African Crop Science Journal, Vol. 21, No. 3, pp. 235 - 244
ISSN 1021-9730/2013 $4.00

EVALUATION OF BIOASSAYS FOR TESTING Bt SWEETPOTATO EVENTS AGAINST SWEETPOTATO WEEVILS

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(Received 24 April, 2013; accepted 24 July, 2013)

ABSTRACT

Sweetpotato weevil (*Cylas puncticollis*) Boheman is a serious pest throughout Sub-Saharan Africa region and is a big threat to sweetpotato cultivation. Ten transgenic sweetpotato events expressing Cry7Aa1, Cry3Ca1, and ET33-34 proteins from *Bacillus thuringiensis* (Bt) were evaluated for resistance against *C. puncticollis*. Four bioassays used were: (i) 1st instar larva in an artificial diet using root powder of transgenic events, (ii) whole root of transgenic events infested with female adults, (iii) root chip, and (iv) small root of transgenic events both infested with egg-plugs. DAS-ELISA analysis showed variation in Cry protein concentration among the events that ranged between 0.1 and 0.394 μg g^{-1} of root fresh weight. The highest protein quantity was observed in the event carrying the ET33-34 transcript. In general, transgenic events had no significant effect on larval survival ($P = 0.28$) and pupal ($P = 0.86$) development, though maximum pupation of 96% was observed on event CIP410009.12 that had very low expression levels of *cry3Ca1* gene. Root chips were prone to damage by fungi and desiccation especially during the first larval instar. The whole root bioassay had less handling injuries to weevils compared with other methods. However, it requires a large number of adult females for oviposition and roots per event to be tested to obtain efficacy results with statistical rigour. The small root egg plug bioassay required much fewer roots and experimental insects to assess larval mortality and development. The root chip method was the least desirable because of susceptibility to fungal and bacterial contamination. In addition, this method was the most labour intensive in terms of frequent replacement of root chips for weevil development. Hence, the most appropriate method for testing Bt efficacy in sweetpotato is the small root egg-plug bioassay. Nonetheless, none of the transgenic events tested provided weevil control probably because of low Cry protein expression in storage roots.

Key Words: *Cry* genes, *Cylas puncticollis, Ipomoea batatas*

RÉSUMÉ

La charançon de la patate douce (*Cylas puncticollis*) Boheman constitue une importante peste à travers la région d'Afrique sub-saharienne et une grande contrainte à la production de la culture. Dix patate douce transgéniques d'événéments exprimant les protéines Cry7Aa1, Cry3Ca1, et ET33-34 des *Bacillus thuringiensis* (Bt) étaient évaluées pour résistance contre *C. puncticollis*. Quatre bioassais utilisés étaient: (i) une larve de premier instar dans une alimentation artificielle utilisant une poudre racinaire d'événement transgénique, (ii) racine entire d'événement transgéniques infestés avec adultes femelles, (iii) un morceau racinaire, et (iv) petite racine d'événements transgéniques tous infestés avec des oeufs. L'analyse DAS-ELISA a montré une variation dans la concentration en protein Cry parmi les événements variant de 0.1 à 0.394 μg g^{-1} de poids de raciness fraîches. La

quqntité la plus élevée de protéines était observée dans l'événement portant le relevé ET33-34. En général, les événements transgéniques n'avaient aucun effet significatif sur la survie de larves (P = 0.28) et le développement de la nimphe (P = 0.86), bien que la pupation maximale de 96% était observée sur l'événement CIP410009.12 qui avait un très bas niveau d'expression du gène *cry3Ca1*. Les morceaux de raciness étaient susceptibles d'être endommagés par les champignons et la dessication spécialement durant le premier instar larvaire. La racine entière bioassay était moins affctée par la charançon et avait moins de blessures en comparaison à d'autres méthodes. Par ailleurs, il nécessite un grand nombre d'adultes femelles pour l'oviposition et raciness par événement devrant être testés pour obtenir des résultats efficaces avec rigueur statistique. Le bioassai de petits oeufs racinaires ongt nécessité un peu moins de raciness et insectes expérimentaux pour évaluer la mortalité larvaire et leur développement. La méthode de morceaux racinaires était la moins desirable à cause de la contamination fongique et bactérienne. En plus, cette méthode exigeait plus de travail intensif en terme remplacement fréquent de morceaux de racines pour le développement de charançon. Ainsi, la méthode la plus appropriée pour tester l'efficacité du Bt dans la patate douce est le bioassai de petis oeufs racinaires. Néamoins, aucun des événements transgéniques testés n'a fourni un control probable de la charançon suite à une basse expression de la protein Cry dans les raciness de stockage.

Mots Clés: Gènes *cry*, *Cylas puncticollis, Ipomoea batatas*

INTRODUCTION

Sweetpotato weevils (SPW) are a major constraint to sweetpotato (*Ipomoea batatas* L.) production worldwide (Fuglie, 2007). In Africa, *Cylas puncticollis* Boheman and *C. brunneus* F. are the major pest species (Kiiza *et al*., 2009); whereas in America and Asia, *C. formicarius* is the major pest species (Smit, 1997). All three *Cylas* species attack sweetpotato both in the field and during storage. On established plants, adult weevils attack leaves while the larvae feed on roots and stems; producing larval tunnels followed by pupal chambers. Stem damage is thought to be the main reason for yield loss, although damage to the vascular system caused by feeding, larval tunneling and secondary rots reduce the size and number of roots substantially (Sorensen, 2009).

Pest damage usually continues during storage; therefore, infested roots cannot be stored for a long time. Severe infestations or slightly damaged roots render the crop unpalatable and, therefore, inedible by humans due to terpenoid produced in response to weevil feeding (Stathers *et al.*, 2003). Together with other beetle pests, *Cylas* spp. can completely destroy sweetpotato fields (Geisthardt and van Harten, 1992).

The nature of attack by sweetpotato weevils and mode of feeding render them difficult to control due to their cryptic habit which reduces the effectiveness of control (Smit *et al.*, 2001). Sex pheromones of *C. formicarius* were effective in controlling weevil populations in mass trapping trials in Taiwan (Hwang and Hung, 1991), Vietnam (Braun and van de Flieart, 1997) and Cuba (Alcazar *et al.*, 1997), but failed in Uganda (Smit *et al.*, 2001). Hence, weevil control remains top priority in sweetpotato breeding especially in countries like Uganda where yield loss often reach as high as 60-100% under heavy infestations (Sorensen, 2009). Despite years of intensive research in conventional plant breeding, varieties with resistance to *C. puncticollis* and *C. brunneus* are not yet available, although low levels of weevil resistance have been recently identified in cultivars (Jackson *et al*., 2012; Muyinza *et al*., 2012). Sweetpotato producers in sub-Saharan Africa are mostly small-scale, resource-poor farmers growing the crop all year round. Hence, management strategies that have been proven successful elsewhere may not be suitable for farmers in sub-Saharan Africa.

Weevil control could, however, be achieved by transferring the insecticidal properties of *Bacillus thuringiensis* (Bt) through genetic engineering. Genes expressing insecticidal crystal (Cry) proteins from Bt have been cloned, modified for expression in plant, and inserted into various cultivated crops (Christou *et al*., 2006).

The activity of some *cry* genes against *Cylas* spp. in sweetpotato has been demonstrated in Cuba (Moran *et al*., 1998; Garcia *et al*., 2000). In Africa, previous artificial diet bioassays found three Cry protoxins (Cry7Aa1, Cry3Ca1, and ET33-34) to be active against *C. puncticollis* and *C. brunneus* with LC_{50} values at less than 1 ppm

(Ekobu *et al.*, 2010). Genes corresponding to the three proteins have been synthesized and used to transform several sweetpotato varieties with the aim of controlling sweetpotato weevils (Kreuze *et al*., 2009). The objective of this study was to evaluate the efficacy of transgenic events with genes expressing Cry toxins, which present potential for control of weevils in sweetpotato.

MATERIALS AND METHODS

Plant material. Transgenic sweetpotato events from the United States variety 'Jewel' expressing *cry* genes used in this study were developed by organogenesis (Luo *et al*., 2006). A total of 10 transgenic events from 'Jewel' were made available for this study: four transgenic events with the *cry7Aa1*; three with *cry3Ca1*; and three with *ET33-34* (Table 1). Non-transformed 'Jewel' was used as control in all the experiments.

Cry protein quantification. Double antibody sandwich enzyme-linked immunosorbent assays (DAS–ELISA) for Cry7Aa1, Cry3Ca1 and ET33-34 proteins were performed as described by Clark and Adams (1977). Plates were pre-coated with polyclonal antibodies, prepared from rabbits, against the purified proteins Cry7Aa1, Cry3Ca1 and ET33-34; and diluted (1:1,000) in carbonate buffer (pH 9.6). Samples (1 g from each root of the 10 roots) in extraction buffer (PBS buffer pH 7.4, 2% polyvinyl pyrrolidone (PVP-40), 2% non-fat milk, 0.5 ml l^{-1} Tween-20) diluted 1/10 (w/v), were added to the polystyrene microtitre plate in triplicate and were incubated overnight at 4°C.

Plates were washed four times, then the conjugated antibodies with alkaline phosphotase from bovine intestinal mucosa (BIORAD) diluted 1:1,000, was added to each well and the plate was incubated for 3 hr at 37 °C. Finally, the plates were washed and p-nitrophenylphosphate diluted in substrate buffer (BIORAD) was added to each well. The absorbance of the yellow product formed was measured at 405 nm with an ELISA plate reader. A non-transformed Jewel used as a negative control was also included in the experiment. The amount of Cry protein was calculated using a standard curve for each Cry protein (Cry7Aa1, Cry3Ca1 and ET33-34), which were prepared by diluting purified proteins obtained from a previous study (Ekobu *et al*., 2010).

TABLE 1. Transgenic sweetpotato events evaluated for resistance to *C. puncticollis*

Event	Gene construct
CIP 410008.7	5'- β-amy/*cry7Aa1*/ -3' β-amy
CIP 410008.9	5'- β-amy/*cry7Aa1*/ -3' β-amy
CIP 410008.14	5'- β-amy/*cry7Aa1*/ -3' β-amy
CIP 410008.17	5'- β-amy/*cry7Aa1*/ -3' β-amy
CIP 410009.12	5'- SPOA1/*cry3Ca1*/ -3' SPOA1
CIP 410009.14	5'- SPOA1/*cry3Ca1*/ -3' SPOA1
CIP 410009.15	5'- SPOA1/*cry3Ca1*/ -3' SPOA1
CIP 410010.14	5'- SPOA1/*ET33-34*/ -3' SPOA1
CIP 410010.18	5'- SPOA1/*ET33-34*/ -3' SPOA1
CIP 410010.19	5'- SPOA1/*ET33-34*/ -3' SPOA1

β-amy and SPOA1 refer to regulatory elements of the sweetpotato β-amylase and sporamin genes, respectively

Sweetpotato weevil rearing. A sweetpotato weevil colony was established from a field-collected population (about 500 insects) and maintained in the laboratory on storage roots, in collapsible cages (1450C, BioQuip Products, Inc, CA) at 25±2 °C and 70±10% RH.

Artificial diet bioassay. Transgenic and non-transgenic storage roots were harvested four months after planting. Samples were frozen at -80 °C following harvest and then lyophilised until dry. Lyophilised material was ground to a fine powder using a laboratory miller and stored at -20 °C until use. Artificial diet was prepared as described by Ekobu *et al*. (2010). For each treatment, 200 ml of the diet was used. The diet + sweetpotato root powder was homogenised to give a final concentration of 8% (wt/vol) lyophilised tissue in the diet.

Formulated diets were poured equally into three 90-mm diameter sterile glass petri dishes and allowed to cool at room temperature. The solidified diet was left at 4°C until the next morning. Ten first-instar, *C. puncticollis* larvae per petri-dish were placed in small (2-3 mm in depth) burrows dug into the diet by using a pair of flat-headed forceps. The set-ups were replicated three times in a completely randomised

design and conducted for 15 days at 22-24 °C and 70±10% RH.

Larval mortality was determined after 15 days, by observing no larval movement. All surviving larvae from a particular treatment were weighed together on an electronic balance to within 0.10 mg (Mettler, Toledo AG204); then this value was divided by the total number of larva weighed, to get average weight.

Whole root assay. Screenhouse-grown storage roots from transgenic and non-transgenic control were used. Roots were harvested approximately four months after planting. Ten *C. puncticollis* female adult (2-3 week-old), were placed in individual 1 litre polystyrene jars, with tissue paper at the base (Stathers *et al*., 2003). Sexing of weevils was conducted using the shape of the distal antennal segment; males have a filiform shape and females have club like shape (Smit, 1997). Single un-infested transgenic and non-transgenic roots were introduced into each jar representing a replicate.

After 24 hr, the adult females were removed. Roots were then incubated until adult weevils emerged. Insect counting commenced when adults started emerging. Newly emerged adults were recorded and removed daily. This was done until weevil emergence stopped.

This bioassay was replicated three times, in a completely randomised design including entire events and the control. The experiment was conducted at 25± 2 °C and 70 ± 10% RH and was repeated three times. In this bioassay, the total number of progeny that emerged and their median development period were derived for each replicate. The median development period was calculated as the number of days from oviposition to first emergence of progeny.

Root chip bioassay. Roots were sliced into thin chips (1 cm thick), and each chip had two small holes (approximately 2-3 mm deep) for introducing eggs (Vasquez and Gapasin, 1980). Eggs were obtained by exposing cultivar NASPOT 1 storage roots (known susceptible variety) to a large number of females for 24 hr for oviposition. Egg plugs were cut from the non-transgenic cultivar and needle nosed forceps were used to gently transfer the plugs onto the transgenic root chips. Root chips were placed between microscope slides. Both ends of the slides (bioassay block) were tied with rubber bands. Each event had six chips and each chip was treated as a replicate.

The bioassay blocks were then placed in covered plastic containers. At 5 days after the eggs were deposited, root chips were examined for hatched eggs. Nonviable eggs or rotten root sections were discarded. Root chips were dissected to locate larvae and pupae. Root chips were changed, when there were signs of contamination or drying. Larval and pupal survival was recorded. Bioassays were replicated four times in a completely randomised design at 25± 2 °C and 70 ± 10% RH.

Small root egg-plug bioassay. Twenty four-hour old eggs were introduced into small transgenic roots measuring 3 cm diameter, as adopted from Mao *et al*. (2001). Roots were provided with initial holes for insertion of eggs. Eggs were obtained from cv. NASPOT 1. Needle nosed forceps were used to gently transfer egg plugs to transgenic roots. Each small root was infested with five egg plugs.

After placing the egg plugs into the holes, they were sealed with masking tape. Roots were placed in 1 litre polystyrene jars with tissue paper at the base for incubation. Five small roots were used per event and control. Each small root was treated as a replicate.

At 5 days after the eggs were deposited, small roots were examined to determine if eggs had hatched and larvae had burrowed into the sweetpotato. Unviable eggs were not considered in this experiment. At 21 days after infestation, root sections were dissected to locate pupae. Larval and pupal survivals were recorded. Roots from all events and controls were tested in a completely randomised experimental design at 25± 2 °C and 70 ± 10% RH.

Statistical analysis. Total mortality, percent mortality and percent pupation of *C. puncticollis* were calculated on artificial diet incorporated with the transgenic root powder. In whole sweetpotato root assay the number of adult weevils that emerged was recorded and; median weevil development period was calculated. Pupation rate was recorded in the small sweetpotato root

infested with 24 hr egg plugs. All percentage data were transformed using the Arcsine square root before analysis. All data collected were subjected to analysis of variance at $p<0.05$ using GenStat 14th Edition software, version 14.1.0.5943 (VSN International Ltd, 2011).

RESULTS

Cry protein quantification. DAS-ELISA analysis showed differences in Cry protein concentration among the sweetpotato events (Fig. 1). Two events carrying ET33-34 had the highest protein quantity among all events tested. CIP410010.18 had a concentration of 0.394 µg g^{-1} of root fresh weight, followed by CIP410010.19 with 0.11 µg g^{-1} of root fresh weight. The rest of the tested events carrying the other proteins had concentration below 0.1 µg g^{-1} of root fresh weight. Cry3Ca1 proteins were not detected in all the three events that were tested.

Artificial diet bioassay incorporated with transgenic root powder. Mortality of larvae in the artificial diet with transgenic sweetpotato powder did not vary from that of the control, reared on a toxin-free diet (P = 0.28; Table 2). There was no adverse effect of powder from the eight transgenic events tested on pupation of *C. puncticollis*. Neither were there significant differences in pupation rate (P = 0.86; Table 2). Larvae were delicate to handle and easily died during placement on diet. In general, the larvae that fed on the artificial diet appeared healthy and active in all treatments. The average weight of *C. puncticollis* larvae ranged between 1.0-1.3 g in all experiments, 15 days post incubation (Table 2).

Whole root bioassays. Seven transgenic events evaluated under laboratory conditions displayed no significant variation in resistance to *C. puncticollis,* as compared to the non-transgenic control (Table 3). Visual observation revealed that the adult weevils fed in the same way on transgenic roots as on non transgenic roots. When roots were incubated after oviposition by female adults, new adult weevils began to emerge 23-24 days post infestation in all events. Peak emergence occurred 26-31 days for all the experimental sweetpotato genotypes. Adult emergence was recorded 40 days after set-up. There was no significant difference (P = 0.94) between the transgenic and non-transgenic storage roots in the total number of weevils that emerged (Table 3). The median development time on Cry-containing plant material was not significant (P = 0.51).

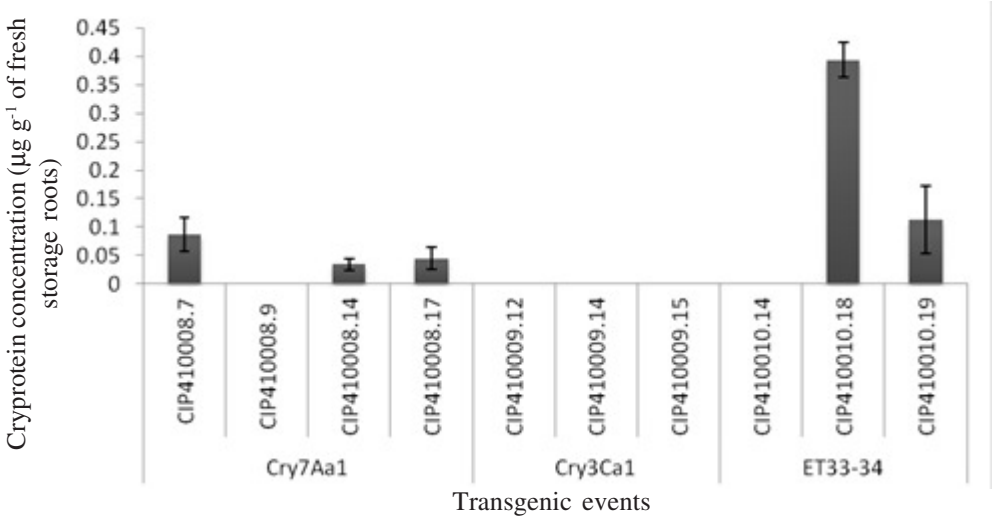

Figure 1. Expression levels of Cry7Aa1, Cry3Ca1 and ET33-34 proteins in storage roots of different transgenic events by using DAS-ELISA assay. Protein levels are expressed in µg g^{-1} fresh root tissue.

TABLE 2. Mortality and development of *C. puncticollis* on artificial diet with lyophilised root powder of transgenic and non transgenic sweetpotato plants

Genotype	Mortality rate (% ±SE)	Pupation rate (% ±SE)	Larval weight (mg)
Transgenic			
CIP410008.7	26.7(±6.7)	23.61 (±6.05)	1.1
CIP410008.9	23.3(±3.3)	30.36(±3.72)	1.2
CIP410008.17	16.7(±3.3	31.94(±3.68)	1.0
CIP410009.5	30.0(±5.8)	28.97(±2.41)	1.2
CIP410009.12	16.7(±3.3)	36.11(±7.35)	1.2
CIP410010.14	26.7(±3.3)	27.38(±1.19)	1.3
CIP410010.18	30.0(±5.8)	24.21(±5.51)	1.0
CIP410010.19	30.0(±5.8)	29.56(±8.96)	1.2
Non-transgenic			
Cv Jewel	23.33(±3.3)	26.19(±7.31)	1.1

There was no significant difference in mortality rate and pupation rate ($P > 0.05$) between the transgenic and non transgenic root powder in artificial diets

TABLE 3. Effect of transgenic and non transgenic sweetpotato roots on *C. puncticollis* emergence and development

Genotype	Number of weevil adults emerged (±SE)	Median development period (days) (±SE)
Transgenic		
CIP 410008.7	75.3±3.18	28.0±0.0
CIP 410008.14	76.3±4.37	27.0±0.6
CIP 410009.12	79.0±4.04	27.0±0.6
CIP 410009.15	77.3±7.22	27.7±0.9
CIP 410010.14	82.0±4.16	26.7±0.3
CIP 410010.18	74.0±10.12	27.7±0.3
CIP 410010.19	71.3±5.17	27.3±0.3
Non-transgenic		
Cv Jewel	77.7±4.67	28.0±0.6

There was no significant difference between number of weevil adults emerged and median development period ($P > 0.05$) between the transgenic events and the control

Root chip bioassay. Mortality rates of up to 100%, in some replicates, were recorded for weevils on root chips making it difficult to compare statistically. In addition, root chips were prone to damage by fungi and desiccation. Mechanical injury inflicted on larvae during examination when the larvae had to be excavated from tunnels of dry root chips and transferred to fresh root chips caused up to 70% mortality. Mortality was highest during the first larval instar, but reduced as the larvae matured. No mortality was observed once the insect reached pupation stage. Very few larvae (up to 30%), which pupated emerged as adults.

Small root egg-plug bioassay. Transgenic events had no significant effect on larval survival and pupal development. Visual observation after 21 days showed that the weevils were not affected by Cry proteins being expressed in roots. Some transgenic lines had higher pupation rate than the non transgenic control. However, there were no significant differences in the pupation rates between the transgenic events and the non

TABLE 4. Pupation rate of *C. puncticollis* at 21 days in the small root egg plug bioassay

Genotype	Pupation rate (%) (±SE)
Transgenic	
CIP 410008.7	92 ± 4.9
CIP 410008.14	82 ± 7.8
CIP 410009.12	96 ± 4.0
CIP 410009.15	87 ± 8.3
CIP 410010.14	95 ± 5.0
CIP 410010.18	86 ± 5.8
CIP 410010.19	91 ± 5.6
Non-transgenic	
Cv Jewel	88 ± 8.0

There was no significant difference in pupation rate ($P > 0.05$) between the events and the control

transgenic Jewel ($F = 0.56$; df = 39; $P = 0.76$). Highest pupation rate of 96% was observed on event CIP410009.12 and low pupation rate (82%) was observed in event CIP410008.14 (Table 4).

DISCUSSION

Cry protein quantification. Although the *cry* genes were expressed under the control of tissue specific promoters, the Cry proteins concentration was low in the roots, and proabaly below detection levels like in the case of Cry3Ca1 events (Fig. 1). Sporamin and β-amylase promoters could not confer high levels of Cry protein accumulation in sweetpotato roots despite reports of enhanced accumulation of other proteins in potato (Hong *et al*., 2008). When Cry proteins were quantified in the storage roots, they were observed to be less than 0.5 μg g^{-1} of fresh storage root flesh. Despite, the presence of the *cry* genes in some events, the Cry protein levels were too low to be detected in the roots. There is a likelihood of very low or total lack of expression. The low or failure of gene expression was probably due to the fact that the sweetpotato root is a low protein storage organ (OECD, 2010).

Artificial diet bioassay incorporated with transgenic root powder. The artificial diet bioassay allowed for relatively easy testing of small quantities of tissue. This would be especially important when test material is limiting (from a single transgenic event). In addition, this technique was relatively fast; it needed only 15 days to observe the effects of Cry proteins on sweetpotato weevil. However, the reagents used for making the artificial diet are relatively expensive and the diet does not adequately account for sweetpotato/Bt interactions. When insects are not handled delicately especially the 1st instar larvae using proper forceps or fine brushes, it is likely that they could be injured and the bioassay effects could be magnified.

Secondly, the effectiveness of this bioassay could be underestimated due to the fact that it involves a high protein dilution rate, where the Cry protein is diluted by about three-fold (80 g in 1 litre of diet compared to about 250 to 300 g dry matter in 1 kg of storage roots according to OECD (2010). Furthermore, due to practical constraints to maintain the media liquid before pouring in petri-dishes, the root powder had to be added at higher temperature (55 °C), which could have inactivated the Cry protein unlike when adding purified Cry protein at 50 °C previously described by Ekobu *et al*. (2010).

The other methods tested used actual fresh root tissue. These had the advantage that the weevil was exposed to the root with the actual amount of Cry protein that will be expressed under certain field conditions. In addition, plant tissues are likely to contain natural defensive compounds, which may act additively or synergistically with the Cry proteins and increase resistance to the pests (Coombs *et al*., 2005).

Whole root bioassays. The whole root bioassay described in this study, has significant advantages when it comes to handling and contamination as compared to all other bioassays. This method, however, does not allow for determining larval mortality directly. It concentrates on the number of weevils that emerge after infestation and the mean development days of the weevil to establish the level of resistance of the events. This is based on the assumption that a few insect progenies would emerge out of a resistant genotype and insect progeny development would take a longer time in a resistant than in a susceptible genotype.

Adults which fed and oviposited on transgenic roots provided no evidence of non-

preference to feed and oviposit on transgenic roots. This could be due to the fact that the adults were not sensitive to the Cry proteins expressed in sweetpotato. Even though we did not observe indication of a feeding deterrent behaviour, this aspect could be given more attention, as reduced feeding and decreased food utilisation could result in reduced fitness leading to population reduction.

Root chip bioassay. The sweetpotato root chip bioassay facilitated observations of the probing activity and other aspects of the weevil's behaviour within the bioassay block other than mortality. Nonetheless, this method was not suitable because larval mortality was too excessive to give reliable results on efficacy of transgenic roots. The high mortality in this study was primarily due to high levels of fungal contamination observed and also the injury inflicted on the weevil during changing of the root chips. Vasquez and Gapasin (1980) also reported high mortality of the weevil due to injury inflicted to the insect during rearing of the sweetpotato weevil on the bioassay blocks.

Small root egg-plug bioassay. This method was an improved version of the root chip method to overcome the above mentioned disadvantages of the root chip bioassay. Escapes and damage of weevil larvae were reduced due to no transfers of the larvae to the new roots and also the contamination by fungi and bacteria was reduced. The eggs used in this technique hatched and larvae went directly into the root for the whole incubation period and developed normally with no hindrance to development. In addition, a major advantage of this technique was the ability to test the efficacy of selected events focusing on mortality and sub lethal effects of the Cry protein to the weevil. This bioassay was suitable for evaluating resistance of transgenic sweetpotato roots to sweetpotato weevil because the number of eggs infested per root was known and the number which hatched and fed on the root flesh would easily be noticed. Additionally, injury inflicted to the insect is reduced, and the weevil is exposed to higher levels of Cry protein than in artificial diet bioassays.

CONCLUSION

An ideal bioassay method, however, is one that is fast, labour efficient, and closely correlated with field control (Perez *et al*., 1997). According to these criteria, small root-egg plug bioassay would be preferable because it is easier to get as many eggs as possible and infest the roots, than the whole root bioassay where a large number of females need to be sexed for the experiment for oviposition on each experimental root. The whole root bioassay, nevertheless, is more suitable for testing a small number of events where a large number storage roots required can be easily handled to reach statistical significance.

Overall, these bioassay methods revealed no significant activity of the three Cry proteins against *C. puncticollis* when transformed sweetpotato storage roots and root powder were used. This lack of efficacy was due to either a low level of accumulation or inactive form of the Cry proteins in these transgenic events (Fig. 1). For the Cry7Aa1 and Cry3Ca1 events, the quantities were too low as compared to the LC_{50} reported by Ekobu *et al*. (2010) to expect control of the weevil. However, for ET33-34, at least one event was close to the LC_{50} (0.394 µg g^{-1} of fresh storage root flesh) previously defined by Ekobu *et al*. (2010) and would be expected to show at least partial activity. Hence, the low accumulation cannot be the sole reason for lack of efficacy.

However, the comparison with the LC_{50} is hazardous because the artificial diet used three fold less dry matter, stressed larva, and pro-toxins which are more stable and typically less toxic than truncated protein. Although several reasons can be responsible for lack of activity of the Cry proteins, information from the literature is scarce concerning expression of Bt Cry proteins in storage roots.

In sweetpotato, the accumulation of the Cry3A1 was estimated to be 0.5 to 1.5 µg g^{-1} fresh storage root flesh (Moran *et al*., 1998). These authors observed partial resistance in the field using *C. formicarius*. Hence, more research is needed on additional transgenic events to determine whether the Cry proteins in sweetpotato roots are functional or alternatively new gene constructs need to be tested in order

to lead to higher accumulation of Cry proteins in the storage root of sweetpotato for effective weevil control.

ACKNOWLEDGEMENT

We acknowledge Mr. Moses Mwonda for taking care of transgenic plants in the screenhouse, Miss Justine Nanteza for assistance in rearing of weevils. The Regional Universities Forum for Capacity Building in Agriculture provided a Ph.D fellowship to the first author. We are grateful to the United States Agency for International Development USAID and the Bill and Melinda Gates Foundation through the SASHA project for funding this work.

REFERENCES

Alcazar, J., Cisneros, F. and Morales, A. 1997. Large-scale implementation of IPM for sweetpotato weevil in Cuba: A collaborative effort. Program Report 1995-1996. International Potato Center, Lima, Peru.

Braun, A.R. and van de Flieart, E. 1997. Implementation of IPM for sweetpotato in Vietnam and Indonesia. Annual Progress Report. International Potato Center, Lima, Peru .

Christou, P., Capell, T., Kohli, A., Gatehouse, J.A. and Gatehouse, A.M. 2006. Recent developments and future prospects in insect pest control in transgenic crops. *Trends in Plant Science* 11:302-308.

Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Coombs, J.J., Douches, D.S., Cooper, S.G., Grafius, E.J., Pett, W.L. and Moyer, D.D. 2005. Combining natural and engineered host plant resistance mechanisms in potato (*Solanum tuberosum* L.) for Colorado potato beetle (Leptinotarsa decemlineata Say): Choice and no choice field studies. *Journal of the American Society for Horticultural Science* 130: 857-864.

Ekobu, M., Solera, M., Kyamanywa, S., Mwanga, R.O.M., Odongo, B., Ghislain, M. and Moar, W.J. 2010. Toxicity of seven Bacillus thuringiensis Cry Proteins Against Cylas puncticollis and Cylas brunneus (Coleoptera: Brentidae) Using a Novel Artificial Diet. *Journal of Economic Entomology* 103:1493-1502.

Fuglie, K.O. 2007. Priorities for sweetpotato research in developing Countries: Results of a survey. *HortScience* 42:1200-1206.

García, R., Morán, R., Mena, J., Somontes, D., Zaldúa, Z., López, A. and García, M. 2000. Sweetpotato (*Ipomoea batatas* L.) regeneration and transformation technology to provide weevil (*Cylas formicarius*) resistance. Field trial results. In: Plant Genetic Engineering: Towards the Third Millennium. Arencibia, A.D. (Ed.). Elsevier Science B.V. pp. 112-117.

Geisthardt, M. and van Harten, A. 1992. Noxious beetles of the Cape Verde Islands with additional reference to West Africa: An illustrated guide to the identification of actual and potential coleopterous pests of crops, stored food, and wood. Verlag Christa, Hemmen Wiesbaden. 242p.

Hong, Y.F., Liu, C.Y., Cheng, K.J., Hour, A.L., Chan, M.T., Tseng, T.H., Chen, K.Y., Shaw, J.F. and Yu, S.M. 2008. The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato. *Plant Molecular Biology* 67:347-361

Hwang, J.S. and Hung, C.C. 1991. Evaluation of the effect of integrated control of sweetpotato weevil, *Cylas formicarius* Fabricius with sex pheromone and insecticide. *Chinese Journal of Agricultural Science* 85:477-493.

Jackson, D.M., Harrison Jr, H.F. and Ryan-Bohac, J.R. 2012. Insect Resistance in Sweetpotato Plant Introduction Accessions. *Journal of Economic Entomology* 105:651-658.

Kiiza, B., Mwanga, R.O.M., Kisembo, L., Kreuze, J., Labarta, R. and Ghislain, M. 2009. Analysis of economic implications of biotech sweetpotato in the Great Lakes Region to control weevil and virus disease damage. Uganda Country Report.

Kreuze, J.F., Valkonen, J.P.T. and Ghislain, M., 2009. Genetic engineering. pp. 41 - 63. In: The sweetpotato. Loebenstein, G. and

Thottappilly, G. (Eds.), Springer-Verlag New York Inc., USA.

Luo, H.R., Santa Maria, M., Benavides, J., Zhang, D.P., Zhang Y.Z. and Ghislain, M. 2006. Rapid genetic transformation of sweetpotato (*Ipomoea batatas* (L.) Lam) via organogenesis. *African Journal of Biotechnology* 5:1851-1857.

Mao, L., Story, R.N., Hammond, A.M. and Labonte D.R. 2001. Effect of sweet potato genotype, storage time and production site on feeding and oviposition behavior of the sweet potato weevil, Cylas formicarius (Coleoptera: Apoinidae). *Florida Entomology* 84:259-264.

Moran, R., Garcia, R., Lopez, A., Zaldua, Z., Mena, J., Garcýa, M., Armas, R., Somonte, D., Rodriguez, J., Gomez, M. and Pimentel, E. 1998. Transgenic sweetpotato plants carrying the delta-endotoxin gene from Bacillus thuringiensis var. tenebrionis. *Plant Science* 139:175-184.

Muyinza, H., Talwana, H.L., Mwanga, R.O. and Stevenson, P.C. 2012. Sweetpotato weevil (*Cylas* spp.) resistance in African sweetpotato germplasm. *International Journal of Pest Management* 58:73-81.

OECD Organisation for Economic Co-operation and Development. 2010. Series on the safety of novel foods and feeds, No. 20. Consensus document on compositional considerations for new varieties of sweet potatoes [*Ipomoea batatas* (L.) Lam.]: Key food and feed nutrients, anti-nutrients, toxicants and allergens: 40. http://www.oecd.org/science/biotrack/46815326.pdf. [Accessed 20 January 2013].

Perez, C.J., Tang, J.D. and. Shelton, A.M. 1997. Comparison of leaf-dip and diet bioassays for monitoring Bacillus thuringiensis resistance in field populations of diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology* 90: 94-101.

Smit, N.E.J.M. 1997. Integrated Pest Management for sweetpotato in Eastern Africa PhD Thesis, Wageningen University, The Netherlands.

Smit, N.E.J.M., Downham, M.C.A., Laboke, P.O., Hall, D.R. and Odongo, B. 2001. Masstrapping male Cylas spp. with sex pheromones: A potential IPM component in Sweetpotato production in Uganda. *Crop Protection* 20: 643-651.

Sorensen, K.A. 2009. Sweetpotato insects: identification, biology and management. pp. 157 - 184. In: The sweetpotato. Loebenstein, G. and Thottappilly, G. (Eds.). Springer-Verlag New York Inc., USA.

Stathers, T.E., Rees, D., Nyango, A., Kiozya, H., Mbilinyi, L., Jeremiah, S., Kabi, S. and Smit, N. 2003. Sweetpotato infestation by *Cylas* spp. in East Africa: II. Investigating the role of root characteristics. *International Journal of Pest Management* 49:141-146.

Vasquez, E.A. and Gapasin, D.P. 1980. Stems and tubers for rearing sweet potato weevil. *Annals of Tropical Research* 2:80-88.