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Screening and partial characterization of δ-endotoxins from some local *Bacillus thuringiensis* isolates for insecticidal activity against the spotted stem borer

PETER GITHAIGA KIMANI[1], HUDSON NYAMBAKA[1,♥], MUO KASINA[2]
[1]Department of Chemistry, Kenyatta University. Nairobi, Kenya. ♥email: nyambaka.hudson@ku.ac.ke
[2]Biotechnology Centre, Kenya Agricultural Research Institute. Nairobi, Kenya

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Abstract. *Kimani PG, Nyambaka H, Kasina M. 2019. Screening and partial characterization of δ-endotoxins from some local* Bacillus thuringiensis *isolates for insecticidal activity against the spotted stem borer. Biofarmasi J Nat Prod Biochem 17: 21-38.* Prolonged use of synthetic chemical pesticides is environmentally undesirable, causing the rapid development of resistance among insect pests. Kenya has unexplored potential in controlling lepidopteran pests by using proteinous *delta*-endotoxins sourced from local isolates of a naturally occurring bacterium, *Bacillus thuringiensis* Berliner (*Bt*). This study attempted to identify the insecticidal proteins in some Kenyan *Bt* isolates, characteristic of Cry1 and/or Cry2 proteins. It also aimed to test their efficacy as affected by different temperatures and their specificity on an invasive and prevalent lepidopteran stem borer, *Chilo partellus* (Swinhoe). Using froth floatation and centrifugation, delta-endotoxin crystals were isolated and purified from cultures of twenty unidentified local Bt isolates. Total protein in the resulting suspensions was quantified using the Bradford assay method, and the approximate protein yield was 3.14 ±0.084 mg/mL of nutrient broth culture with a purity level of 54.8 % ±15.3 %. Leaf-dip bioassays were used for testing the efficacy of the δ-endotoxins against *C. partellus*. Among the isolates evaluated, *Bt* 44 and *Bt* 48 had the most potent δ-endotoxin crystals towards the 1st instar larvae, leading to mortality of 62.6 % and 64.8 %, respectively, after 72 h. The effect of the δ-endotoxins' concentration and temperature on larval mortality was examined for 72 hours at temperatures of 24°C, 27°C, and 31°C and levels of 0.01 mg/mL, 0.1 mg/mL, and 1.0 mg/mL. The resulting LC_{50} was 52.3 µg/mL and 42.0 µg/mL, while LT_{50} values were 76.7h and 60.9h for *Bt* 44 and *Bt* 48, respectively. Higher efficacy was found at 24°C and 31°C than at 27°C, an indication that these δ-endotoxins are tolerable for local conditions where temperatures are higher than in temperate regions. The relationship between concentration and temperature was significant for δ-endotoxins of *Bt* 48 but not those of *Bt* 44. A major protein component of the δ-endotoxins had a molecular weight of M_r ~ 130 kDa, which generates a trypsin-resistant core of M_r ~ 70 kDa. Cry protein analysis detected more Cry1 in *Bt* 44 than *Bt* 48 δ-endotoxins and no Cry2 in either. However, *cry* gene analysis using PCR detected the presence of both *cry1* and *cry2* genes in the DNA of *Bt* 44 but none in *Bt* 51, a negative control from toxicity tests against the pest. The chromatographic analysis revealed some differences in the elution profiles of δ-endotoxins of both *Bt* 44 and *Bt* 48, indicating that there may be different types and amounts of Cry toxins in the crystals or even novel proteins. These findings indicate that the two local *Bt* isolates expressed Cry1 and probably Cry2 proteins can control *C. partellus* and may, therefore, become promising sources for δ-endotoxins for biopesticide development for controlling the pest.

Keywords: δ-endotoxins, *Bacillus thuringiensis*, insecticidal activity, stem borer

INTRODUCTION

Lepidopteran stem borers are the most important group of insect pests that attack sorghum, maize, and sugarcane in many parts of the world (Mugo et al., 2001; Tende et al., 2010). Stem borer species that attack cereal crops in Kenya include *Chilo orichalcociliellus*, *Chilo partellus*, *Busseola fusca*, *Sesamia calamities*, and *Eldana saccharina* (Songa et al. 2002). *C. partellus* (Lepidoptera: Pyralidae) is Africa's most prevalent stem borer pests, which has colonized much of Eastern and South Africa (Hutchison et al. 2008) due to its high invasiveness.

Chilo partellus may be displacing native stem borer species in several areas of Kenya, including the low-altitude and coastal maize regions (Mbapila et al. 2002; Ofomata 2003). In severe infestations, *C. partellus* attacks entire parts of the maize plant but the roots and causes losses of up to 75% of maize seedlings (Khan and Amjad 2000). In Kenya, pest populations are abundant not only in the dry mid-altitude and dry coastal areas but also in the moist-transitional and moist mid-altitude (ca.<1500 m) agro-ecological zones. In this area, yield losses fall at US$ 34-75/hectare (De Groote et al. 2003; Muhammad and Underwood 2004). These losses have made up a large portion of the estimated yield loss of 13.5% annually in Kenya (De Groote 2002), translating to 400,000 tonnes of maize, equivalent to the amount imported annually; besides, the areas are most prone to food insecurity.

The maize ruined by pests is highly susceptible to mycotoxin contamination causing aflatoxin and fumonisin poisoning generally experienced in Kenyan maize growing zones (Betran and Isakeit 2003; Campa et al. 2005). Nowadays, Kenya lost a bumper harvest of maize to aflatoxin contamination in the Coast and Eastern provinces, which the government had to mop up to decrease eminent poisoning (Muthomi et al. 2010; Ngetich 2010).

Broad-spectrum synthetic chemical pesticides have been mainly used to manage agricultural pests (Muhammad

and Underwood 2004), but their application has not been maintained due to their high cost for small-scale Kenyan farmers (Bonhof et al. 2001; Tarus et al. 2010). Other issues of concern have been environmental degradation, disruption of the pests' natural enemies, and the development of secondary pests. Many reports showed cases of resistance to target insects. It is, therefore, essential to develop alternative pest management strategies that are safe, environmentally friendly, and can be used in the Integrated Pest Management systems (Camilla 2000). Biological control using entomopathogenic bacterium such as *Bacillus thuringiensis (Bt)* is a promising option.

The microbial pesticide, based on *Bacillus thuringiensis* (*Bt*), can, in an integrated control program, be used rapidly and efficiently to control insect pests (Dulmage 1993). Products based on *Bt* are the most successful microbiological pesticides used in forestry, agriculture, and public health (Burges 1982). Despite their success, local people use it in a limited amount, accounting for only about 2% of the world's insecticide market (Cetinkaya 2002). The main limiting factors include the fact that many of the well-characterized *Bt* strains have been isolated in temperate zones and may not be well suited for application in pest management programs in the tropics. Therefore, local isolates may be better because they possess useful attributes such as greater field persistence at high temperatures (Brownbridge 1991). Further, the impact of the environment on *Bt* efficacy is not unexpected because secondary products are induced or increase in concentration during periods of physiological stress or physical damage (Dixon and Paiva 1995).

Specific objectives: (i) To determine the insecticidal activity of local *Bt* isolates against the spotted stem borer, *C. partellus*, (ii) To assess the effect of temperature and concentration of δ-endotoxin from local *Bt* isolates on insecticidal activity, (iii) To characterize the local *Bt* isolates for the presence of Cry1 and Cry2 proteins.

MATERIALS AND METHODS

Research design

The study was carried out at three levels. First, determine the most active isolates by culturing each *Bt* isolate sampled on agar plates and multiplying it by fermentation in liquid broth. The two most active strains were identified by leaf-dip insect bioassay. Second, to determine the effects of *Bt* isolate, δ-endotoxin concentration, temperature, and their interaction on larval mortality using leaf-dip insect bioassay on neonate *Chilo partellus* (Lepidoptera: Pyralidae). Third, protein profiles such as molecular weights, relative quantity, and identity in each active isolate were examined using denaturing gel electrophoresis (SDS-PAGE), liquid chromatography (RP-HPLC), and Enzyme-linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR) experiments as illustrated in Figure 1.

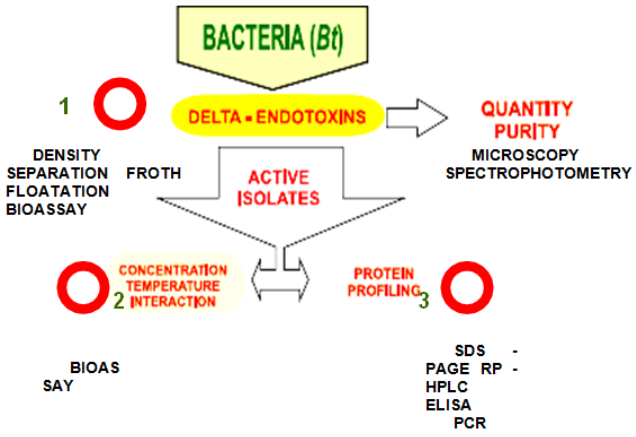


Figure 1. Illustration of the research design

Bt isolates and the sampling procedure

Local *Bt* isolates were isolated from KARI Biotechnology Centre (Mwathi 2007). Meanwhile, the standard control reference isolates, *B. thuringiensis* subsp. *aizawai* HD-133 (*Bta*) and *B. thuringiensis* subsp. *kurstaki* HD-1 (*Btk*) was kindly provided by Prof. Zeigler, D. R. of *Bacillus* Genetic Stock Centre, USA. Simple random sampling was used to select twenty local *Bt* isolates from a pool of 68.

Activation and multiplication of *Bt* isolates

Twenty local *Bt* samples and a reference standard *Bt. aizawai* (*Bta*) isolate was renewed by streaking aseptically on agar plates following the manufacturer's instructions and earlier report (Poinar and Thomas 1978). The *Bt* inoculants were incubated at 30ºC for seven days. The formation of bi-pyramidal-shaped protein crystals by a phase-contrast microscope confirmed the growth of inoculants. A loopful of the sporulated culture of *Bt* was inoculated into 50 mL liquid nutrient broth (Biotec Laboratories Ltd Ipswich, United Kingdom). Separate inoculation processes were repeated for all the isolates and the standard. The inoculated flasks were incubated on a rotary shaker for 96 h at 30ºC at 200 rpm.

Isolation of the delta-endotoxin crystals

The δ-endotoxin crystals were collected by centrifugation at 1,300 rpm for 10 min at 4ºC (Eppendorf 5810C®, Eppendorf-Nether-Hinz, Hamburg, Germany). The pelleted crystals were rinsed by low-speed centrifugation (4,000 rpm, 4ºC, 5 min) in sterile 0.85 % saline, air-dried, weighed, and suspended in 5-mL 0.85 % saline to minimize exoprotease activity, and stored at-20ºC. The purity of the crystals was confirmed by phase-contrast microscopy.

The total protein concentration of the crystals was measured using the Bradford-protein determination method (Bradford 1976) using a UV-Vis spectrophotometer (SmartSpec Plus®, Bio-Rad laboratories Inc. USA). The Bradford reagent consists of 10 mg coomassie brilliant blue (G-250) in 5 mL of 95 % ethanol, adding 10 mL of 85 % (w/v) phosphoric acid, diluting it to 100 mL, and filtering

the solution through Whatman's no.1 before use. Bovine Serum Albumin (BSA, fraction V) served as a standard at a stock solution concentration of 2 mg/mL. Into appropriately labeled test tubes, 0.1 mL of each standard and selected *Bt* samples were mixed, 5.0 mL of the Bradford reagent added, mixed well, and incubated at room temperature for five minutes. Absorbance was calculated at 595 nm for dilutions ranging from 0.1 to 2 mg/mL of standard. A calibration curve of the standard protein was made to determine the total protein concentration of *Bt* isolates. These measurements resulted in protein yield and percentage of total protein in the pellets of the *Bt* isolates.

Tests for insecticidal activity

Experimental insects

Yellow *C. partellus* eggs were collected from the International Centre for Insect Physiology and Ecology (ICIPE) and Kenya Agricultural Research Institute (KARI), Katumani insectaries. The eggs were surface sterilized with a 10% sodium hypochlorite solution and placed in plastic hatching jars at 28°C. It required 5-6 days for eggs to hatch to neonate larvae. The physiological age of larvae in all bioassays was determined to be 1-day-old old neonates.

To ensure a continuous supply of neonate larvae, a colony was maintained at 28°C and 65 ± 5 % at a photo-period of 12L:12D hours without exposure to any xenobiotics in the Insect Bioassay Laboratory, Biotechnology section of KARI, NARL, Nairobi from the initial eggs to minimize physiological variability of the larvae.

Experimental diet

The natural diet for the larvae was maize leaves collected from two to four-leaved CML 216 maize plants grown in the KARI Biosafety Greenhouse Level 2. All bioassay experiments refer to the leaf-dip method by Yee et al. (2008) and Insecticide Resistance Action Committee (IRAC) method No. IV, with some modifications. The neonates were collected using a camel hairbrush to avoid injury and were classified as dead when they did not respond to prodding utilizing the brush. The corpses were surface sterilized and crushed in a Petri dish to confirm that the larvae had died from Bt infection. The homogenate was inoculated on a nutrient agar plate and incubated for 24 h at 30°C. At the end of each bioassay, all dead larvae were sterilized in 70 % ethanol, autoclaved at 121°C, and disposed of. The Petri dishes and other reusable pieces of equipment were cleaned by soaking in sodium hypochlorite for one hour and washed with a detergent. All plant tissues, dead insects, and other disposable items were placed in biohazard bags, autoclaved for one hour, and burnt in an open pit.

Preliminary bioassay

A preliminary study was carried out to select the optimum concentration treatments for screening the toxicity of local *Bt* isolates. At the 2-4th leaf stage (2 cm), Maize leaf disks were dipped in the *Bta* δ-endotoxin suspension for 3 minutes at various concentrations (13.5, 1.35, 0.135, 0.0135, and 0.00135 mg/mL) and allowed to air dry on for 30 minutes. 0.85 % saline in place of δ-endotoxin suspension served as the negative control. One larva per leaf disk grew in a Petri dish (diameter = 8.5 cm) lined with a wet filter paper aseptically. All Petri dishes were sealed with an absorbent paper towel for proper aeration. The room temperature was maintained at 27 ± 1°C and relative humidity (R. H.) at 65 ± 5 % at a photoperiod of L12: D12.

Three replicate assays were carried out on alternate days for a total of 30 larvae at each dose level. The monitoring of the experiment was carried out daily for seven days, with larval mortalities recorded every 24 h. The mean lethal concentration causing 50 % larval mortality (LC_{50}) was determined by averaging the replicates, converting mortalities to percentage mortality, transforming percentage larval mortalities to probits, and plotting these against log-transformed concentration values, then reading off the concentration corresponding to the probit = 5 using probit analysis. The LC_{50} obtained was used as the toxin concentration in the screening assay.

Screening bioassay

Nineteen *Bt* isolates were screened to determine the two most effective strains against *C. partellus*. The larvae were subject to a treatment of one dose of toxin. Maize leaf discs were treated with 11.0 µg/mL of *Bt* δ-endotoxin suspension (LC_{50} at 72 h obtained for *Bta* standard), and the bioassay setup is referred to as above mentioned. The average lethal time taken for 50 % larval mortality (LT_{50}) was determined by transforming percentage larval mortalities to probits and plotting these against log-transformed time values. The LT_{50} result was used to rank the *Bt* isolates to increase adequate median lethal time, which suggests decreasing toxicity of δ-endotoxin to *C. partellus* neonate larvae. The two isolates showing the highest larval mortality and the shortest LT_{50} were selected for further bioassay and protein analysis.

Concentration and temperature bioassays

The principal technique used to assess concentration-mortality relationships was similar to Yee et al. (2008), with slight modifications to accommodate the temperature variable. Neonate *C. partellus* larvae were exposed to maize leaf disks dipped in *Bt* suspensions with concentrations ranging from 1.0, 0.1, and 0.01 mg/mL. The duration of exposure was maintained for 72 h, after which untreated leaf disks replaced the inoculated ones for the remainder of the assay. The larvae were incubated at 24°C. Thirty larvae were examined per concentration per isolate per temperature. The entire experiment was carried out on two other alternate days resulting in three replicate assays for each temperature variation. The same was repeated for two different temperatures (27 and 31°C), giving about 270 larvae being exposed to each *Bt* isolates.

For each bioassay, 90 larvae (in groups of 30 per temperature treatment) were treated similarly as a negative control by exposing them to distilled water. The larvae were assigned to treatments in a complete randomized design. Dead larvae were documented daily up to six days after treatment (DAT). Probit analysis was applied to

estimate median lethal concentration (LC_{50} values) and median lethal time (LT_{50} values) for each isolate. To determine the effects of level, temperature, and their interaction for each strain on percentage mortality of *C. partellus* neonate larvae, one and two-way analyses of variance were carried out using Statistical Package for Social Scientists (SPSS Inc. 2004) program.

Protein profiling

The characteristics of *Bt* δ-endotoxins from the efficacious isolates were investigated using electrophoresis, ELISA, liquid chromatography, and polymerase chain reaction (PCR).

Electrophoresis

Electrophoresis using discontinuous 12 % SDS-PAGE was conducted under reducing conditions on a vertical electrophoresis unit (Vertical slab gel unit, Hoefer scientific instruments, SE 600 series) following Laemmli (1970) with modifications. The running buffer comprises 15.0 g/l Tris base, 72.0 g/l glycines, and 5.0 g/l SDS at pH 8.3. The polyacrylamide gel slab was discontinuous with an upper stacking gel to concentrate the sample, increase band resolution, and resolve the lower gel for protein separation. The resolving gel was cast first using 30 mL of a solution containing acrylamide mix (30 % total monomer concentration and 2.67 % cross-linking monomer concentration, 7.5 mL of 1.5 M Tris-HCl (pH 8.8), 0.3 mL of 10 % SDS, 9.9 mL distilled water, 0.3 mL of 10 % APS, 12 µL TEMED) and allowed to polymerize at room temperature (25ºC) for 90 minutes. The stacking gel was cast on top of the resolving gel using a 10 mL solution containing acrylamide mix (1.7 mL), 1.0 M Tris-HCl (pH 6.8) of 1.25 mL, 10 % SDS (100 µL), 6.8 mL distilled water, 10 % APS (100 µL), 10 µL TEMED and allowed to polymerize at room temperature for 90 minutes.

Samples which contain the 15 µL *Bt* δ-endotoxin suspensions of *Bt* 44, *Bt* 48, *Bt* 51 (negative control) and *Bta* (standard control) were diluted at a ratio of 1:3 with sample/loading buffer containing 10 % SDS (1.6 mL), 2-β-mercaptoethanol (0.4 mL), glycerol (0.8 mL), 0.2 mL of 0.05 % (w/v) bromophenol blue dye (tracker dye), 0.5 M Tris-HCl pH 6.8 (1.0 mL), 1.6 mL of 1 M 1,4-dithiothreitol (DTT) in 4.0 mL distilled water and repeated using 20 µL and 30 µL of samples for optimization. Samples were boiled for five minutes and centrifuged at 10000g for 5 min before loading 60 µL per well alongside a standard molecular weight marker (See Blue® Plus 2 prestained standard, Range: 4-250 kDa, Invitrogen).

Electrophoresis was performed at 80mA for about two hours. The gel was submerged in a staining solution for 30 minutes at room temperature with very gentle shaking on a rocker. The staining solution was made of 0.1 % (w/v) Coomassie Brilliant Blue (R250) in 40 % methanol, and 10 % (v/v) acetic acid and then filtered after the dye had dissolved. Destaining the gel was carried out in an excess solution containing 40 % (v/v) methanol, and 10 % (v/v) acetic acid at shallow speed shaking while making three changes to the destaining solution until a transparent background was obtained. The procedure was repeated the same as above, using samples containing solubilized *Bt* δ-endotoxins.

The samples were solubilized by suspending the *Bt* δ-endotoxins crystals in 50 mM Na_2CO_3.$NaHCO_3$ buffer (pH 9.5) containing ten mM DTT and incubated for 30 h at 37ºC. The solution was centrifuged for 15 minutes at 10000g. The supernatant was mixed with porcine trypsin solution (cell-culture grade, Sigma Chemical, St. Louis, USA) at an enzyme/toxin ratio of 1:20 (w/w) for 24 h at 37ºC to obtain trypsin-resistant fragments. The suspension was centrifuged for 10 minutes at 10000 g at 4ºC, and the supernatant was diluted with the loading buffer as previously described.

Crystal protein solubilization and tryptic digestion

The *Bt* δ-endotoxins crystals of the local isolates (*Bt* 44 and *Bt* 48) and the standard strain *Bta*) were solubilized and digested as described previously in section 3.6.1 to obtain trypsin-resistant fragments. The resulting solution was centrifuged at 15000 g for 15 minutes before injection into the HPLC system.

Reverse phase-High-performance liquid chromatography

A protein mapping method was modified from the original method, according to Fullmer and Wasserman (1979), that included a Beckman 126 HPLC system equipped with a reversed-phase C_{18} (Beckman ODS, 250 mm x 4.6 mm i.d.) analytical column. A 100 µL aliquot of trypsin-digested δ-endotoxins was combined with 25 µL acetic acid, then 20 µL of the sample, which had been centrifuged (15000 g, 15 min.), manually injected into the HPLC apparatus. After optimization, chromatographic separations were done by isocratic elution using a mobile phase containing acetonitrile and water at a ratio of 70 %: 30 %, respectively (the water was acidified with 0.2 % acetic acid) at ambient room temperature (25ºC). The flow rate was set at 1 mL/min at 10 MPa, and the separations were monitored by UV absorption at 254 nm using a photodiode array detector for 10 minutes. The data obtained was analyzed automatically using the integrated computer program.

Sandwich enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA analysis was carried out according to the EnviroLogix QualiPlate® kit (EnviroLogix Inc, Portland, USA) for non-quantitative laboratory detection of Cry1 and Cry2 proteins. The kit set was supplied with micro-titer plates, enzyme conjugates, substrates, and positive controls. The samples of δ-endotoxin suspensions of *Bt* isolates (*Bt* 44, *Bt* 48, *Bt* 51, and *Bta*) were made by adding a buffer solution containing phosphate-buffered saline (PBS) / 0.55 % Tween-20 at a ratio of 1:10. The solution was left to stand overnight, centrifuged at 15,000 rpm for 10 mins, and the supernatant was decanted and retained. The experiment was carried out following the manufacturer's kit protocol.

In brief, separate microtitre plates with wells precoated with antibodies raised against Cry1 and Cry2 proteins. Cry enzyme conjugate (horseradish peroxidase) (50 µL) was mixed immediately, followed by 50 µL or buffer blank, 50 µL of Cry positive control, and 50 µL of *Bt* δ-endotoxin

supernatant solution. The solutions of the wells were thoroughly mixed by shaking the plates in a rapid circular motion on the benchtop for 30 seconds. The high precaution was observed to avoid cross-contamination between wells. The wells were sealed with Parafilm to prevent evaporation and incubated at ambient temperature (25ºC) for 2 hours. The Parafilm was carefully removed, and the contents of the wells were vigorously shaken off into a sink. The wells were soaked with PBS / 0.05 % and Tween-20 wash buffer, then shaken off to empty contents. This wash step was repeated three times. The plates were dried using a blotting paper towel to remove as much water as possible.

100 µL of the substrate was added to each well, and the contents were thoroughly mixed. The plates were sealed with new parafilm and incubated further for 30 minutes at ambient temperature. Finally, 100 µL of 1 M hydrochloric acid stop solution was mixed thoroughly in each well. The experiment was carried out in three replicates (three wells per sample, blank, and positive control). The plates were read using UV absorption at 450 nm using a microtitre plate reader (Biohit® BP 800, Biotek Instruments Inc. USA) within 30 minutes of the addition of the stop solution, and the absorbance was recorded.

Polymerase Chain Reaction

The more toxic local *Bt* isolate *Bt* 44 was analyzed for genes encoding Cry1, Cry2, and Cry3 proteins. *Bt aizawai (Bta)* and *Bt kurstaki* (*Btk)* isolates served as positive control. A local strain, *Bt* 51, which had shown no toxicity against *C. partellus* at the screening stage and had previously been used in screening assays on coleopterans (Mwathi 2007), served as a negative control in the experiments.

DNA extraction

High purity plasmid DNA was isolated from *Bt* isolates using Pure Link® plasmid miniprep Kit (Invitrogen). *Bt* culture was incubated at 30ºC overnight in LB media while shaking at 200 rpm. Cells were pelleted by centrifugation for 5 minutes at 14000 g and resuspended in 250µL of suspension buffer (R3) with RNase A until no cell clumps remained. An equal amount of lysis buffer was mixed with the cells, and the solutions were mixed gently, five times without vortexing. The suspension was incubated for 5 minutes at room temperature (25ºC), and after that, 350µL of precipitation buffer was added until the solution was homogenous. Centrifugation of the mixture was carried out at 12000 g for 10 minutes to separate lysate from lysis debris. The supernatant was loaded on a spin column, and DNA was purified using centrifugation as per the manufacturer's instructions.

In brief, the supernatant in the spin column was placed into a wash tube. About 700 µL of wash buffer (W9) with Ethanol was added to the column. The mixture was centrifuged for 1 minute at 12,000g. The flow-through was discarded, and the column was placed back into the wash tube. The same step was repeated for a minute. Both the wash tube and the flow-through were discarded. Plasmid DNA was eluted using 75µL of preheated TE buffer in the middle of the spin column. The DNA concentration was estimated using Nanodrop. This plasmid DNA was used for all Polymerase Chain Reactions (PCR).

Oligonucleotide PCR primers

Amplification of the *cry1 and cry2* homology groups utilized one pair of universal primers designed to amplify a specific fragment by simultaneous alignment with all previously described genes in that group by using the Amplify 1.0 program (Bill Engels, University of Wisconsin, Madison) as a defined by Ben-Dov et al. (1997). The primers and the expected sizes of their PCR products are displayed in Table 1. A single universal primer and several specific primers for each *cry* class (selected from their highly variable regions) were applied together in two reactions. The oligonucleotide primers were obtained from Ransom Hill Bioscience, Inc. (Ramona, Calif., USA); each pair was highly specific and yielded a PCR product of the predicted size that was easily identified by electrophoresis in agarose gels (0.8 to 2.5 %).

DNA templates and PCR analysis

Template DNA was denatured for 5 min at 94oC, annealed to primers for 40 s at 55oC, and extended at 72oC for 90 s for 2.15 h in a DNA Thermocycler (MJ Research, Inc., Watertown, Mass.) by 30 reaction cycles. Each reaction was carried out in 25 µL; (1 µL of template DNA was mixed with 2 X GC of 12.5 µL reaction buffer, 150 mM (each) deoxynucleoside triphosphate (3.75 µL), ten µM (each) primer (Forward and reverse), and 0.25 U of *Taq* DNA polymerase (Appligene).

Table 1. The characteristics of the universal primers for Cry1 and Cry2 and specific primers for Cry2Aa1, Cry2Ab2, and Cry2Ac protein-encoding genes

Gene	Primer[a]	Primer sequence[b]	Product size (Bp)
cry1	Un1 (d)	5'-CATGATTCATGCGGCAGATAAAC-3'	277
	Un1(r)	5'-TTGTGACACTTCTGCTTCCCATT-3'	
cry2	Un2 (d)	5'-GTTATTCTTAATGCAGATGAATGGG-3'	698
	Un2(r)	5'-CGGATAAAATAATCTGGGAAATAGT-3'	
cry2Aa1	Un2 (d)	5'-GTTATTCTTAATGCAGATGAATGGG-3'	498
	EE-2Aa(r)	5'-GAGATTAGTCGCCCCTATGAG-3'	
cry2Ab2	Un2 (d)	5'-GTTATTCTtAATGCAGATGAATGGG-3'	546
	EE-2Ab(r)	5'-TGGCGTTAACAATGGGGGGAGAAAT-3'	
cry2Ac	Un2 (d)	5'-GTtATtCTTAATGCAGATGAATGGG-3'	725
	EE-2Ac(r)	5'-GCGTTGCTAATAGTCCCAACAACA-3'	

Note: **a** (d) and (r) direct and reverse primers, respectively; **b** Bases that do not match the appropriate sequences are shown by lowercase letters

The reliability of the primers was verified with the following *B. thuringiensis* reference strains: *B. thuringiensis* subsp. *kurstaki* HD-1 (*Btk*) and *B. thuringiensis* subsp. *aizawai* HD-133 (*Bta*) for the *cry1* and *cry2* classes against samples (*Bt* 44 and *Bt* 51). Electrophoresis of the PCR products was carried out in agarose gels (1.5%) for 30 minutes, followed by staining in Coomassie Blue for 15 minutes. Gels were photographed under UV.

Statistical analysis

The bioassay data were compiled, tabulated, and analyzed using Microsoft Office Excel 2003 (Microsoft Corporation, 1983-2003) and Statistical Package for Social Sciences (SPSS) version 13 (SPSS Inc. 2004, USA). The calibration curve using BSA as the standard protein was used to estimate the total protein concentration of the *Bt* δ-endotoxins. Dixon's Q-test was used to reject outliers, while the accuracy of results was expressed using standard deviation, standard error, coefficient of variance, and confidence intervals (Harvey 2000; Rorabacher 1991; Skoog et al. 1992). The percentage of larval mortalities was calculated every 24 h for 144 h in replicates. Where the mortalities of the control were between 5 % and 10 %, the larval mortalities were corrected using Abbott's formula (Equation below) before analysis, while those >10% were excluded (Abbott 1925).

$$P_t = \frac{P_o - P_c}{100 - P_c} \times 100\ \%$$

Where;
P_t = corrected mortality (%);
P_o = observed mortality (%);
P_c = control mortality (%):

Mean lethal time causing 50 % larval mortality (LT_{50}) and mean lethal concentration causing 50 % larval mortality (LC_{50}) values were measured using the probit analysis method by transforming percentage cumulative larval mortalities to probits and plotting these against log-transformed time and concentration values, respectively. The data were subjected to one and two-way ANOVA to evaluate the effect of each treatment and their interactions and thus test the null hypothesis. The average value was separated across different isolates, temperature levels, and concentrations levels with Tukey's HSD (Honestly Significant Difference) test and used to determine the main effects and interactions of the treatments (DeCoster and Claypool 2004). Where no statistical significance was observed, data were subjected to Bonferroni *post hoc* tests. The main factors and interaction effect size were calculated using partial *Eta* squared (η^2) and categorized as 0.01 for a small effect, 0.06 for a medium effect, and 0.14 for a large effect, according to Cohen (1988). The correlations were investigated using a two-tailed Pearson product-moment correlation coefficient, and the strength of the relationship was categorized as 0.5-1.0 for strong, 0.3-0.49 for medium, and 0.1-0.29 for weak relationships (Cohen 1988). The significance level of all tests was p = 0.05. For chromatograms, peak properties were measured by the integrated HPLC computer program and aligned for comparison between samples. For Electrophoresis, a calibration curve was used to determine the molecular weights of the separated proteins in the samples.

RESULTS AND DISCUSSION

Isolation of *Bt* delta-endotoxin

During the period of activation and multiplication, the growth of the local *Bt* isolates and *Bta* was fast, producing smooth creamish-white colonies that were rough-edged and slightly raised from the nutrient agar (Figure 2.A), except for *Bt* 20, which showed yellowish fluffy growth. The Bt grew with slight foaming in the nutrient broth to form a pale yellow opaque suspension that thickened over time and settled to reveal white sediment. However, isolate *Bt* 20 developed a fast-settling gelatinous yellow suspension whose pellet mass weighed 0.873 g. The Dixon's Q-test revealed that pellet mass from *Bt* 20 was an outlier as Q_{exp} = 0.592, which was higher than Q_{crit} = 0.342 at a 95 % confidence level and hence excluded from further analysis due to its different characteristics (Figure 2.B).

The autolysis of the *Bt* cells was complete; thereby, most of the released protein crystals precipitated at the bottom of the flask. Nearly complete separation of the δ-endotoxin protein crystals from the spores and cell debris was carried out by decantating the frothy spent culture and low-speed centrifugation at 1,300 rpm. The crystals precipitated as a white pellet at the bottom of the tube, separating the spores and cell debris in the supernatant fraction. Serial washing, decanting, and centrifugation removed the crystals of spent culture components, spores, and cell debris.

Microscopy showed a high concentration of the bi-pyramidal-shaped crystalline inclusions in the pellets obtained. Table 2 displayed the pellet masses, total protein concentrations, and yields. The mass of the resulting pellets r from 0.489 g for *Bt* 16 to 0.225 g for *Bt* 47. The protein mass of the pellets was different significantly among the isolates. The percentage of total protein content in the pellets ranged from 26.5 to 92.1 %. Isolates *Bt* 31 and *Bt* 47 recorded higher contents, while *Bt* 5, *Bt* 16, and *Bt* 8 had low protein content. The protein yield from the nutrient broth ranged from 2.223 mg/mL to 4.603 mg/mL of nutrient broth and was significantly different across the different *Bt* isolates.

A **B**

Figure 2.A. Agar plate of *Bt* culture. B. Suspended pellets of *Bt* after centrifugation (yellow one is *Bt* 20)

The growth of the Kenyan *Bt* isolates on nutrient agar, and the nutrient broth was similar to that reported by others (Brownbridge 1991; Brownbridge and Onyango 1992; Wang'ondu 2003; Mwathi 2007), which illustrates the viability of these isolates at KARI germplasm. Nevertheless, the growth of *the Bt* 20 strain showed a deviation from the rest to its exclusion from further analysis. The protein purity and yield values reported in this study varied, although the culturing conditions were similar, indicating that the local isolates tested were diverse. Mohan and Gujar (2001) reported that although *Bt* strains in their study were cultured under the same conditions, their total yield spore counts differed even for the same strain obtained from two different sources.

The total protein yield reported in the literature also varies significantly from as low as 0.634 mg/mL (Ghribi et al. 2004) of culture broth to 15.33 mg/mL (Liu et al. 2000) using different culture conditions and crystal protein extraction protocols. Furthermore, it is documented that δ-endotoxin yields are affected by culture conditions and may vary within different *Bt* strains cultured in similar circumstances due to the enormous diversity of isolates that may have differences in optimum growing conditions and the variety of insecticidal crystal protein produced (Aronson et al. 1995).

Insecticidal activity of *Bt* δ-endotoxins

Toxicity of Bta

The neonate larvae range was fed from the underside of the leaf disk soon after placing them into the Petri dish. However, feeding frequency slowed with time, and some larvae moved away from the meal after 24 h. Most larvae, especially with higher δ-endotoxin concentrations, stopped feeding after 48 h, appeared weak, and stunted in growth compared to the control upon where death was also observed. For example, with the 13.5-mg/mL δ-endotoxin treatments, 60 % of the larvae were found to be away from the diet after 24 h. After 48 h, only 10 % of larvae were still feeding, 40% were located away from the leaves, 20 % looked weak, and 40 % of the larvae were dead. By contrast, all the larvae were actively feeding in the control group g.

Leaf damage was observed to be less on treated leaf disks than on the control. The larvae appeared dark and shrunk upon death compared to the live larva in control (Figure 3.A). A dead larva was washed, ground, and aseptically inoculated onto a nutrient agar plate. Creamish growth was observed around the larva, confirming that larval mortality was due to the ingestion of *Bt* endotoxins (Figure 3.B). In the set of starved larvae, mortality was 100 % in 48 h.

On the control treatment, a 10 % larval mortality was observed after 72 h, while 30 % was recorded after 144 h (Table 3). The LC_{50} value estimate for reference isolates *Bta* was 11.0 µg/mL after 72 h when larval mortality was 10 to 90 %.

Screening of Bt isolates

Among the different *Bt* treatments, only *Bta* and *Bt* 48 recorded 10% mortality at 24 h of observation. Although *Bt* 44 marked the first mortality of 40 % at 48 h, it is the only isolate that recorded 100 % mortality by 120 h. The *Bta* recorded 100 % mortality at 96 h. No larval mortality was found with *Bt* 51 and the control throughout the observation period. Calculations of LT_{50} showed that *Bt* 44 had the most toxic δ-endotoxins, causing 50 % mortality after only 56 h, followed by *Bta* at 64 h and *Bt* 48 at 73 h. The activity of δ-endotoxins from sample isolates compared to standard *Bta* (LT_{50} standard/LT_{50} sample) was 1.13 and 0.87 for *Bt* 44 and *Bt* 48, respectively, compared to the standard isolate *Bta* (Table 4), which was 1.00. One-way ANOVA (for repeated measures) revealed that the difference in percentage larval mortalities of the standard isolate *Bta* and the *Bt* isolates was statistically significant except for the two strains, *Bt* 48 and *Bt* 44, which were retained for further investigation.

$$* \text{Activity} = \frac{LT_{50}(\text{standard})}{LT_{50}(\text{sample})}$$

The toxicity of the local isolates tested towards the first instar of *C. partellus* varied from 70 % for *Bt* 44 to 0 % for *Bt* 51 after 72 h. Thus, reject the null hypothesis that there are no differences in the relative toxicity of δ-endotoxins from local *Bt* isolates against *C. partellus*. Besides, there was no correlation between the endotoxin yield and toxicity in this study, which illustrates that toxicity is probably due to the type of Cry proteins present in the crystals and not so much the protein amount. Indeed, this agrees with the finding that different *Bt* isolates produce different δ-endotoxins, which may differ in toxicity against different target pests (Uribe et al., 2003).

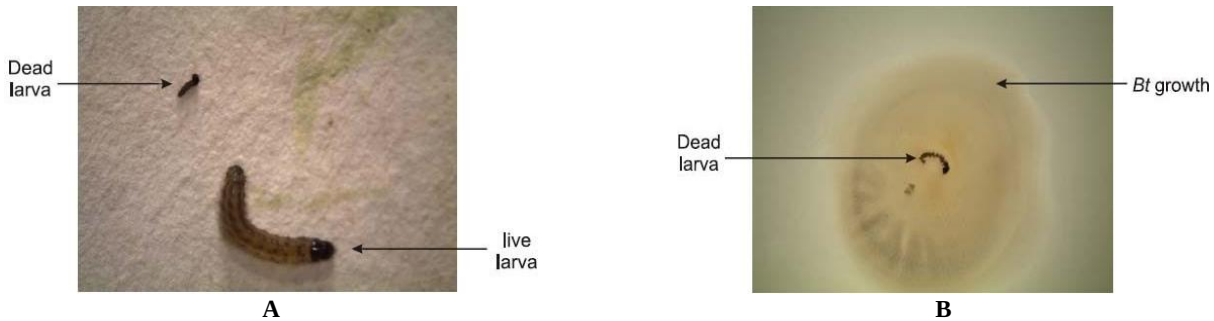


Figure 3.A. *C. partellus* larvae (live and dead). B. Culture of dead *C. partellus* larva

Therefore, culturing conditions need to be optimized for each *Bt* strain for large-scale production. More information is required on standardized culturing techniques, endotoxin extraction, and purification if comparisons are made. For example, Ghribi et al. (2004) reported that for *Bt var kurstaki*, the toxin yield increased by 30 % after 20 min of heat shock at 42 °C together with the addition of 5 g/l NaCl in a gruel-based culturing medium.

The δ-endotoxins from two isolates (*Bt* 44 and *Bt* 48) were selected for further investigation since they exhibited the highest larval mortalities. Although these endotoxins differed significantly from the other samples, they did not vary significantly with the standard reference isolate used (*Bta*). This selection was later confirmed by ranking the LT_{50} values obtained and retaining those that did not differ considerably from the standard reference isolate. This study also revealed that the δ-endotoxins from *Bt* 44 were 1.13 times more toxic than the standard control (*Bta*). Interestingly, an earlier study by Mwathi (2007) revealed that *Bt* 44 showed significant insecticidal activity against *P. truncatus* (the larger grain borer), suggesting that this isolate expressed toxins that may have efficacy towards both lepidopterans and coleopterans. The *Bt* strains in commercial formulations for control of lepidopterans are primarily *Bt kurstaki* and *Bt aizawai*, with the latter strain showing better larval control in situations where *Bt kurstaki* has become less effective due to the resistance development of the pests like the diamond black moth (Schnepf et al. 1998; Polanczyk et al. 2000).

Table 2. Masses of pellets of δ-endotoxins from *Bt* isolates and their protein quantities

***Bt isolates* no.**	**Mass of pellet (g)**	**Protein Conc. (mg/mL)**	**Protein in pellet (%)**	**Protein yield per mL broth (mg)**
31	0.250	1.151	92.1	4.603
19	0.318	1.070	67.3	4.282
70	0.460	1.007	43.8	4.042
44	0.338	0.930	55.0	3.718
74	0.351	0.910	51.9	3.642
47	0.225	0.867	77.1	3.468
48	0.267	0.864	64.7	3.456
5	0.452	0.856	37.9	3.426
41	0.245	0.831	67.8	3.324
12	0.346	0.829	47.9	3.316
16	0.489	0.824	33.7	3.295
Bt aizawai	0.270	0.807	59.8	3.227
66	0.258	0.780	60.5	3.121
3	0.277	0.767	55.3	3.066
24	0.265	0.732	55.2	2.926
58	0.230	0.722	62.8	2.888
45	0.279	0.692	49.6	2.770
8	0.443	0.588	26.5	2.350
51	0.234	0.556	47.5	2.223
60	0.280	0.556	39.7	2.223
Mean	0.314		54.8	3.268
SD	0.084		15.3	0.627
CV	26.8 %		27.9 %	19. 2%

Table 3. Percent mortality of neonate *C. partellus* larvae on treatment with different levels of *Bt aizawai* δ-endotoxins and the LT_{50} values from each concentration treatment

Toxin conc. (mg/mL)	**Mean larvae mortality (%) after time (h)**							
	0 h	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**LT_{50} (h)**
0	0	0	0	10	10	20	30	nd
13.5	0	0	40	90	100	100	100	51.4
1.35	0	0	50	70	90	100	100	53.6
0.135	0	0	10	50	70	100	100	73.1
0.0135	0	0	20	60	60	90	100	70.7
0.00135	0	0	20	40	40	60	60	105.0

Table 4. Percent cumulative mortality of neonate *C. partellus* larvae exposed to 0.011 mg/mL δ-endotoxins from *Bt* isolates, LT_{50} values, and relative activity of each isolate

Treatment	**Percentage mortality after time (h)**							**LT_{50} at 72 h**	***Activity**
	0	**24**	**48**	**72**	**96**	**120**	**144**		
44	0	0	40	70	80	100	100	56.3	1.13
Bta	0	10	30	60	100	100	100	63.5	1.00
48	0	10	30	50	50	50	50	73.1	0.87
24	0	0	10	30	40	70	90	90.8	0.70
31	0	0	10	30	30	50	60	90.8	0.70
60	0	0	10	20	30	50	70	121.7	0.52
66	0	0	10	10	30	30	90	314.4	0.20
70	0	0	10	10	30	30	30	314.4	0.20
41	0	0	10	10	10	20	20	314.4	0.20
16	0	0	0	10	10	10	10	567.0	0.11
45	0	0	0	10	10	10	10	567.0	0.11
12	0	0	0	0	20	40	40	-	-
8	0	0	0	0	20	20	20	-	-
19	0	0	0	0	20	20	20	-	-
47	0	0	0	0	10	40	70	-	-
74	0	0	0	0	10	30	60	-	-
58	0	0	0	0	10	10	10	-	-
5	0	0	0	0	0	30	40	-	-
3	0	0	0	0	0	20	40	-	-
51	0	0	0	0	0	0	0	-	-
Control	0	0	0	0	0	0	0	-	-

Note: -: not determined

Information on its efficacy on local *C. partellus* or its comparison with local isolates has not been widely reported, despite numerous reports on the high toxicity of Bta on various lepidopterans. Consequently, this study emphasized the need to include it as a standard reference isolate when screening for toxicity.

Effect of temperature and concentration of δ-endotoxins on insecticidal activity

Bt isolates-mortality response

After exposing the neonate larvae for 72h to the *Bt* δ-endotoxins, the cumulative mean mortality recorded for *Bt* 48 was 64.8 %, and Bt 44 was 62.6 % compared to the standard control *Bta* of 87.0 %. The two isolates did not differ significantly concerning larval mortalities (Tukey HSD); however, the standard isolate (*Bta*) differed considerably with both isolates *Bt* 44 and *Bt* 48 (Bonferroni test). The LT_{50} values were 76.7 h and 60.9 h for *Bt* 44 and *Bt* 48, respectively (Table 5).

Table 5. Larval mortality, LC_{50}, and LT_{50} values for δ-endotoxins from local *Bt* and standard *Bt* isolates

Isolate	No. of larvae	Mortality (%)	(SE)	LC_{50} (µg/mL)	(SE)	Slope (SE)	LT_{50} (h)	(SE)
Bt 44	270	62.6	(7.1)	52.3	(31.2)	1.6 (0.3)	76.7	(13.7)
Bt 48	270	64.8	(6.6)	42.0	(21.8)	1.1 (0.4)	60.9	(9.0)
Bta	270	*87.0	(4.1)	*6.0	(3.5)	0.7 (0.3)	*37.0	(5.0)
Control	90	**0.0	(0.0)	nd	(nd)	nd (nd)	nd	(nd)

Note: Means (SE) in the same column differ significantly (p = < 0.05, Tukey HSD test) nd: not determined

Concentration-mortality response

There was a general increase in larval mortality as the δ-endotoxin level increased from 0.01 mg/mL to 1.00 mg/mL for the tested *Bt* isolates and standard control (Figure 4). The effect of the δ-endotoxin level on the percentage cumulative mean larval mortality was primarily significant for *Bt* 44 and *Bt* 48 compared to *Bta*. This trend also appeared as a strong positive considerable correlation (using Pearson product-moment correlation coefficient) between mean percentage larval mortality and concentration of δ-endotoxins from isolates *Bt* 44, *Bt* 48, and *Bta*, respectively. There was no significant difference between the effect of δ-endotoxin concentration from isolates *Bt* 44 and *Bt* 48 on larval mortality. Still, they differed significantly from the standard *Bta* at 0.01 mg/mL and 0.10 mg/mL.

Temperature-mortality response

The larval mortality was lowest for the δ-endotoxins from the two local isolates at 27ºC (*Bt* 44: 47.8 %; *Bt* 48: 51.1 %) and differed significantly from the mortality at 24ºC (*Bt* 44: 71.1 %; *Bt* 48: 68.9 %) and 31ºC (*Bt* 44: 68.9 %; *Bt* 48: 74.4 %). However, this trend was reversed for *Bta*, having the highest larval mortality at 27ºC (92.2 %) but did not differ significantly with mortality at 24ºC (77.8 %) and 31ºC (91.1 %). Generally, although larval mortalities due to δ-endotoxins from local isolates did not differ significantly between themselves, they differed significantly from those of the standard, *Bta* at 27ºC (Figure 5).

The correlation between temperature and mean cumulative percentage mortality due to *Bt* δ-endotoxin was negative, weak, and not statistically significant for *Bt* 44, positive, weak, and not statistically significant for *Bt* 48, and positive, weak, and not statistically significant for *Bta* (Pearson product-moment correlation coefficient).

Interaction between concentration and temperature effects

There was no statistically significant interactive effect between the δ-endotoxin concentration from *Bt* 44 and temperature on mean cumulative larval mortality (Two-way ANOVA: $F_{4,18}$ = 1.797, p = 0.173) (Figure 6). There was a significant interaction between temperature and concentration of δ-endotoxin from *Bt* 48 on the percentage mean cumulative larval mortality (Figure 7). The interaction between temperature and concentration of δ-endotoxin from *Bta* on the percentage mean cumulative larval mortality was not significant (Figure 8).

The susceptibility observed for neonate *C. partellus* larvae to *Bt* δ-endotoxins was in line with findings of Obonyo and Ogola (2011), that reported a five-day larval mortalities of 81.9 % for neonates and 25.6, 28.0, 14.9 and 9.2 % for 2nd, 3rd, 4th and 5th instar of *C. partellus* larvae fed on *Bt* maize expressing Cry1Ab. Furthermore, he observed that neonate larvae of *C. partellus* and *S. calamistis* showed significantly higher mortalities compared to later instars. At the same time, there was no significant difference between the mortalities of the 2nd to 5th instar larvae. Others have reported higher neonatal stem borer larvae susceptibility to *Bt* δ-endotoxins (Mugo et al. 2004). Yee et al. (2008) found that in most cases, the third instar larvae required a lesser amount of *Bt* and a shorter time to kill compared to the fifth instar larvae satisfactorily. Two *Bt* Cry proteins Cry1Ab and Cry1Ba, controlled *C. partellus* and showed stability in control, with no indication of a change in susceptibility over generations (Tende et al. 2010).

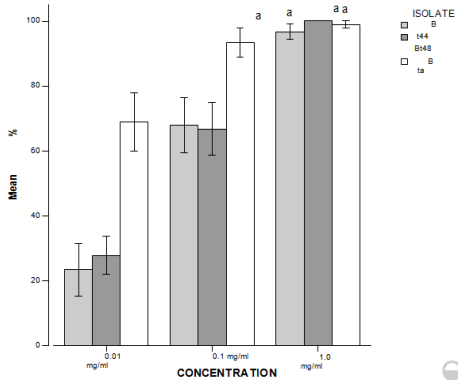


Figure 4. Mean cumulative larval mortality (%) due to concentration effects after 72 h of *Bt* δ-endotoxin exposure. For each isolate, bars labeled with the same letter are not significantly different (p < 0.05, Tukey HSD)

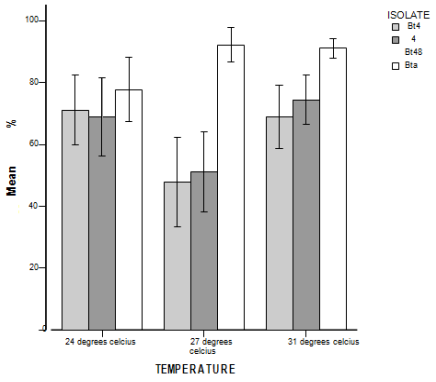


Figure 5. Mean cumulative larval mortality (%) due to temperature effects after 72 h of *Bt* δ-endotoxin exposure. For each isolate, bars labeled with the same letter are not significantly different (p < 0.05, Tukey HSD)

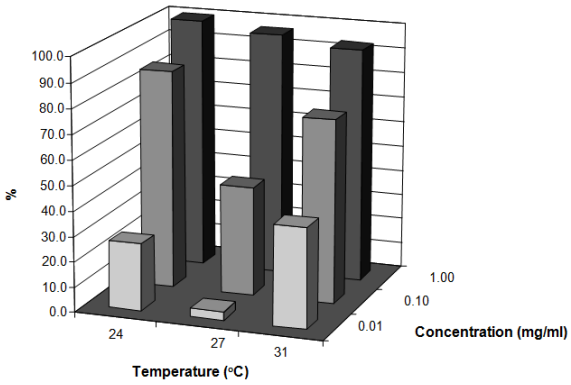


Figure 6. Mean cumulative larval mortality (%) due to interactions between *Bt* 44 δ-endotoxin concentration and temperature effects after 72 h of larval exposure

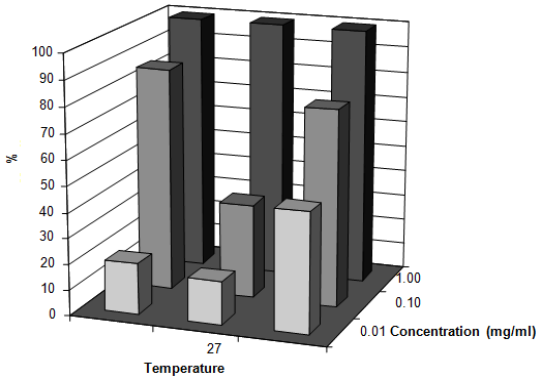


Figure 7. Mean cumulative larval mortality (%) due to interactions between *Bt* 48 δ-endotoxin concentration and temperature effects after 72 h of larval exposure

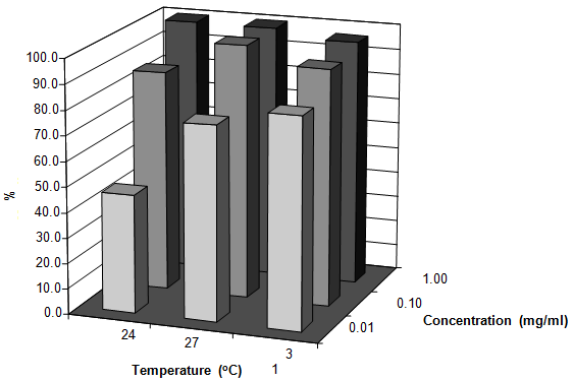


Figure 8. Mean cumulative larval mortality (%) due to interactions between *Bta* δ-endotoxin concentration and temperature effects after 72 h of larval exposure

There was a large and significant main effect of δ-endotoxin concentration on the percentage of cumulative average larval mortality for the *Bt* isolates used in this study. Dose-mortality relationship of *C. partellus* neonates to *Bt* δ-endotoxins also revealed a strong positive correlation between the larval mortality and δ-endotoxin concentration from the *Bt* isolates used. This observation was consistent with most lepidopteran species studied, where an increase in δ-endotoxin concentration resulted in increased mortalities. The phenomena had been observed for *C. partellus* (Osir and Vundla 1999), *C. partellus, S. calamistis, B. fusca* (Wang'ondu 2003), *P. truncatus* (Mwathi 2007), and *M. plana* (Yee et al. 2008). Mean cumulative larval mortalities for the isolates used revealed that, generally, δ-endotoxins from *Bt* 48 were equally toxic to *C. partellus* neonates as those from *Bt* 44. However, both were less toxic than the reference standard Bta, which showed a significantly higher larval mortality of 87.0 % after 72 h, as expected. The high toxicity of *Bta* had been previously demonstrated by Basri et al. (1994; 1996) and Polanczyk et al. (2000), which formed the basis of its choice as a standard reference isolate for this study.

Temperature similarly affected larval mortality for the local isolates tested but differed from the control as expected. These results indicated that the larval mortality was significantly higher at 24 and 31°C, at dose levels of 0.01 and 0.1 mg/mL of local *Bt* δ-endotoxins. At the same time, there were no differences at the highest dose level tested (1.00mg/mL). This observation differed from that of Van Frankenhuyze et al. (2008). Using a different method, they reported that neonate larval mortality of gypsy moth, *Lymantria dispar* L. increased when the temperature was raised from 13 to 25°C at each dose level tested (0.0125-0.049 IU). However, the control in this study had 0% mortality and, therefore, did not exhibit any temperature susceptibility within the study period.

Generally, the neonate larvae showed higher susceptibility to the tested isolates at temperatures higher or lower than 27°C, indicating that the larvae were more tolerant of tested strains at 27°C. The fact that the larvae were reared at 27°C before δ-endotoxin exposure may have conferred tolerance or enhanced immunity towards *Bt* efficacy at this temperature. This phenomenon may be explained in various ways; either the *C. partellus* gut proteases fully activate local isolates' δ-endotoxins faster and/or more entirely at 24 and 31 than at 27°C, or the reduced toxicity at 27°C may result from a reduced rate of protein site activation and reduced toxin binding to the larvae midgut. It may also indicate a situation of altered larvae physiology at temperatures higher or lower than 27°C, which probably suppresses the immunity of the larvae towards *Bt* δ-endotoxins.

These findings, however, do not agree with those of Van Frankenhuyzen et al. (2008), who reported that the final level of mortality attained by each instar decreases with increasing rearing temperature. Rearing temperature is well known to affect mortality progression in larvae infected with *Bt*, which is believed to be mediated by the rate of bacterial cell multiplication (septicemia) in the diseased host, at least in some species (Van Frankenhuyzen et al. 1994); this effect across instars may need to be investigated further for *C. partellus*. The Cry proteins administered to the larvae were also unlikely to be thermally denatured at the highest temperature used (31°C). It has been reported that 90 % of Cry1Ab protein was

denatured after heat treatment at 77°C for 10 mins (Perferoen 1998) and that 75 and 77°C for 2 min were sufficient to denature 50 and 70 % of Cry1Ab, respectively (De Luis et al. 2008). Further, no decrease in the insecticidal activity of Cry9C was found after heat treatment at 90°C for 10 min although, Cry1Ab completely lost its insecticidal activity after heating at 80°C for 10 min (Perferoen 1998).

Therefore, Cry proteins could lose their insecticidal activity at the temperatures experienced in the maize adaptation zones in Kenya, which were tested in this study. Deviation of findings from those of *Bta* δ-endotoxins may be because of a difference in Cry protein composition between that in the tested isolates from *Bta*, necessitating protein profiling or gene characterization of these isolates. Even minor differences in the amino acid sequences of the same toxin produced by different *Bt* strains may influence the specific toxicity of a test insect (Crickmore et al. 1998; Feldmann et al. 1995). Indeed, different Cry proteins may act synergistically or antagonistically on various larvae, or the same cry proteins may behave differently on different larvae (Rouis et al. 2008).

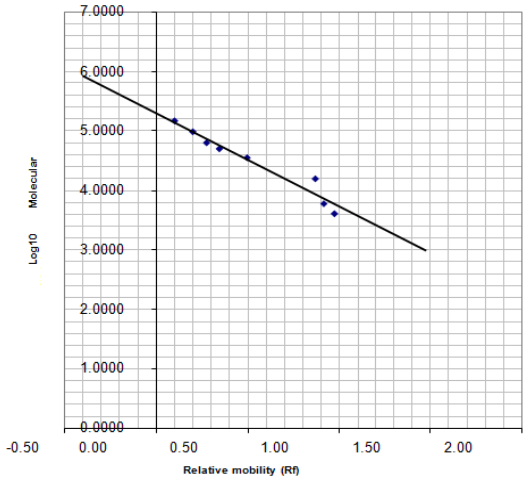
The interactive effect between temperature and δ-endotoxin concentration was statistically significant for *Bt* 44 but not for *Bt* 48. However, the tested isolates were significantly different from the control of the dose/temperature interactive effect on larval mortality. This study hypothesized no difference in the relative toxicity at different concentrations and temperatures of δ-endotoxins from local *Bt* isolates of *C. artellus*. This hypothesis was thus rejected as these effects were found to be significant. It might indicate that the tested isolates *Bt* 44 and *Bt* 48 appeared to have δ-endotoxins whose toxicity was more susceptible to temperature effects than the standard reference *Bta* with *Bt* 48 appearing the more vulnerable. The study, therefore, indicated that δ-endotoxins from tested isolates might be better suited for use in high and low-temperature regions of Kenyan maize adaptation zones. However, field tests would be needed to investigate these findings further.

Characterization of Cry proteins in *Bt* isolates

SDS-PAGE

The theoretical gel exclusion limit was 197000 Daltons determined from the y-intercepted (5.2944) of the gel calibration curve (Figure 9). Analysis of native *Bt* δ-endotoxin suspensions revealed a major protein band of M_r approximately 130 kDa in *Bt* 44 (lanes 6, 7, and 11), *Bt* 48 (lane 12), and *Bta* (lanes 4 and 9) (dark arrow) that was absent in *Bt* 51 (lanes 5 and 10). However, all these isolates revealed numerous minor molecular weight protein bands between ~ 98-35 kDa (Figure 10).

The trypsin activated δ-endotoxins from *Bt* 44 (Lanes 1 and 5), *Bt* 48 (Lanes 2 and 6), and *Bta* (Lane 5) were composed of a major protein band at M_r ~ 70 kDa (arrow A, Fig.13). However, trypsin digests of δ-endotoxins from Bt 51 (Lane 3 and 8) revealed a distinct protein band of M_r ~ 64 kDa (arrow B), as shown in Figure 11.



Equation: y =-1.5705x + 5.2944; R^2 = 0.9476

Figure 9. Gel calibration curve showing a plot of Log_{10} molecular weight against relative mobility of the standard marker proteins.

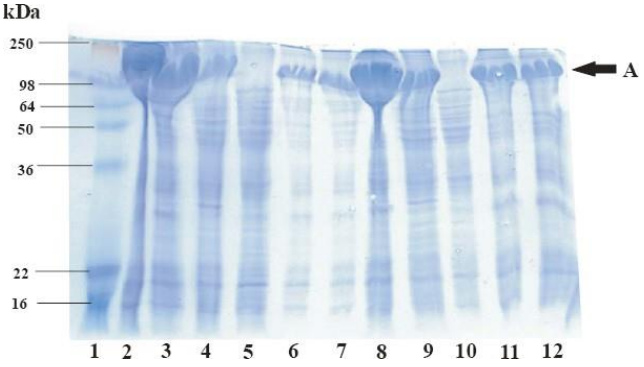


Figure 10. The 12 % SDS-PAGE gel of δ-endotoxins from local *Bt* samples. Lane 1: molecular weight markers. Lane 2: *Bt* 44 (30 µL). Lane 3: *Bt* 48 (30 µL). Lane 4: *Bta* (30 µL). Lane 5: *Bt* 51 (30 µL). Lane 6: *Bt* 44 (15 µL). Lane 7: *Bt* 44 (15 µL). Lane 8: *Bt* 48 (15 µL). Lane 9: *Bta* (15 µL). Lane 10: *Bt* 51 (15 µL). Lane 11: *Bt* 44 (20 µL). Lane 12: *Bt* 48 (20 µL). Arrow (A) indicates a significant protein band position.

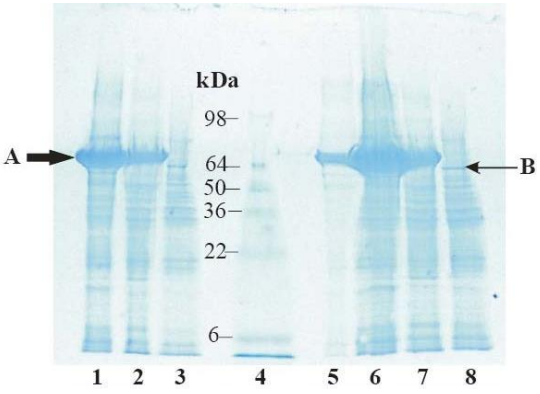


Figure 11. The 12 % SDS-PAGE gel of trypsin activated δ-endotoxins from local *Bt* samples. Lane 1: *Bt* 44 (20 µL). Lane 2: *Bt* 48 (20 µL). Lane 3: *Bt* 51 (20 µL). Lane 4: molecular weight marker. Lane 5: *Bta* (10 µL). Lane 6: *Bt* 44 (30 µL). Lane 7: *Bt* 48 (30 µL). Lane 8: *Bt* 51 (30 µL). Major protein bands (arrows A and B)

Liquid chromatography

The HPLC equipment used had a sensitivity of ±0.02 minutes peak deviation and the limit of detection in the order of 10^{-6} g of injected sample. The elution pattern of trypsin-digested δ-endotoxins from *Bt* 44 revealed 11 peaks resolved into four distinct peaks; A, B, C, and D (dotted lines at the base of each, Figure 12). Peak B consisted of a broad overlap of six peaks (2-7), while peak D, had three peaks (9-11). Peak 3 showed the highest absorbance and the highest amount of protein, which was 21.5 x 10^{-3} peak height, 29.9 % of peak height, and a peak area of 85.3 x 10^{-1}, 46.3 % of peak area of the profile, respectively, at a retention time (t_R) of 2.8 minutes. Other distinctly resolved peaks were peak 1(t_R = 2.1 mins; peak area = 6.5 x 10^{-1}), peak 8 (t_R = 4.7 mins; peak area = 3.6 x 10^{-1}) and peak 10 (t_R = 6.2 mins; peak area = 16.6 x 10^{-1}) as shown in Table 6.

Table 6 shows the retention times of the eleven peaks, their peak area, and height percentages. The elution pattern of trypsin-digested δ-endotoxins from *Bt* 48 revealed 12 peaks resolved into four distinct peaks; A, B, C, and D (dotted lines at the base of each) shown in Figure 13. Peak B consisted of a broad overlap of seven peaks (2-8), while peak D, had three peaks (10-12). Peak 3 showed the highest absorbance and the highest amount of protein, which was 41.8 x 10^{-3} peak height, 19.0 % of peak height, and a peak area of 131.4 x 10^{-1}, 36.7 % of peak area of the profile, respectively, at the retention time of 2.8 minutes. Other distinctly resolved peaks were peak 1 (t_R = 2.2 mins; peak area = 21.5 x 10^{-1}), peak 9 (t_R = 3.9 mins; peak area = 11.6 x 10^{-1}) and peak 11 (t_R = 6.2 mins; peak area = 40.9 x 10^{-1}) as shown in Table 7.

Table 7 shows the retention times of the twelve peaks, their peak area, and height percentages. The elution pattern of the standard isolate *Bta* trypsin-digested δ-endotoxins showed nine peaks resolved into five distinct peaks; A, B, C, D, and E (dotted lines at the base of each, Figure 14). Peak E consisted of a broad overlap of three peaks (7-9). Peak 1 showed the highest absorbance and the highest amount of protein, which was 44.4 x 10^{-3} peak height, 41.1 % of peak height, and a peak area of 23.0 x 10^{-1}, 17.2 % of peak area of the profile, respectively, at a retention time of 2.3 minutes. Other resolved peaks were peak 2 (t_R = 2.8 mins; peak area = 25.4 x 10^{-1}), peak 5 (t_R = 3.6 mins; peak area = 3.3 x 10^{-1}), peak 6 (t_R = 3.7 mins; peak area = 12.8 x 10^{-1}) and peak 8 (t_R = 6.3 mins; peak area = 43.1 x 10^{-1}) as shown in Table 8.

Table 8 shows the retention times of the twelve peaks, their peak area, and height percentages. The chromatograms of the trypsin-digested δ-endotoxins of the local isolate *Bt* 44 (A) and standard isolate *Bta* were aligned and compared (Figure 15). Peaks of eluents observed at similar elution times trypsin-digested δ-endotoxins of both isolates (*Bt* isolate; peak number, peak area) were at 2.8 mins (A; 3, 85.3 x 10^{-1} and *Bta*; 2, 25.4 x 10^{-1}), 3.0 mins (A; 4, 35.5 x 10^{-1} and *Bta*; 3, 2.1 x 10^{-1}), 6.1 mins (A; 9, 2.3 x 10^{-1} and *Bta*; 7, 4.4 x 10^{-1}) and 6.5 mins (A; 11, 4.5 x 10^{-1} and *Bta*; 9, 17.7 x 10^{-1}) (Tables 7 and 8). Nevertheless, the elution patterns differed at peaks 1, 2, 5, 6, 7, 8, and 10 of A and peaks 1, 4, 5, 6, and 8 of *Bta*.

Table 6. Peak properties of RP-HPLC elution profile of trypsin-digestedmδ-endotoxins of *Bt* 44

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.1	7.4	10.2	6.5	3.5
2	2.4	3.6	5.0	3.1	1.7
3	2.8	21.5	29.9	85.3	46.3
4	3.0	13.3	18.5	35.5	19.3
5	3.4	7.0	9.7	19.2	10.4
6	3.8	3.3	4.5	3.0	1.6
7	3.9	2.9	4.0	5.0	2.7
8	4.7	2.4	3.3	3.6	2.0
9	6.1	2.1	3.0	2.3	1.2
10	6.2	5.7	7.9	16.6	9.0
11	6.5	2.8	3.9	4.1	2.2
Totals		71.9	100.0	184.2	100.0

Table 7. Peak properties of RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 48

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.2	40.0	18.2	21.5	6.0
2	2.4	6.3	2.9	4.4	1.2
3	2.8	41.8	19.0	131.4	36.6
4	2.9	32.2	14.6	30.6	8.5
5	3.0	29.8	13.6	57.7	16.1
6	3.3	14.6	6.7	22.8	6.4
7	3.5	8.9	4.0	11.6	3.2
8	3.6	7.0	3.2	6.4	1.8
9	3.9	10.4	4.7	11.6	3.2
10	6.0	4.8	2.2	4.6	1.3
11	6.2	15.1	6.8	40.9	11.4
12	6.4	9.2	4.2	14.8	4.1
Totals		220.1	100.0	358.4	100.0

Table 8. Peak properties of RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bta*

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.3	44.4	41.1	23.0	17.2
2	2.8	13.2	12.2	25.4	19.0
3	3.0	1.7	1.6	2.1	1.6
4	3.3	1.9	1.8	2.0	1.5
5	3.5	3.8	3.5	3.3	2.5
6	3.7	12.1	11.2	12.8	9.6
7	6.1	4.7	4.3	4.4	3.3
8	6.3	15.7	14.4	43.1	32.2
9	6.6	10.6	9.8	17.7	13.2
Totals		108.1	100.0	133.7	100.0

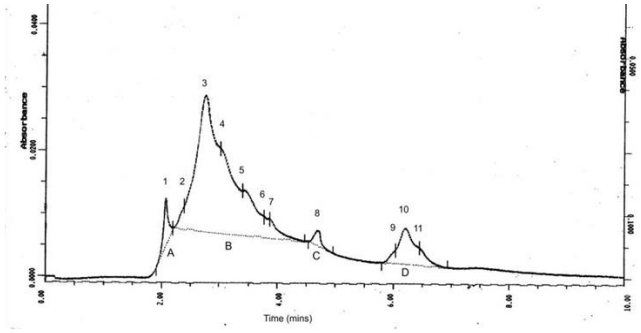


Figure 12. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 44

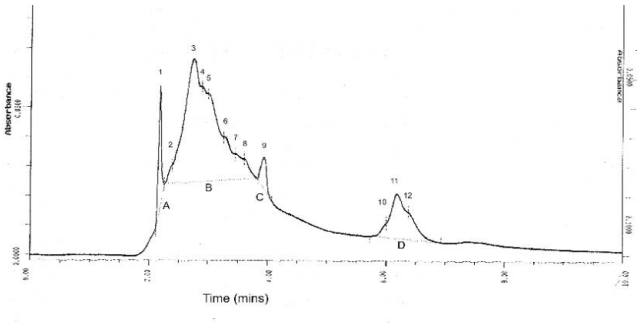


Figure 13. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 48

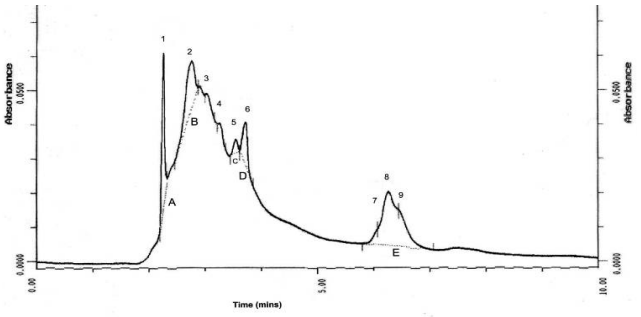


Figure 14. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bta*

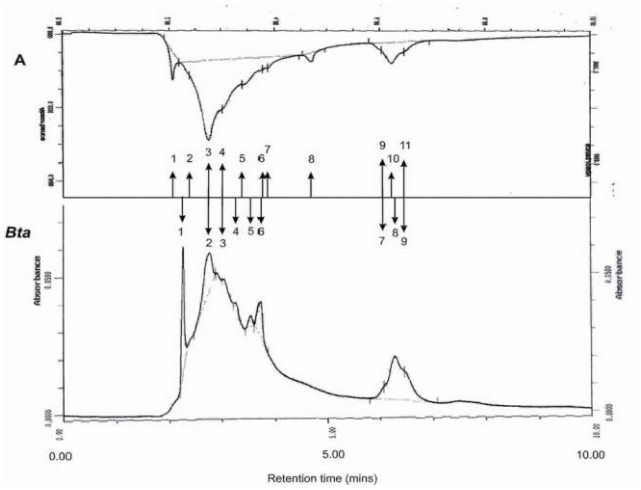


Figure 15. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 44 (A) and *Bta*

The chromatograms of the trypsin digests of δ-endotoxins from the two local isolates were aligned and compared (Figures 16-17), with A representing that of *Bt* 44 and B that of *Bt* 48. Peaks of eluents observed at similar elution times for both isolates (*Bt* isolate; peak number, peak area) were 2.4 mins (A; 2, 3.1 x 10^{-1} and B; 2, 4.4 x 10^{-1}), 2.8 mins (A; 3, 85.3 x 10^{-1} and B; 3, 131.4 x 10^{-1})3.0 mins (A; 4, 35.5 x 10^{-1} and B; 5, 57.7 x 10^{-1}) and 6.2 mins (A; 10, 16.6 x 10^{-1} and B; 11, 40.9 x 10^{-1}). However, the elution patterns differed at peaks 1, 5, 6, 8, 9, and 11 of A and peaks 1, 4, 6, 7, 8, 10, and 12 of B.

ELISA

The ELISA technique had a limit of detection of 5 ng/mL. The contents of the microtitre plate testing for Cry1 proteins turned yellow for positive proteins. In contrast, Cry2 proteins turned blue with the intensity of the colors corresponding to absorbance values and thus relative Cry protein concentration. The positive control ratio (PC ratio) for each sample was calculated from mean absorbance values using the mean absorbance for the positive control as 0.393 for Cry1 and 0.886 for Cry2, with the latter having produced a more considerable background (Table 9).

The results revealed that the isolates *Bt* 44, *Bt* 48, and *Bta* contained Cry1 proteins while none contained Cry2 protein. The quantity of Cry1 proteins reduced the order of *Bt* 44 > *Bt* 48 > *Bta* as indicated by the PC ratio values of 4.529, 3.997, and 3.573, respectively. The isolate *Bt* 51 did not have either Cry1 proteins or Cry2 proteins in its crystals, indicating PC ratio < 0.5 and PC ratio < 1.0 for the proteins, respectively. The local isolates tested did not differ among themselves in Cry protein content, nor did they differ from the positive control (*Bta*) in terms of Cry1 and Cry2 protein content. However, they differed from the negative control (*Bt* 51) in terms of Cry1 though they were not different in terms of Cry2 content.

Polymerase Chain Reaction

The more efficacious local isolate (*Bt* 44) was analyzed alongside two positive and one negative reference isolates (*Bta*, *Btk*, and *Bt* 51, respectively). The local *Bt* isolate (lane 6; *Bt* 44) yielded a reliable amplification product of M_r = 277 bp with universal primers for the gene encoding Cry1 protein, similar to the positive standards *Btk* and *Bta* (lanes 3 and 4) respectively indicating the presence of *cry1* gene (Figure 18).

Table 9. Mean absorbance and PC ratio values for the sandwich ELISA test of δ-endotoxins

Sample	Cry1		Cry2	
	Mean absorbance	PC ratio *	Mean absorbance	PC ratio *
Bt 44	1.780	4.529**	0.129	0.146
Bt 48	1.571	3.997**	0.099	0.112
Bt 51	0.003	0.008	0.053	0.060
Bta	1.404	3.573**	0.396	0.447

Note:

$$\text{* PC ratio} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of positive control}}$$

** The *Bt* samples positive for Cry proteins tested

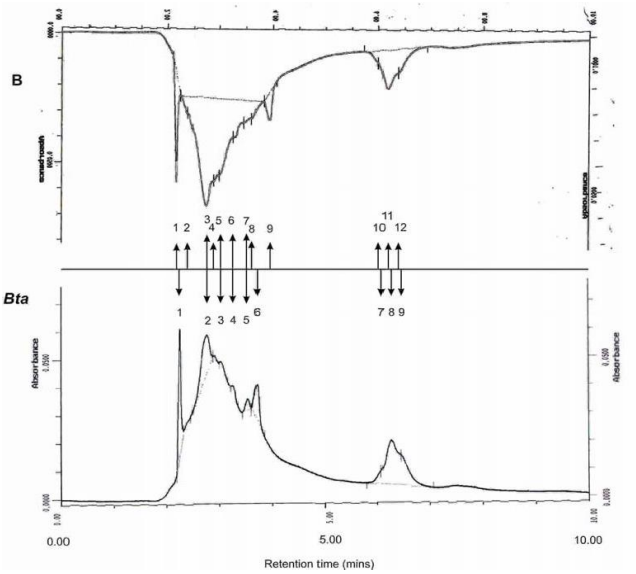


Figure 16. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 48 (B) and *Bta*

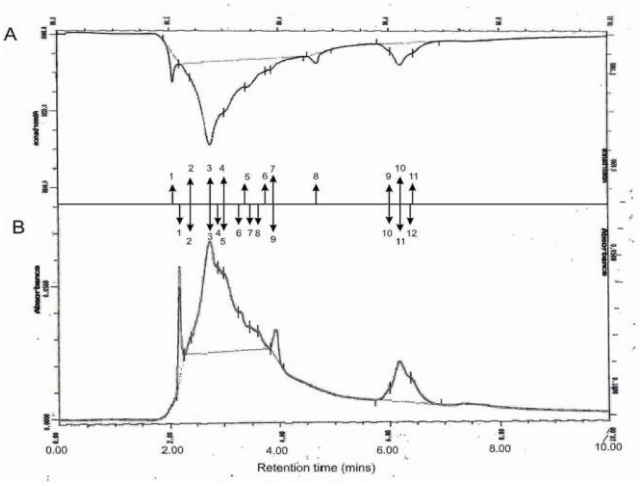


Figure 17. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 44 (A) and *Bt* 48 (B)

The negative control local isolate (lane 5: *Bt* 51) gave a similar result as the negative control (lane 7: water), indicating the absence of the *cry1* gene. However, these two lanes 5 and 7 revealed a faint band (M_r = 277 bp), which may be false bands due to the high number of PCR cycles (35 cycles).

The local *Bt* isolate (lane 6: *Bt* 44) yielded a strong amplification product band of M_r = 689 bp with universal primers for *cry2* gene, similar to the positive standards *Btk* and *Bta* (lanes 3 and 4) respectively indicating the presence of the gene encoding Cry2 protein (Figure 19). The negative control isolate (lane 5: *Bt* 51) gave a similar result as the negative control (lane 7: water), indicating the absence of the *cry2* gene.

The PCR analysis using specific *cry2Aa1* primers for the amplified product of M_r = 498 bp revealed a strong band for *Bt* 44 and the positive controls (*Btk* and *Bta*) (Figure 20). A weak amplified product band of M_r = 546 bp was observed for *Btk* and *Bt* 44 but not for *Bta using cry2Ab2 primers.*However, analysis using specific *cry2Ac* primers for an amplified product expected at M_r = 725 bp yielded non-specific bands for isolates (*Btk*, *Bta*, and *Bt* 44) and no band for *Bt* 51 (NC) and water controls (data not shown). These results suggest that the presence or absence of the gene encoding Cry2Ac protein was not confirmed for *Bt* 44 and the standard reference isolates.

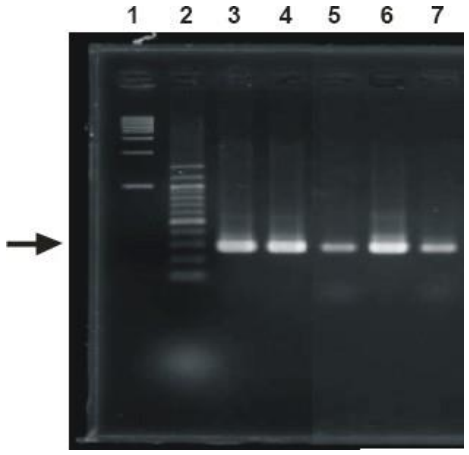


Figure 18. The 1.5 % agarose gel of PCR products amplified from the *Bt* isolates with *cry1* universal primers: Lane 1, high molecular weight marker (>1200 bp); Lane 2, low molecular weight marker (<1500 bp); Lane 3 and 4, *Bt kurstaki* HD-1 and *Bt aizawai* HD-133 respectively, positive controls; Lane 5, *Bt* 51; Lane 6, *Bt* 44; Lane 7, negative control. Note: Bold arrow showing the position of M_r of expected PCR product (277 bp)

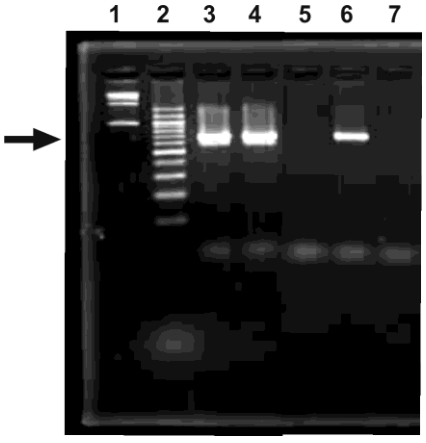


Figure 19. The 1.5 % agarose gel of PCR products amplified from the *Bt* isolates with *cry2* universal primers: Lane 1, high molecular weight marker (>1200 bp); Lane 2, low molecular weight marker (<1500 bp); Lane 3 and 4, *Bt kurstaki* HD1 and *Bt aizawai* HD133 respectively, positive controls; Lane 5, *Bt* 51; Lane 6, *Bt* 44; Lane 7, negative control. Note: Bold arrow showing the position of Mr of expected PCR product (698 bp)

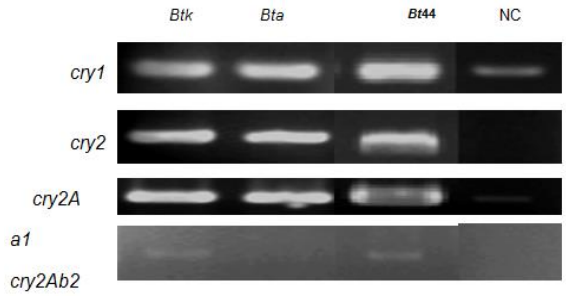


Figure 20. The 1.5 % agarose gel summary of PCR products amplified from the *Bt* isolates with *cry1* and *cry2* universal primers and *cry2Aa1* and *cry2Ab2* specific primers

Table 10. Summary of distribution of profile of genes encoding Cry proteins as analyzed by PCR

***cry* gene**	***Bt* isolate**			
	Btk	***Bta***	***Bt* 44**	***Bt* 51 (NC)**
cry1	+	+	+	-
cry2	+	+	+	-
cry2Aa1	+	+	+	-
cry2Ab2	+	-	+	-
cry2Ac	Ns	Ns	Ns	-
cry3	+	-	Ns	+

Note: +: Presence of gene encoding Cry protein, -: Absence of gene encoding Cry protein, Ns: Non-specific bands detected

Table 10 showed the presence and/or absence of genes encoding the Cry proteins tested. The local isolate *Bt* 44 contained *cry1* and *cry2* genes, which were absent in *Bt* 51 (NC). The two standard reference isolates (*Btk* and *Bta*) were mostly similar except for the *cry2Ab2* gene that was present in *Btk* but absent in *Bta*. Thus, these results showed that *Bt* 44 might be more similar to *Btk* than *Bta* for the tested genes, while *Bt* 51 announced uniqueness with the primers used not detecting any of the *cry* genes tested. A further test showed the presence of *the cry3* gene, which was identified in the *Btk* and *Bt* 51, and was absent in *Bta* and with non-specific bands in *Bt* 44 (data not shown).

This study employed a four-stage protein characterization strategy to investigate the presence or absence of Cry1 and Cry2 proteinous δ-endotoxins from *Bt* isolates with significant activity against *C. partellus*. Gel electrophoresis, ELISA, and HPLC techniques were used to investigate the δ-endotoxins at protein level while PCR was used to investigate the *cry* genes present at a molecular level.

The SDS-PAGE technique investigated the molecular mass of the δ-endotoxins present in the isolates. The results using the native proteins revealed that δ-endotoxins of *Bt* 44 and *Bt* 48 had a major protein band of M_r ~ 130 kDa characteristic of the protoxins of lepidopteran specific δ-endotoxins. This protoxin appeared to yield a trypsin resistant protein M_r ~ 70 kDa after trypsin digestion. Similar findings have been reported in a process where alkaline solubilization at high pH preceded enzymatic cleavage (Dulmage 1993; Hickle and Fitch 1990). Such a sequential and slow enzymatic digestion using commercial trypsin removes approximately 600 amino acids residues from the C-terminus and the first 28 N-terminal residues of the protoxin, producing a protease-resistant toxin core (Lightwood et al. 2000) does not result to over digestion as observed using the gut juice of *Choristoneura fumiferana* in the Eastern spruce budworm (Bah et al. 2004).

Several minor bands of molecular weight around ~ 98-35 kDa were also seen on both samples, probably due to crystal-associated endogenous proteolytic degradation processes. This observation was similar to that of *Bta* in all aspects but distinct from *Bt* 51 (negative control due to 0 % larval mortality observed during bioassay screening), which lacked the 130-kDa band. However, *Bt* 51 revealed a distinct band starting of M_r ~ 80 kDa, which appeared to be digested to a protein of M_r ~ 64 kDa with a series of other lower molecular weight proteinous products. Such numerous smaller molecular weight fragments are a common observation after a proteolytic digestion process (Hickle and Fitch 1990). However, the protein-banding pattern was not discriminatory enough within *Bt* isolates tested and could not reveal minute differences in the protein contents in the crystal inclusions necessitating further characterization.

The trypsin digests were further analyzed using RP-HPLC, which revealed that although the two uncharacterized *Bt* isolates may appear to have similar protein content, some differences revealed by different peaks. Due to their smaller size, the proteolysis products of the proteins were used because larger protoxins tend to undergo self-adsorption in the analytical column due to their larger molecular weights making elution difficult (Hickle and Fitch 1990). Five peaks from *Bt* 44 and *Bt* 48 trypsin-digested δ-endotoxins eluted at similar retention times while their percentage peak areas showed tiny differences, probably representing related proteins. However, the 1st peak for each isolate differed in their elution time and abundance. This observation could either be associated with concentration difference or a slight difference in hydrophobicity of different Cry proteins in the same Cry class (Cry1). Further, peak 8 for *Bt* 44 was unique for the isolates tested.

The chromatogram results made it possible to compare the protein content of the δ-endotoxins of the *Bt* isolates. The differing peaks from each strain may well represent different cry proteins in each isolate or even existence of novel Cry proteins. The two native isolates appeared more similar to each other (*Bt* 44, 11 peaks; *Bt* 48, 12 peaks) but distinctly different from the standard strain *Bta* (9 peaks). Furthermore, five peaks from both isolates eluted at the same time, with the rest showing minor differences. The δ-endotoxins from both *Bt* 44 (A) and *Bt* 48 (B) appeared to have four proteins, similar to those of the standard. Nevertheless, while *Bt* 44 showed peaks of proteins eluted both earlier and later as with the standard, *Bt* 48 showed related proteins eluted only in retention times below 3.5 minutes. The peaks observed could not be associated with any particular Cry protein nor their specific quantities determined, as protein standards were not used.

The ELISA analysis was used to evaluate the presence and relative abundance of Cry1 and Cry2 proteins. The findings detected only Cry1 proteins in the isolates tested. These results did not differ qualitatively from the standard *Bta* used. In the order of quantity, the amount of Cry1 proteins reduced as follows, *Bt* 44 > *Bt* 48 > *Bta*, as was validated by the PC ratio values of 4.529, 3.997, and 3.573, respectively. The isolate *Bt* 51, which served as the negative control, did not have either Cry1 proteins or Cry2 proteins in its crystals. The findings from the ELISA analysis corroborate those obtained from SDS-PAGE, where the major protein band (M_r ~ 130 kDa) observed with electrophoresis may be due to the presence of Cry1 proteins.

Furthermore, the relative amounts of the Cry1 protein in the isolates did correlate with the toxicity of the strains against *C. partellus* observed with the bioassay. The higher toxicity of *Bt* 44 may be due to the higher quantity of Cry1 proteins therein. It may indicate that that the two tested isolates may be expressing similar proteins in many aspects but only differ in expression levels of the Cry proteins. However, although the standard strain *Bta* appeared to have the least amount of Cry1 protein, it exhibited significantly higher larval mortality than the tested isolates. Indeed, a common trypsin-digested protein eluted at 2.8 minutes and appeared to be least in δ-endotoxins of the standard, *Bta*, while most in those of *Bt* 44.

As for the standard, *Bta* showed a unique chromatographic peak at a retention time of 6.3 minutes, which was the most abundant of its constituent proteins, as shown by its high percentage peak area of 32.2 %. Still, the trypsin digests from the local isolates showed peaks that only slightly deviated in height and domain from that of the standard. The results may be due to the standard expressing Cry1 proteins different from those expressed by the isolates tested. Masson et al. (1998) reported that the standard used (*Bta* HD-133) formed a proteinous crystal containing Cry1Ab, Cry1C, and Cry1D composed at 60 %, 37 %, and 3 % respectively and that the toxicity of *Bta* δ-endotoxins against *Mamestra configuration* Walker (bertha armyworm) was associated to Cry1Ab and Cry1C and not Cry1D.

A molecular approach using PCR was used to determine the genes present in the isolates tested. In this aspect, we tested only the more toxic *Bt* 44 because it appeared similar primarily to *Bt* 48 from the earlier tests carried out. The results revealed that *Bt* 44 contained *cry1* and *cry2* genes that produced strong bands at 277 bp for *cry1* and 689 bp for *cry2* when using universal primers. It contained *cry2Aa1* and *cry2Ab2* genes as well when specific primers were used. Although these results differed from those of the standard *Bta* in that the *cry2Ab2* was missing, *Bt* 44 was similar to the other standard isolate used (*Btk*) for the genes tested. Further, *Bt* 44 differed entirely from the negative standard (*Bt* 51) in that; the *cry1* and *cry2* genes tested were found to be absent in *Bt* 51. Also, *Bt* 51 was found to contain the *cry3* gene; thus, it may explain why *Bt* 51 showed no toxicity against *C. partellus* in the bioassay screening, as it did not include the lepidopteran-specific δ-endotoxins.

From this study, it appears that *Bt* 44 contains *cry1* and *cry2* genes, but only Cry1 proteins were expressed. Perhaps, the *cry2* gene being silent or its expression levels were below the detection limit of the ELISA technique used (Adugna and Mefsin 2008). Further, the use of universal primers for *cry1* genes may not reveal the specific *cry1* genes present, nor can they detect the presence of novel *cry1* genes. Therefore, it necessitates performing tests using specific *cry1* primers to determine the actual *cry1* gene present therein at least at the tertiary classification level, where differences in toxicity are distinguishable among proteins (Van Frankenhuyzen 2009). Another approach that may be used to reveal the specific identity of the δ-endotoxins expressed would be to sequence those proteins and compare them with a data bank of known Cry proteins.

Although none analytical technique sufficed in the detailed and complete characterization of the unknown native Cry proteins, each of the methods chosen contributed vital information about the δ-endotoxins tested. The analyses were largely qualitative due to the lack of appropriate standard proteins, and the results obtained were only relatively quantitative.

The challenge of appropriate standards as exacerbated by the necessity to use many pure compounds of the same Cry protein class, because the analyte was a mixture of closely related proteins, which were yet to be known in the *Bt* isolates tested. Furthermore, obtaining these standards was either expensive, or the sources (usually private multinational companies) carefully controlled their access. However, each of these techniques can be used quantitatively either when used individually or when combined with other methods, for example, SDS-PAGE-densitometry (Crespo et al. 2008) and HPLC-MS (Nakayama 2001). Indeed, the use of internal toxin standards needs to be promoted to increase confidence in comparisons between laboratories, insect colonies, and toxin preparations (Van Frankenhuyzen 2009). The phenomena are due to the reference standards would permit researchers to calibrate toxin protein estimates, by using the ratio of the known toxin protein content of the standard (based on mg of protein) to toxin protein content acquired with their method of choice.

This study had hypothesized that the relative toxicity of δ-endotoxins from local *Bt* isolates on *C. partellus* does not depend on the nature of the crystal protein. The results showed that the crystal inclusions of the two isolates (*Bt* 44 and *Bt* 48) with the highest toxicity against the pest contained Cry1 and Cry2 proteins while crystals from *Bt* 51, which caused 0 % larval mortality, did not contain Cry1 and/or Cry2 proteins. Besides, *Bt* 51 contained *a cry3* gene, which was absent in both *Bt* 44 and *Bt* 48 (data not shown). It explains why *Bt* 51 exhibited significant efficacy against the larger grain borer *P. truncatus* observed in a previous study by Mwathi (2007). These results, therefore, indicated that Cry1 and Cry2 proteins and not Cry3 were the toxins responsible for the mortality of *C. partellus*; therefore, the hypothesis was thus rejected.

Conclusions

The *B. thuringiensis* (Berliner) δ-endotoxins offer an array of advantages over conventional pest control approaches, including reduced reliance on chemical pesticides, increased crop yields, and reduced cases of aflatoxin poisoning from pest-damaged maize, a severe problem in Kenya. The conclusions drawn from this study were: (i) The yield of total protein in the crystal inclusions was 3.268 ± 0.627 mg/mL of culture broth with a protein purity level of 54.8 ± 15.3 %. This information may be useful when considering the mass production of the toxin. (ii) Two local *Bt* isolates (*Bt* 44 and *Bt* 48) had δ-endotoxins that showed significant insecticidal activity against neonate *C. partellus* larvae. They caused larval mortality of 62.6 (Bt 44) and 64.8 % (*Bt* 48) after 72 h. (iii) The isolate *Bt* 51 showed no insecticidal activity (0% mortality) against neonate *C. partellus* larvae. (iv) The effect of concentration of δ-endotoxins from *Bt* 44 and *Bt* 48 on larval mortality was significant, with LC_{50} values of 52.3 and 42.0 µg/mL, respectively. (v) Larval mortality due to δ-endotoxins from *Bt* 44 and *Bt* 48 was significantly higher at 24°C and 31°C and only differed considerably from the standard (*Bta*) at 27°C. These two local isolates might, therefore, be better suited for domestic use where temperatures are generally higher compared to the temperate regions, as they did not show loss of insecticidal activities at these elevated temperatures (31°C). (vi) The interaction effect between δ-endotoxin concentration and temperature on larval mortality was significant for *Bt* 48 but not significant for *Bt* 44. These results indicated that different levels and temperatures of endotoxins from *Bt* 44 and *Bt* 48 against *C. partellus* affect their relative toxicity. (vii) The major protein component on the endotoxins from *Bt* 44 and *Bt* 48 had a molecular weight of M_r ~ 130 kDa, which was digested to a trypsin-resistant core of M_r ~ 70 kDa. (viii) The isolates *Bt* 44 and *Bt* 48 expressed Cry1 proteins but not Cry2 proteins, although they both contained *cry1* and *cry2* genes. The isolate *Bt* 51 did not express Cry1 or Cry2 proteins; neither did it include *cry1* or *cry2* genes. (ix) Besides, only *Bt* 51 did not show any insecticidal activity on *C. partellus*. Furthermore, *Bt* 51 contained *a cry3* gene, which was absent in *Bt* 44 and *Bt* 48, which explains why *Bt* 51 showed significant efficacy against *P. truncatus* (Coleoptera). These results suggest that the toxicity of *Bt* δ-endotoxins from *Bt* 44, *Bt* 48, and *Bt* 51 depended on the nature of Cry proteins. (x) Reversed-Phase-HPLC chromatograms demonstrated five similar peaks in the trypsin-digested δ-endotoxins from *Bt* 44 and *Bt* 48 and one unique peak in *Bt* 44. These results revealed that the isolates *Bt* 44 and *Bt* 48 are mostly similar but not identical and thus might contain distinct quantities of related Cry proteins, other Cry proteins that were not tested, and probably novel Cry proteins.

REFERENCES

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. J Econ Entomol 18: 265-267. DOI: 10.1093/jee/18.2.265a

Adugna A, Mesfin T. 2008. Detection and quantification of genetically engineered crops. J SAT Agric Res 6: 1-10.

Aronson AI, Wu D, Zhang C. 1995. Mutagenesis of specificity and toxicity regions of a *Bacillus thuringiensis* protoxin gene. J Bacteriol 177 (14): 4059-4065. DOI: 10.1128/JB.177.14.4059-4065.1995

Bah A, van Frankenhuyzen K, Brousseau R, Masson L. 2004. The *Bacillus thuringiensis* Cry1Aa toxin: effects of trypsin and chymotrypsin site mutations on toxicity and stability. J Invertebr Pathol 85: 120-127. DOI: 10.1016/j.jip.2004.02.002

Basri MW, Siti Ramlah AA, Ramle M, Othman A. 1996. Biological efficacy of three products of *Bacillus thuringiensis* for the control of bagworms, *Metisa plana* and *Mahasena corbetti* (Lepidoptera: Psychidae) of oil palm. In: Proc of the 1996 PORIM Int Palm Oil Congress: Competitiveness for the 21st Century.

Basri WM, Ramlah AAS, Norman K. 1994. Status report on the use of *Bacillus thuringiensis* in the control of some oil palm pests. Elaeis 6 (2): 82-101.

Ben-Dov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N, Margalith Y. 1997. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. Appl Environ Microbiol 63 (12): 4883-4890. DOI: 10.1128/AEM.63.12.4883-4890.1997

Betran FJ, Isakeit T. 2003. Aflatoxin accumulation in maize hybrids of different maturities. Agron J 96: 565-570. DOI: 10.2134/agronj2004.5650

Bonhof MJ, Van Huis A, Kiros FG, Dibogo N. 2001. Farmers' perceptions of importance, control methods and natural enemies of maize stem borers at the Kenyan coast. Insect Sci its Appl 21: 33-42. DOI: 10.1017/S1742758400020026

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254. DOI: 10.1016/0003-2697(76)90527-3

Brownbridge M. 1991. Native *Bacillus thuringiensis* isolates for the management of lepidopteran cereal pests. Insect Sci its Appl 12: 57-61. DOI: 10.1017/S1742758400020531

Brownbridge M, Onyango T. 1992. Screening of exotic and locally isolated *Bacillus thuringiensis* (Berliner) strains in Kenya for toxicity of the spotted stem borer Chilo partellus (Swinhoe). Trop Pest Manag 38: 71-81. DOI: 10.1080/09670879209371651

Burges HD. 1982. Control of insects by bacteria. Parasitology 84: 59-117. DOI: 10.1017/S0031182000053610

Camilla CN. 2000. Managing resistance to *Bacillus thuringiensis* toxins. B.A. [Thesis]. University of Chicago, Chicago, USA.

Campa R, Hooker DC, Miller JD, Schaafsma AW, Hammond BG. 2005. Modelling effects of environment, insect damage, and Bt genotypes on fumonisin accumulation in maize in Argentina and the Philippines. Mycopathologia 159: 539-552. DOI: 10.1007/s11046-005-2150-3

Cetinkaya FT. 2002. Isolation of Bacillus thuringiensis and investigation of its crystal protein genes. [Thesis]. Izmir Institute of Technology, Izmir, Turkey.

Cohen J. 1988. Statistical power analysis for the behavioral sciences (2d ed.). Hillsdale NJ. Lawrence Earlbaum Associates. Find this resource.

Crespo ALB, Spencer TA, Nekl E, Pusztai-Carey M, Moar WJ, Siegfried BD. 2008. Comparison and validation of methods to quantify Cry1Ab toxin from *Bacillus thuringiensis* for standardization of insect bioassays. Appl Environ Microbiol 74 (1): 130-135. DOI: 10.1128/AEM.01855-07

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 807-813. DOI: 10.1128/MMBR.62.3.807-813.1998

DeCoster J, Claypool H. 2004. Data Analysis in SPSS. Retrieved on 19/03/2010 from http://www.stat-help.com/notes.html.

De Groote H. 2002. Maize yield losses from stem borers in Kenya. Insect Sci its Appl 22 (2): 89-96. DOI: 10.1017/S1742758400015162

De Groote H, Overholt W, Ouma JO, Mugo S. 2003. "Assessing the impact of Bt maize in Kenya using a GIS model." Paper presented at the International Agricultural Economics Conference, Durban, August 2003.

De Luis R, Perez MD, Sanchez L, Lavilla M, Calvo M. 2008. Kinetic and thermal parameters for heat denaturation of Cry1A(b) protein from transgenic maize (*Zea mays*). J Food Sci 73: 447-451. DOI: 10.1111/j.1750-3841.2008.00798.x

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoic metabolism. Plant Cell 7: 1085-1097. DOI: 10.1105/tpc.7.7.1085

Dulmage HT. 1993. Development of isolates of *Bacillus thuringiensis* and similar aerobic microbes for use in developing countries. In: Salama HS, Morris ON, Rached E (eds) the biopesticides *Bacillus thuringiensis* and its applications in developing countries. Al-Ahran Press, Cairo.

Feldmann F, Dullemans A, Waalwijk C. 1995. Binding of the CryIVD Toxin of *Bacillus thuringiensis* subsp. israelensis to larval dipteran midgut proteins. Appl Environ Microbiol 61 (7): 2601-2605. DOI: 10.1128/AEM.61.7.2601-2605.1995

Fullmer CS, Wasserman RH. 1979. Analytical peptide mapping by high-performance liquid chromatography. Application to intestinal calcium-binding proteins. J Biol Chem 254 (15): 7208-7212. DOI: 10.1016/S0021-9258(18)50305-7

Ghribi D, Zouari N, Jaoua S. 2004. Improvement of bioinsecticides production through mutagenesis of *Bacillus thuringiensis* by UV and nitrous acid affecting metabolic pathways and/or delta-endotoxin synthesis. J Appl Microbiol 97 (2): 338-346. DOI: 10.1111/j.1365-2672.2004.02323.x

Harvey D. 2000. Modern Analytical Chemistry. McGraw-Hill Book Company, International Edition, Singapore.

Hickle LA, Fitch WL. 1990. Analytical Chemistry of *Bacillus Thuringiensis*. ACS Symposium Series, American Chemical Society, Washington DC. DOI: 10.1021/bk-1990-0432

Hutchinson D, McDonald I. 2008. Laser ablation ICP-MS study of platinum-group elements in sulphides from the Platreef at Turfspruit, northern limb of the Bushveld Complex, South Africa. Mineralium Deposita 43 (6): 695-711. DOI: 10.1007%2Fs00126-008-0190-6

Khan MS, Amjad M. 2000. Chemical control of maize stem borer (*Chilo partellus*, Swinhoe). Pak J Biol Sci 3 (12): 2116-2118. DOI: 10.3923/pjbs.2000.2116.2118

Laemmli K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685. DOI: 10.1038/227680a0

Lightwood DJ, Ellar DJ, Jarrett P. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin. Appl Environ Microbiol 66: 5174-5181. DOI: 10.1128/AEM.66.12.5174-5181.2000

Liu BL, Tzeng Y, Wei CT. 2000. Recovery of solubilised δ-endotoxin from *Bacillus thuringiensis* subsp. Kurstaki fermentation broth. Pest Manag Sci 56: 448-454. DOI: 10.1002/(SICI)1526-4998(200005)56:5<448::AID-PS146>3.0.CO;2-O

Masson L, Erlandson M, Carey MP, Brousseau R, Perez VC, Frutos R. 1988. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl Environ Microbiol 64: 4782-4788. DOI: 10.1128/AEM.64.12.4782-4788.1998

Mbapila JC, Overholt WA, Kayumbo HY. 2002. Comparative development and population growth of an exotic stemborer, *Chilo partellus* (Swinhoe), and an ecologically similar congener, *C. orichalcociliellus* (Strand)(Lepidoptera: Crambidae). Int J Trop Insect Sci 22 (1): 21-27. DOI: 10.1017/S1742758400015022

Mohan M, Gujar GT. 2001. Toxicity of *Bacillus thuringiensis* strains and commercial formulations to the diamondback moth, *Plutella xylostella* (L.). Crop Prot 20: 311-316. DOI: 10.1016/S0261-2194(00)00157-5

Mugo SN, Bergvinson D, Hoisington D. 2001. Options in developing stem borer-resistant maize: CIMMYT's approaches and experiences. Insect Sci Appl 21 (4): 409-415.DOI: 10.1017/S1742758400008535

Mugo S, Taracha C, Bergvinson D, Odhiambo B, Songa J, Hoisington D, Gethi M. 2004. Screening cry proteins produced by Bt maize leaves for activity against Kenyan maize stem borers. In: Friesen DK, Palmer AFE (eds) 102-105.

Muhammad L, Underwood E. 2004. The maize agricultural context in Kenya. In: Hillbeck A, Andow DA (eds) Environmental Risk Assessment of Genetically Modified Organisms. CAB International, Wallingford, UK.

Muthomi JW, Mureithi BK, Chemining'wa GN, Gathumbi JK, Mutitu EW. 2010. Aspergillus and aflatoxin B1 contamination of maize and maize products from Eastern and North-rift regions of Kenya. Proceedings of the 12th KARI Biennial Scientific Conference, Nairobi, 8th-12th November 2010.

Mwathi JW. 2007. Isolation, evaluation and molecular characterisation of *Bacillus thuringiensis* isolates against *Prostephanus truncatus*, a major storage pest in maize. M.Sc. [Thesis]. Kenyatta University.

Nakayama H. 2001. Protein functional analysis using liquid chromatography-tandem mass spectrometry in post-genomic era. Chromatography 22 (3): 201-207.

Ngetich J. 2010. New cure for aflatoxin maize still miles. The Daily Nation. Retrieved from http://nationmedia.com

Obonyo DN, Ogola JBO. 2011. Transgenic Pesticidal Crops and the Environment: The Case of Bt Maize and Natural Enemies. In Pesticides in the Modern World-Risks and Benefits. IntechOpen.

Ofomata VC, Overholt WA, Van Huis A, Egwuatu RI, Ngi-Song AJ. 2003. Niche overlap and interspecific association between *Chilo partellus* and *Chilo orichalcociliellus* on the Kenya coast. Entomol Exp Appl 93: 141-149. DOI: 10.1046/j.1570-7458.1999.00572.x

Osir EO, Vundla WRM. 1999. Characterisation of the δ-endotoxin of a *Bacillus thuringiensis* isolates active against tsetse, *Glossina morsitans*, and a stem borer, *Chilo partellus*. Biocontrol Sci Technol 9: 247-258. DOI: 10.1080/09583159929820

Perferoen M. 1998. EPA Data Evaluation Report. *In vitro* digestability and heat stability of the endotoxin Cry9C of *Bacillus turiengensis*. Retrieved10th December from 2010 http://www.epa.gov.oppbppd1/biopesticides/pips/old/cry9c/der-4258108a.htm.

Poinar GO, Thomas GM. 1978. Diagnostic manual for the identification of insect pathogens. Plenum Press, New York, USA.

Polanczyk RA, Silva RFPD, Fiuza LM. 2000. Effectiveness of *Bacillus thuringiensis* strains against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Braz J Microbiol 31 (3): 164-166. DOI: 10.1590/S1517-83822000000300003

Rorabacher DB. 1991. Statistical treatment for rejection of deviant values: Critical values of Dixon q parameter and related subrange ratios at the 95 percent confidence level. Anal Chem 63: 636-638. DOI: 10.1021/ac00002a010

Rouis S, Chakroun M, Jaoua S. 2008. Comparative study of *Bacillus thuringiensis* Cry1Aa and Cry1Ac d-endotoxin: Activation, inactivation and insitu histopathological effect in *Ephestia kuehniella* (Lepidoptera: Pyralidae). Mol Biotechnol 38: 233-239. DOI: 10.1007/s12033-007-9021-8

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 775-806. DOI: 10.1128/MMBR.62.3.775-806.1998

Skoog DA, West DM, Holler FJ. 1992. Fundamentals of Analytical Chemistry. (6th ed). Harcourt Brace Jovanovich Publisher, Orlando.

Songa JM, Overholt WA, Okello RO, Mueke JM. 2002. Control of lepidopteran stemborers in maize by indigenous parasitoids in the semi-arid areas of Eastern Kenya. Biol Agric Hortic 20: 77-90. DOI: 10.1080/01448765.2002.9754949

Tarus SJ, Nyambati EM, Kituyi JL, Segor FK, Chebii FJ. 2010. Pesticide residue levels in Nzoia river catchment area. Proceedings of the 12th KARI Biennial Scientific Conference, Nairobi, 8th-12th November 2010. Poster presentation.

Tende R, Tefera T, Mugo S. 2010. Mass rearing and use of stem borers at the KARI-Katumani insectary. Proceedings of the 12th KARI Biennial Scientific Conference, Nairobi, 8th-12th November 2010.

Uribe D, Martinez W, Ceron J. 2003. Distribution and diversity of cry genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. J Invertebr Pathol 82 (2): 119-127. DOI: 10.1016/S0022-2011(02)00195-7

Van Frankenhuyzen K. 1994. Effect of temperature on the pathogenesis of *Bacillus thuringiensis* Berliner, in larvae of the spruce budworm *Horistoneura fumiferana* L (Lepidoptera: Tortricidae). Can Entomol 126: 1061-1065. DOI: 10.4039/Ent1261061-4

Van Frankenhuyzen K, Regniere J, Bernier-Cardou M. 2008. Response of *Lymantria dispar* L. (Lepidoptera: Lymantriidae) to *Bacillus thuringiensis* subsp. kurstaki at different ingested doses and temperatures. J Invertebr Pathol 99: 263-274. DOI: 10.1016/j.jip.2008.06.011

Van Frankenhuyzen K. 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr Pathol 101: 1-16. DOI: 10.1016/j.jip.2009.02.009

Wang'ondu VW. 2001. Isolation and partial characterization of *Bacillus thuringiensis* from Kakamega and Machakos districts in Kenya. [Thesis]. University of Nairobi, Kenya.

Yee TS, Ibrahim Y, Omar D. 2008. Efficacy of *Bacillus thuringiensis* (Berliner) subspecies kurstaki and aizawai against the bagworm, metisa plana walker on oil palm. J Biosci 19 (1): 103-114.