13.8	6.8	91.3
2	13.8	8.8	62
3	14.3	8.5	77.3
4	11.5	5.8	61.3
Means	13.3	7.4	70.4
CV	18	18	30.3
LSD	ns	2.14	34.16

however event 223**::** *cry1Ab* was observed to be more effective in controlling the stem borers. The study further revealed that Bt maize once adapted by farmers, will reduce the losses attributed to stem borer damage and also reduce the dependency by farmers on chemical pesticides for control of stem borer.

There was no resistance development to the studied species of stem borer within the specified time of this study.

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