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Full Length Research Paper

Testing public Bt maize events for control of stem borers in the first confined field trials in Kenya

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Transgenic maize (*Zea mays* L), developed using modified genes from the bacterium *Bacillus thuringiensis* (Bt), controls stem borers without observable negative effects to humans, livestock or the environment, and is now sown on 134 million hectares globally. Bt maize could contribute to increasing maize production in Kenya. Nine public Bt maize events of *cry1Ab* and *cry1Ba* genes were tested in confined field trials site (CFTs) to assess the control of four major Kenyan stem borer species. Leaf damage rating, number of exit holes and tunnel length were scored in the field evaluations. Leaf area consumed and mortality rates among stem borers were scored in the leaf bioassays in a Biosafety Level II laboratory, located at the Kenya Agricultural Research Institute (KARI), National Agricultural Research Laboratories (NARL). Field evaluations showed that Bt maize controlled *Chilo partellus* with mean damage scores of 1.2 against 2.7 for the non-Bt CML216 control. Laboratory bioassays showed high control for *Eldana saccharina* and *Sesamia calamistis*, with mean larval mortality of 64 and 92%, respectively. However, substantial control was not observed for *Busseola fusca*. These results showed that Bt maize could control three of the four major stem borers in Kenya with mortality records of 52.7% for *B. fusca*, 62.3% for *E. saccharina* and 85.8% for *S. calamistis.* Additional Bt genes need to be sought and tested for effective stem borer control in all maize growing ecologies in Kenya.

Key words: Maize, Bt, stem borers, confined field trials.

INTRODUCTION

The world average maize yield in 2008 was 5.1 t/ha, with an average yield in the industrialized countries of 8 t/ha but only 1.3 t/ha in sub-Saharan Africa (FAOSTAT, 2007). Though Africa grows 29 million hectares of maize (16% of global maize area), it produces only 7% of global maize harvest. Biotic constraints including stem borer insect pests contribute to this very low productivity and production of maize in Africa.

The most prominent maize stem borers in eastern and southern Africa are the spotted stem borer (*Chilo partellus* Swinhoe), the African stem borer (*Busseola fusca* Fuller), the sugarcane borer (*Eldana saccharina* Walker) and the pink stem borer (*Sesamia calamistis* Hampson). *C. partellus* and *B. fusca* are of greater economic importance in eastern and southern Africa. Stem borers are most destructive in the larval stage. They tun-

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Abbreviations: Bt, *Bacillus thuringiensis*; **IPM,** integrated pest management; **CFTs,** confined field trials; **KEPHIS,** Kenya Plant Health Inspectorate Service.

nel inside the stalk after hatching, and are therefore, difficult to control. Once inside, their feeding may lead to death of the central growing shoot (dead heart), reductions in the number of ears, or structural damage increasing the likelihood of lodging in high winds. In some cases, a second generation of the pests also attack maize ears making the cob vulnerable to ear rots, such as *Fusarium spp* and *Aspergillus* spp., which produce harmful mycotoxins including fumonisins and aflatoxins, respectively (Nderitu, 2000). In Kenya, stem borers destroy an estimated 400,000 metric tons of maize equivalent to 13.5% of farmers' annual harvest, costing over 72 million US dollars (DeGroote, 2002).

With the advent of genetic engineering, genes conferring resistance to insects have been inserted into crop plants such as maize, cotton, potato, tobacco, rice, broccoli, lettuce, walnuts, apples, alfalfa, and soybean (Bennett, 1994; Federici, 1998; Griffiths, 1998; USDA, 1995). Among the biological pesticides, bacteria have been the most successful group of organisms identified as a source of biological insecticide for commercial crops. The best example comes from the soil bacterium, *Bacillus thuringiensis* (Bt) (Gill et al., 1992). Insecticidal crystal proteins, called δ-endotoxins, produced by Bt are highly toxic to specific insect species, yet cause no harm to humans, animals, or other non-target organisms such as beneficial insects (Croft, 1990). Field studies confirm that the abundance and activity of parasitoids and predators were similar in Bt and non-Bt crops (Dutton et al., 2002; Dutton et al., 2003; Obrist et al., 2006; Romeis et al., 2006). The Bt toxins are made potent by midgut proteases, the Bt proteins bind to epithelial brush border membrane vesicles (Lee et al., 1995) creating pores that result in cell lyses (Gill et al., 1992). Insertion of genes encoding δ-endotoxins into the maize genome has provided astonishing levels of host plant resistance to insect pests (Koziel et al., 1993).

Bt transgenic plants containing insecticidal proteins have featured prominently in agricultural systems in both developed and developing countries. The global area of approved transgenic crops in 2009 was 134 with 45 Mha grown to transgenic maize varieties (James, 2003; James, 2009). The benefits of growing Bt crops to farmers are substantial across a number of geographies and economic strata, especially in developing countries. These benefits include increased crop yields, reduced pesticide use, reduced exposure by farmers to pesticides, less environmental damage, less fungal contamination, and reduced labor (Huesing and English, 2004). Transgenic crops can lead to substantial reductions in insecticide use in some crops and contribute to integrated pest management (IPM) systems with a strong biological control component (Romeis et al., 2006).

Various Bt *cry* genes (*cry1Ab, cry1Ac, cry1B, cry1E, cry1Ca,* and *cry2Aa*) were used at CIMMYT to develop constructs where the Bt gene was under the control of either the maize ubiquitin or the rice actin promoters (Bohorova et al., 2001). These constructs were used to transform embryos from a CIMMYT maize hybrid (CML216 x CML72). Backcrosses were made to CML216 to develop an inbred line carrier of the Bt genes, resulting in a number of Bt maize events (Bohorova et al., 1999). As the transformation program at CIMMYT progressed and in light of public concern regarding the use of selectable markers, such as herbicide or antibiotic resistance to assist in the identification of events, CIMMYT strived to develop “clean events” that did not carry the selectable Basta herbicide resistance marker (the *bar* gene), thus addressing some of the concerns raised earlier about this technology (Mugo et al., 2005). The public events studied here were from two Bt genes (*cry1Ab* and *cry1Ba*). *Cry1Ab* was sourced from the University of Ottawa, while *cry1Ba* was synthesized by CIRAD, France and CIMMYT.

Bt maize leaves were first introduced into Kenya in 2002 following National biosafety regulations (Mugo et al., 2005). This was followed by the first introduction of Bt maize seeds in 2004, after completion of biosafety facilities including the first Biosafety Level II green house and the first confined field trial site for growing the transgenic maize plants in Kenya (Mugo et al., 2005). The expression of cry1Ab Bt protein had been shown to be influenced by the environment in which the maize plants are growing (Dutton et al., 2004), hence the need for CFTs. The studies presented here assessed the control of major Kenyan stem borers by public Bt maize events in confined field trials (CFTs) in Kenya.

MATERIALS AND METHODS

Bt maize germplasm

Confined field testing was carried out using nine second generation Bt maize events, which carried only the trait of interest and no selectable markers. This was accomplished through co-transformation of the Bt gene and selectable markers that allowed segregating out the selectable marker genes. These were the Bt maize events used in the confined field trial at Kiboko during 2005B (March planting) and 2006A (October planting) seasons.

Confined field trials site

A confined field trials site (CFTs) was developed and approved for research with genetically modified crops at KARI’s Kiboko field station located at 02°15’ S and 37°75’ E at an elevation of 975 m above sea level. The site has sandy clay soils and receives 530 mm annual rainfall, spread over two very short rainy seasons. The

CFTs was developed within the national biosafety framework, and meets Biosafety Level II international standards. The major biosafety features present at the CFTs include trained staff, isolation distance of more than 400 m from other maize fields, secure perimeter fencing to restrict access by people and animals, disinfectant-treated stepping mat and drive-through, and a locked gate. Other features are 24 h surveillance, a signboard stating access restrictions and safety measures, appropriate structures for destruction and disposal of plant material and other waste.

Seed management

The transgenic seeds were stored in a metallic storage seed cabinet, placed in an air conditioned secured seed store in the Biosafety Level II green house at KARI Biotechnology Center in Nairobi. Eighty-four seeds were counted per plot for three replications and packaged in a three tier system. The seeds were placed in seed envelopes which were placed in plastic zip-lock bags then packaged in a small metal urn. The urn was carried in a locked metal box, escorted by the Kenya Plant Health Inspectorate Services (KEPHIS) plant inspectors from the seed store in Nairobi to the CFTs at KARI Kiboko.

Experimental design

The experimental trial was under a 0, 1 alpha lattice design with, ten entries, three replications and two five meter long rows for each plot. The row spacing was 75 cm while the plant spacing was 25 cm giving a plant density of 53,000 plants/ha. Planting was carried out under the supervision of KEPHIS personnel. Ziploc bags and other materials used for seed transportation were destroyed by burning. The ashes were then buried in trenches dug within the CFTs. Fertilizers were applied at the rate of 60 kgN and 60 kgP_2O_5/ha^{-1}as recommended for the maize growing ecology. Nitrogen was applied in two splits of 30 kgN basal application at planting and 30 kgN top dressing at the 5-leaf stage. The trial was rainfed but supplemental irrigation was applied when needed. The fields were kept free of weeds by hand weeding.

Field and laboratory infestation and damage assessment

The stem borer responses in the laboratory bioassays using leaves from the CFT was determined by the leaf area consumed by the stem borer larvae and the mortality rate observed among larvae infested to the leaves. At the four-leaf stage, every maize plant in the first row was infested with 40 black head eggs of *C. partellus* by placing two egg masses of 20 eggs each in the whorl. The second row was neither treated nor infested. Stem borer damage on each plant in the infested row was assessed two weeks after infestation using a 1-9 scale (1 with no damage, and 9 completely damaged) (CIMMYT, 1989; Tefera et al., 2010).

Leaf bioassays were carried out to determine responses to other major stem borer species in Kenya, including *B. fusca*, *E. saccharina* and *S. calamistis*. One leaf was harvested from each of the representative plants from the non-infested row at the time of infestation. The leaves were wrapped in wet hand paper towels, packaged and transported to Biosafety Level II Green House (BGH) escorted by Kenya Planters Health Inspectorate (KEPHIS) personnel. The leaves were cut into 3-cm square sections across the leaf blade. Each section was placed in a five centimeter diameter petri-dish containing moistened filter paper with the abaxial side facing up.

Ten neonates were placed on the leaf tissue using a camel hair brush. This was repeated for each of the respective stem borer species: *C. partellus, S. calamistis, E. saccharina* and *B. fusca*. The petri-dishes were sealed with parafilm. Ten replicates were set up for each of the nine events. A control was set up using the susceptible non-transgenic tropical inbred line (CML216). The petri-dishes were kept at room temperature (25°C) and total darkness in the biosafety laboratory. After five days, the mortality of the larvae was assessed and recorded by counting the number of live and dead larvae. Leaf damage was assessed and recorded by measuring the leaf area consumed using a millimeter grid. After these evaluations, all larvae, leaf tissue, the petri-dishes and other reusable equipment were autoclaved at 121 Psi for 15 minutes and disposed of by incineration and the ashes buried in a dug up trench (Mugo et al., 2004b).

Data analysis

Analysis of variance (ANOVA) on data from insect resistance, agronomic traits which include plant stand, 50% anthesis, ear height, and leaf bioassays from the confined field trials was done using PROC General Linear Model (GLM) of Statistical Analysis Software (SAS, 2003) program and least significant difference (LSD) used to separate means.

RESULTS AND DISCUSSION

Leaf damage assessment and agronomic traits

Leaf damage scores in the field showed Bt maize control of *C. partellus* with mean scores of 1.2 against 2.7 for the non-Bt CML216 control (Table 1). All Bt maize events showed significantly lower leaf damage compared to the control.

Event 6 displayed slightly more leaf feeding damage than the other events with a score of 1.6. The number of exit holes showed a mean of 1.2 exit holes for Bt maize compared to 10.5 for the check. All Bt maize events showed significantly fewer exit holes compared to the check (Table 1).

Shorter mean tunnel lengths, 2.7 cm were observed in Bt maize plants, compared to 18.6 cm observed in the check. All Bt maize events showed significantly lower damage response compared to the non-Bt check. All agronomic traits measured showed no significant differences from the non-Bt check (Table 2).

Leaf bioassays in the biosafety laboratory

B. fusca and *E. saccharina* larvae consumed the same leaf area on the Bt maize leaves as in the non-Bt control (Table 3). However, *S. calamistis* larvae feeding on Bt maize leaves consumed an average of 8.8 mm^2, which is

Table 1. *Chilo partellus* responses to nine Bt maize public events in confined field trials during 2005B and 2006A seasons at Kiboko, Kenya.

Entry	Pedigree	Leaf Damage (1-9)	No. of Exit Holes No.	Tunnel Length cm
1	Event 3:cry1Ba::Ubi	1.2±0.07	1.9±0.22	2.8±0.15
2	Event 6:cry1Ba::Ubi	1.6±0.26	1.8±0.51	4.2±0.7
3	Event 10:cry1Ba::Ubi	1±0	0.2±0.11	0.1±0.09
4	Event 58:cry1Ba::Ubi	1.1±0.05	1.5±0.21	2.9±0.07
5	Event 93:cry1Ba::Ubi	1.4±0.16	2.2±0.21	7.1±1.88
6	Event 127:cry1Ba::Ubi	1.1±0.02	0.6±0.41	1.4±0.9
7	Event 216:cry1Ab::Ubi	1.1±0.03	0.8±0.39	2.2±0.95
8	Event 223:cry1Ab::Ubi	1.2±0.08	0.70.22	1.5±0.24
9	Event 396:cry1Ab::Act	1.3±0.16	1.20.33	2.4±0.78
	Mean Bt	1.2	1.2	2.7
10	CML216	2.7	10.5	18.6
	Mean	1.4	2.1	4.3
	CV	25	42.0	30
	LSD	0.39	5.25	7.08
	Significance	**	*	**
	Stderr	0.14	1.77	2.38

Table 2. Agronomic traits of nine Bt maize public events in confined field trials during 2005B and 2006A seasons at Kiboko, Kenya.

Entry	Predigree	Ear height (infested) cm	Ear height (normal) cm	50% Anthesis days
1	Event 3:cry1Ba::Ubi	73±4.5	74±4.3	74±1.7
2	Event 6:cry1Ba::Ubi	74±4.3	74±4.6	74±1.7
3	Event 10:crv1Ba::Ubi	81±3.7	79±3.7	76±1.9
4	Event 58:crv1Ba::Ubi	72±2.9	80±4.4	75±2.3
5	Event 93:crv1Ba::Ubi	74±1.8	75±3.2	76±1.8
6	Event 127:crv1Ba::Ubi	71±4.3	68±6.3	78±2.0
7	Event 216:crv1Ab::Ubi	70±7.0	72±8.1	79±3.5
8	Event 223crv1Ab::Ubi	60±6.5	62±3.3	75±1.7
9	Event 396:crv1Ab::Act	79±3.9	82±5.2	72±2.1
	Mean Bt	72	74	76
10	CML216	71±2.0	82 ±12.8	74±1.6
	Mean	72	75	75
	CV	15	21	7
	LSD	-	-	-
	Significance	ns	ns	ns

much lower compared with 57.7 mm^2 consumed in the CML216 check. Larval mortality due to Bt maize of *E. saccharina* and *S. calamistis* were 64 and 92%, respectively, which were significantly higher than the control with 44.8 and 30.1% (Table 3). There were no significant differences in larval mortality for *B. fusca* in all Bt maize events, except for event 3 which had 26.8% mortality.

The large leaf area consumed and the low larval mortality rate observed for *B. fusca* point to very low resistance among the Bt maize events against this pest. However, *E. saccharina* and *S. calamistis* were well controlled by both cry1Ab and cry1Ba δ-endotoxins. These results also agree with those by Mugo et al. (2005, 2004a) who found that Bt cry1Ab, cry1Ba and other tested proteins were not effective in the control of *B. fusca*, and that *S. calamistis* was affected by cry1Ab, cry1Ba and other tested proteins.

The results from the bioassays showed that a prospective control was identified for *C. partellus*, the most destructive and most widely distributed stem borer in

Table 3. Results of leaf bioassays on nine Bt maize public events in confined field trials during 2005B and 2006A seasons at Kiboko, Kenya.

Entry	Predigree	Area (mm^2)			Mortality (%)		
		Busseola fusca	***Eldana saccharina***	***Sesamia calamistis***	***Busseola fusca***	***Eldana saccharina***	***Sesamia calamistis***
1	Event 3:cry1Ba::Ubi	78.5±7.6	8.2±3.3	15.2±5.4	26.8±6.9	68.9±4.7	87±5.4
2	Event 6:cry1Ba::Ubi	59.3±10.7	13±6.3	13.2±4.2	56.9±7.8	39.1±5.0	82.9±7.9
3	Event 10:crv1Ba::Ubi	51.4±6.9	4.4±0.4	2.6±1.1	61.2±3.4	76.4±9.2	96.3±3.4
4	Event 58:crv1Ba::Ubi	66±21.2	14±5.8	18.9±7.7	39.5±8.9	58.6±4.6	83±7.8
5	Event 93:crv1Ba::Ubi	49.4±11.3	11±4.1	6.7±3.6	67.5±9.5	72.7±7.9	93.7±4.6
6	Event 127:crv1Ba::Ubi	43.4±10.6	5.6±0.9	8.1±4.4	65.3±5.8	72.7±7.9	93.7±4.6
7	Event 216:crv1Ab::Ubi	44±0.4	8.1±3.9	6.5±3.3	43.4±6.8	51.3±8.3	95±2.6
8	Event 223crv1Ab::Ubi	49.4±11.2	7.2±1.4	3.5±1.9	55.3±8.8	81.8±5.3	95.5±1.9
9	Event 396:crv1Ab::Act	38.1±7.8	10.2±2.4	4.3±1.8	62±7.3	71.9±7.2	99.1±.8
	Bt mean	55.3 ±6.2	33.9 ±15.3	57.7±11.3	48.7±10.9	44.8±5.2	30.1±4.1
10	*CML216*	55.3±6.2	33.9±15.3	57.7±11.3	48.7±10.9	44.8±5.2	30.1±4.1
	Mean	53.5	11.5	13.7	52.7	62.3	85.8
	CV	50	124	94	36	27	13
	LSD	-	-	15	21.9	19.9	13.2
	Significance	ns	ns	**	**	**	**

Kenya. The *cry* proteins effectively controlled *E. saccharina* and *S. calamistis*. These results are in agreement with those from Mugo et al.(2005, 2004a) who found complete control for *C. partellus* from cut leaves expressing cry1Ab and cry1Ba δ-endotoxins of maize grown in CIMMYT-Mexico.

Similarly, the cry1Ab and cry1Ba δ-endotoxins from Bt maize effectively controlled the *C. partellus* in the field. These results are also in agreement with observations made in the laboratory and greenhouse. Greenplate (Greenplate, 1999) and Coviella et al. (2000) reported that both genetic and environmental factors control variation in protein expression. For crops expressing genes encoding Bt-toxins, changes in protein production have been shown to be affected by factors including growth in different environments, nitrogen fertilization and/or atmospheric carbon dioxide (CO_2) concentrations.

These results demonstrate that the level of expression of δ-endotoxins in the maize tissues tested was adequate in control of the three stem borer species. The amount of *Bt*-toxin in transgenic plants can also vary depending on the plant growth stage (Archer et al., 2000; Onstad and Gould, 1998). However, infestations in the field using tissue before the 6-leaf stage, may explain the similar level of control observed in the greenhouse and field conditions. It has been reported that different commercial varieties of Bt-maize have been shown to express variable amounts of toxin in different plant parts (Dutton et al., 2003). All these tests were done in the CML216 genetic background and cultivar differences were not expected.

None of the Bt proteins tested were effective in the control of *B. fusca*. Different Cry Bt genes or events will need to be sourced and tested for effective stem borer control in all maize growing ecologies in Kenya. Not all the δ-endotoxins are effective against all the lepidopteran pests. Therefore, bioassays should be conducted to determine which toxins are effective, and which ones could be used in combination to ensure pest control. These results also demonstrated the specificity of δ-endotoxins even among lepidopteran stem borer insects.

Conclusions

The importation of the first Bt maize seeds into Kenya, were carried out successfully, demonstrating that transgenic materials can be safely imported and managed in the secure seed store in the biosafety greenhouse and handled in the CFTs in Kenya (Mugo et al., (2011). The evaluations of the second generation Bt maize events under field conditions in confined field trials confirmed the findings from the laboratory and greenhouse evaluations (Mugo et al., 2005; Mugo et al., 2004a). These results indicated that a prospective control was identified for the most destructive borer, the spotted stem borer (*C. partellus*), which is the most widely distributed stem borer in Kenya. Similarly, cry proteins to control *E. saccharina* and *S. calamistis* stem borers were identified. None of the tested Bt proteins could control *B. fusca*. The tests showed that Bt maize controlled three of the four major stem borers in Kenya under field conditions. Additional effective Bt genes or events will need to be identified and tested for effective stem borer control in all maize growing ecologies in Kenya.

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