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ISOLATION, EVALUATION AND MOLECULAR CHARACTERISATION OF *Bacillus thuringiensis* ISOLATES AGAINST *Prostephanus truncatus*, A MAJOR STORAGE PEST IN MAIZE

By

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(Bsc Hons)

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology in the School of Pure and Applied Sciences of Kenyatta University

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Isolation, evaluation and molecular

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or for any other award.

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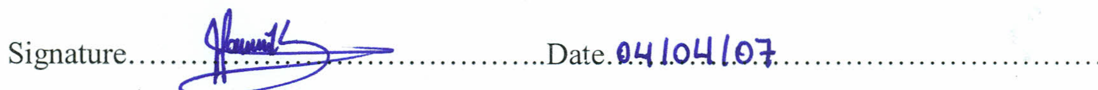

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DEDICATION

To

My son Antony Mwathi for his patience, perseverance and endurance during the preparation of this thesis. To all my family members, my brothers and sisters; My dad Mwathi Munene and my late mother Jacinta Waithera Mwathi. Your encouragement was outstanding.

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ABSTRACT

The study was initiated to investigate the potential of locally isolated *Bacillus thuringiensis (Bt)* isolates for the control of the larger grain borer (LGB) (*Prostephanus truncatus)*, a major storage pest of maize and cassava in Kenya. Samples of soil, grain and dead insects were collected from various maize growing areas in Kenya and screened for the presence of *Bt*. The isolates were tested for toxic activity against *P. truncatus* in the laboratory. *Bacillus thuringiensis* var. *tenebrio (Btt),* toxic to the Colorado Potato Beetle (CPB) was included in the bioassays as a Standard control reference. *Bt* precipitate obtained from broth was mixed with maize flour at a ratio of 2:1 to make an artificial diet. Pellets of 2g were cut from the artificial diet and offered to insects placed in plastic containers. A total of 68 isolates of *Bt* were recovered out of 320 samples screened, and of which 85 % were from grains, 12 % from soils and 3 % from dead insects. Grains proved to be a better source of *Bt* isolates. The screening for *Bt* isolates in the laboratory revealed that different *Bt* isolates from different samples were either bipyramidal, irregular or square shaped. Ninety five (95 %) of the *Bt* isolates from grain samples were bi-pyramidal shaped and (5 %) from grains were square shaped. Analysis of soil samples showed that 12.5 % were square shaped and 87.5 % were bipyramidal shaped. The *Bt* isolates from insect samples were all irregular shaped (100 %). Among the *Bt* isolates, 82 % were positive and 18 % were negative for Voges Paskeur test. Methyl Red tests revealed that 24 % of the *Bt* isolates were negative and 76 % were positive, whilst indole tests showed that 72 % of the *Bt* isolates were negative and 28 % were positive. The biochemical characteristics of isolated pathogens confirmed that these isolates were *Bt*. Mortality in the controls was 0 % and 3.3 %, 7 and 30 days after treatment, respectively Isolates of *Bt* were toxic to adult *P. truncatus* but mortality varied from 1.7 to 53 % and 3.3 to 85 %, 7 and 30 days after treatment between the isolates, respectively. Seven *B. thuringiensis* isolates (*Bt* 41, *Bt* 61, *Bt* 51, *Bt* 60, *Bt* 46, *Bt* 45 and *Bt* 44) were more toxic to adult *P. truncatus* than other isolates, causing mortalities of 50 and 80 %, 7 and 30 days after treatment. However, there was no significant difference between these isolates ($P \geq 0.05$). The standard reference isolate, *Bt tenebrio* was also toxic, causing mortality of 37 % and 60 %, 7 and 30 days after treatment, and was significantly different ($P \leq 0.05$) from the most toxic isolates listed above. The lethal time 50 % mortality (LT_{50}) values were calculated for isolates that achieved more than 50 % mortality after 30 days following treatment. The LT_{50} ranged from 7 to 37 days and varied significantly ($P \leq 0.05$) among isolates. *Bacillus thuringiensis* isolates 41, 51, 61, 60, 45, 44, 46 and 23 had the shortest LT_{50} values of between 8 and 11 days. The standard reference strain, *Bt tenebrio,* had LT_{50} of 16 days, which was significantly different ($P \leq 0.05$) from the most toxic isolates. *Bacillus thuringiensis* 41 and *Bt* 51 molecular sizes varied between 67 to 74 KDa while the Standard control isolate, *Btt,* had a molecular size of 67 KDa. There was a common protein band of 67 KDa in the two isolates (*Bt* 41 and 51). These results suggest that a further DNA study was necessary to ascertain the presence of *cry* 111 genes that are known to be toxic to coleopterans. These results demonstrated the potential of discovering isolates of *B. thuringiensis* that are toxic to adult *P. truncatus* and could be, therefore, developed as biopesticides. Preservation of these isolates in a germplasm bank for pathogen biodiversity should be considered since these pathogens could be utilized as sources of new *cry* genes for genetic transformation.

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ACRONYMS AND ABBREVIATIONS

AMC	Acetyl Methyl Carbinol.
ANOVA	Analysis of Variance
AS	After solubilisation
ATA	After Trypsin Activation
BS	Before solubilisation
BSA	Bovine Serum Albumin
CPB	Colorado potato beetle
cry	Crystal
DTT	Dithriolthreitol
DNA	Deoxy-ribonucleic-acid
HCl	Hydrogen chloride
ICIPE	International Centre of Insect Physiology and Ecology
ICP	Insecticidal Crystal protein
IPM	Integrated Pest Management
KARI	Kenya Agricultural Research Institute
KDa	Kilo Dalton
KU	Kenyatta University
L12:D12.	Twelve hours light: Twelve hours dark
LT_{50}	Lethal Time causing 50% mortality
LGB	Larger Grain Borer
Min	Minute
MM	Molecular marker
MR	Methyl red test
MRVP	Methyl red, Voges Proskauer medium
NA	Nutrient Agar
ODA	Oversees Development Authority
PEG	Polyethylene glycol
PSB	Phosphate Saline Buffer
R.H	Relative humidity
rpm	Rotation per minute
SAS	Statistical Analysis Software
SNK	Student Newman's Keul's test
SRI	Standard control reference Isolate
SDS-PAGE	Sodium Duodecyl Sulphate Polyacrylamide Gel Electrophoresis
TB	Tryptone broth
USA	United States of America
USAID	United States of America International Development
v/v	Volume per volume
VP	Voges-Proskauer test
w/w	Weight per weight

CHAPTER ONE

1. INTRODUCTION

1. 1 Background

Maize, *Zea mays* L., is the main staple food for most of the Kenyan households. Because of its high demand in the urban and export markets, small-scale farmers in Central and Western districts of the country have intensified its cultivation (Ayaga, 2003). Its production, however, is limited by various factors, the major ones being infestation and damage by storage insect pests (Songa *et al.*, 2004). The Larger Grain Borer (LGB), *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), is currently the most destructive pest of stored maize in Kenya and other countries in East and West Africa (Boxall, 2002).

The most commonly used method for the management of this pest has been synthetic chemical control. This approach has, however, not proved to be sustainable due to problems of pesticide resistance, high cost of the insecticides, and increasing concern among consumers about the health safety of this approach (White and Leesch, 1995). In addition, the effect of synthetic chemicals on the environment and applicators are well documented. For example, long-term exposure to synthetic insecticides has been associated with cancer, liver damage, immunotoxicology, birth defects, reproductive problems in humans and other animals (Wright *et al.*, 1993). It is therefore important to develop alternative pest management strategies that are safe, environmentally friendly, as part of an integrated pest management system (Camilla, 2000). Biological control using entomopathogenic bacterium such as *Bacillus thuringiensis (Bt)* is a promising option.

Bacillus thuringiensis is being used as a biopesticide worldwide for the control of many insect pests and disease vectors. However, no attempt has so far been made to evaluate its potential for the control of *P. truncatus. Bacillus thuringiensis* isolates that are pathogenic to coleopterans are rare and belong to the subspecies *tenebriosis* (Krieg *et al.*, 1983), *donegani* (Cidaria *et al.,* 1990) and *San Diego* (Payne, 1988).

There is therefore a need to collect local isolates of *B. thuringiensis* and evaluate their efficacy against *P. truncatus* in order to select the most potent isolate that could be used as a biopesticide. The present study investigated the possible use of local isolates of *B. thuringiensis* as a component of Integrated Pest Management (IPM) for *P. truncatus* in Kenya. The toxin of an effective *Bt* isolate can be utilised as a source of a gene for engineering *Bt* maize that is resistant to *P. truncatus.*

1. 2 Problem statement

Losses in stored maize due to infestation and damage by the larger grain borer, *P. truncatus,* in Africa have been severe and average 30-90 % (Hodges *et al.*, 1983; Keil, 1988; Adda *et al.,* 1996; Bell *et al.*, 1999; Farrell, 2000; Odour *et al.*, 2000). For instance in Tanzania, *P. truncatus* has been reported to cause losses as high as 41.2 % of maize in storage, within six months (Dunstan and Magazini, 1981; Hodges *et al.*, 1983; Keil, 1988). Dry weight losses of 17.4 % have been recorded in Kenya after six months of storage (Muhihu and Kibata, 1985). In Togo, surveys carried out before and after the introduction of *P. truncatus* suggested that losses might have risen from around 7 % to over 30 % (Pantenius, 1988).

The attack of grains by *P. truncatus* constitutes the point of entry of mould, thus resulting in production of aflatoxins produced by the fungus *Aspergillus flavus* (Goldbatt, 1969). Aflatoxins consumption in human leads to aflatoxicosis, a primary hepatic disease. Cases of acute aflatoxicosis have been reported in many parts of the world, especially in developing countries like the Taiwan, Uganda, India and Kenya (Ngindu *et al.,* 1982; Nyikal *et al.,* 2004) among others. Aflatoxins poisoning will likely continue to be a public health problem until culturally appropriate storage methods for dry maize are implemented by the local population.

1. 3 Justification of the study

One of the major post harvest constraints to maize production in Kenya is infestation and damage by *P. truncatus*. Sustainable management of this pest has been a major challenge. Farmers who can afford synthetic chemical insecticides use them extensively in order to bring the pest under control. This often leads to insecticide resistance by the pest (Nang'ayo, 1996). Consequently, due to the high cost of chemicals, most farmers cannot afford. Furthermore, there is also increasing concern by consumers about the safety use of pesticides in maize (White and Leesch, 1995) and their effects on the environment (Hodges, 1986).

This underscores the need to rationalise and minimise pesticide use in maize and also to explore safer pest management options. A biological control agent like *B. thuringiensis* would constitute an alternative option for management of the *P. truncatus*. An effective

B. thuringiensis can also be utilised as a source of genes for engineering maize that is resistant to the *P. truncatus*.

1. 4 Null Hypotheses

1. *Bacillus thuringiensis* does not occur naturally in different geographical regions of Kenya.
2. There are no differences in the relative toxicity of local isolates of *B. thuringiensis* against *P. truncatus*.
3. The relative toxicity of *B. thuringiensis* does not depend on the nature of the Crystal protein.

1. 5 Alternative Hypotheses

1. *Bacillus thuringiensis* occurs naturally in different geographical regions of Kenya.
2. There are differences in the relative toxicity of local isolates of *B. thuringiensis* against *P. truncatus*.
3. Different isolates of *B. thuringiensis* have different Crystal proteins, which determine their relative toxicities against *P. truncatus*.

1. 6 Objectives of the study

1. 6. 1 General objective

To contribute to the reduction in post-harvest losses in maize by identifying local *B. thuringiensis* isolates which are effective on *P. truncatus*.

1. 6. 2 Specific objectives

1. To identify isolates of *B. thuringiensis* from stored grains and dead insects and carry out their characterization.
2. To evaluate the toxicity of different local isolates of *B. thuringiensis* against *P. truncatus*.
3. To carry out molecular characterization of the most effective isolates of *B. thuringiensis*.

1. 7 Significance and anticipated output

1. Effective local isolates of *B. thuringiensis* against *P. truncatus* will be isolated, identified and preserved in the germplasm as genetic resource.
2. Identification of *B. thuringiensis* genes that could be used for possible genetic transformation of maize.

CHAPTER TWO

2. LITERATURE REVIEW

2. 1 Post-harvest constraints to Maize production

Harvested maize grain is stored for various periods of time depending on the quantity of farmers' production, needs and the market demand. The major post-harvest constraints of maize include infestation and damage by storage pests, and losses due to improper post-harvest practices such as threshing, drying, transportation and poor storage conditions, that may often lead to infestation by fungal pathogens (McFarlane, 1988).

In storage, insects cause physical damage mainly through direct feeding on the whole grain, on the endosperm causing loss in weight and quality of the grain, and on the germ, resulting in poor seed germination and viability (Santos *et al.,* 1990). In addition, the insect pests also contaminate the grain with their faecal matter, thus reducing their market value and rendering them unfit for human consumption (Santos *et al.,* 1990).

There is a wide range of insect pests that attack maize in stores, for example, the maize weevil, *Sitophilus zeamais* (L), granary weevil, *Sitophilus granarius* (L), the lesser grain borer, *Rhizopertha dominica* and the larger grain borer, *P. truncatus,* with the latter being the most destructive of the four (Boxall, 2002).

2. 2 The Larger Grain Borer, *Prostephanus truncatus*

The larger grain borer, *P. truncatus,* belongs to the family Bostrichidae, members commonly known as the false powder post beetles (Browne, 1968; Booth *et al.*, 1990).

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Adult *P. truncatus* is 2 to 3.5 mm in length and 1 to 1.5 mm in width (Fig. 1) (Birkinshaw, 1998). Shires and McCarthy (1976) developed a key for sexing live *P. truncatus* adults based on clypeal tubercles. It has also been shown that females are significantly heavier (range 3.4 - 6.8 mg) than males (range 3.4 - 5.8 mg) (Guntrip *et al.*, 1997) and have a sex ratio of 1:1 (Shires, 1979).

Prostephanus truncatus is an important pest of traditional maize stores in sub-Saharan Africa and is currently the most destructive pest of stored grains (Hodges *et al.*, 1983; Dick, 1988; Markham *et al.*, 1994).

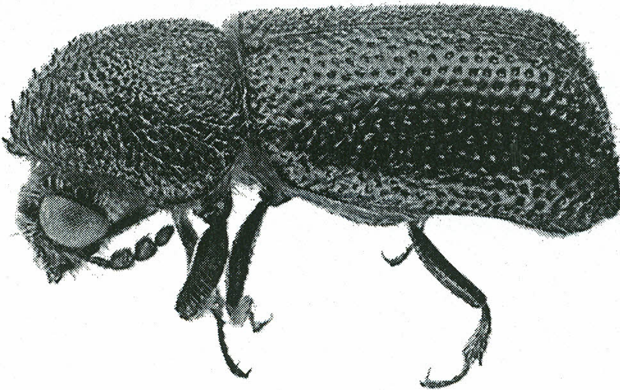

Fig 1: Picture of the *Prostephanus truncatus*

(Source: http:stemborer.icipe.org/projects/LGB.pdf)

2. 3 Geographical distribution of *Prostephanus truncatus*

The current distribution of *P. truncatus* includes countries in both the new world and Africa, but it is considered to be a major storage pest in Mexico, Central America and Sub-Saharan Africa only (Delgado and Hernandez Luna, 1951; Quintana *et al.*, 1960; Kumar, 2001). In East Africa, *P. truncatus* is believed to have been accidentally introduced into Tanzania in 1970s (Dunstan *et al.*, 1981).

Prostephanus truncatus has subsequently spread into other parts of Eastern and Western Africa (Harnish and Krall, 1984; Anon, 1986; Dick and Rees, 1989). In West Africa, *P. truncatus* was first reported in Togo and later in Benin in 1986 where it has become a serious pest of rural grain stores (Anon, 1986; Meikle *et al.*, 2002). Outbreaks were also noted in Israel (Calderon and Donahaye, 1962), in Iraq (Al- Sousi *et al.*, 1970; Wright *et al.*, 1984), and in Thailand (Sukprakarn, 1976), presumably from grain shipments. These outbreaks were, however, contained so that the pest did not become established.

In Kenya, *P. truncatus* was first observed in 1983 in Taita Taveta district (Kega and Warui, 1983; Muhihu and Kibata, 1985**)**. The pest was later recorded in Loitoktok in Kajiado District in 1987, and has since spread to other parts of Kenya including the Eastern parts around Kiboko, Kitui and Machakos areas, and in Kiambu district in Central Kenya, as well as in Western Kenya along South Nyanza. At the Kenyan Coast, the *P. truncatus* has been recorded in Voi, Mwatate and Wundayi areas (Mutambuki *et al.*, 1990; Kibata *et al.*, 1996). In October 2004, *P. truncatus* was found to have spread to

the major maize growing areas of Western Kenya in Kitale and Kakamega (Songa *et al.*, 2004).

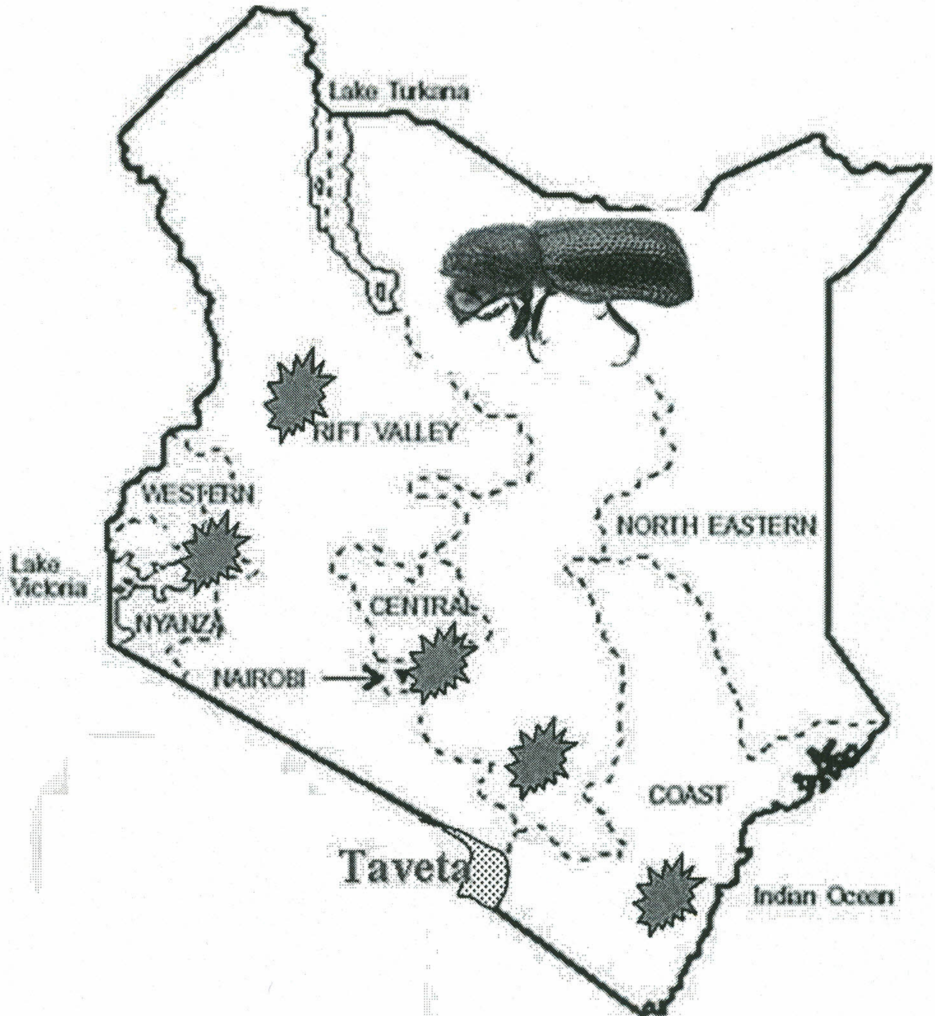

Fig 2: Geographical distribution of *Prostephanus truncatus* in Kenya

Source: Songa *et al.*, 2004

2. 4 The pest status

Prostephanus truncatus continues to be an escalating problem for farmers in sub-Saharan Africa despite the various management options that have been used in the last 25 years (Farrell and Schulten, 2002; Meikle, 2002). On-farm losses in maize often exceed 70-80% in the tropics including sub-Saharan Africa (Golob and Hodges, 1982; Hodges *et al.*, 1983; Boxall, 2002).

Further spread of *P. truncatus* is anticipated due to: - (i) Its ability to reproduce under a wide range of temperature and humidity regimes (Shires, 1979; Bell and Watters, 1982); (ii) Its considerable flight mobility (Farrell and Key, 1992; Fadamiro, 1996); (iii) Its longevity (35 weeks) (Nansen *et al.*, 2002); and (iv) Its ability to survive and breed on non-agricultural hosts, for example, wood (Boeye, 1990; Rees *et al.*, 1990; Ramirez - Martinez *et al.*, 1994; Nang'ayo, 1996 and Nansen, 2002).

2. 5 The hosts of *Prostephanus truncatus*

Prostephanus truncatus has a wide host range, and exists both in stores feeding on stored grain (Howard, 1983) and also in the forest feeding on various wood species (Nangayo *et al.*, 1993). Early records associated *P. truncatus* with various other alternative hosts. It has been shown to damage economically important foods like the wheat, paddy, pulses, groundnuts, cocoa, beans and coffee beans (Shires, 1977), dried tubers of yam and sweet potatoes (Jalloh, 1989) and various confectionary nuts (Bangaly, 1993, Nansen, 2002) woody and starchy roots and tubers (Chittenden, 1911; Lesne, 1939; Detmers, 1990; Helbig and Schulz, 1994; Nang'ayo, 1996), natural forest (Browne, 1968;

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Ramirez- Martinez *et al.*, 1994; Nang'ayo, 1996) seeds of trees (Li, 1988; Nansen, 2002) and even plastics (Li, 1988).

Symbiotic associations of both intracellular and extracellular bacteria have been observed both in Africa and Meso-America (Kleespies *et al.*, 2001). However, *P. truncatus* is mainly a pest of stored cereals (Howard, 1983) preferably maize (Shires, 1977; Jalloh, 1989; Bangaly, 1993) and to a lesser extent, stored starchy tubers especially cassava (Nyakunga, 1982; Jalloh, 1989).

2. 6 Life cycle of *Prostephanus truncatus*

A male *P. truncatus* produces its own sex pheromone on a suitable substrate, in the absence of a female (Scholz *et al.*, 1998b). The pheromone attracts the female, after which mating occurs. Females are generally known to mate repeatedly with the same male (Birkinshaw, 1998).

The females have a pre-oviposition period of 5-10 days and an oviposition period of 95-100 days (Shires, 1979; Shires, 1980; Burkholder and Ma, 1985; Demianyk and Sinha, 1988; Nissen *et al.*, 1991; Guntrip *et al.*, 1996 and Meikle *et al.*, 1998). The females lay most of the eggs within kernels in blind ending chambers bored at right angles to the main tunnels (Hodges, 1982; Howard, 1983). The eggs are laid in batches of up to 20 (termed *sub clutches* by Howard, 1983) with a mean of 10.5 eggs per clutch. They are covered with finely chewed maize dust called *frass*, whose function is to protect eggs and developing larva from dehydration.

The optimum conditions for development of the *P. truncatus* on maize are 32° C and 70-80 % relative humidity (R.H.) (Shires, 1980; Bell and Watters, 1982). From egg to adult development, it takes approximately 25 to 35 days (Shires, 1979; Bell and Watters, 1982; Meikle *et al.*, 1998, Nansen and Meikle, 2002).

Prostephanus truncatus undergoes complete metamorphosis. There are three larva instar, which bore and feed while developing as (reviewed by Demianyk and Sinha, 1988). The first instar larva is predominantly found in the "floury endosperm", while the second and third instar larva are found in the "germ tissue" (Nansen and Meikle, 2002). The last larva instar constructs pupal cases from frass stuck together with larval secretion either within the grain or in the surrounding dust. Pupation takes approximately 4-5 days (Bell and Watters, 1982). *Prostephanus truncatus* individuals are described as "internal feeders" because female's oviposition inside the host and the entire egg to emergence cycle is completed within the same host.

2. 7 Damage and loss caused by *Prostephanus truncatus*

Damage by the *P. truncatus* in maize is caused by both the larva and adults, and it starts in the field on maturing maize, and continues in the store. *Prostephanus truncatus* is known to cause more damage on maize on the cob than on shelled maize (Hodges, 1986). Adult *P. truncatus* tunnels in the grain extensively, reducing it to a "floury dust" (Wright *et al.*, 1993). The beetle has also been observed boring into a range of other materials without feeding, including wood, beans, groundnuts, perspex, plastic and polythene (Li, 1988).

The impact of these losses to the farmer is that there is less food for the household as the damaged grain is often unfit for human consumption. The farmer also experiences loss of income through early sales, and the need to buy grain at higher prices later in the season. Trading with neighbouring countries due to phytosanitary regulations is also restricted and consequently the farmer may experience real food shortages (Lugg and Ndibalema, 1988; Schulten and Toet, 1988; Wright *et al.*, 1993; Semple and Kirenga, 1994; Magrath *et al.*, 1996, 1997).

2. 8 Control of *Prostephanus truncatus*

A number of management strategies have been used for the control of *P. truncatus* since its outbreak in Africa (Schulten and Toet, 1988). These include chemical, physical, cultural, legislative and more recently biological control. However, to date, it has been difficult to provide economic control of the pest without an effective chemical insecticide.

2. 8. 1 Cultural control

Farmers harvest the crop early and expose maize cobs either with or without husks, to heat and smoke from cooking fires and to sunlight to dry it properly. The advantage of this method is that it is practical, affordable and safe. However, the practice has not proved effective hence the method has not given sustainable control against *P. truncatus* (Golob and Hodges, 1982; Giles *et al.*, 1995; Nang'ayo, 1996).

The other indigenous control method is the use of plant products with insecticidal, repellent, or antifeedant activities such as the legume plants *Crotalaria juncea*, *Tephrosia vogalii*, leaves of Chinaberry *Melia azedarach*, and the neem tree *Azadirachta indica* as reviewed by (Nang'ayo, 1996). Mineral products such as lime, sand or ash dust have also been used to control the *P. truncatus* but with little success (Heckles, 1992). The method is not cost effective and is labour intensive, which is the reason why it has low adoption by farmers as reviewed by (Markham *et al*., 1991).

2. 8. 2 Physical control

This method involves the use of airtight drums (hermetic storage), which prevent the entry of oxygen or carbon dioxide and insects. A shortcoming to the use of drums is that it requires the grains to be completely dry before storage, in order to avoid fungal growth and associated condensation that leads to the production of mycotoxins. The drums are also generally expensive, a factor which limit their use in rural grain storage. An alternative is to store the grain in plastic bags, because they are cheap and easily available; however, they are limited by the ability of some insects to perforate them (Giles and Leon, 1974).

2. 8. 3 Quarantine or legislative control

In this control method, strict regulations are imposed upon movement of maize from infested areas to uninfested areas in an effort to prevent the spread of *P. truncatus*. In Kenya, this has been done in prevention of outbreaks in uninfested areas. The spread can occur due to intra-regional trade in maize, or occasional food-aid lifts. Close scrutiny is

maintained to ensure that maize originating from *P. truncatus*-infested areas and destined for long distance trade is adequately disinfested before transit (Schulten and Toet, 1988; Tyler *et al.*, 1990). The major drawback with this method is that the success in the enforcement of these measures largely depends on good co-operation between the local administration, police force, custom officials and rural market supervisors; hence the logistics involved are complicated to fully eliminate further spread of *P. truncatus* (Tyler *et al.*, 1990).

2. 8. 4 Chemical control

Various synthetic chemical insecticides belonging to classes of pyrethroids, organochlorines and organophosphates have been used. Insecticides have a high degree of efficacy and rapid kill, which has been a strong incentive for farmers to use them for the control of *P. truncatus* (Nang'ayo, 1996; Meikle *et al.*, 2000). However, use of these insecticides has proved to be unsustainable due to increased resistance in *P. truncatus* populations, which has in turn necessitated increased dosages and more frequent applications (Meikle *et al.*, 2000).

Fumigants have also been used with success. They are low molecular weight chemicals that are highly toxic and volatile. They are commercially available in a solid, liquid, or gaseous state and include phosphine gas, methyl bromide, carbon dioxide and biogas amongst others. The advantage is that they are very effective and fast in action. When used in stored grain, they kill all insect life stages found in the produce. Phosphine use on the *P. truncatus* in Togo proved successful in the disinfestations of maize stores (Krall,

1984). However, the method proved unpopular for adoption by farmers since it was technically not feasible (Taylor and Harris, 1989). In addition, there are records of phosphine resistance in pests of stored produce (Tyler *et al.*, 1983). Methyl bromide on the other hand, is effective against *P. truncatus* in killing all stages of the borer but has recently been outlawed as it pollutes the environment, more so, it depletes the ozone layer.

2. 8. 5 Biological control

Biological control involve the use protozoans, fungi and bacteria to control *P. truncatus*. For example, protozoa *Ascogregarina* spp. (Eugregarinidia: Lecudinidae), *Mattessia* spp. (Neogregarinida: Ophryocystidae) and two species of *Nosema* (Microsporidia: Nosematidae) from Tanzania (Purrini and Keil, 1989; Laborious, 1990) and *Pleistophora* spp. (Microsporidia: Nosematidae) from Costa Rica were tested against *P. truncatus* in the laboratory (Laborious, 1990). One isolate of *Mattesia* caused 90 % mortality in first-instar larva (Henning-Helbig, 1994). The microsporidia *Pleistophora* species, *Nosema* species., *Ascogregarina bostrichidorium* protistans could kill up to 90 % of first-instar *P. truncatus* larva in the laboratory; however, mortality was generally low in the field and ranged from 1-6 % (Markham *et al.*, 1991).

Fungal entomopathogens have also been tested against *P. truncatus*. They include entomopathogenic fungi of the genera *Metarhizium* and *Beauveria* (Burde, 1988; Laborious, 1990, Meikle *et al.*, 2001, Bourassa *et al.*, 2001). The screening results showed > 90 % mortality on *P. truncatus* (Bourassa *et al.*, 2001; Meikle *et al.*, 2001),

however, the major drawback towards the use of entomopathogens is the threat of contaminating food with toxic secondary metabolites such as destruxins (Strasser *et al.*, 2000)

The parasitoids *Theocolax* (=*Choetopsila*) *elegans* (Westwood), *Anisopteromalus calandrae*; *Lariophagus distinguendas* (Forster) (Hymenoptera: Pteromalidae) and *Pteromalus cerealellae* (Walker) (Hymenoptera: Otromalidae), respectively, were reported attacking *P. truncatus* in stores in Germany and in Yucatan, Mexico (Boeye, 1988; Rees *et al.*, 1990; Leliveldt, 1990). They were, however, not fully able to suppress the pest's population leading to extensive damage (Helbig, 1998; Brower, 1991; Martinez Cerdas *et al.*, 1992).

The predatory beetle, *Teretrius nigrescens*, Lewis, (Coleopteran: Histeridae) was found to be commonly associated with *P. truncatus* in Meso-America. *Teretrius nigrescens* uses the *P. truncatus* pheromone as a kairomone (Rees *et al.*, 1990), and exhibits a strong preference for feeding on *P. truncatus* in choice tests (Ayertey *et al.*, 1999), indicating a relatively close association between the predator and its prey. *Teretrius nigrescens* have been widely used in Africa as a biological control agent for *P. truncatus*, for example, in Togo (Ritcher *et al.*, 1997) in Benin, Ghana (as reviewed by Nansen, 2002), and Kenya (Giles *et al.*, 1996) among others. However, it has been shown that *P. truncatus* is still capable of inflicting significant damage on maize stores even in presence of the predator *T. nigrescens* (Meikle *et al.*, 2002).

Teretrius nigrescens was introduced in Kenya in May 1992 as a classical biological control (Giles *et al.*, 1996, Nang'ayo and Hill, 1996) at Makueni in Kibwezi Division of Eastern Province, and at Mgange in Wundayi Division of Coast province. *Teretrius nigrescens* established itself in natural woodland habitats in the area where it was released and was caught repeatedly in *P. truncatus* pheromone traps, to which it was readily attracted (Giles *et al.*, 1995; Scholz *et al.*, 1998a).

2. 8. 6 Host resistance

Due to the above post-harvest losses of maize to insect pests, there is an accelerated effort towards increasing host resistance (Guthrie and Russel, 1989). This approach is safe, practical and affordable. However, for conventional resistance, there is a need to have resistant varieties. This has been made possible through conventional breeding, but it takes a long duration. Moreover, there is no *P. truncatus* resistant germplasm for conventional breeding so far. Suggested options are search for pathogen-derived resistance. The focus has been through the use of bacteria mainly *B. thuringiensis.*

2. 8. 7 *Bacillus thuringiensis*

Bacillus thuringiensis represents the major class of microbes used for insect biocontrol (Klausner, 1984). It is a Gram positive, rod-shaped, aerobic, motile, spore-forming, soil bacterium. This bacterium is also found in grain dust from silos and other grain facilities, and has various strains. Each strain produces its own unique insecticidal crystal protein (ICPs) which are segregated in parasporal bodies (also known as *δelta*-endotoxins) (Bulla *et al.*, 1977). The genes encoding ICPs normally occur on large plasmids in the bacterium

and direct the synthesis of a family or related proteins classified as *cry* 1-28 and *cty* 1-2 groups (Bulla *et al.*, 1977; Fast, 1981; Camilla, 2000). The insecticidal activity of the toxins from each *Bt* strain differ. Nevertheless, the set of *Bt delta*-endotoxins show host specificity to a wide variety of insects. This includes Coleoptera (Kreig *et al.,* 1983, 1987a) Lepidoptera and Diptera (Gould and Anderson, 1991).

The mode of action of *B. thuringiensis* is that a susceptible insect ingests the bacterium; the latter is dissolved in the midgut of the insect, liberating protoxins. The protoxin is activated within the insect's gut by a combination of an alkaline (pH 7.5 - 8.0) and the specific digestive proteases, which then convert the protoxins into an active toxin. This is proteolytically processed by midgut proteases to yield smaller toxin fragments, one of which binds to cells of the midgut epithelium (Hofte and Whiteley, 1989). The activated toxin interacts with the midgut epithelial cell, generating pores in the cell membrane and disturbing the osmotic balance. The cells, therefore, loose the ability to control permeability barrier of ions and protons. Influx of water accompanies entrance of ions and intestinal cells consequently causing the cells to swell and lyse (Gill *et al.*, 1992). This leads to spilling of their cytoplasm content into the lumen (Heimpel and Angus, 1959). The gut becomes paralysed and the insect stops feeding. The insect finally dies within a few hours of ingestion due to starvation or septicaemia (blood poisoning) (Hofte and Whiteley, 1989).

Bacillus thuringiensis has been reported to be safe to humans and other non-target organisms; it is very specific in its mode of action, it does not accumulate along food

chains hence it is environmentally safe, and is often suitable for integrated pest control programs (Luthy *et al.*, 1993). For these reasons, use of *B. thuringiensis* is seen as a possible option to synthetic pesticides.

Because of quarantine regulation, however, the use of local *Bt* isolates does not pose constrains as compared to exotic isolates; but there is a need to have effective *B. thuringiensis* isolates. It is also of paramount importance to understand their molecular basis in order to facilitate utilisation of the effective gene.

CHAPTER THREE

3. MATERIALS AND METHODS

3. 1 Collection of soil, grain and insect samples

Soil samples were collected from some maize growing areas in Kenya namely Embu, Murang'a, Nyeri and Kiambu districts. These districts were selected randomly within Central Province. Soil samples (20g each) were randomly collected from ten farms in each of the four regions. The soil samples were taken using a soil auger, from a depth of 0-5 cm. Each of the samples was kept in labelled plastic bags before being transferred to the laboratory for isolation of *B. thuringiensis*. A total of 120 soil samples were collected.

The sampling of grains was conducted on-station in the KARI Kiboko sub-centre and from farmers' granaries and posho mills in Kericho. Grain samples (50g) were randomly picked from the bottom of the storage sack using a grain poker. Each of the samples was passed through a set of three sieves (1-4mm) stacked over each other; the top sieve retained the large grains; the middle sieve retained the fine grain dust; while the lower sieve trapped the dead insects. Sampling of the grain was done in the middle sieve where 20g samples were scooped and put in a labelled plastic bag before taking them to the laboratory for isolation of *Bt*. A total of 150 grain samples were collected from Kiboko and Kericho sites.

Insect's samples were collected from the bottom sieve. In each case, 5g samples of dead insects were scooped and put in labelled sterile plastic vials before taking them to the

laboratory for isolation of *Bt*. A total of 50 insect samples were collected from Kiboko and Kericho sites.

3.2 Media preparation

The standard method described by Poinar and Thomas (1978) was used in media preparation.

3. 2. 1 Nutrient agar plates

The method used in media preparation (nutrient agar plates) was as described by Poinar and Thomas (1978) and by the manufacturer's instruction. 28g of commercial nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, England) comprised of '*Lab-Lemco*' powder (1g), yeast extract (2g), peptone (5g), sodium chloride (5g) and agar (15g) were weighed and put into a 2L conical flask. One litre of distilled water was added. The mixture was uniformly mixed for 5 minutes by shaking with a magnetic stirrer, then heated to dissolve completely for another 5 minutes. The mouth of the conical flask was firmly closed with cotton wool that was rolled into a ball and covered with aluminium foil. The media was autoclaved for 20 minutes at 121^{0}C 15 *lb* pressure, cooled to 45^{0}C and poured into Petridishes. The plates were stored in the refrigerator at - 4 ^{0}C, and then kept for use later during isolation of *B. thuringiensis*.

3. 2. 2 Nutrient broth

Nutrient broth (NB) (Medium A) was prepared according to the manufacturer's instructions. 13g grams of NB that composed of '*Lab-Lemco*' powder (1g), yeast extract

(2g), peptone (5g) and sodium chloride (5g) were weighed and put in a two litre conical flask. One litre of distilled water was added. The media was mixed thoroughly and dispensed into 250 ml conical flasks and was covered with cottonwool and aluminium foil. Autoclaving was done at 121^{0}C, 15 *lb* pressure for 20 minutes. The Nutrient broth was kept and used for preservation of *B. thuringiensis*.

Nutrient broth (NB) (Medium B) (600 ml) of NB comprised of 2g/l peptone, 15g/l dextrose, 2g/l yeast extract, 10g/l corn steep liquor, 0.3 g/l $MnSO_4.7H_2O$, 0.02 g/l $FeSO_4.7H_2O$, 0.02g/l $ZnSO_4.7H_2O$ and 1g/l $CaCO_3$.

3. 3 Sample preparation

Isolation of *B. thuringiensis* was based on the technique developed by Travers *et al.*, 1987 with slight modifications. One gram of soil or grain was suspended in 9 ml distilled water in 50 ml conical flasks. The flasks were placed in a shaker- incubator (200 rpm, 30 ^{0}C) for 60 minutes. The mixture was allowed to settle for 10 minutes. Aliquots of 1 ml of the supernatant from each flask were diluted with 9 ml of distilled water to attain a ten-fold dilution. Successive serial dilutions were prepared. The last two dilutions (10^{-3} and 10^{-4}) were heat shocked in a thermal bath at 80^0 C for 15 minutes in order to remove all the vegetative forms of both spore and non-spore forming bacteria.

Dead insects were first surface sterilized by washing in 0.5 % sodium hypochlorite for 2 minutes, then in sterile distilled water for 5 minutes and finally in 0.85 % sterile saline. The insects were crushed in 0.5 ml sterile saline using a homogenizer. The homogenate

was allowed to settle for 30 minutes and the supernatant solution removed. The solution was heat shocked in a thermal bath at 80^0 C for 15 minutes in order to remove all the vegetative forms of both spore and non-spore forming bacteria.

Approximately 100µl aliquot of the above heat-shocked samples was streaked using spread plate method on two nutrient agar plates per sample and incubated as outlined by Poinar and Thomas (1978). The inoculating loop was flamed along its entire length until it was red-hot, and the holder was passed quickly a few times through the flame. The loop was dipped into the inoculants and used to streak on the nutrient agar prepared above, and spread at several angles. The plates were incubated at 30^0 C for 48h and examined for colonies that showed *B. thuringiensis* morphology. These samples were further sub-cultured on NA plates and incubated at 30^0 C for 72h. The resulting colonies were examined for the presence or absence of spore or crystal in the cells under phase contrast microscope (Magnification X 1000) after Gram staining and Smirnoff staining (Smirnoff, 1962).

3. 4 Morphological characterization of *Bacillus thuringiensis*

3. 4. 1 Gram staining

Gram staining was carried out according to Poinar and Thomas, 1978) to ascertain the gram stain reaction of the culture after 24 and 48h. Young cells (18h old) cultures were used for the Gram staining experiment. A thin smear of bacterial culture was made on a clean glass slide by first air-drying and heat fixing for one second. The primary stain (crystal violet) was added and the slide was kept for 1-2 minutes before washing it with distilled water. The slide was covered with a mordant (iodine solution) for one minute

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and washed off with a decolouriser (alcohol) until no more colour came out of the smear. A counter stain (safranine red) was applied for one minute and washed off with distilled water. The slide was blot dried using blotting paper, air-dried and observed under a microscope (Magnification X 1000).

3. 4. 2 Smirnoff staining

This was done for the parasporal bodies as outlined by Smirnoff (1962). A few drops of solution A (that contained 1.5g Amido black dissolved in 50 parts of 90 % Methanol, 40 parts distilled water, and 40 parts acetic acid) was added to the heat fixed slides of single colony isolates and washed off after 70 seconds. Solution B (that contained 1g of basic fuschin dissolved in 10 ml of 95 % ethanol and 5g of phenol dissolved in 90ml of distilled water) was added to the slide and left for 20 seconds. This was washed in cold tap water and wiped with a filter paper. The crystals that stained dark-violet colour and spores that appeared pale-pink were observed in a standard light microscope (Magnification X 1000). Those that were positive for *B. thuringiensis* were preserved in nutrient agar slants and kept at -4 ^{0}C.

3. 5 Characterisation of *B. thuringiensis* by biochemical tests

3. 5. 1 Voges-Proskauer (VP) and Methyl Red (MR) test

Voges-Proskauer (VP) test and Methyl Red (MR) test were used to differentiate two major types of facultative anaerobic enteric bacteria that produce lower amount of acid or non- acidic neutral end products like acetyl methyl carbinol (AMC). Methyl red, Voges Proskauer (MRVP) medium (5g polypeptone, 5g glucose, and 5g sodium chloride) were

mixed with a litre of distilled water. The media was heated gently and the pH adjusted to 7.0. The media was dispatched into 5 ml screw cap tubes and sterilized at 121^{0}C for 20 min at 15 *lb* pressure. Tubes containing the media were inoculated with a single colony of the different isolates of *B. thuringiensis* and incubated at 30 ^{0}C for 48h.

For VP test, 1 ml of culture was mixed with 0.6 ml VP reagent A (40g potassium hydroxide and 100 ml distilled water) and 0.2 ml VP reagent B (6g alpha-napthol and 100 ml absolute ethanol). The tubes were placed open on a slant to increase contact with air. For MR test, methyl red indicator was added after incubation of different *B. thuringiensis* isolates to the broth that was used for VP test. In each of the above experiments, a Standard control reference isolate of *Bt tenebrio* was included.

3. 5. 2 Indole test

Tryptophan is an essential amino acid which when oxidized by some bacteria by the enzyme *tryptophanase* results in the formation of indole, ammonia and pyruvic acid.

Tryptophan ⟶ indole + ammonia + pyruvic acid.
Tryptophanase

The indole produced can be detected by adding Kovac's reagent (dimethyl aminobenzaldehyde) which produces a cherry red compound due to production of a compound Rosi-Indole.

Indole + (dimethyl aminobenzaldehyde) ⟶ cherry red compound (Rosi-Indole)

HCl + Butanol

Tryptone broths (TB) were prepared by dissolving 20g of peptone in a litre of distilled water before adding 5g of NaCl. The pH was adjusted to 7.0. Five (5) ml of TB was dispensed in 10 ml screw cap tubes and sterilized at 121^0C for 20 min, 15 *lb* pressure. After sterilisation, the TB medium was inoculated with the *Bt* culture. *Bt tenebrio* was included as a Standard control reference. The tubes were incubated overnight at 37^0 C for 24hrs. One millilitre of Kovac's reagents was added after incubation. The tubes were labelled and shaken gently until the two layers of the culture and reagent got separated.

3. 6 Toxicity of *Bacillus thuringiensis* isolates to adult *Prostephanus truncatus*

3. 6. 1 Insects

Adult *P. truncatus* were obtained from colonies maintained at KARI-Kiboko Sub-Centre, Kenya. About 100 adults were introduced into 1L-glass jars (9cm-wide mouth) and fed with 400g-shelled maize (Katumani Composite B) (Cowley *et al.*, 1980; Howard, 1983; Meikle *et al.*, 1998). All the culture jars were covered and sealed with a lid consisting of three parts; a piece of filter paper 9cm in diameter, a circle of 60 meshes/inch (0.3mm openings), brass screen (9cm in diameter) and a metal ring that screws down onto the mouth of the jar. The entire lid assembly was tightly secured to the jar. The room temperature was controlled at 27 ^{0}C ± 2 ^{0}C, 65 ± 5 % R. H. A photoperiod regime of L12:D12 was maintained. Each culture jar was labelled accordingly.

3. 6. 2 *Bacillus thuringiensis* isolates

All the *B. thuringiensis* isolates used for bioassays were locally isolated in Kenya from different sources (Table 1).

Table 1: *Bacillus thuringiensis* used in bioassays

Bt isolate	Host Species/Substrate	Locality	Year of isolation
41	Grain	Kiboko	2004
51	Grain	Kiboko	2004
61	Grain	Kericho	2004
45	Grain	Kiboko	2004
54	Grain	Kiboko	2004
60	Grain	Kericho	2004
50	Grain	Kiboko	2004
44	Grain	Kiboko	2004
46	Grain	Kiboko	2004
23	Grain	Kiboko	2004
47	Grain	Kiboko	2004
48	Grain	Kiboko	2004
58	Grain	Kiboko	2004
52	Grain	Kiboko	2004
49	Grain	Kiboko	2004
35	Grain	Kiboko	2004
36	Grain	Kiboko	2004
59	Grain	Kiboko	2004
34	Grain	Kiboko	2004
335	Soil	Kiambu-Githunguri	2003
295(3)	Soil	Murang'a-Kariti	2003
53	Grain	Kiboko	2004
33	Grain	Kiboko	2004
330	Soil	Kiambu-Githunguri	2003
323	Soil	Kiambu-Githunguri	2003
6(4)	Grain	Kiboko	2004
25	Grain	Kiboko	2004
29	Grain	Kiboko	2004
12i	Insects	Kiboko	2004
13	Grain	Kiboko	2004
57	Grain	Kiboko	2004
30	Grain	Kiboko	2004
21	Grain	Kiboko	2004
K1	Soil	Nyeri	2003
305	Soil	Murang'a-Kariti	2003
9i	Insects	Kiboko	2004
40	Grain	Kiboko	2004
295(1)	Soil	Murang'a-Kariti	2003
24	Grain	Kiboko	2004
6(1)	Grain	Kiboko	2004
37	Grain	Kiboko	2004
321	Soil	Nyeri	2003

43	Grain	Kiboko	2004
14	Grain	Kiboko	2004
K-2126	Soil	Icipe germplasm	1995
Icipe 58	Soil	Icipe germplasm	2005

A Standard control reference of *Bt tenebrio* kindly supplied from *Bt* laboratory (USA) was included. The *Bt* isolates preserved on NA slants were revived on NA plates as described by Poinar and Thomas (1978).

3. 6. 3 Isolation of the *δelta*-endotoxins crystals

The isolation of the δ*elta*-endotoxins crystals involved fermentation, harvesting of the *Bt* δ*elta* -endotoxins crystals and *Bt* precipitation.

The fermentation of the initial culture of *Bt* was done in two steps. In step one, 600 ml Nutrient broth (Medium A) described in section 3.2.2 was poured into six 250 ml Erlenmeyer flasks and autoclaved at 121^0C for 20 min at 15 *lb* pressure. A loopful of *Bt* colony grown on nutrient agar slant was inoculated into the flasks and incubated in a shaker incubator (200 rpm, 30° C) for 24h.

In step two, 600 ml Nutrient broth (Medium B) described in section 3.2.2 was autoclaved at 121^0C for 20 min at *15 lb* pressure and poured into six 250 ml Erlenmeyer flasks. Medium B was aseptically mixed with medium A and incubated for a further 48h. The viability of the bacteria was confirmed as described by Thomas and Stevenson (1984).

The *δelta-endotoxin* crystals were harvested from the 72h-old culture by low speed centrifugation (1300 rpm, 4 ^{0}C, 2 min) with a GSA rotor (DuPont, Newton, CT, USA) and Sorvall RC5C centrifuge (Beckham, USA) for 10min. The crystals that settled at the bottom of the tube as *Bt*-pellet were washed three times with distilled water. Each washing cycle included centrifugation at 1300 rpm at 4 ^{0}C. The purity of the crystals was ascertained by phase contrast microscopic examination after Smirnoff staining (Smirnoff, 1962).

The *Bt* crystal-pellets were precipitated in 200 ml of water. Ten gram of ά-lactose was added into the suspension. High purity acetone was also added to make a final volume of 1L. The acetone; the water and the *Bt* suspension were filtered through Whatman filter paper No 1 to collect precipitates containing lactose, *Bt* spores and crystals. The precipitate was used immediately to make pellets for bioassays or air-dried in a fume hood for 24h and stored in plastic vials at room temperature.

3. 6. 4 Viability tests

To determine the viability of bacteria, serial dilutions of samples were carried out up to dilution 10^{-4}. Suspensions (0.1 ml) of each dilution were spread using spread plate method and incubated at 37° C for 12h. The viable colonies were counted using a Quebec colony counter. *Bt tenebrio* was included as the reference standard. The number of bacteria per ml of sample was calculated by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to the nutrient broth. In each sample, four replicates were used.

3. 6. 5 Bioassays

Maize variety, Katumani Composite B, susceptible to *P. truncatus* and free from insecticides, was obtained from Kiboko and ground to obtain maize flour. *Bt* precipitate was mixed with flour at a ratio of 1:2 (50 ml: 20g) to make an artificial diet. When necessary, the latter was supplemented with nutrient broth to facilitate easier mixing of the pathogen. Pellets of approximately 2g were cut from the artificial diet using the top of a 1000 ml-Eppendorf tube (150 µl) and dried in an oven at 4^0 C for 30 minutes. For the controls, the pellets were prepared as above but were mixed with distilled water and with the Standard control reference *Bt tenebrio*. Ten pellets each weighing 20g were placed in small containers. Ten 14-day-old insects were placed in each small container. Each treatment was replicated six times. The experiment was maintained at room conditions (27 ^{0}C and 65 ± 5 % R.H). Mortality was recorded after every three days for 30 days.

To ensure that the insects died from *Bt* infection, the dead cadavers were surface sterilized and crushed in a Petri dish. The homogenate was inoculated on NA and incubated for 24h at 30^0 C. The mortality of the insects from *Bt* infection was confirmed by growing the insects on Nutrient Agar.

3. 7 Dose-mortality relationship experiments

Based on the results on time-mortality relationships, the two isolates of *B. thuringiensis* (*Bt* 41 and *Bt* 51) that were more toxic as compared to the Standard control reference (*Bt tenebrio*) were subjected to dose-mortality response experiment. The *Bt* crystal protein samples were initially activated with 10 % Trypsin. The concentration of the activated *Bt*

crystal proteins was determined using the Bovine Serum Albumin (BSA) kit according to manufacturer's instructions (Pierce, Rockford, IL, USA) (Appendix 1). This was done by checking the absorbance of the *Bt* isolates in a spectrophotometer at a wavelength of 562 ηm for dilutions ranging from 0.1-2.0 mg/ml. *Bt tenebrio* was included as a Standard control reference. The absorbance values of *Bt* isolates 51 and 41 obtained were interpreted alongside a BSA standard curve. Based on this curve, the protein concentration was calculated.

Serial dilutions were prepared to attain five *Bt* concentrations (25, 12.5, 6.25, 3.125 and 1.5625 µg/ml protein) that were used in the dose mortality experiment. *Bt* pellets used for bioassays were prepared using the different concentrations in the same way as described in Section 3.6.5.

3. 8 Statistical analysis

The insect mortality was corrected for natural mortality using Abbott's formula (Abbott, 1925). Percentage mortality data were arcsine-transformed before analysis of variance (ANOVA) and means were separated by Student Newman's Keul's test

(SNK) test (P=0.05). Regression analyses were used to estimate the lethal time to 50 % mortality (LT_{50}) and the lethal concentration causing 50 % mortality (LC_{50}), using the LIFEREG procedure of Statistical Analysis Software (SAS). All analysis was carried out with the SAS (1999) computer package.

3.9 Molecular characterisation of *δelta*-endotoxins *δelta*-endotoxins of the most active *Bacillus thuringiensis* isolates against the *Prostephanus truncatus*

3.9.1 Solubilisation of *Bt δelta* -endotoxin crystals.

The *Bt δelta* endotoxin crystals were harvested as described in Section 3.6.3. The purified *δelta*-endotoxin crystals were suspended in 50mM Na_2 $C0_3$.$NaHC0_3$ (pH 9.5) solubilisation buffer. The buffer contained 10mM dithriolthreitol (DTT) and was incubated at 37^0 C for 30h. The suspension was centrifuged (10,000 rpm, 10min at 4^0 C) using an Eppendorf 5415C centrifuge (Eppendorf-Netheler-Hinz, Hamburg, Germany). The supernatant fraction was collected in a sterile 50 ml-Falcon tube and its volume was marked. The pellet and the supernatant were stored at -20 ^{0}C in a refrigerator.

The protein content of the supernatant was determined after solubilisation using the Bovine Serum Albumin (BSA, fraction V) as the protein standard according to the manufacturer's instructions (Pierce, Rockford, IL, USA) as described in Appendix 1.

3.9.2 Enzymatic activation of the solubilised *Bt δelta*-endotoxin crystals

The solubilised supernatant fraction was dialysed against 50mM Na_2 $C0_3$. $NaHC0_3$ (pH 9.5) solubilisation buffer (DTT-free) using a dialysing membrane and incubated in a water bath at 37^0 C for 30 minutes with 10 % Commercial Bovine Trypsin (50:1 v/v, w/w). The protein content was determined as described in Appendix 1. The activated protein was equilibrated in a dialysis membrane overnight using Phosphate Saline Buffer (PSB) at pH 7.0. The dialysis was done in polyethylene glycol (PEG) until all the water

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molecules on the protein were extracted. The protein that concentrated inside the dialysis tube was flushed out using the PSB until the membrane was clean and stored at -20 ^{0}C in 1 ml Eppendorf tube. The volume of PSB that was used to clean out the protein was recorded to take care of the dilution factor. The concentration of the dialysed protein was determined using the BSA protein determination method according to manufacturer's instructions (Pierce, Rockford, IL, USA).

3. 9. 3 Gel electrophoresis

Electrophoresis on denaturing gels was carried out by the method of Laemmli (1970). Gradients (4-20 %) were cast using a gradient maker (BRL, Gaithersburg, MD, USA). The high and low molecular weight standards that were used were peqGOLD pre-stained protein marker 111 (Peq-Gold Biotechnologie GmbH, Germany) (Appendix 3). Gels were stained for protein with Coomasie Brilliant Blue (R250). Molecular weights were determined from plots of $\log_{10}$ of molecular weight versus the relative migration of the protein standards.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4. 1 Isolation of *Bacillus thuringiensis*

The search for *Bt* from soils, grains and insects samples around Kenya yielded 68 *Bt* isolates out of the 320 samples screened. These showed that *Bt* is a widespread bacterium in Kenya and occurs naturally in different geographical regions. These results agree with Bernard *et al.*, 1997 who also found that *Bt* is a widespread bacterium and has a worldwide distribution. Out of these *Bt* isolates, 85.3 % were from grains, 11.8 % from soil and 2.9 % from insects (Table 2). The results showed that the origin of the sample was an important factor as most of the *Bt* isolates (85.3 %) were isolated from grains. Similar results were reported by other workers (Delucca *et al.*, 1982; Bernard *et al.*, 1997). It can therefore be concluded that grains are a better source of *B. thuringiensis*.

Table 2: Percentage number of *Bt* isolates obtained from different sources.

Sample type	**No. of samples screened**	**No. of *Bt* isolates**	**Percentage No. of *Bt* isolates**
Soil	120	8	11.8%
Grain	150	58	85.3%
Insects	50	2	2.9%
Total	**320**	**68**	**100.0%**

4. 2 Characteristic shapes of *Bacillus thuringiensis* isolated from different sources and localities

The screening for *Bt* isolates in the laboratory revealed that different *Bt* isolates from different samples were either bipyramidal, irregular or square shaped. Ninety five (95 %) of the *Bt* isolates from grain samples were bi-pyramidal shaped and included K10, K11, K13, K15 - K51 and Ke52 -Ke58. These results agreed with (Delucca *et al.*, 1982; Ohba and Aizawai, 1986; Martin and Travers, 1989; Wang'ondu, 2001 and Thumbi, 2002) who also found that majority of their *Bt* isolates from soils and stored grain and grain dust were bipyramidal in shape. The other *Bt* isolates (5 %) from grains were square shaped and included K9, K12 and K14 (Table 3).

Analysis of soil samples showed that *Bt* K295 (3) (12.5%) was square shaped and *Bt* isolates (K1, K268, K295 (1), K305, K326, K330 and K335) (87.5 %) were bipyramidal shaped. The *Bt* isolates from insect samples were all irregular shaped (100%) and included *Bt* 9i and *Bt* 12i (Table 3).

Bacillus thuringiensis crystals with square and irregular shape have been generally reported to be toxic to coleopteran insects (Krieg *et al.*, 1983; Hernsdat *et al.*, 1986). However, the results showed that the majority of the *Bt* isolates toxic to *P. truncatus* were bi-pyramidal shaped. According to Hernsdat *et al.*, 1986, bi-pyramidal shaped *Bt* isolates are mainly toxic to lepidopteran and dipterans. However, Tailor *et al.*, 1992 showed that although Cry 1 bipyramidal shaped proteins were active on lepidopteran generally; Cry 1Ba and Cry 11a bipyramidal shaped proteins showed some activity on coleopterans,

though their toxicity on Colorado potato beetle was much lower than that of Cry 111Aa square shaped proteins. This strengthens the need for further screening of *Bt* isolates toxic to *P. truncatus*.

Table 3: Characteristic shapes of *Bacillus thuringiensis* isolated from different sources and localities

No	Isolate code	Source	Shape	Locality
1	K1	Soil	Bipyramidal	Nyeri
2	K268	Soil	Bipyramidal	Murang'a
3	K295 (1)	Soil	Bipyramidal	Embu
4	K295(3)	Soil	Square	Embu
5	K305	Soil	Bipyramidal	Embu
6	K326	Soil	Bipyramidal	Kiambu
7	K330	Soil	Bipyramidal	Kiambu
8	K335	Soil	Bipyramidal	Kiambu
9	K9	Grain	Square	Kiboko
10	K10	Grain	Bipyramidal	Kiboko
11	K11	Grain	Bipyramidal	Kiboko
12	K12	Grain	Square	Kiboko
13	K13	Grain	Bipyramidal	Kiboko
14	K14	Grain	Square	Kiboko
15	K15 to K51	Grain	Bipyramidal	Kiboko
16	Ke52 to Ke58	Grain	Bipyramidal	Kericho
17	*Bt* 9i	Insects	Irregular	Kiboko
18	*Bt* 12i	Insects	Irregular	Kiboko

4. 3 Colony morphology of - *Bacillus thuringiensis* isolates

The growth of the *Bt* isolates on nutrient agar plates was entire with irregular raised edges. The colony colour was cream and the surface was either smooth or dull (Plate 1).

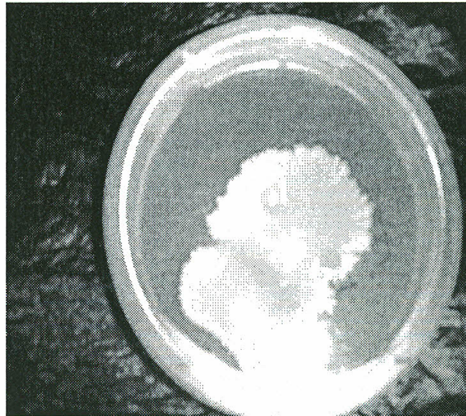

Plate 1: *Bacillus thuringiensis* colonies on Nutrient agar with entire and irregular raised edges.

4. 4 Staining

4. 4. 1 Gram staining

The Gram positive bacteria appeared dark violet whereas the Gram negative bacteria appeared pink. The isolates submitted to staining had a positive dark violet colour, 24h after staining hence were Gram positive. At 48h, the cells had fully sporulated, hence the spores did not fix the Gram stain and appeared colourless from the background of the cell. The crystals appeared dark violet.

4. 4. 2 Smirnoff staining

The presence of parasporal bodies (crystals) in *B. thuringiensis* isolates were confirmed by staining with Smirnoff stain. At 72h, the bacterial cells had fully lysed and the crystals were clearly visualized. All the *Bt* crystals stained dark violet while the spores stained pink. The bacterial cells and their fragments assumed a light lilac tint colour. The shapes of the spores of all bipyramidal crystals were mainly ellipsoidal and terminally positioned.

4. 5 Biochemical characteristics of the *Bacillus thuringiensis* isolates

The biochemical characterisation of the *Bt* isolates was done using Voges Proskauer (VP), Methyl Red (MR) and Indole tests. The presence of acetyl methyl carbinol (AMC) produced from pyruvic acid in the course of butylenes-glycolic fermentation was indicated by a positive AMC test (Red colour) whilst the absence of AMC was indicated by a yellow colour. For VP test, *B. thuringiensis* isolates that reacted to produce AMC changed the colour to pink within 10-30 minutes, an indication that the test was positive. Those that did not change colour were scored as negative.

For MR test, those isolates that changed colour to red due to production of AMC were considered MR positive, whilst those that had no colour change were considered MR negative. The presence of an indole ring was scored as indole positive and the absence as indole negative.

Among the *Bt* isolates, 82 % were positive and 18 % were negative for VP test. MR tests revealed that 24 % of the *Bt* isolates were negative and 76 % were positive, whilst indole tests showed that 72 % of the *Bt* isolates were negative and 28 % were positive. The biochemical characteristics of isolated pathogens confirmed that these isolates were *Bt*. This concurred with findings of Sergio *et al.* (1992), Kwan-Hee *et al.* (1996) and Hossain *et al.*(1997).

4. 6 Evaluation of the toxicity of isolates of *Bacillus thuringiensis* on *Prostephanus truncatus*

Mortality in the controls was 0 % and 3.3 %, 7 and 30 days after treatment, respectively. Isolates of *Bt* were toxic to adult *P. truncatus* but mortality varied from 1.7 to 53 % and 3.3 to 85 %, 7 and 30 days after treatment between the isolates, respectively (Table 4.). Seven *B. thuringiensis* isolates (*Bt* 41, *Bt* 61, *Bt* 51, *Bt* 60, *Bt* 46, *Bt* 45 and *Bt* 44) were more toxic to adult *P. truncatus* than other isolates, causing mortalities of 50 and 80 %, 7 and 30 days after treatment. However, there was no significant difference between these isolates ($P \geq 0.05$) (Table 4). The standard reference isolate, *Bt tenebrio* was also toxic, causing mortality of 37 % and 60 %, 7 and 30 days after treatment, and was significantly different ($P \leq 0.05$) from the most toxic isolates listed above (Table 4) (Appendix 4).

The seven most toxic *Bt* isolates took half the time required to cause pathogenesis as compared to *Bt tenebrio*. The reason is probably because *Bt tenebrio* is effective on coleopteran larva of *Leptinortasa decemlineata* (Colorado potato beetle-CPB) (Krieg *et al.*, 1987a) and not the CPB adult. However, *Bt* strain HD 695 from ICIPE germplasm

was among the least toxic isolates causing mortality of 1.3 and 3.3 %, 7 and 30 days after treatment (Table 4). The intraspecific variations in pathogenic activity of *B. thuringiensis* isolates against *P. truncatus* observed in this study is similar to those reported on coleopterans by other workers (Krieg *et al.*, 1983; Hernsdat *et al.*, 1986; Donovan *et al.*, 1988). These differences in pathogenic activity strengthens the need for further screening to select the best isolate for *P. truncatus* adult since it is the stage that causes the greatest damage.

There was a significant difference ($P \leq 0.05$) in mortality rates among (*Bt* isolates 59, 23, 50, 335, 52, 35, 34, 48, 33, 9i 49, 43, 36 and 14) while no significant difference ($P \geq 0.05$) occurred between each of the groups of (*Bt* (295 (3) and *Bt* 58); (6(4), and 54) (*Bt* 47, 29, 12i and 53); (*Bt* 13, 57 and 40); (*Bt* 21, 30, 24, 25 and 47); (*Bt* 295 (1), 55, 326 and 305); (*Bt* 6(1), 37, 56, 330, 16, Icipe 58 and K(1)) and (*Bt* K268, K21-26 and HD695) respectively (Table 4).

The lethal time 50 % mortality (LT_{50}) values were calculated for isolates that achieved more than 50 % mortality after 30 days following treatment. The LT_{50} ranged from 7 to 37 days and varied significantly ($P \leq 0.05$) among isolates (Table 5). *Bacillus thuringiensis* isolates 41, 51, 61, 60, 45, 44, 46 and 23 had the shortest LT_{50} values of between 8 and 11 days (Table 5). The standard reference strain, *Bt tenebrio,* had LT_{50} of 16 days, which was significantly different ($P \leq 0.05$) from the most toxic isolates.

Table 4: Mortality of adult *P. truncatus* following exposure to artificial pellets treated with different isolates of *B. thuringiensis*

		Percentage mortality± SE	
	***Bt* isolate**	**7 DAT**	**30 DAT**
1	41	53.3 ± 2.6 a	85.0 ± 2.2 a
2	61	51.7 ± 3.1 a	80.0 ±.0.0 ab
3	51	51.7 ± 3.1 a	80.0 ±.5.0 a
5	60	51.7 ± 1.0 a	78.3 ± 7.0 ab
6	46	50.0 ± 7.0 a	80.0 ±.0.0 abc
7	45	50.0 ± 7.3 a	83.3 ± 3.0 ab
8	44	48.3 ± 3.1 a	83.3 ± 1.0 abc
9	59	46.7 ± 2.1 ab	55.3 ± 2.4 bcde
10	23	45.0 ± 2.2 abc	78.3 ± 7.0 abc
11	50	43.3 ± 4.9 abcd	78.3 ± 7.0 ab
12	335	41.7 ± 1.7 abcde	41.7 ±.7.0 defg
13	295(3)	35.0 ± 3.4 abcdef	38.3 ±.1.0 defgh
14	58	38.3 ± 3.1 abcdef	70.0 ± 0.0 abc
15	52	28.3 ± 1.7 bcdefg	60.6 ± 2.0 abcd
16	35	26.7 ± 2.1 cdefg	60.5 ± 2.0 abcd
18	34	25.0 ± 2.2 defg	50.3 ± 7.0 cdef
19	48	25.0 ± 3.4 efg	70.1 ± 3.0 abc
20	33	23.3 ± 3.3 efgh	28.3 ± 1.0 fghij
21	6(4)	21.7 ± 1.7 fghi	23.3 ± 1.0 ghijk
22	54	20.0 ± 0.0 fghi	22.3 ± 2.1 ghijk
23	47	18.3 ± 1.7 ghij	73.3 ± 2.1 abc
24	29	18.3 ± 1.7 ghij	....
25	12i	18.3 ± 3.1 ghij	20.0 ± 3.7 ghijk
26	53	18.3 ± 4.0 ghij	31.7 ± 1.7 efghi
27	13	16.7 ± 2.1 ghijk	18.3 ± 1.7 ghijkl
28	57	15.0 ± 2.2 ghijk	....
29	40	15.0 ± 2.2 ghijk	....
31	21	15.0 ± 3.4 ghijkl	16.7 ± 3.3 ghijkl
32	30	10.0 ± 2.6 ghijkl	16.7 ± 2.1 ghijkl
33	24	13.3 ± 3.3 ghijkl	16.7 ± 4.9 ijklm
34	25	10.0 ± 0.0 ghijkl	10.0 ± 0.0 ijklm
35	47	10.0 ±.0 0 ghijkl	....
36	295(1)	10.0 ± 0.0 ghijklm	10.0 ± 0.0 ijklm
37	55	10.0 ± 0.0 ghijklm	....
39	326	10.0 ± 0.0 ghijklm	25.0 ± 4.3 ghij
40	305	10.0 ± 0.0 ghijklm	15.0 ± 2.2 hijklm
41	9i	8.3 ± 1.7 hijklmn	11.7 ±1.7 ijklm
42	49	8.3 ± 1.7 hijklmn	60.0 ± 2.6 abcd
43	43	6.7 ± 3.3 ijklmn	....
44	36	6.7 ± 2.1 jklmno	58.3 ± 4.8 abcd
45	6(1)	6.7 ± 3.3 klmno	10.0 ± 2.6 jklm
46	37	6.7 ± 3.3 klmno	11.7 ± 1.7 jklm

47	56	6.7 ± 2.1 lmno	……………
49	330	6.7 ± 2.1 lmno	28.3 ± 6.0 fghij
50	16	6.7 ± 2.1 lmno	………….
51	Icipe 58	3.3 ± 2.1 mno	………….
52	K(1)	3.3 ± 2.1 mno	16.7 ± 3.3 ghijkl
53	14	3.3 ± 2.1 m-o	6.7 ± 4.9 lm
54	K268	3.3 ± 2.0 n-o	………….
55	K21-26	3.3 ± 2.0 n-o	6.8 ± 2.0 m
56	HD695	1.7 ± 1.7 n-o	3.3 ± 2.0 m
57	*Bt tenebrio*	36.7 ± 3.3 a-f	60.0 ± 3.7 abcd
58	Control	0.0 ± 0.0 o	3.3 ± 2.1 m

Means within the same column followed by the same letter(s) are not significantly different by Student Newman-Kuels test at ($P \geq 0.05$). Means were angularly transformed before analysis but values represent un-transformed data. A dash in the 30 DAT column means that 50% mortality was not observed. This was because the *P. truncatus* escaped out of the experimental pots by boring through the small plastic containers.

Table 5: LT_{50} values of adult *P. truncatus,* 30 days after treatment following exposure to artificial pellets treated with different isolates of *B. thuringiensis*.

	***Bt* isolate**	**LT_{50} (days) (95% ficudial limit is)**
1	41	7.6 (7.3-7.9) g
2	51	7.4 (7.0-7.7) g
3	61	8.9 (8.5-9.3) g
4	60	8.2 (7.8-8.5) g
5	45	8.0 (7.7-8.3) g
6	44	9.2 (8.8-9.6) g
7	46	8.5 (8.2-8.8) g
8	23	8.7 (8.3-9.1) g
9	50	10.7 (10.3-11.1) fg
10	58	13.4 (12.8-14.1) ef
11	48	13.5 (12.0-15.2) e
12	52	23.7 (22.1-25.6) d
13	47	27.5 (26.2-29.2) cd
14	35	26.1 (24.2-28.3) cd
15	49	27.7 (26.5-29.1) bc
16	36	31.4 (29.9-32.9) b
17	34	37.3 (33.1-42.2) a
18	*Bt tenebrio*	15.8 (14.7-17.2) e

Means within the same column followed by the same letter(s) are not significantly different by Student Newman-Kuels test at ($P \geq 0.05$). Means were angularly transformed before analysis but values represent un-transformed data

4. 7 SDS - PAGE of the two isolates of *Bacillus thuringiensis* (*Bt* 41 and *Bt* 51) most toxic to *Prostephanus. truncatus*

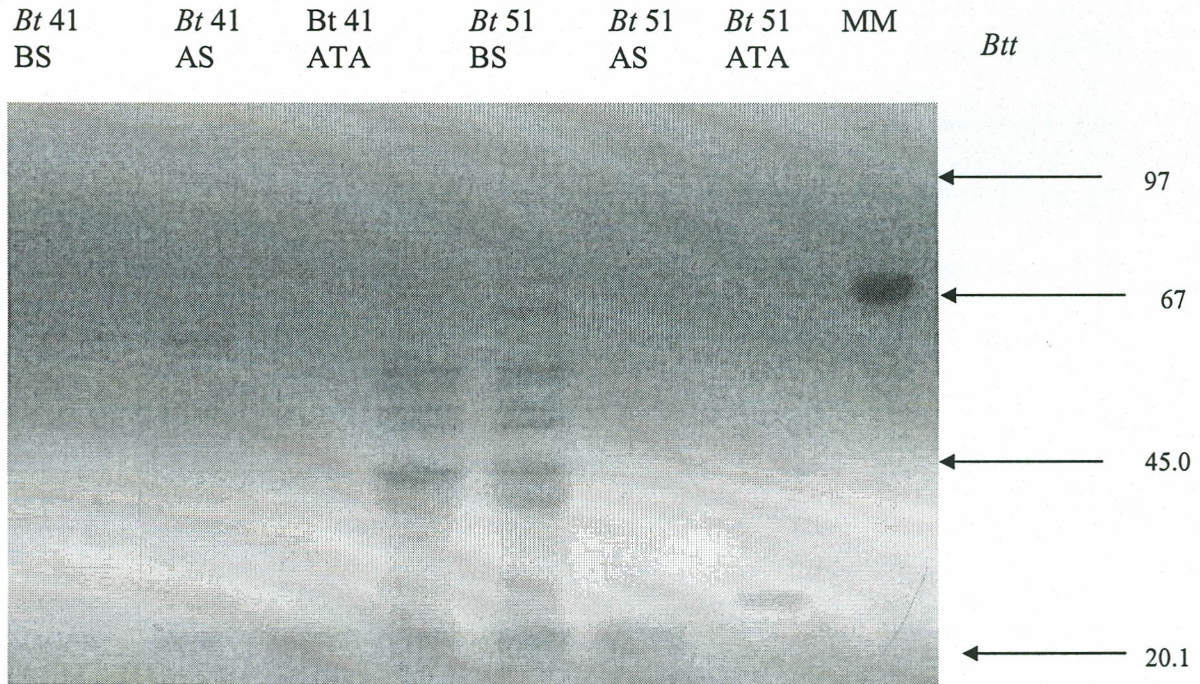

Plate 2: SDS-PAGE gel of *Bt* 41 and *Bt* 51 alongside a Standard *(Btt)*/Molecular Marker
Key: - BS-Before solubilisation, AS-After solubilisation, ATA-After Trypsin activation
MM-Molecular marker, *Btt- Bacillus thuringiensis tenebrio*

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The results of the two most toxic isolates on a SDS-PAGE gel revealed that there were differences in the molecular weights of the *Bt* isolates 41 and 51. Before solubilisation of the *∂elta*-endotoxin crystals, *Bt* 41 had two major bands of 67 and 65 KDa while *Bt* 51 had four major bands of 85, 75, 73 and 67 KDa. After solubilisation there were no bands in both *Bt* 41 and *Bt* 51. After trypsin activation, *Bt* 41 had two major bands of 73 and 67 KDa and minor bands of 54, 46, 44, 35, 20, 17 and 15 KDa while *Bt* 51 had two major bands of 97 and 68 KDa and two minor bands of 44 and 17 KDa. The *Bt tenebrio* (*Btt*) had only one major band of 67 KDa. According to Krieg *et al.*, 1983 the weight of the crystal proteins of most toxic *Bt* isolates to coleopterans is in the range of 67 to74 KDa, suggesting that they may contain *cry* 111 related genes. This strengthens the need for a further DNA study to confirm the presence of the available genes from *Bt* 41 and 51 isolates.

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATION

5. 1 Conclusions

This study demonstrated that Kenya has a rich diversity of *B. thuringiensis* isolates. It has also shown the existence of effective indigenous *Bt* that could be developed as biological control agents of insect pests of agricultural importance including coleopterans, and could also be used as a source of *cry* genes for use in genetic transformation programmes. Among the different sources collected, grain samples were found to be the best sources of *Bt* than soils or insects. It was also demonstrated that it is possible to find *Bt* isolates that are toxic to against adult coleopterans.

5. 2 Recommendations

The development of resistance to chemical pesticides and concerns over the deleterious effect of inorganic pesticides on the environment and human safety makes the utilization of biopesticides more appealing. This study has demonstrated the potential of discovering isolates of *B. thuringiensis* that are toxic to adult *P. truncatus* and could be, therefore, developed as biopesticides. Although no isolate caused 100 % mortality in this study, there is likelihood to discover more toxic isolates through further screening. There is a need, therefore, to undertake further screening of *Bt* isolates. Preservation of these isolates in a germplasm bank for pathogen biodiversity should be considered since these pathogens could be utilized as sources of new *cry* genes for genetic transformation. Further investigations to determine the performance of *Bt* under various regimes of temperature and humidity also require further attention.

Further screening of *B. thuringiensis* isolates against other group of insects like lepidopteran and dipterans is also necessary and could result in the development of local biopesticide industries.

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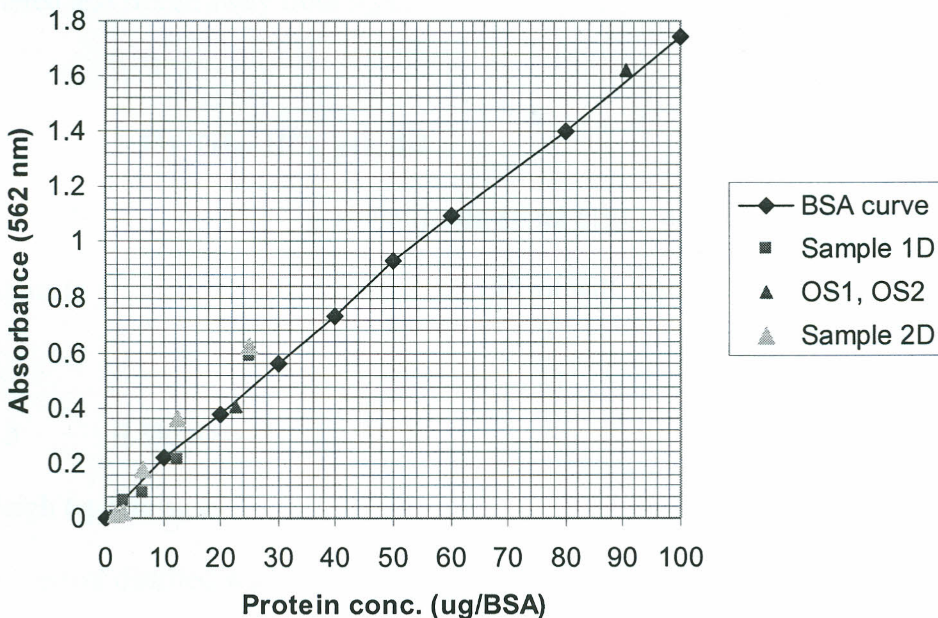
APPENDICES

APPENDIX 1: PROTEIN DETERMINATION BY (BCA/BSA) PROTEIN ASSAY METHOD

One part of BCA Reagent B was added to 50 parts of BCA Reagent A and mixed thoroughly. Two ml of the mixture was put in test-tubes. A 100µl of the Standard control reference (*Bt tenebrio*), a blank and *Bt* samples were each added separately to the mixture and vortexed for 2 sec. The tubes were incubated at 37^0 C for 30 minutes and allowed to cool at room temperature for 5 minutes. The results were interpreted using the BSA curve shown below.



APPENDIX 2: SDS-GEL ELECTROPHORESIS STOCK SOLUTIONS

2. 1 Acrylamide/bis (30% T, 2.67 C)

Weigh 87.6g acrylamide (29.2g/100 ml)

2.4g N' N'- bis-methylene-acrylamide (0.8g/100 ml)

Make to 300 ml with distilled water. Filter and store at 4 0C in the dark (30 days maximum).

Or

Use **Bio-rads Stock acrylamide (used as monomer, comprising 30% acrylamide stock solution)**

30g acrylamide was dissolved in about 50 ml of distilled water was dissolved by stirring while covered. After dissolving, the volume was made up to 100 ml and the solution was filtered and stored away from light.

2. 2 1.5M Tris- HCl, pH, 8.8

27.23g Trizma base (18.15g/100 ml), add 80 ml distilled water

Adjust to pH 8.8 with 1N HCl. Make to 150 ml with distilled water and store at 40 ^{0}C.

2. 3 0.5M Tris- HCl, pH 6.8

Weigh 6g Tris base

Add 60 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 40 ^{0}C.

2. 4 10% SDS, buffers

10g SDS was dissolved in distilled water to a final volume of 100ml and then stored in light tight container at room temperature.

2. 5 10% Ammonium persulfate (APS)

0.1g/ml of APS was dissolved in 1 ml of distilled water. This was made fresh for each use and kept at 4 0C and was covered with a Para film in a refrigerator.

(Use glass distilled water for everything. Keep TEMED in a fridge and seal with parafilm).

2. 6 GLASS PLATES

Wash plates with detergent and scrub brush. Rinse with water and 70% methanol. Air dry and use gloves while washing.

2. 7 separating gel (20 %), (4 %)

Recipe % gel	4 %	20 %
Acry/Bis (ml)	2.4	12.0
Buffer(1.5M Tris pH 8.8)ml	4.6	4.6
Distilled water (ml)	10.8	1.2
Mix and degas		
10% SDS (μl)	180	180
TEMED (μl)	10	10
APS (μl)	130	90

2. 8 Stacking gel

Reagents	To make 3.13 %.
30 acrylamide mix (ml)	1.02
Buffer(1MTris pH 6.8) (ml)	2.5
10% SDS (ml)	0.1
10% APS (ml)	0.05
TEMED (ml)	0.01
Distilled water (ml)	6.1

2. 9 Sample buffer (SDS reducing buffer) (stored at room temperature)

Distilled water	4ml
0.5M Tris- HCl, pH 6.8	1 ml (0.08g)
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml (0.16g)
2-b-mercapoethanol	0.4 ml
0.05% (w/v) Bromophenol blue	0.2 ml
Total	**8 ml**

Dilute the sample at least 1:4 with sample buffer, and heat at 95 ^{0}C for 4 minutes

2. 10 Preparation of samples for loading

Dilute 1:1 with sample buffer. The same was done on the low/high molecular weight marker. The samples were boiled in a water bath for 5 minutes before loading into the

sample wells using a suitable a micro-syringe (Hanington, USA, 50µl). All the wells were well labelled.

2. 11 Electrophoresis buffer (4L of 4X)

For use; 375ml + 15ml SDS (10%), make up to 1500ml total

Trizma	48g
Glycine	230.4g

2. 12 5X electrode (Running buffer), pH 8.3 (enough for 10 runs)

Tris base	9g	(15g.l)
Glycine	43.2g	(72g/l)

Store at 4^0 C.

Warm at 37^0 C before use if precipitation occurs.

Dilute 60ml 5X stock with 240ml distilled water for one electrophoresis run.

(*Use a small amount of Bromophenol blue as tracking dye (0.1% in water). Run at 20mA, should not go above 200V. (Regular length gels take 3-3.5 h*))

2. 13 Stain

Coomasie Brilliant Blue; Made by dissolving 0.025g of Coomasie Brilliant blue stain in 25ml methanol, 10ml acetic acid and 65ml of distilled water with several changes of the destaining solution.

2. 14 Destain

Done in excess of 50ml Methanol, 10ml acetic acid and 40ml of distilled water with several changes of the destaining solution until a clear background is obtained.

APPENDIX 3: PEQGOLD PRE-STAINED PROTEIN MARKER 111

Protein	Molecular weight
Myosin	250
Phosphorylase	148
BSA	98
Glutamic dehydrogenase	64
Alcohol Dehydrogenase	50
Carbonic anhydrase	36
Myoglobin red	22
Lysozyme	16
Aprotinin	6
Insulin Chain	4

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APPENDIX 4: ANOVA PROCEDURE

4. 1 The ANOVA Procedure 7 Days after Treatment

Class Level Information

Class	Levels	Values
trt	53	A B C D E F G H I J K L M N O P Q R S T U V W X Y Z a b c d e f g h i j k l m n o p q r s t ten u v w x y z
reps	6	1 2 3 4 5 6

Number of observations 318

Dependent Variable: t_mort

Source	DF	.Sum of Squares	Mean Square	F Value	Pr > F
Model	57	60197.68840	1056.09980	25.07	<.0001
Error	260	10953.18847	42.12765		
Corrected Total	317	71150.87687			

R-Square	Coeff Var	Root MSE	t_mort Mean
0.846057	26.70470	6.490581	24.30501

Source	DF	Anova SS	Mean Square	F Value	Pr > F
trt	52	60113.37750	1156.02649	27.44	<.0001
reps	5	84.31090	16.86218	0.40	0.8484

Student-Newman-Keuls Test for t_mort

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis
but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	260
Error Mean Square	42.12765

Student-Newman-Keuls Test for t_mort
Means with the same letter are not significantly different.

SNK Grouping	Mean	N	trt
A	48.846	6	f
A			
A	47.926	6	i
A			
A	46.923	6	k
A			
A	45.961	6	n
A			
A	45.961	6	o
A			
A	45.000	6	h
A			
A	44.038	6	g
A			
B A	43.077	6	m
B A			
B A C	42.116	6	e
B A C			
B D A C	41.070	6	j
B D A C			
E B D A C	40.193	6	L
E B D A C			
E B D A C F	38.186	6	C
E B D A C F			
E B D A C F	38.186	6	l
E B D A C F			
E B D A C F	37.183	6	ten
E B D C F			
E B D G C F	32.103	6	y
E D G C F			
E D G C F	30.996	6	s
E D G F			
E D G F	29.888	6	p
E D G F			
E D G F	29.641	6	w
E G F			
E H G F	28.676	6	a
H G F			
H G I F	26.565	6	B
H G I F			
H G I F	26.565	6	u

```
    H     G     I
    H  J  G     I        25.210   6  v
    H  J  G     I
    H  J  G     I        25.210   6  X
    H  J  G     I
    H  J  G     I        24.963   6  G
    H  J  G     I
    H  J  G     I        24.715   6  q
    H  J  G     I
K  H  J  G     I        23.855   6  V
K  H  J  G     I
K  H  J  G     I        23.608   6  b
K  H  J  G     I
K  H  J  G     I        22.500   6  Z
K  H  J  G     I
K  H  J  G     I  L     22.253   6  U
K  H  J  G     I  L
K  H  J  G     I  L     21.145   6  Y
K  H  J  G     I  L
K  H  J  G     I  L     20.898   6  d
K  H  J  G     I  L
K  H  J  G     I  L     19.790   6  W
K  H  J  G     I  L
K  H  J  G  M  I  L     18.435   6  c
K  H  J  G  M  I  L
K  H  J  G  M  I  L     18.435   6  D
K  H  J  G  M  I  L
K  H  J  G  M  I  L     18.435   6  x
K  H  J  G  M  I  L
K  H  J  G  M  I  L     18.435   6  P
K  H  J  G  M  I  L
K  H  J  G  M  I  L     18.435   6  K
K  H  J     M  I  L
K  H  J  N  M  I  L     15.362   6  F
K  H  J  N  M  I  L
K  H  J  N  M  I  L     15.362   6  r
K     J  N  M  I  L
K     J  N  M  I  L     13.645   6  J
K     J  N  M     L
K  O  J  N  M     L     12.290   6  t
K  O     N  M     L
K  O     N  M     L     10.572   6  A
K  O     N  M     L
K  O     N  M     L     10.572   6  O
K  O     N  M     L
K  O     N  M     L     10.572   6  T
```

```
O     N  M    L
O     N  M    L     9.217    6   R
O     N  M
O     N  M         6.145    6   M
O     N  M
O     N  M         6.145    6   I
O     N  M
O     N  M         6.145    6   Q
O     N  M
O     N  M         6.145    6   H
O     N
O     N           4.427    6   S
O     N
O     N           3.072    6   z
O     N
O     N           3.072    6   N
O
O                0.000    6   E
```

4. 2 The ANOVA Procedure 30 Days after Treatment

Class Level Information

Class Levels Values

trt 48 12I 13 14 21 23 24 25 29 295(1) 295(3) 30 305 321 326 33 330 335 34 35 36 37
40 41 43 44 45 46 47 48 49 50 51 52 53 54 57 58 59 6(1) 6(4) 60 61 9I C HD695
K1 K2126 ten

Reps 6 1 2 3 4 5 6

Number of observations 318

Dependent Variable: t_mort

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	52	112193.3731	2157.5649	28.14	<.0001
Error	265	20320.5146	76.6812		
Corrected Total	317	132513.8877			

R-Square	Coeff Var	Root MSE	t_mort Mean
0.846654	24.37689	8.756779	35.92247

Source	DF	Anova SS	Mean Square	F Value	Pr > F
trt	47	112099.2127	2385.0896	31.10	<.0001
reps	5	94.1604	18.8321	0.25	0.9418

Student-Newman-Keuls Test for t_mort

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis
but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	265
Error Mean Square	76.68119
Harmonic Mean of Cell Sizes	6.32967

Student-Newman-Keuls Test for t_mort

Means with the same letter are not significantly different.

SNK Grouping			Mean	N	trt
	A		66.392	6	41
	A				
B	A		64.177	6	51
B	A				
B	A		63.930	6	61
B	A				
B	A		63.930	6	45
B	A				
B	A		63.682	6	54
B	A				
B	A		62.327	6	60
B	A				
B	A		62.327	6	50
B	A				
B	A	C	61.220	6	44
B	A	C			
B	A	C	61.220	6	46
B	A	C			

	B		A	C		60.216	6	23
	B		A	C				
	B		A	C		59.004	6	47
	B		A	C				
	B		A	C		57.537	6	48
	B		A	C				
	B		A	C		56.789	6	58
	B		A	C				
	B	D	A	C		52.921	6	52
	B	D	A	C				
	B	D	A	C		50.999	6	49
	B	D	A	C				
	B	D	A	C		50.852	6	ten
	B	D	A	C				
	B	D	A	C		50.810	6	35
	B	D	A	C				
	B	D	A	C		49.995	6	36
	B	D		C				
	B	D	E	C		46.909	12	59
		D	E	C				
	F	D	E	C		45.000	6	34
	F	D	E					
	F	D	E	G		40.193	6	335
	F	D	E	G				
H	F	D	E	G		38.186	6	295(3)
H	F		E	G				
H	F	I	E	G		34.214	6	53
H	F	I		G				
H	F	I	J	G		31.999	6	33
H	F	I	J	G				
H	F	I	J	G		31.501	6	330
H		I	J	G				
H	K	I	J	G		29.536	6	326
H	K	I	J	G				
H	K	I	J	G		28.780	6	6(4)
H	K	I	J	G				
H	K	I	J	G		28.683	12	25
H	K	I	J	G				
H	K	I	J	G		26.565	6	29
H	K	I	J	G				
H	K	I	J	G		26.070	6	12I
H	K	I	J	G				
H	K	I	J	G	L	25.210	6	13
H	K	I	J	G	L			
H	K	I	J	G	L	24.963	6	57
H	K	I	J	G	L			

Duncan Grouping	Mean	N	trt
H K I J G L	23.855	6	30
H K I J G L			
H K I J G L	23.608	6	21
H K I J G L			
H K I J G L	23.608	6	K1
H K I J L			
H K I J M L	22.500	6	305
K I J M L			
K I J M L	19.790	6	9I
K I J M L			
K I J M L	19.790	6	40
K I J M L			
K I J M L	18.435	6	295(1)
K I J M L			
K I J M L	17.773	12	24
K J M L			
K J M L	16.717	6	6(1)
K J M L			
K J M L	16.237	12	37
K J M L			
K J M L	15.362	6	321
K J M L			
K J M L	15.362	6	C
K M L			
K M L	12.109	12	43
M L			
M L	8.608	6	14
M			
M	6.145	6	HD695
M			
M	6.145	6	K2126

4. 3 LT $_{50}$ 30 Days after Treatment

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	18	23 34 35 36 41 44 45 46 47 48 49 50 51 52 58 60 61 tene
reps	6	1 2 3 4 5 6

Number of observations 108

Dependent Variable: weevils

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	10138.46572	460.83935	38.98	<.0001
Error	85	1004.94124	11.82284		
Corrected Total	107	11143.40697			

R-Square	Coeff Var	Root MSE	weevils Mean
0.909817	20.58190	3.438435	16.70611

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	17	10066.77300	592.16312	50.09	<.0001
reps	5	71.69272	14.33854	1.21	0.3102

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	17	10066.77300	592.16312	50.09	<.0001
reps	5	71.69272	14.33854	1.21	0.3102

The GLM Procedure

t Tests (LSD) for weevils

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	11.82284
Critical Value of t	1.98827
Least Significant Difference	3.9471

Means with the same letter are not significantly different.

t Grouping		Mean	N	trt
	A	37.875	6	34
	B	31.468	6	36
	B			
C	B	29.420	6	49
C				

		Mean	N	Treatment
C	D	27.475	6	47
C	D			
C	D	26.010	6	35
	D			
	D	24.080	6	52
	E	16.467	6	tene
	E			
	E	15.935	6	48
	E			
F	E	13.527	6	58
F				
F	G	10.875	6	50
	G			
	G	9.263	6	44
	G	9.098	6	61
	G			
	G	8.943	6	23
	G			
	G	8.458	6	45
	G			
	G	8.372	6	46
	G			
	G	8.158	6	60
	G			
	G	7.807	6	41
	G			
	G	7.478	6	51

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	11.82284
Critical Value of t	1.98827
Least Significant Difference	2.2788

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