ISOLATION, CHARACTERIZATION AND EVALUATION OF POTENCY OF NATIVE *Bacillus thuringiensis* AGAINST MAIZE INSECT PESTS AND AFLATOXIN PRODUCING FUNGI

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DECLARATION

This is my original work and has not been presented for the award of any degree at any other University or educational institution.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

AMC – Acetylmethylcarbinol

ANOVA – Analysis Of Variance

BSA – Bovine Serum Albumin

Bt – Bacillus thuringiensis

CPB – Colorado potato beetle

Cry – Crystal

Hr/hr – hour

IPM – Integrated Pest Management

ICIPE- International Centre of Insect Physiology and Ecology

KARI - Kenya Agriculture Research Institute

KEBS- Kenya Bureau of Standards

KG-Kenya Gazi

KM- Kenya Machakos

KU – Kenyatta University

LD_{50} – Lethal Dose causing 50% mortality

LGB – Larger Grain Borer

Log- logarithm

LSD-List Significant Difference

Min – minutes

MR – Methyl Red test

MRVP– Methyl Red, Voges Proskauer medium

NA – Nutrient agar

) – Optical Density

M – Rotation per Minute

S PAGE – Sodium duodecyl sulphate Polyacrylamide Gel Electrophoresis

– Species

– Tryptone Broth

– Variety

LIST OF UNITS AND SYMBOLS

NITS

a – kilo dalton

– nanometer

– Degrees Centigrade

- Seconds

– micrograms

- micrometer

SYMBOLS

β- Beta

δ- Delta

ABSTRACT

Bio-control of pests using *Bacillus thuringiensis* represents the most potential use of biological control agents to date. Among major constraints to maize production, safety and hence food sufficiency in Kenya is infestation, damage and contamination by insect pests. Maize grains are adversely damaged by *Prostephanus truncatus* which occasionally paves way for the growth of aflatoxin producing fungi. A number of management strategies have been employed for the control of maize insect pests including the use of synthetic insecticides all of which however, have limitations. The focus of this study was to isolate and establish the efficacy of native *B. thuringiensis* against *P. truncatus*, *Chilo partellus*, *Aspergillus flavus* and *Aspergillus niger* for use in both pre-harvest and post harvest maize protection. The isolated *B. thuringiensis* strains were classified according to their morphological appearance, growth and biochemical characteristics. Based on the preliminary potency tests, isolates KG 411, KG 12-0, KG 20, KG 420, KM 31 and KM 24 caused over 50 % mean mortality at the first predetermined discriminate dose of 10 mg/ml. With subsequent log probit analysis KG 411 was significantly more toxic at 95% confidence limit ($p < 0.001$) than all the other isolates, with a median dose of 0.30 mg/ml causing 77.1 % mean mortality. Log probit analysis for toxicity of the native *B. thuringiensis* isolates on the second instar larvae of *C. partellus* showed significant differences at 95% confidence limit ($p<0.001$), with isolate KM 12 causing the highest mean mortality of 76 %. Evaluation of effects of the *B. thuringiensis* isolates against the above mentioned two fungal strains showed that isolate KM 31 was the most inhibitory, causing 83.33 % and 78.33 % inhibition against *A. niger* and *A. flavus* respectively at 95% confidence ($p < 0.001$). In conclusion, this result

demonstrates the availability of novel native *B. thuringiensis* which would constitute an alternative biological control option for management of adult *P. truncatus*, *A. niger* and *A. flavus* in both pre-harvest and post harvest maize protection.

CHAPTER ONE

1.1.0 GENERAL INTRODUCTION

There is continuing need to increase food production in developing countries of Africa, Latin America and Asia. This increase has to come from increased yields from major crops grown on existing cultivable lands. Nevertheless, several factors impact retrogressively on the realization of this goal. Among such setbacks are the negative effects of climatic change such as global warming and flooding; land degradation and pest associated losses. While a lot of measures are being put in place to curb the effect of climate change, there is even an increasing need to fight against food losses and food contamination caused directly or indirectly by pests. Maize is the most important grain among many African nations. It is the staple food for most of the Kenyan population. The production of maize in Kenya fluctuates between 2.2 and 2.7 million metric tons per annum (Anon, 2001) of which 80% are stored on the farm and the 20% at the central storage system. Losses in maize produce due to pest results from the activity of insect pests both in pre-harvest and post-harvest (Harris, 1989). Among the most important pests are the coleopterans such as *Prostephanus truncatus* and lepidopterans such as *Chilo partellus*, *Buseola fusca* and *Sesamia calamistis* (Harris, 1989). In subsequent sections, *Prostephanus truncatus* will be referred to as *P. truncatus* while *Chilo partellus* will be referred to as *C. partellus* for purposes of simplicity. *C. partellus* cause the dead heart symptoms characterized by dead central leaves in young host plants. Among the coleopterans, the most notorious is the larger grain borer (LGB). It was first identified in 1983 in Taveta (Muhihu and Kibata, 1985). Maize storage is adversely affected by weevil damage. In Kenya (De Lima, 1979) reported the main causes of damage and weight loss in maize due to insect pests to be 4.5% and 2.7 million metric tones of total

harvest respectively. The real losses caused by *P. truncatus* to unprotected maize during one storage season has been estimated to be between 10% and slightly over 30% by weight, significantly more than 2-3 times of what is normally lost due to attacks by indigenous insect pests (Tyler and Boxal, 1986). Muhihu and Kibata, (1985) reported 35% weight loss, making the pest a more serious threat to food security in the country. Moreover in developing maize particularly during reproductive growth, drought, excessive heat, inadequate plant nutrition, weeds, excessive plant populations, plant diseases and more worse, insects feeding on developing kernels produce plant stress which facilitates the infection of maize grain by mycotoxin producing (Arnold, 2003).

Aflatoxin and fumonisin are mycotoxins produced primarily by fungi of the genus *Aspergillus* and *Fusarium* respectively. These toxins are very potent carcinogens in both humans and livestock. They can readily contaminate maize grain in the field and in storage as was witnessed in Eastern and Central Provinces of Kenya. In 2004, 125 people died and nearly 200 others were treated after eating aflatoxin contaminated maize, (Lewis, 2005). The deaths were mainly associated with home-grown maize that had not been treated with fungicides or properly stored. In 2007, over a hundred deaths were reported in the Eastern part of the country. Therefore, insect control is one of the ways to reduce mycotoxin contamination of maize.

The fight against maize borer pests has been largely through the use of pesticides whose use has been under increasing scrutiny because of environmental contamination, destruction of the atmospheric ozone layer, potential carcinogenic effects and general consumer aversion to the use of chemicals. These shortcomings have necessitated the need for alternative forms

of pest control which are socially acceptable, sustainable and environmentally safe. As such integrated pest management systems have been developed whereby chemical, biological and cultural controls are used together. Furthermore more progress and discovery is being made in agricultural entomology and microbiology to exploit the potential use of microbial pesticides in insect pathology. The current use of microbial pesticides such as those based on *Bacillus thuringiensis* in integrated control programs attests to this. In subsequent sections, this bacterium will be referred to as *Bt* for simplicity. The *Bt* bacterium produces large proteinaceous parasporal crystals called insecticidal crystal proteins or ICPs which may be extremely toxic to certain species yet virtually non-toxic to insects in the same Linnaen family or even genus (MacIntosh *et al.,* 1990; Ge *et al.*, 1991; Peacock *et al.*, 1998). This combination of remarkable specificity and extreme toxicity makes *Bt* one of the most environmentally 'friendly' and widely used microbial insecticides (Feitelson *et al.,* 1992; Lambert and Peferoen, 1992). Products based on *Bt* are the most successful microbial pesticides used in agriculture, forestry and public health (Drummed and Pinnock, 1994).

Even though the *Bt* serotype discovered about two decades ago (*tenebrionis*; Krieg *et al*, 1983), and *San diego* (Herrnstadt *et al.*, 1986) produce δ endotoxins specific in their activity against coleopteran larvae; few species other than *Chrysomelids* such as the Colorado potato beetle and *Leptinotarsa decemlineata* are reported to be highly susceptible. There is also lack of native *Bt* strains which could possess better quality and a wide activity spectrum and more so against coleopterans as well as aflatoxin producing fungi. This causes a challenge of screening for more *Bt* strains that are highly potent against the voracious adult *P. truncatus* and aflatoxin producing fungi. The focus of this study was to isolate and characterize native *Bts* effective against coleopterans and also inhibitory to fungal growth

for use against maize insect pests as well as in reducing mycotoxin contamination of maize. Novel native *Bt* strains may also help to enrich the country's genetic *Bt* germplasm as a source for *Bt* genes for insect pest resistant transgenic maize and; above all, production of microbial pesticides to curb such constraints in maize production as well as food security.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The discovery and study of insecticidal bacteria

Discovery and study of insecticidal bacteria began over a century ago leading to the development of commercial bacterial insecticides in the subsequent years. Bacterial insecticides have become the first successful and widely used microbial agents (Federici, 2005). Most of these products were based on *Bt* although they were only effective against lepidoptera pests, their success led in the 1970's and 1980's to the discovery of strains effective against the larvae of coleopterans pests and nematocerous, dipterans, such as the nuisance mosquitoes and black flies (Drummond and Pinnock, 1994; Cooper, 1994; Federici, 2005).

The cloning in 1981 of the first gene encoding a cry protein led to an explosion of basic and applied research that culminated in new strains of recombinant insecticidal bacteria, and even more importantly, the development, commercialization and wide scale deployment of insecticidal transgenic crops based on Cry proteins(Federici, 2005). This new environmentally safe technology has revolutionized agricultural pest control, yielding a multibillion dollar industry that is then paving way to new types of plants that will dominate food and fiber production as the 21st century progresses (Federici, 2005).

Apart from feeding on plants and other organisms, insects feed on bacteria too. Some of the bacteria in the long run have developed defense mechanisms against such predation for example by causing diseases in insects. Interest in such insect diseases especially the

beneficial insects such as the silkworm, formed the foundation for insect pathology. Of the various pathogens that attack insects and other vertebrates, insecticidal bacteria have proved to be the easiest and most cost effective to mass produce and this were the first commercially microbial insecticides (Federici, 2005). They produce crystals which may include several related proteins. Within each protein 'sub family', individual ICPs, or mixtures thereof, may be extremely toxic to certain species yet virtually nontoxic to insects in the same Linnean family, or even genus (MacIntosh *et al.*, 1990; van Frankenhuyzen *et al.*, 1991, 1993; Ge *et al* 1991; Peacock *et al.*, 1998).

2.2.1 Morphological characteristics and habitat of *Bt*

Bt is an aerobic, spore forming, Gram positive and rod shaped bacterium. It produces proteinaceous parasporal inclusions with insecticidal properties (Bulla *et al.*, 1977; Fast, 1981). *Bt* is widely distributed in the environment and can be isolated from soil, insects and their habitat, stored product material and the leaves of certain deciduous and coniferous trees (Ohba and Aizawa, 1986; Martin and Travers, 1989); from phylloplanes (Smith and Couche, 1991, Ohba 1996; Mizuki *et al.*, 1999). Recently several investigations have demonstrated that *Bt is* also a common member in microflora of fresh water (Climatsu *et al.*, 2000; Iriarte *et al.*, 2000) and marine sediments (Maeda *et al.*, 2001). A study examining a subtropical mangrove environment of Japan with the aim of isolating *Bt* with novel toxicity spectral revealed sediments in mangroves as a good source for the recovery of the bacteria (Maeda *et al.*, 2001).

2.2.2 Classification of *Bt*

Bt strains can be classified by a number of techniques such as on the basis of flagellar serotype (de Barjac, 1981), crystal antigens (Kry-wienczyk *et al.*, 1978; Krywienczyk and Fast, 1980), crystal morphology, crystal protein profiles, serology peptide mapping DNA probes and insecticidal activity. Classification at variety level can also be based on the antigenic reactions of the flagellar associated with the young actively motile cells of the *Bacillus* (Dulmage, 1993). Depending on the antigenic reactions, isolates are either assigned an "H – number" "serotype or subspecies" in consecutive order or placed in a group of other isolates (Dulmage, 1993). On the basis of their flagellers (H) antigens, *Bt* has been classified into 69 serotypes comprising 82 serovars (Lecadet *et al.*, 1999). Crystal proteins antibodies have been used to differentiate among the numerous *Bt* isolates in attempt to categorize variants with similar characteristics (Kry-wienzyk *et al.*, 1978). Use of crystal serology in identification of *Bt* on basis of toxicity toward insects is useful in detecting specificity among orders but not within genera. In addition to cry proteins, some *Bt* strains produce other insecticidal proteins, for example β– exotoxins which are associated with certain *Bt* subspecies such as *darmstadiensis, gallariae, tenebrionis* and *thuringiensis* (Drummond and Pinnock, 1994). Considering crystal morphology, crystal shapes can be classified into bipyramidal, spherical, rectangular, irregularly pointed or irregularly spherical. However the typical shape of most crystal is bipyramidal (Fast, 1981). In view of the protein composition in crystals, the predominant proteins in lepidoptera-active strains are those of 130 – 140kDa (Calabrese *et al.*, 1980) although some isolates contain 65kDa protein. Dipteran-active strains are 130 – 140 kDa, 65 and 25 kDa proteins (Chilcott and Ellar, 1988) however; the crystal protein profile does not always indicate the toxicity of the isolate.

2.2.3 Insecticidal toxins produced by *Bt*

The most important group of toxins produced by *Bt* is composed of delta endotoxins or the crystal proteins (Drummond and Pinnock, 1994). A second group of toxins is thuringiensin or β exotoxin which rates second in importance to the crystal proteins (Drummond and Pinnock, 1994), α- exotoxin which is heat labile and water soluble and; the louse factor (Dulmage *et al.*,1981).

2.2.3.1 Crystal proteins

Cry proteins are encoded by *Cry* genes that are mostly carried on plasmids. To date nearly 300 *Cry* genes have been identified and classified into 51 groups and subgroups on the basis of amino acid sequence similarity (Crickmore *et al.*, 2007). Other forms of Classification are based on Crystal protein structure (deduced from the DNA sequence) and also on the host range (Dulmage, 1981). The more than 300 distinct crystal protein (*Cry*) genes also specify a family of related insecticidal cry proteins and are divided into four major classes: - Lepidoptera specific – I, Lepidoptera and Diptera specific – II, Dipteral – specific - IV and Coleopteran specific – III . *Cry* I gene comprises of 20 distinct genes which encode 130 – 140 kDa proteins and have typical pyramidal crystals. *Cry* II genes which encode 65kDa proteins have cuboidal inclusions earlier known as P2 proteins in classifications by (Aronson *et al.*, 1986). High molecular weight i.e. 130kDa produced by same strain were referred to as P1 proteins (Aronson *et al.*, 1986). The genes in *Cry* III category encode 72 kDa proteins and have variable crystal shapes i.e., flat diamond shaped or rhomboid, while *Cry* IV genes are heterogeneous group and encodes protein molecules with predicted molecular weights of 128, 135, 78, 72 kDa. They form ovoid crystal complexes. In some subspecies of *Bt* more than one crystal protein can occur (Hofte and Whiteley, 1989).

2.2.3.2 α and β exotoxins

Beta and alpha exotoxins are non protenacious toxins produced by *Bt*. β-exotoxin is a heat stable nucleotide, fly – factor and adenine containing compound. It is an ATP analogue and inhibits RNA synthesis displaying toxicity to a wide range of insects while alpha-exotoxin, also identified as lecithinase c is heat labile, water soluble and toxic to insects as well (Drummond and Pinnock, 1994; Dulmage *et al.*,1981).

2.2.4 Mode of action of *Bt* toxins

The mode of action of *Bt* entomopathogenic toxins has been extensively studied. According to (Fiuza *et al.*, 1996) the mode of action tends to take a trend of stimulation within the midgut of insects. The mechanism primarily involves solubilization of the crystals in the insect's midgut via breakage of disulfide bonds in the cry protein monomers. This is followed by proteolytic processing of the protoxin by midgut proteases, releasing the cry toxin in its active form. The active proteins then bind to specific receptors on the apical brush border of the midgut microvillae of susceptible insects resulting to insertion of the toxins into apical membrane. As a result ion channels or pores are created through which leakage of the intercellular contents take place. This is then followed by the death of the insect (Schepf *et al.*, 1998). Some *Bt* strains produce other insecticidal proteins e.g. β – exotoxin which is associated with *Bt* subspecies *dermstadiensis*, *galleriae*, *tenebrionis* and *thuringiensis*. β – exotoxin inhibits RNA polymerases by acting competitively with ATP.

2.2.5 Application of *Bt* microbial pesticide

Bt has been successfully employed to control 'filth' flies in Kenya (Carberg *et al.*, 1991) and in Australia strains are being developed to control the sheep blowfly *Lucilia cuprina*

(Cooper *et al.*, 1985). *Bt* is also reported to produce nematocidal toxin (s) (Bone *et al.*, 1988) active against various stages of free-living plant and animal parasitic nematode species (Bradfisch *et al.*, 1991). The coleopteran active insecticidal proteins produced by strains of *Bt* subsp *tenebrionis* have been well characterized (Li *et al.*, 1988, 1991, Mc Phersch *et al.*, 1988; MacIntosh *et al.*, 1990) and used in potato plants to confer resistance to the Colorado potato beetle (Perlak *et al.*, 1993).

2.2.6 Advantages of *Bt* as a biological control agent

Members of the genus *Bacillus* have great potential as biological control agents because they keep their viability when stored for long time and currently accounts for the highest total world microbial insecticide in the market (Feitelson *et al.*, 1992; Lambert and Perferoen, 1992). According to (Luthy *et al.*, 1982), *Bt* biopesticide have the following advantages: it has no phytotoxicity, does not harm predators and non-target insect populations. Moreover the δ endotoxin as well as the spores incorporated in the products has no mammalian toxicity. Its combination of remarkable specificity and extreme toxicity makes *Bt* biopesticide one of the most environmentally 'friendly'insecticides and the most widely used microbial insecticide (Feitelson *et al.*, 1992; Lambert and Perferoen, 1992).

2.2.7 Disadvantages of *Bt* as a biological control agent

Two of the problems which have prevented large scale use of *Bt* have been its limited activity spectrum and its low toxicity to many important cosmopolitan insect pests. Another emerging problem is resistance to toxins by some pests e.g. resistance by *Plodia interpunctella* and *Plutella xylostella* against *Bt* var *kurstaki* (McGaughey, 1985; McGaughey and Beeman, 1988), resistance by *Heliothis virescens* to *Bt* var *kurstaki* and

resistance produced in *Pseudomonas fluorescens* and *Plutella xylostella* from Hawaii against *Bt* subspecies *kurstaki* (Tabashnik *et al.*, 1990).

2.3 *Bt* variety effective against coleopterans, their source and toxins

A particular good source of *Bt* has been stored product material and dead insects. Slanley *et al.*, (1991) reported a new strain of *Bt* var *kumamotensis* which is toxic to the southern corn rootworm and Mexican bean beetle. Analysis of *Bt* Crystal using SDS– PAGE has revealed that coleopteran active crystal is a 73 – kDa protein (Donovan *et al.*, 1988). *Bt* strains producing insecticidal proteins with activity against coleopterans have been described (Kreig *et al.*, 1983; (Herrnstadt *et al.*, 1986; Donovan *et al.*, 1988; Bernahard, 1986). This important insect group includes pests such as the Colorado potato beetle (*Leptinotarsa decemliniata* and *P. truncatus*). The coleoptera – active insecticidal proteins produced by strains of *Bt* subspecies *tenebrionis* have now been well characterized (Li *et al.* 1988, 1991; McPherson *et al.*, 1988; MacIntosh *et al.*, 1990) and the active protein in use is a 68 – kDa protein produced by translation initiation at an internal initiation site in the native DNA sequence (McPherson *et al.*, 1988). The gene has been introduced into potato plants to confer resistance to the Colorado potato beetle (Perlak, 1993). Even though the more recently discovered *Bt* serotypes *tenebrionis* (Krieg *et al*, 1983), and *Bt* subsp. *san diego* (Herrnstadt *et al.*, 1986) produce – endotoxins specific in their activity against coleopteran larvae; few species other than Chrysomelids such as the Colorado potato beetle and *Leptinotarsa decemlineata* are reported to be highly susceptible (Drummond and Pinnock, 1994). *Bt* subsp *tenebrionis* produces flat, rhomboid crystals composed of proteins of approximately 68 kDa.

Hernstadt *et al.*, (1986) reported the isolation of a strain of *Bt* that is toxic to coleopterans, designated as strain M7 of *Bt* subsp. *san diego* that appeared to be identical to *Bt* subsp *tenebrionis*. (Donovan *et al.*, 1988) reported the isolation of a strain of *Bt* (strain EG21158) that was toxic to coleopterans and that differed from *Bt* subsp. *tenebrionis*, as judged by plasmid array and by the production of certain crystal proteins. All these three organisms contain an identical gene (*Cry* IIIA) encoding a 73-kDa protein toxic to the larvae of the Colorado potato beetle (Donovan *et al.*, 1988; Herrnstadt *et a l.*, 1987; Jahn *et al.*, 1987; Mcpherson *et al.*, 1988 and Sekar *et al.*, 1987). Conclusively at least three distinct types of *Bt* toxic to colepterans exist in nature. These three types can be distinguished by the following properties; flagellar serotype, native plasmid complement, sizes of *Cry* IIIA-hybridizing restriction fragments, crystal proteins, and insecticidal activity (Donvan *et al*, 1991). Strain EG2158 that is toxic to coleopterans belongs to H serotypes 8a, 8b (*Bt* var *morrisoni*).

2.4 Kenyan *Bt* isolates effective against *P. truncatus* and *C. partellus* and their source.

In Kenya a range of materials have been sampled from different sites and ecological zones such as Nyeri, Embu, Murang'a, Kiambu, Kericho, Njoro, Lambwe valley, Mombasa area, Busia Mfangano Island, (Brownbridge, 1991) Kakamega, Machakos, (Wang'ondu, 2001), Kiboko, Murang'a- Kariti, Kiambu-Githuguri, Kericho, Nyeri (Wamaitha, 2007) and used as source of *Bt*. These include insect frass, dead insect materials, soil, barks of trees and maize grains. Some of the toxic strains isolated include A-3, B1-1, A-C-2, L1-5, L1-6, M44-7, BUS-3/7, MF-3A-1 (Brownbridge, 1991), 1M, VM-10, 44M,12F-K and K10-2 (Wang'ondu, 2001). Currently a lot of research is being undertaken to isolate effective native *Bt* strains against destructive coleopterans and more so the larger grain borer. Results

of a recent study showed two of the most toxic isolates obtained (i.e. *Bt* 41 and *Bt* 51) against *P. truncatus* to have two major bands of 73 and 67 kDa and minor bands 54, 46, 35 20, 17 and15 for *Bt* 41 while *Bt* 51 had two major bands of 97 and 68 kDa and two minor bands of 44 and17 kDa; after trypsin activation (Mwathi, 2007). According to (Krieg *et al.*, 1983) the weight of the crystal proteins of the most toxic *Bt* to coleopterans is in the range of 67 to74 kDa, which suggests that the isolates mentioned above may contain *Cry* III related genes. The search for more native *Bt* strains with high potency against coleopterans is therefore very paramount in war against *P. truncatus*.

2.4 Antifungal effects of *Bt*

Investigations on the effect of toxins and some proteinaceous substances produced by some varieties of *Bt* such as *Bt* var *israelensis* reveals some antifungal effects attributed to the enzyme chitinase which they produce (Reyes-Ramirez *et al.*, 2004). The antifungal chitinase activity on phytopathogenic fungi was investigated in growing cultures and on soybean seeds infested with *Sclerotium rolfsii*. Fungal inhibition was found to be 100% for *S. rolfsii*; 55% to 82% for *A. terreus*, *A. flavus*, *Nigrospora* sp, *Rhizopus* sp, *A. niger*, *Fusarium* sp, *A. candidus*, *Absidia* sp, and *Helminthosporium* sp; 45% for *Curvularia* sp; and 10% for *A. fumigatus* ($P < 0.05$). When soybean seeds were infected with *S. rolfsii*, germination was reduced from 93% to 25%; the addition of chitinase (0.8 µ/mg proteins) increased germination to 90%. *Bt* chitinase may contribute to the bio-control of *S. rolfsii* and other phytopathogenic fungi in soybean seeds in IPM (Reyes-Ramirez *et al.*, 2006). In another study by (Knaak *et al.*, 2007) on in vitro effect of *Bt* strains and cry proteins in phytopathogenic fungi of paddy rice-field, *Cry*1Ab and *Cry*1Ac strains and proteins

synthesized by *Bt* var *kurstaki* was assessed in the following phytopathogens: *Rhizoctonia solani, Pyricularia grisea, F. oxysporum* and *F. solani*, which had their mycelial growth decreased after incubation in the presence of the bacterial strains although as for cry proteins, there were no inhibition development in the assessed concentrations.

2.5 Justification of the study

Among major constraints to maize production, safety and sufficiency in Kenya is infestation, damage and contamination by insect pests in both the field and in post harvest preservation. Attack in the field may occur fairly early when the drying maize still has a moisture content of 40-50% (Giles, 1975). Under such conditions, feeding on developing kernels by pests facilitate the infection of maize grains by aflatoxin producing fungi. There is unsustainability in the use of chemicals pesticides such as pyrethroids or organochlorins and organophosphates due to insect resistance and hence increased dosages and more frequent applications (Meikle *et al.*, 2000). Other delimitations in the use of synthetic insecticides include environmental contamination, depletion of the atmospheric ozone, and potential carcinogenic effects by the chemicals. There is also lack of native *Bt* strains which could possess better quality and a wide activity spectrum and more so against coleopterans. Novel native *Bt* strains may help to enrich the country's genetic *Bt* germplasm as a source for *Bt* genes for insect pest resistant transgenic maize as well as production of microbial pesticides. A lot of work on *Bt* field application trials and isolation has been concentrated in some parts of Kenya such as Western province, Central province and in the Rift valley unlike in some parts of the coastal province such as the Southern coast. Yet the first

incidence of *P. truncatus* was in the coastal province hence the need for search of native *Bt* strains from such ecological niches.

2.6 Problem statement

P. truncatus destroys stored maize grains, converting it into powder, with losses estimated between 10% and slightly over 30% by weight (Giles, 1975). In storage 50% damage of the grain occurs within 3 months of infestation while six month's effect amounts to 100% damage. Destruction by such pests is recurrent and results into overwhelming losses to both subsistence and large-scale farmers due to reduced crop productivity, damage and contamination of grains in storage hence reduced market prices. The physical damage caused by the weevil further increases the chances of fungal infestation by mycotoxin producing species and hence mycotoxin contamination. Loss of human lives on ingestion of such maize often occurs. For example in 2004 and 2007 over 225 deaths were reported in Kenya due to ingestion of aflatoxin contaminated maize CDC, (2004). Currently this pest has spread to various parts of Kenya, greatly affecting farmers in areas such as Taita Taveta, Kajiado district, Loitoktok, Eastern parts around Kitui, Kiboko and Machakos; Kiambu district, Western Kenya along Kitale and Kakamega and; along the Kenyan Coast- Voi, Mwatate and Wundanyi (Muhihu and Kibata, 1985; Mutambuki *et al.*, 1990; Kibata *et al.*, 1996). It is in this respect that the study investigated the use of novel native *Bt* isolates with higher toxicity and wider activity spectrum against both maize insect pests and aflatoxin producing fungi. At this point it worth noting that insect control is one of the ways to reduce mycotoxin contamination of maize.

2.7 Research hypotheses

Toxins produced by *Bt* isolates from brackish sediments and savanna grassland soils from Machakos district are potent against insect maize pests *P. truncatus* and *C. partellus* and; aflatoxin producing fungi, *A. flavus* and *A. niger*.

2.8 Broad objective

To isolate characterize and evaluate the potency of native *Bt* against *P. truncatus*, *C. partellus* and; two fungal species i.e. *A. flavus* and *A. niger*.

2.8.1 Specific objectives

1. To isolate native *Bt* from Coastal intertidal brackish sediments and savanna grassland soils from Machakos district in Kenya.
2. To evaluate the potency of the isolates against *P. trancutus* and *C. partellus*.
3. To evaluate growth inhibition effect of *Bt* isolates toxic to *P. truncatus* gainst *A. flavus* and *A. niger*.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Description of sample collection site

Soil samples used were collected from two different ecological regions in Kenya. These areas were: Machakos District in Eastern province; an area that was chosen because of the high prevalence of *P. truncatus* and from the South Coast (Gazi Mangroves- fig 1, 2 and *3*) in the Coastal province. Four different sites were chosen for soil sampling in the Gazi mangroves. Three of these sites were each under a different type of mangrove species i.e. *Avicennia marina* (designated as KG 1), *Rhizophora mucronata* (designated as KG 2) and *Sonneratia alba* (designated as KG 3) while the fourth one was from the intertidal brackish sediments along river Kidogoweni (designated as KG 4). These mangroves were chosen as a new ecological zone for *Bt* isolation since no *Bt* isolation had ever been done here before. Yet subtropical mangrove sediments are a good source for the recovery of *Bt* with novel toxicity spectral (Maeda *et al.*, 2001).

3.2 Collection of soil samples

A total of 63 soil samples, each weighing approximately 10g were randomly collected from the following areas: Kari Katumani in Machakos District – 21 samples; Gazi mangroves along the Kenyan South Coast; *A. marina* -7 samples, *R. mucronata* -7 samples, *S. alba*– 7 sample and from along the banks of river Kidogoweni - 7 samples. The banks had a mixture of both *R. mucronata* and *A. marina* mangroves. Sampling was done during low tides from randomly selected sites that were near and away from mangrove trees; at a depth of 0 – 4 centimeters after removal of the surface layer of soil. The surface layer of soils was removed

to avoid the detrimental effect of UV radiation on *Bt* (Addison, 1993). The soil samples were then kept in labeled sterile polythene paper bags for isolation of the bacteria.

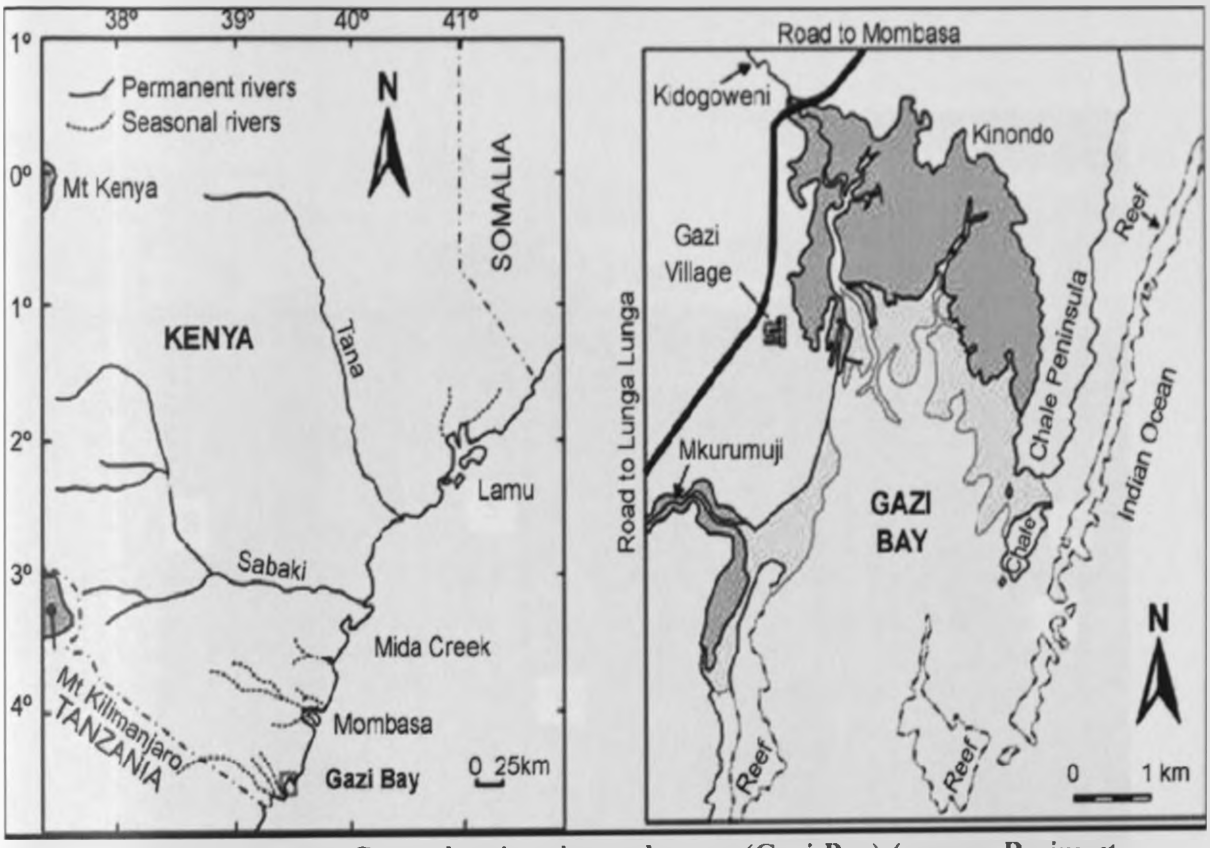


Fig. 1 Map of the Kenyan Coast showing the study area (Gazi Bay) (source: Bosire *et al.*, 2003).

Fig. 2 ***Avicennia marina*** **(KG I); (S) Site of soil sample collection**

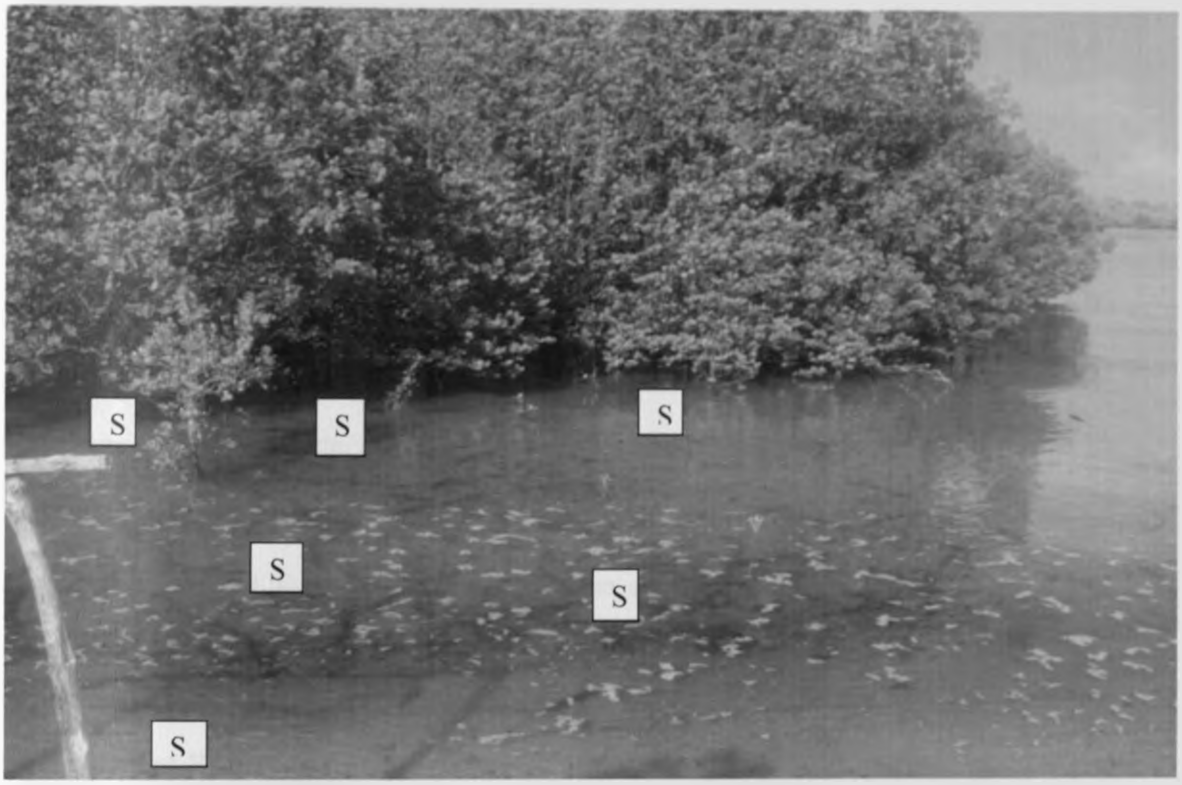


Fig. 3 *Sonneratia alba* (KG 2); (S) Site of soil sample collection

3.3 Collection of pests for bioassays

Untreated maize grains susceptible to target insects (adult *P. truncatus*) were collected from farmers' granaries. Larvae for *C. partellus* were obtained from I.C.I.P.E and sample adult *P. truncatus* from KARI- Kiboko. *P. truncatus* was reared on sterilized maize, at 28° C. Standard *Bt* strains, i.e. *Bt* var *tenebrionis*, var *israelensis* and var *kurstaki* were obtained from Kenya Agriculture Research Institute (KARI). While 4-5 weeks old maize leaves were obtained from a green house in the University of Nairobi, Chiromo Campus.

3.4 Media Preparation

3.4.1 Nutrient agar

The standard method described by (Poinars and Thomas, 1978) together with manufacturer's instructions was used in media preparation. 15.5 g of nutrient agar composed of: Meat extract 1.0 g, peptone 5.0, Yeast extract 2.0, Sodium Chloride 8.0 and Agar 15.0; pH-7.1(+0.2); was suspended in 500 ml of demineralized water. The suspension was boiled while stirring until completely dissolved. The mixture was then autoclaved at 121° C and *l b* (pressure) for 15 minutes. The agar was left to cool to 45-50° C, mixed well and 20 ml poured into sterilized disposable Petri dishes under aseptic condition; left to cool and stored in the refrigerator at 4° C, awaiting use.

3.4.2 Nutrient Broth

Nutrient broth was prepared by adding 28 g of nutrient broth composed of 'Lab- Lemco' powder (1 g), yeast extract (2g) to one litre of distilled water in a conical flask. The media was mixed thoroughly and distributed into 250 conical flasks, covered with cotton wool and aluminium foil and autoclaved at 121° C, 15 *l b* pressure for 15 minutes. It was then allowed to cool for preservation at 4° C to be used in the culturing and preservation of the obtained *Bt* isolates.

3.5 Isolation procedure

Isolation of *Bt* was based on the techniques developed by (Travers *et al.*, 1987) with slight modifications. One gram of each soil sample was suspended in 10 ml of sterile distilled water in 20 ml universal bottle and mixed by vortexing for 1 min. The mixture was then heat shocked in a thermal bath at 80° C for 15 minutes; in order to remove all the

vegetative form of both spore and none spore forming bacteria. After shaking and allowing the mixture to settle for about 3 minutes aliquots of 1 ml of the supernatant from each bottle was diluted with 9 ml of sterile distilled water to attain a tenfold dilution. Successive serial dilutions of up to 10^{-6} were prepared. From the last three dilutions (10^{-4}, 10^{-5} and 10^{-6}) approximately 100 µl aliquot of the above heat-shocked samples were cultured in triplicates using spread plate method. Incubation was done at 30° C for 48 hrs and examined for colonies that showed *Bt* morphology. Further sub- culturing of the prospective isolates using streak plate method on nutrient agar plates and incubation at 30° C for 96 hrs then followed. Resulting colonies were examined for the presence or absence of spores and parasporal bodies/ crystals under oil emersion (magnification x1000) of light microscope after Gram staining and Smirnoff staining (Smirnoff, 1962). Isolates that were rod shaped, Gram positive and indicated the presence of insecticidal crystal proteins were selected for preliminary bioassays with *P. truncatus*.

3.6 Characterization of *Bt* isolates based on microscopy and staining

Morphological characterization of *Bt* isolates was based on the method by (Poinar and Thomson, 1978), while staining of parasporal bodies was as outlined by (Smirnoff, 1962).

3.6.1 Gram staining

This was done to ascertain the Gram stain reaction of the cultures after 24 hrs and 48 hrs. Thin smears of bacterial cells were made on a clean slide by first air-drying and heat fixing for one second. The primary stain (crystal violet) was added and the slide kept for one minute before washing it with distilled water. The slide was then covered with a mordant dye (iodine solution) for one minute and washed off with 80% alcohol until no more colour

came out of the smears. Each smear was then flooded with a counter stain (Safranine) for a minute. Excess counter stain was drained off using absorbent capillarity method. Each slide was blot dried and observation done using a standard light microscope (Magnification x1000).

3.6.2 Smirnoff staining

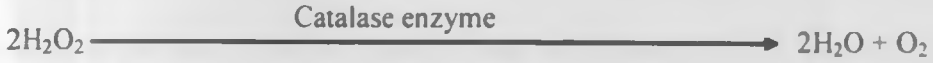
A few drops of solution A containing 1.5 g Amido black dissolved in 50 parts of 90% methanol, 40 parts distilled water 40 parts acetic acid was added to the heat fixed slides of single colony isolates and washed off after 70 seconds. A solution (B) containing 1 g of basic fuschin dissolved in 10 ml of 95% of ethanol and 5 g of phenol dissolved in 90 ml of distilled water was added to each slide and left for 20 seconds before draining it off using absorbent capillarity technique and; dried on a filter paper. Observation was made using a standard light microscope with an oil immersion objective lens, a standard filter being used.

3.6.3 Biochemical tests

These were done to ascertain further the presence of *Bt* isolates obtained as pure cultures.

3.6.3.1 Catalase test

The isolates that were rod shaped and Gram positive were subjected to catalase test for the presence of catalase enzyme. Individual colonies were scraped from a plate with a metallic instrument and suspended in 1 ml of 3% hydrogen peroxide (three ml in a 100 ml of sterile distilled water) in an Eppendorf tube and sealed under aseptic conditions. Each tube was examined immediately and then after between 5 and 10 minutes for bubbles. The presence of oxygen as evidenced by air bubbles which rekindled a glowing splint showed the presence of catalase enzyme contained in the bacterial cells.



3.6.3.2 Voges-Proskauer (VP) test

During the intermediate steps of glucose metabolism, acetylmethylcarbinol (AMC) is produced by certain strains of bacteria (from Pyruvic acid or during the course of butylenes glycolic fermentation). Detection of this substance is a useful phenotypic test in identification of *Bt.* MRVP medium (polypeptone 5 g, Glucose 5 g, sodium chloride 5 g and 1 litre of distilled water) was used. The media was dissolved gently by heating, and adjusted to pH 7. The media was then dispatched into 10 ml – screw cap tubes and sterilized at 105° C for 30 min. Tubes containing the media were inoculated with a single colony of the different *Bt* isolates and incubated at 30° C for 48 hrs. One ml of the culture was mixed with 0.6 ml VP reagent A (Potassium hydroxide 40 g, Distilled water 100 ml) and 0.2 ml VP reagent B (Alpha-napthol 6 g, absolute ethanol 100 ml). The tubes were placed open on a slant to increase contact with air. Change of colour to pink within 10-30 min was a clear indication that the test is positive. Those that did not change colour were scored as negative for the test. Standard control reference *Bt* var *tenebrionis, kurstaki* and *israelensis* were included in each of the above tests.

3.7. Early growth patterns of *Bt* isolates under different parameters.

The different *Bt* isolate obtained were tested for their tolerance to pH, and temperature in their early growth (12-48 hours).

3.7.1 Determination of tolerance of the *Bt* isolates to pH

Each *Bt* isolate was grown on nutrient broth at different pH (4.5, 5.5, 6.5, 7.5 and 8.5) in screw cap tubes and incubated for 24 and 48 hours at 30° C. Optical density was then measured using a light spectrophotometer at 600 nm. Optical density of a blank containing nutrient broth at the same pH value but not inoculated with the inocula for each *Bt* isolate was also taken. To determine the bacterial growth pattern, optical density of the blank at the start was subtracted from the optical density of each *Bt* isolate at different pH values, after 24 and 48 hrs.

3.7.2 Determination of the tolerance of the *Bt* isolates to temperature

Each *Bt* isolate was grown on nutrient broth at varied temperature (25° C, 30° C, 35° C, 40 ° C) in screw cap tubes and incubated for 48 hrs. Optical density was then measured using a light spectrophotometer at 600 nm after every 12 hours. Optical density of a blank containing nutrient broth at the same temperature but not inoculated with *Bt* isolates was also taken. To determine the bacterial growth pattern, optical density of the blank at the start was subtracted from the optical density of each *Bt* isolate at different salinity values, after every 12 hours.

3.8 Growth of *Bt* with parasporal crystals for bioassay

Sporulating cultures of *Bt* isolates were used to inoculate 50 ml of liquid broth in 250 ml fluted Elrenmeyer flask. The inoculated flask was incubated for 96 hrs at 30° C and 200 rpm. At the end of the incubation period the spores and endotoxins were harvested by centrifugation at 4000 rpm for 10 min. The supernatant was discarded and pellet washed three times by centrifugation in sterile 0.85% saline. The final product was suspended in 5 ml saline for storage at -20° C (Wang'ondu, 2001).

3.8.1 Estimation of protein content for the *Bt* isolates

The protein content of each isolate was determined using the UV protein method determination with Bovine Serum Albumin (BSA) as a standard; at a concentration of one mg/ml. 1ml of the non clear *Bt* stock proteins suspended in 0.85% saline was centrifuged for 5 minutes, 14,000 rpm, prior to taking any readings. The concentration of stock protein solution was diluted in the buffer to a concentration ranging from 0.1 to 1mg/ml. Absorbance of the protein solution was measured at 280 nm, using 100 µl quartz cuvets. A standard curve (Appendix 1) was obtained and employed in determining the protein concentration used for bioassays.

3.9 Screening assays

All screening bioassays were carried out using *P. truncantus*, *C. partellus Aspergillus sp A. niger* and *A. flavus*.

3.9.1 Screening bioassays for *Bt* toxicity against *P. truncatus*

For a start for each treatment, 10 mg/ml of *Bt* toxin suspension was mixed with 10 g of each of the 3 different form of toxin delivery diet, i.e. Maize grains (GR), Maize Grain Particles (PT) and Maize flour pellets. Maize flour was mixed with toxin suspension to form pellets. For maize particles and grains, maximum absorption of the toxins was allowed followed by a drying period of 24 hrs at 30° C. Each treatment had 10 adult (not less than 30 days old) *P. truncatus* per Petri dish replicated three times on separate days. Each treatment was set on the same day but sampling for destruction of the maize by the pest and pest mortality done at different times for a period of 21 days after every 3 days. This process was followed for each of the *Bt* isolates. Isolates and form of diet for toxin delivery causing more than 50% mean

mortality of the pests at 95% confidence limit were selected for further tests to determine LD_{50} using five different concentrations (i.e.10.0 ,7.5, 5.0 2.5 and 1.0 mg/ml), of each of the *Bt* isolates. Mortality for *P. truncatus* was recorded after every three days for a period of approximately three months.

3.9.2 Leaf disc bioassay for toxicity of the *Bt* isolates against *C. partellus*

This was done according to method by (Wang'ondu, 2001). By appropriate dilution of stock suspension of each type of *Bt* toxins in sterile saline solution (0.85%), five concentrations (i.e.10.0, 7.5, 5.0 2.5 & 1.0 mg/ml) were prepared for bioassays. Treatments using each concentration were done in replicates of three per day for three different days to cater for variation. For every treatment 10 *C. partellus'* second instar larvae were subjected to the *Bt* toxins. Control treatments consisting of sterile distilled water and two *Bt* standard control references i.e. *Bt* var *tenebrionis* as negative control and *Bt* var *kurstaki* as positive control were included. Four pieces of excised maize leaves each 3 cm long were immersed in a specific amount of the *Bt* toxins corresponding to each particular concentration for three minutes. The treated pieces of maize leaves were then moved to another sterile Petri dish and allowed to dry before the introduction of 10 larvae and a piece of wet filter paper soaked in distilled water to provide moisture. Each Petri dish was sealed with two separate straps of stretched parafilm and incubated at 26° C +2° C. Larval mortality was recorded after every one day for a period of five days.

3.9.3 Fungal growth inhibition test

Bt growth inhibition against *A. flavus and A. niger* was done in dual culture.

3.9.3.1 Growth of *Bt* isolates for fungal inhibition test

The growth of *Bt* isolates was obtained in nutrient broth, at 28 ± 2° C and 200 rpm, until 90 % of cell lyses was achieved. The culture was then centrifuged at 5000 rpm, 5° C, for 15 min, and the obtained isolates in form of pellets washed with phosphate buffer (0.1M $NaH_2PO_4.H_2O$ + 0.1M NaCl, pH 6.0) (Reyes- Ramirez., *et al,* 2004).

3.9.3.2 Bioassay for fungal inhibition

Using an isolation needle, cultures of *A. flavus and A. niger* were transferred to Petri plates containing Malt extract Agar at a central site. Each of the obtained *Bt* bacterial strain was then inoculated in striations using a platinum loop at 1.5 cm far from where the fungus was previously inoculated. Control plates without bacteria were maintained. This was done in triplicates for each of the toxic bacterial isolates on four different occasions against each of the fungal strains. Temperature was maintained at 28° C. Fungal growth inhibition effects of *Bt* in dual culture was assessed on the 3rd, 4th, 7th and 14^{th} day after the onset of the experiment.

3.9.4 Data analysis

Data from all bioassay replicates for *P. truncatus* and *C. partellus* was pooled for ANOVA at 95% confidence limit for means comparison; and for log- probit analysis. For log - probit analysis concentrations were transformed into logarithm to the base 10 while percentage mortality was transformed into probits. Probits or probability units are used in transforming the sigmoid dose-mortality curve to a straight line (Finney, 1952; Bliss, 1935). Median doses were calculated at 95% confidence limit of the means using StatsDirect statistical software package. Log dosage- probit analysis graphs for mortality were plotted using

Minitab statistical software. Data analysis for inhibition tests went through ANOVA at 95% confidence limit for means comparison; in which differences between effects of the *Bt* isolates was based on least significance difference (LSD).

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *Bt*

A total of 28 isolates of *Bt* were obtained from the 63 soil samples collected. This showed that *Bt* is a widely spread bacterium in Kenya and occurs naturally even within the Coastal mangroves. The abundance of the *Bt* isolates with respect to the site of isolation was as follows:

- Kari Katumani in Machakos District – 14 *Bt* isolates
- Gazi mangroves along the Kenyan South Coast÷
- (i) *Avicennia marina* (KG1) – 2 *Bt* isolates

  (ii) *Rhizophora mucronata* (KG 2) –3 *Bt* isolates

  (iii) *Sonneratia alba* (KG 3) –0

  (iv) Along the banks of river Kidogoweni (KG 4) - 9 *Bt* isolates

4.2 Characterization of *Bt* isolates based on microscopy and staining.

The *Bt* isolates when incubated at 30° C for 48 hrs revealed smooth colonies which were cream in colour (Fig. 4).

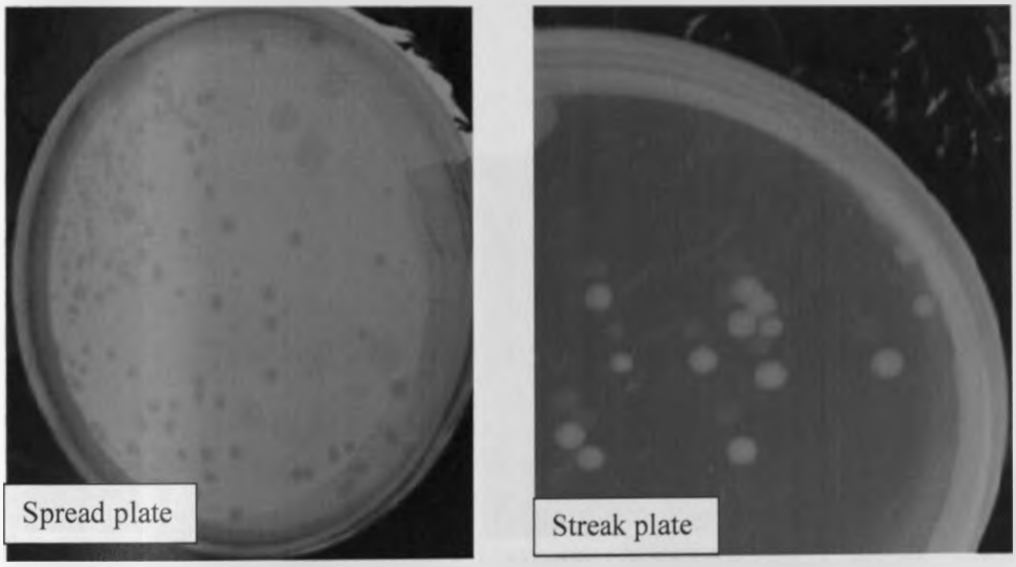


Fig. 4: Colonies of *Bt* isolate (KM 24) using spread & streak plate isolation methods respectively.

4.2.1. Gram staining

The obtained *Bt* isolates stained Gram positive within both 24 and 48 hrs of incubation. (Fig. 5: slides A, B and C).

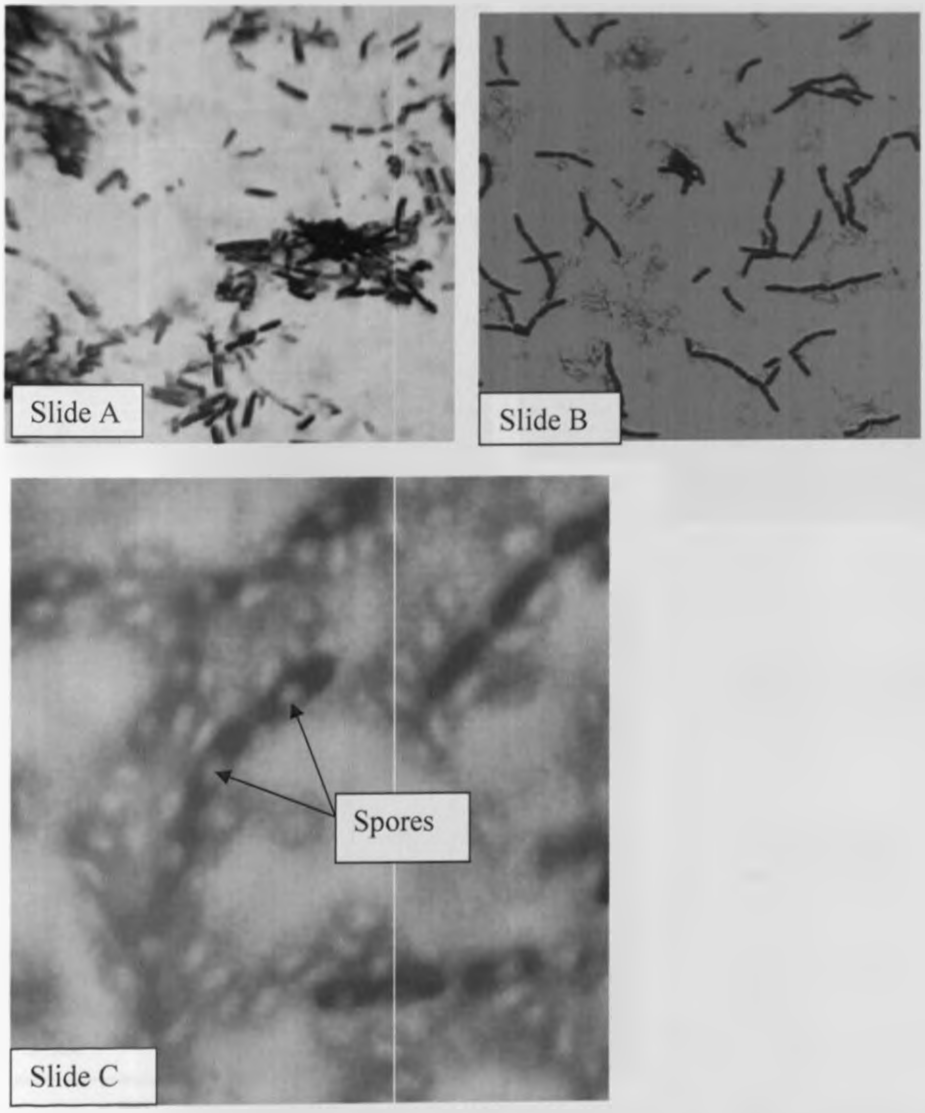


Fig. 5: Positive results for Gram stain reactions of the obtained *Bt* isolates. Slide (A) KG 413 – 24 hrs old; (B) KG 12-0 - 24 hrs old; and Slide (C) KG 411- 48 hrs old sporulating cells with unstained spores. Mg x1000

4.2.2. Smirnoff staining

The *Bt* isolates obtained produced parasporal crystals with varied shapes (Fig. 6: Slides D, E and F; Table 1).

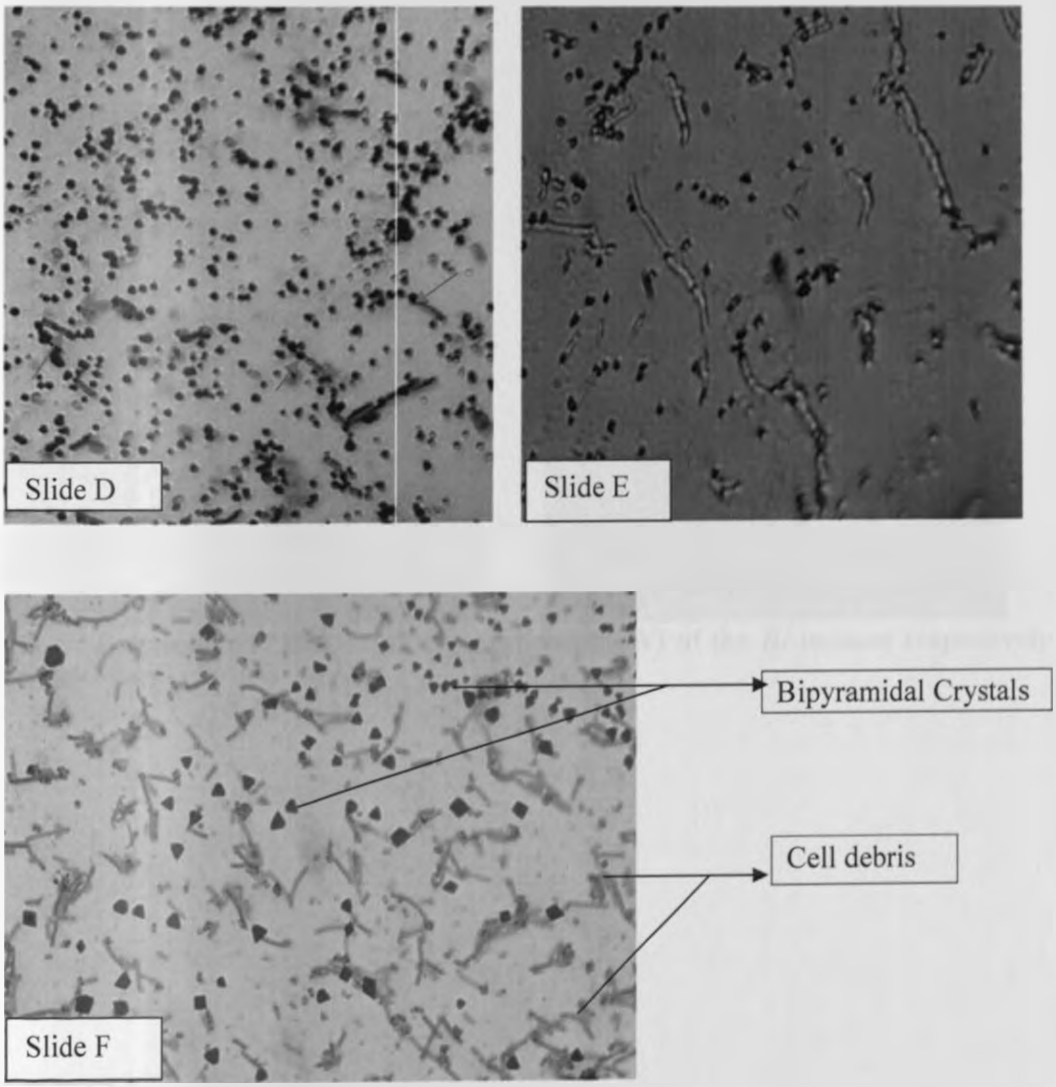


Fig. 6: Positive results for Smirnoff staining of parasporal crystals produced by some of the obtained *Bt* isolates. Slides: (D) KG 12-0 and (E) KG 411; Arrows pointing at crystal proteins within and outside cells respectively; Slide (F) KM 31. Mg x1000

4. 2.3. Cell size estimation, cell shape and spore site within the cell

The cells were rod shaped, with the spores terminally located within the cells. The cells had varying length within the range of 3.5 to 4.0; and width ranging between 1.5 µm and1.6 µm. The average length for *Bt* isolates from Gazi was 3.6 µm while those from Machakos were 3.85 µm (Fig. 7 and Table 1).

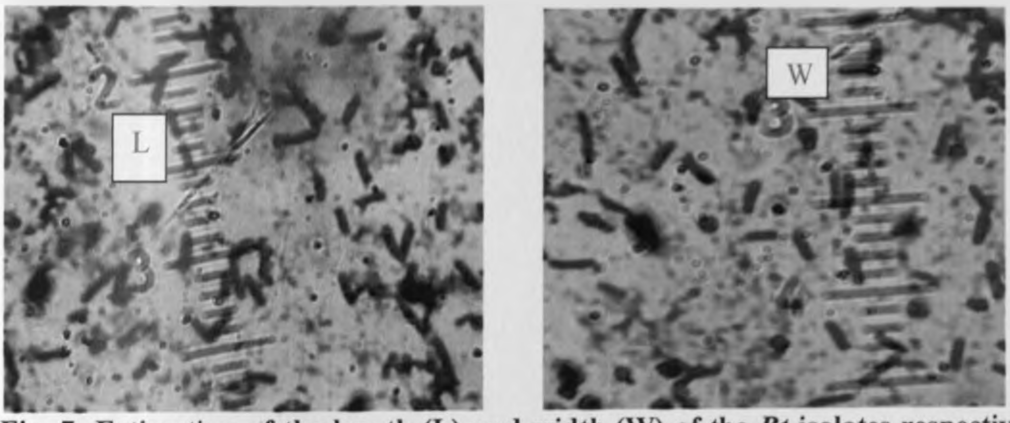


Fig. 7: Estimation of the length (L) and width (W) of the *Bt* isolates respectively. Mg x1000

4.3. Characterization of the *Bt* isolates based on biochemical tests

Biochemical tests included catalase test and Voges-Proskauer (VP) test. The production of oxygen as evidenced by air bubbles which rekindled a glowing splint showed the presence of catalase enzyme contained in the obtained *Bt* isolates. 90% of the *Bt* isolates obtained were positive for Voges-Proskauer (VP) test (Fig. 8; Table 1). The results for biochemical tests confirmed that the isolates were *Bt* in agreement with the findings of (Sergio *et al.*, 1992), and (Hossain *et al.* 1997).

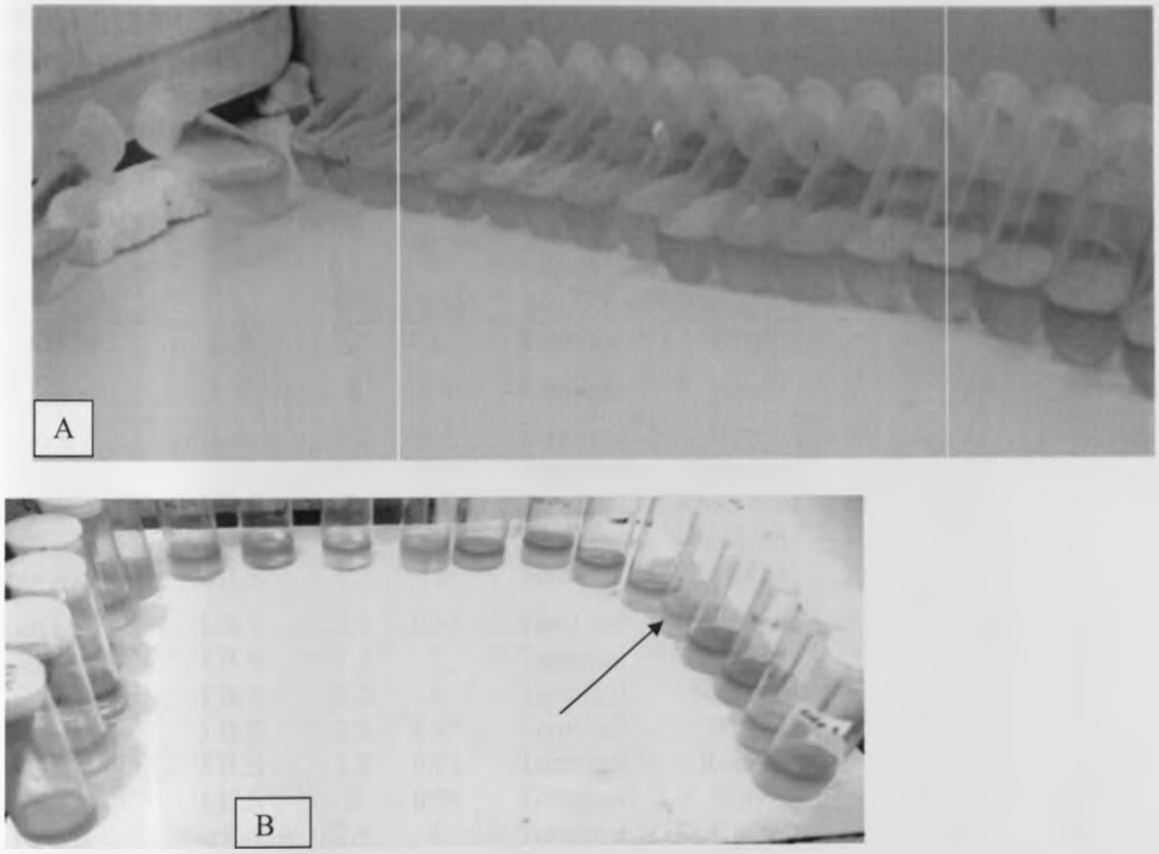


Fig. 8: Voges-Proskauer (VP) test. Result for *Bt* isolates from Gazi mangroves; (B) Result for the *Bt* isolates from Machakos. The colour change is due to production of acetylmethylcarbinol (AMC). The arrow points at negative result for the VP test

Table 1: Morphological and biochemical characteristics of the obtained *Bt* isolates. Length and width are averages of 10 cells. (+) Minimal; (++) Moderate; (+++) Very Positive; (-) Negative; (Bipy)-Bipyramidal; (I.S) Intertidal sediments; (I.B.S) Intertidal Brackish sediment (V.P) Voges-Proskauer; (G.S) Gram stain

Isolate. Code	Soil source	Morphological Characteristics					Biochemical Tests	
		L (µm)	W (µm)	Spore position	Crystal Shape	G.S	Catalase	V.P
1.KG10	I. S	3	1	Terminal	Bipy	+	+	+
2 .KG12-0	I. S	3	1.5	Terminal	Bipy	+	+	+
3. KG.20	I. S	2.2	1.5	Terminal	Bipy	+	+	++
4 KG 21	I. S	1.6	0.9	Terminal	Circular	+	+	++
5 KG 420	I.B.S	2	0.9	Terminal	Rhomboid	+	+	++
6 KG.410	I.B.S	2.3	1	Terminal	Squared	+	+	++
7.KG.411	I.B.S	1.7	1.6	Terminal	Squared	+	+	+
8 KG 412	I.B.S	2.1	0.97	Terminal	Rhomboid	+	+	++
9 KG 22	I.B.S	2.6	1	Terminal	Bipy	+	+	++
10.KG 413	I.B.S	2 .5	1	Terminal	Squared	+	+	+
11.KG.14	I.B.S	2.5	0.97	Terminal	Bipy	+	+	+++
12.KG 415	I.B.S	1.5	0.83	Terminal	Round	+	+	++
13.KG 416	I.B.S	2	0.98	Terminal	Bipy	+	+	++
14 .KM 10	Machakos	2.9	1	Terminal	Bipy	+	+	++
15.KM.11	Machakos	3	1	Terminal	Bipy	+	+	++
16.KM.12	Machakos	1	0.9	Terminal	Bipy	+	+	++
17.KM.13	Machakos	2	0.8	Terminal	oval	+	+	+
18.KM.14	Machakos	2	1	Terminal	oval	+	+	++
19.KM.15	Machakos	2	1	Terminal	Bipy	+	+	++
20.KM.16	Machakos	2	1	Terminal	Bipy	+	+	++
21.KM.17	Machakos	3	0.9	Terminal	Squared	+	+	++
22.KM.18	Machakos	2.5	1	Terminal	Bipy	+	+	++
23..KM.20	Machakos	2.8	1	Terminal	Round	+	+	+++
24.KM.21	Machakos	3	1	Terminal	oval	+	+	++
25.KM.22	Machakos	1.5	1	Terminal	oval	+	+	++
26.KM.24	Machakos	2.3	0 .9	Terminal	Bipy	+	+	+++
27.KM.31	Machakos	2.5	1	Terminal	Bipy	+	+	++
28.KM.30	Machakos	2	1	Terminal	Bipy	+	+	++

4.4. Determination of early growth patterns under different parameters

4.4.1. Determination of tolerance to temperature

The *Bt* isolate KM 31 from Machakos recorded high average growth both at 25° C and 30° C while isolate KM 24 had moderate growth at all temperatures except at 30° C. *Bt* isolates KG 411, KG 12-0 and KG 20 from Gazi had highest growth at 30° C whereas low optical densities were observed at 25° C and 40° C. Optical density for isolates KG 12-0 and KG 411 was minimal until after 36 hrs at both 25° C and 40° C, (Table 2).

Table 2: Effect of temperature on early growth of the *Bt* isolates.

(+) Minimal growth; (++) Moderate growth; (+++) High growth; (-) No growth

SOURCE AREA OF SOIL SAMPLE	*Bt* ISOLATE	GROWTH DURATION(HRS)	TEMPERATURE C			
			25	30	35	40
MACHAKOS	KM 31	12	++	+	+	++
		24	+++	+	++	+
		36	+++	+++	+	++
		48	+++	++	+	++
	KM 24	12	++	++	+	++
		24	+	+	+	+
		36	+	+++	++	+
		48	++	++	+	+
GAZI	KG 411	12	_	+++	+	_
		24	_	++	+	+
		36	+	+++	+	+
		48	+	++	+	+
	KG 12-0	12	_	+++	+	_
		24	+	++	+	+
		36	+	++	+	+
		48	+	++	+	+
	KG 20	12	_	++	+	+
		24	+	++	+	+
		36	+	+++	+	+
		48	+	+	_	_

4.4.2 Determination of tolerance of *Bt* isolates to pH

Analysis for sensitivity to pH by isolates from Machakos revealed a high level of sensitivity with very minimal optical density (0.05) at pH 4.5 while highest optical density of 4.9 was at pH 7.5. Isolate KM 24 required a pH range of 6.5 - 7.5 for average growth with optical density of 0.5 and 0.4 respectively. The lowest optical density was 0.07 at pH 4.5. The maximum optical density for isolate KG 411 within 24 and 48 hours of inoculation was 0.37 and 0.33 respectively at pH 7.5. The lowest optical density of 0.02 was attained at pH 4.5. This indicates that isolate KG 411 had a high sensitivity to pH with optimal pH of 7.5. The optimal pH for KG 12-0 was 7.5 with an average optical density of 0.36 and 0.33 at 48 and 24 hrs respectively. KG 20 had a wide pH range of 6.5 to 8.5 with optical density of 0.57 and 0.45 respectively. Minimum optical density of 0.09 was recorded at pH 4.5 (Fig. 9).

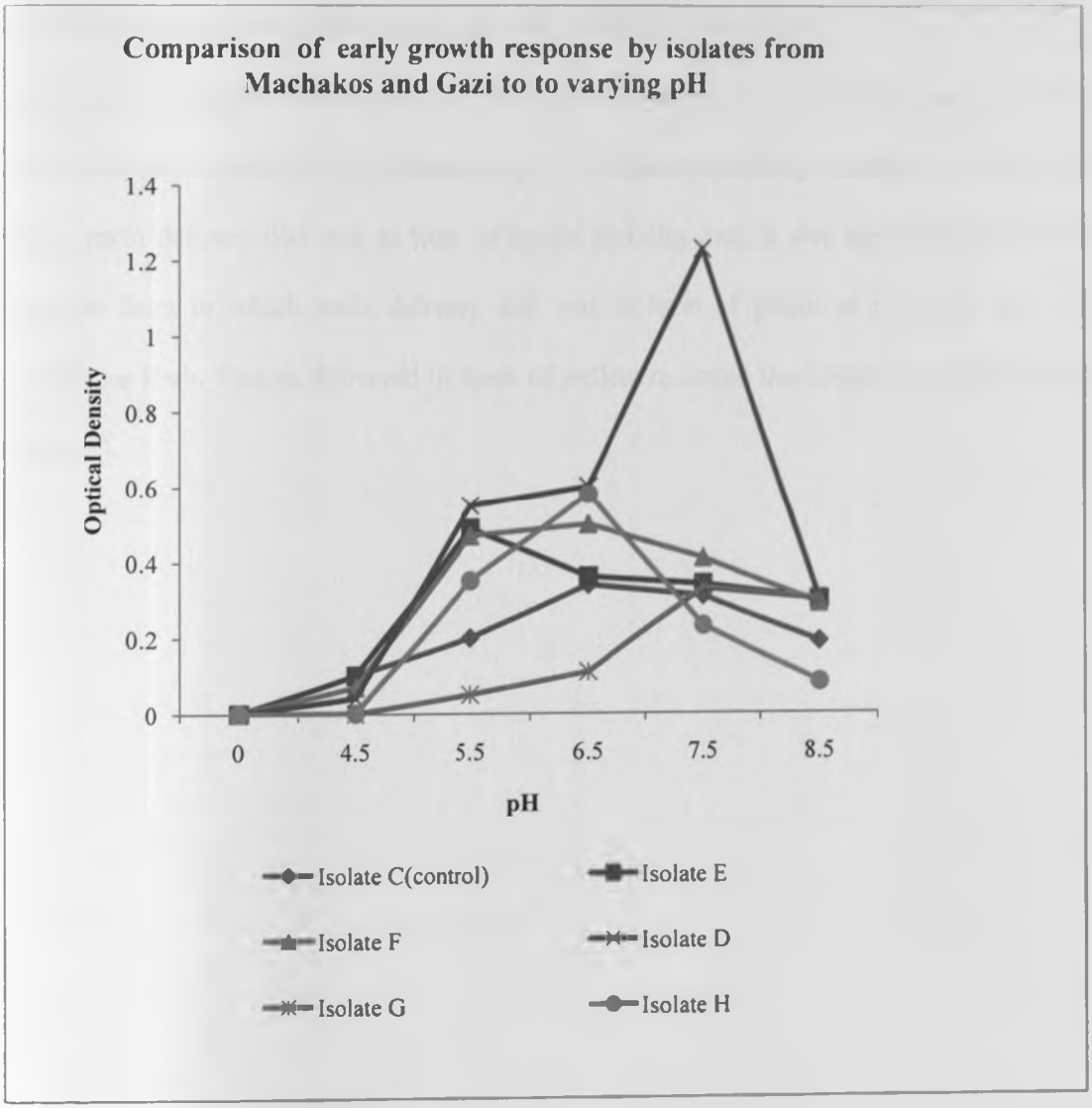


Fig. 9: Effect of varying pH on early growth of the *Bt* isolates

KEY: Isolates - (C) *Bt* var *tenebrionis*, (E) *Bt* KM 31, (F) *Bt* KM 24, (D) KG 411, (G) KG 12-0 and (H) KG 20

4. 5. Determining best type of maize diet for delivery of *Bt* toxins

There were significant differences at 95 % confidence limit and $p< 0.001$ in mean mortality due to different modes of toxin diet delivery. The highest percentage mortality was recorded where toxin delivery diet was in form of maize particles and; it was significantly different from the form in which toxin delivery diet was in form of grains at $p< 0.001$ and 95% confidence limit. Toxins delivered in form of pellets recorded the lowest average mortality (Table 3).

Table 3: Mean percentage mortalities with different type of *Bt* toxin delivery diet within 21 days of post treatment.

ISOLATE	TYPE OF MAIZE DIET AND PERCENTAGE MEAN MORTALITY OF *P. truncatus* GRAINS	PARTICLES	PELLETS
Saline water	2.5	2.5	2.3
Bt var israelensis	28.8	33.8	28.8
Bt var tenebrionis	46.5	57.5	34.6
KG 411	66.3	84.8	45.8
KM31	58.8	75.8	48.6
KM 24	58.3	71.3	43.1
KG 12-0	64	75.8	51.8
KG 20	56.3	79.3	50.4
KG420	47.8	66.9	46.3
KM 12	25.4	45.4	25.8
KG12	20.8	31.7	17.5
KG 11	16.7	29.6	3.3
KG 14	26.2	30.4	9.6
KG 15	22.5	24.6	15.8
KG 10	7.9	22.1	2.5
KG 415	20.8	20.4	12.9
KG413	17.9	22.5	3.3
KM 23	18.9	23.8	12.1
L.s.d	0.14		
P	<0.001		

4.5.1 Toxicity of *Bt* isolates against 30-days old *P. truncutus*

There were significant differences in cumulative average mortalities in the *Bt* isolates tested. The highest average mortalities regardless of the form of toxin diet delivery were due to *Bt* isolate KG 411 followed by KG 12-0, KG 20 and KM 31.The Average mortalities due to *Bt* isolate KG 420 and KM 24 were moderate but still higher than the positive control *Bt tenebrionis* which caused 46.2% cumulative average mortalities. The rest of the isolates registered low mortalities which were still higher than mortalities recorded in saline water. All the isolates with above 50% average mortality were selected for further analysis to determine 50% lethal dose concentration (LD_{50}). These included *Bt* isolates KG 411, KG 12-0 and KG 20 (from Gazi); isolates KM 31and KM 24 from Machakos (Fig. 10).

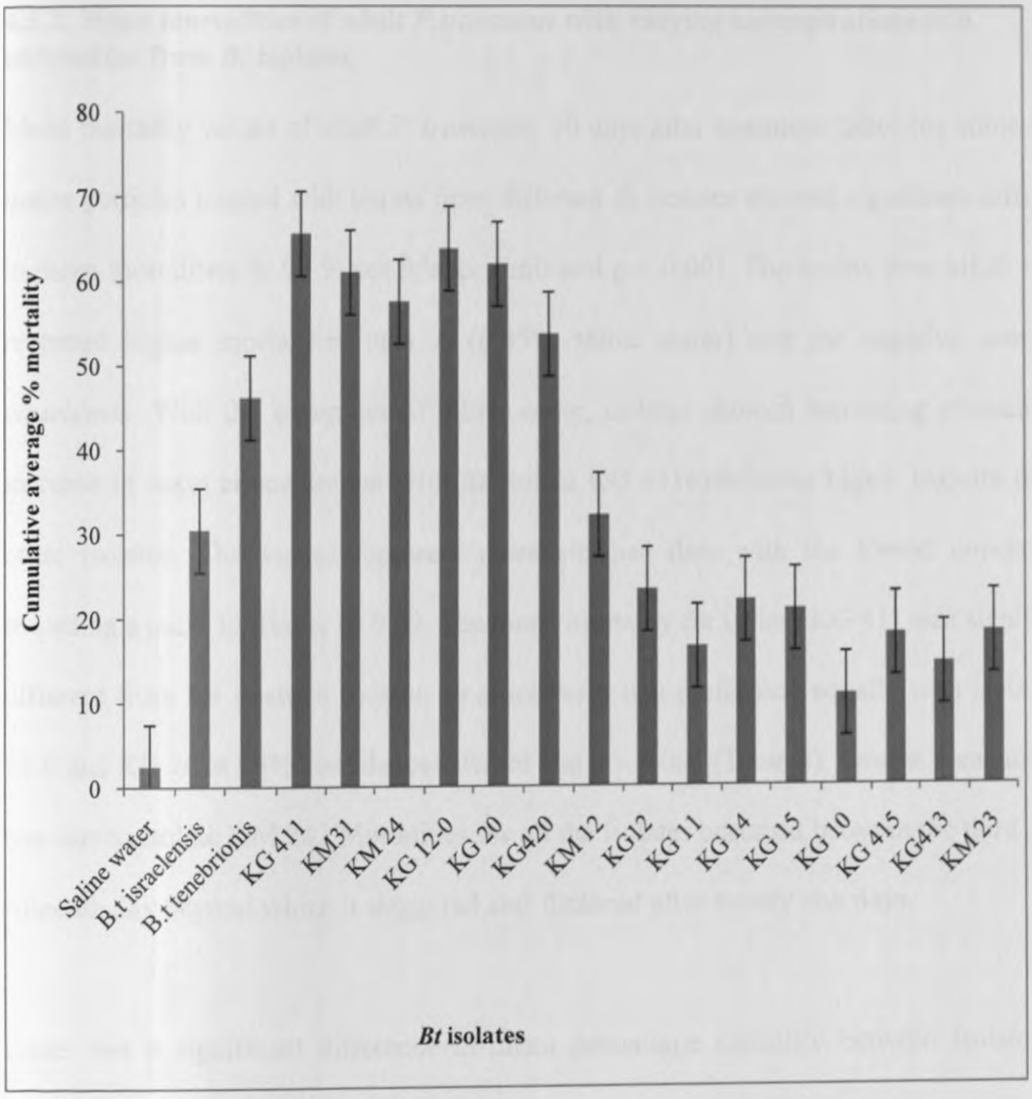


Fig. 9: Toxicity of *Bt* isolates against 30-days old *P. truncutus*

4.5.2. Mean mortalities of adult *P.truncatus* with varying concentrations of δ endotoxins from *Bt* isolates

Mean mortality values of adult *P. truncatus,* 30 days after treatment following subjection to maize particles treated with toxins from different *Bt* isolates showed significant differences in mean mortalities at 95 % confidence limit and $p < 0.001$. The toxins from all *Bt* isolates recorded higher mortalities than A (0.85% saline water) and the negative control, *Bt israelensis*. With the exception of saline water, isolates showed increasing mortality with increase in toxin concentration with *Bt* isolate KG 411exihibiting higher toxicity than the other isolates. The values represent untransformed data with the lowest concentration recording a mean mortality of 0.39. The mean mortality for isolate KG 411 was significantly different from the positive control, *Bt tenebrionis* that performed equally with isolates KG 12-0 and KG 20 at 95% confidence interval and $p < 0.001$ (Table 4). Lowest mean mortality was due to isolate KM 24. Mortalities for all the isolates occurred between the third and the fifteenth day beyond which it staggered and flattened after twenty one days.

There was a significant difference in mean percentage mortality between isolates from Machakos. Isolate KM 31 caused the highest mortality while KM 24 registered the lowest mortality at 95% confidence interval $p < 0.001$. Analysis to determine LD_{50} for isolates KM 31 and KM 24 showed significant slopes at 95% confidence limit and $p< 0.001$, indicating increase in deaths with increase in toxin concentration (Fig. 11 and Table 4).

Table 4: Mean mortality of *P. trancutus* with increasing concentrations of *Bt* δ-endotoxins from the different *Bt* isolates

	P. truncatus Mean mortalities with increasing concentration of *Bt* toxins (mg/ml)				
Bt isolate	1	2.5	5	7.5	10
Sterile distilled water	0	0	0.08	0.21	0.29
Bt israelensis	0.43	1.04	1.94	2.04	2.3
Bt tenebrionis	2.51	4.35	4.67	5.33	6.49
KG 411	5.93	7.07	7.3	7.4	7.97
KM 31	4.68	5.24	6.14	6.84	7.23
KM 24	4.28	4.42	4.54	4.98	5.6
KG 12-0	4.34	5.07	5.68	6.63	6.72
KG 20	4.81	5.32	5.75	6.25	6.65
Grand mean		4.46			
d.f		1160			
L.s.d		0.23			
C.v		29.1			
P		<0.001			

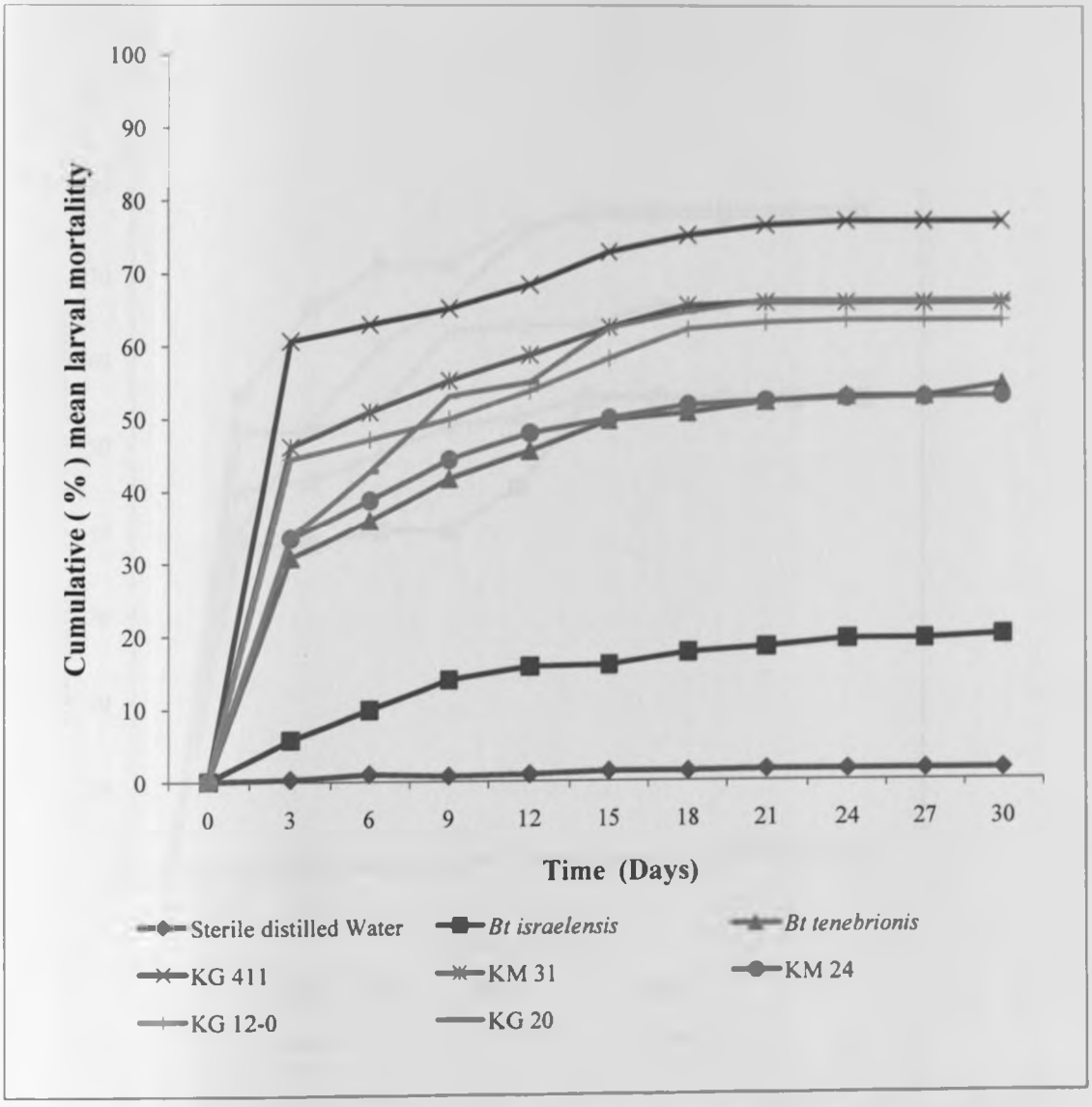


Fig. 11: Mean percentage mortality of adult *P. truncutus* exposed to different *Bt δ* - endotoxins from different isolates against time.

Where control mortality exceeded 5%, but not higher than 10%, data was kept and analyzed by probit analysis after adjusting for control mortality (Abbott's, 1925)

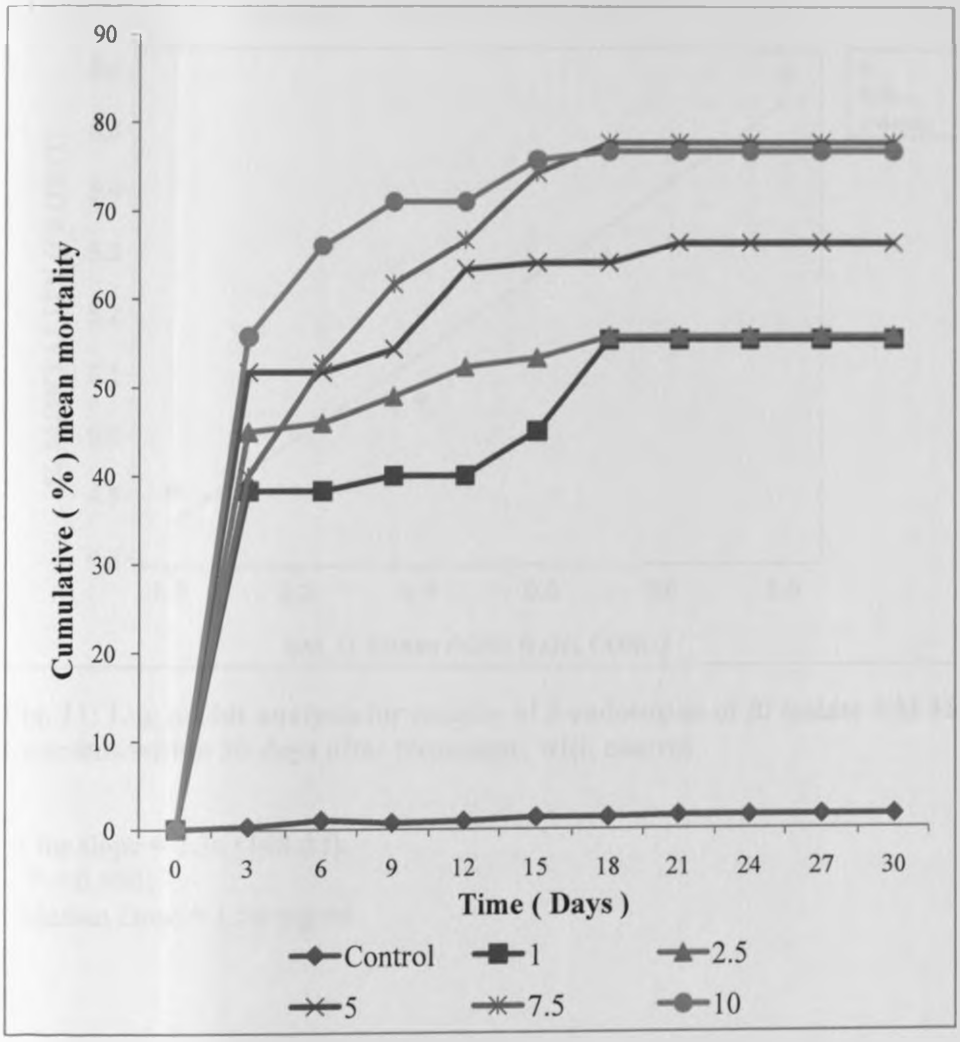


Fig. 10: Percentage mortality of adult *P. trancutus* exposed to different concentrations of *Bt* δ-endotoxin of *Bt* isolate KM 31.

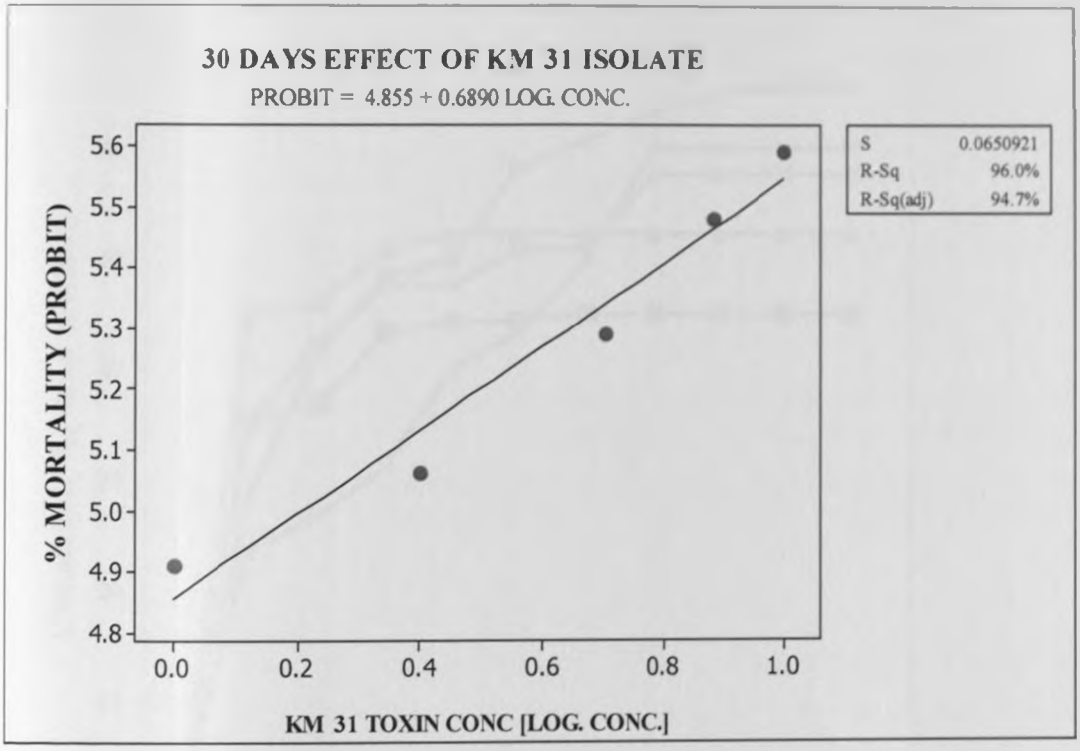


Fig. 11: Log probit analysis for toxicity of δ endotoxins of *Bt* isolate KM 31 on adult *P. truncatus* within 30 days after treatment; with control

t for slope = 7.36 (148 d f)
P < 0.0001
Median Dose = 1.58 mg/ml

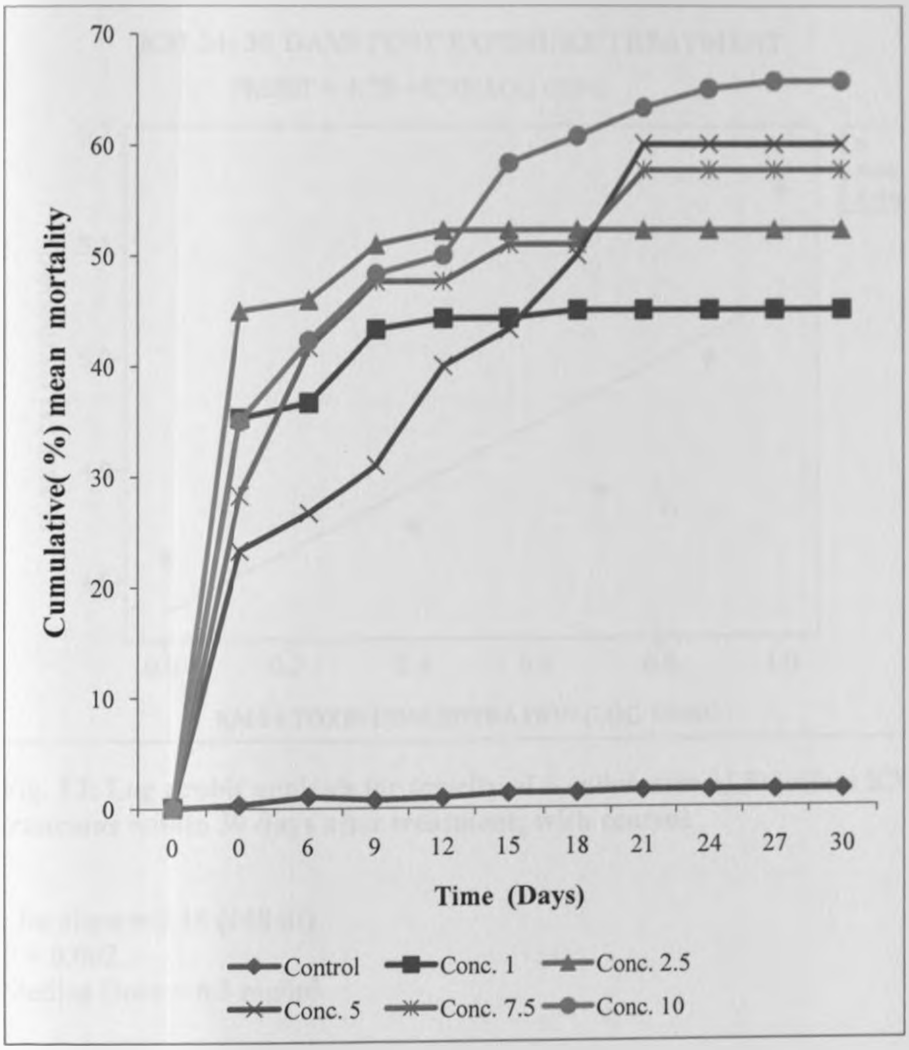


Fig. 12: Percentage mortality of adult *P. trancutus* exposed to different concentrations of δ-endotoxin of *Bt* isolate KM 24.

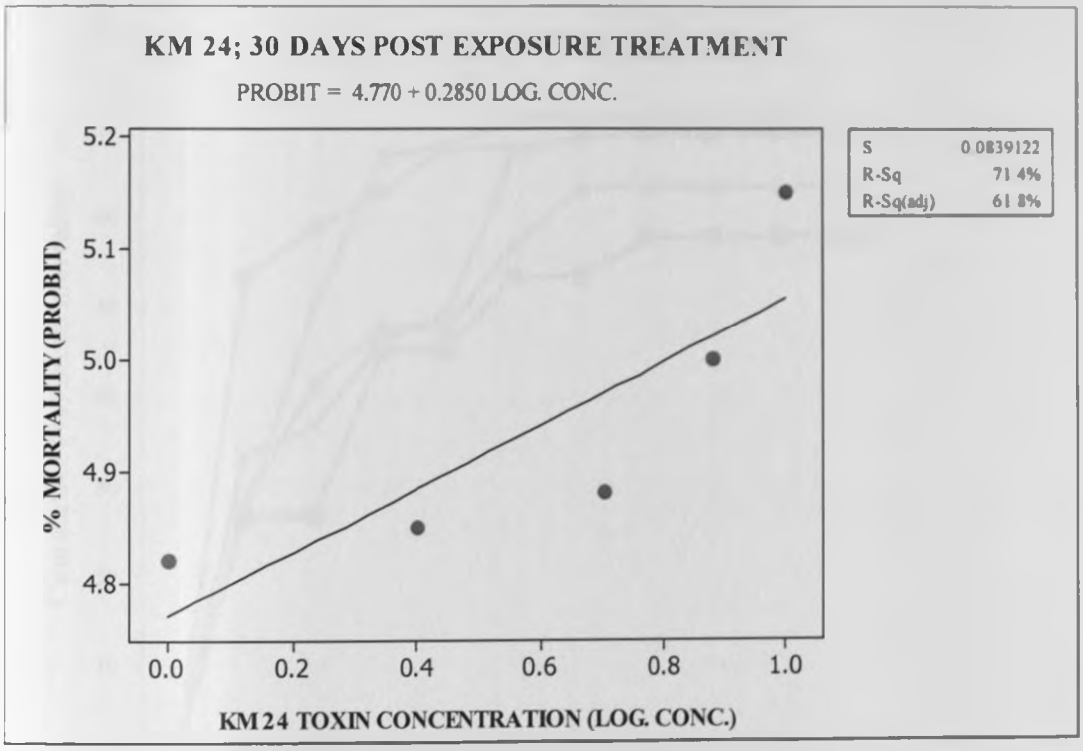


Fig. 13: Log probit analysis for toxicity of δ endotoxins of *Bt* isolate KM 24 on adult *P. truncatus* within 30 days after treatment; with control.

t for slope = 3.16 (148 df)
P = 0.002
Median Dose = 6.3 mg/ml

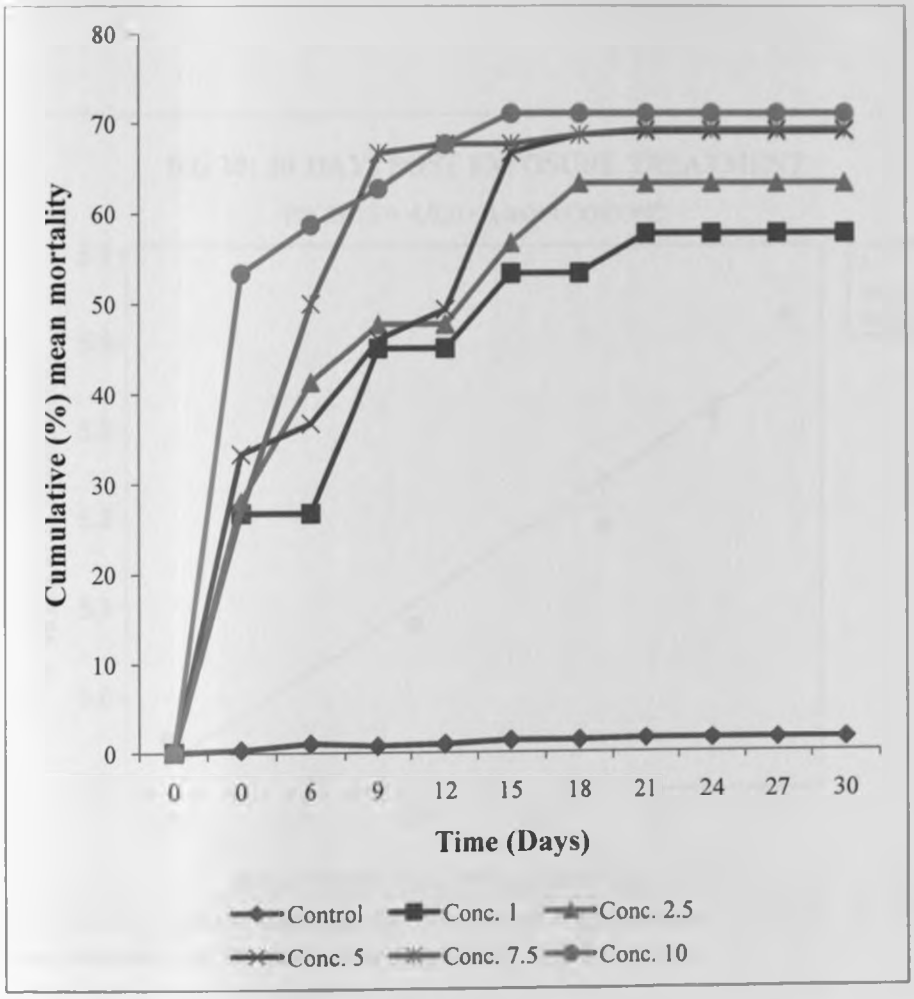


Fig. 14: Percentage mortality of adult *P. trancutus* exposed to different concentrations of δ-endotoxin of *Bt* isolate KG 20.

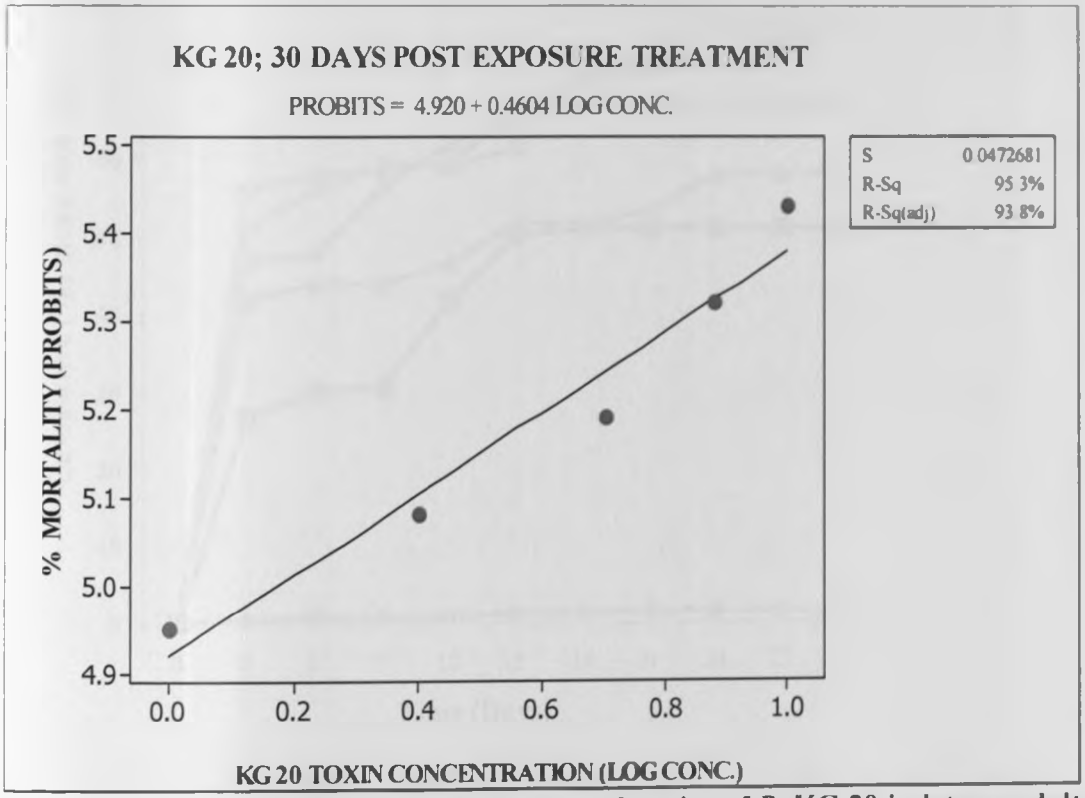


Fig. 15: Log probit analysis for toxicity of δ endotoxins of *Bt* KG 20 isolate on adult *P. truncatus* within 30 days after treatment; with control.

Median Dose = 4.01 mg/ml
t for slope = 4.9 (148 df)
P < 0.0001

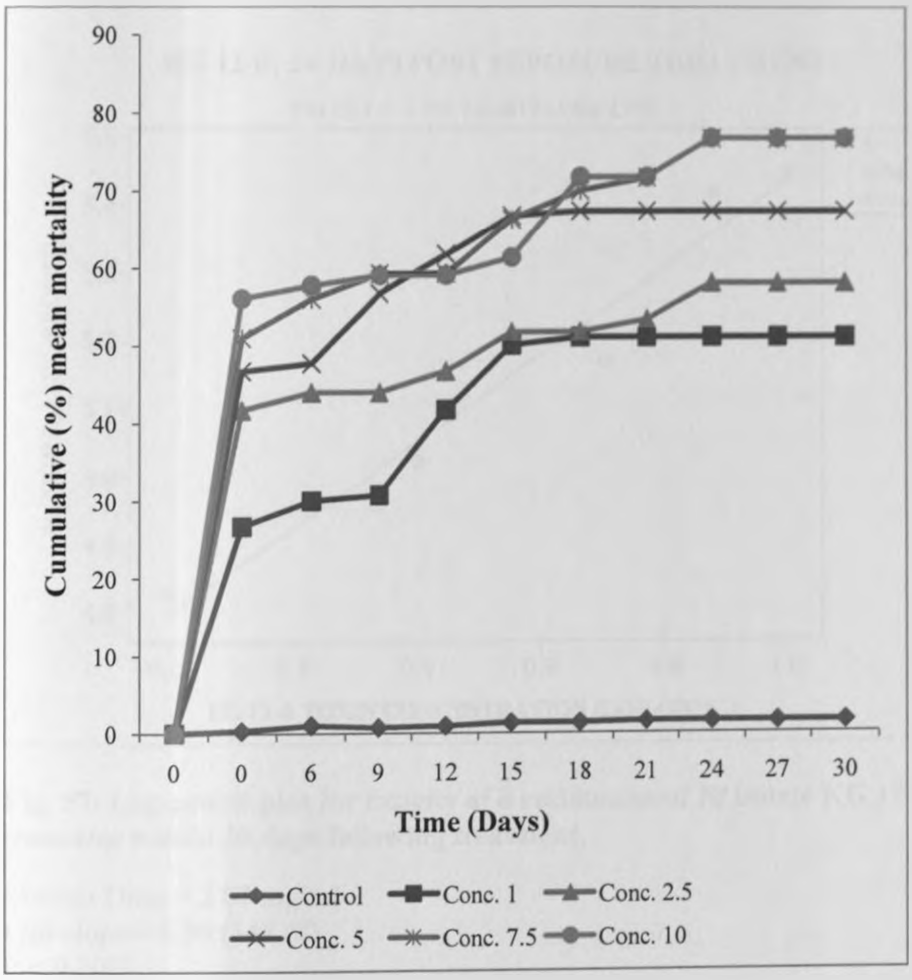


Fig. 16: Percentage mortality of adult *P. truncatus* exposed to different concentrations of δ-endotoxin of *Bt* isolate KG 12-0.

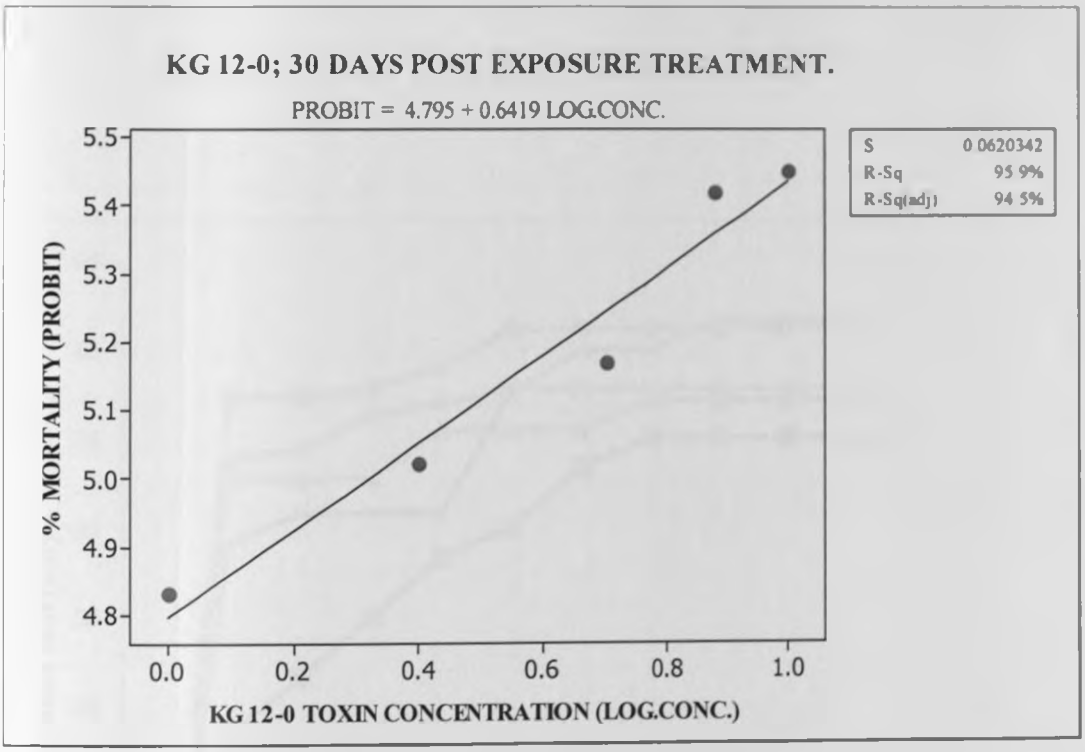


Fig. 17: Log-probit plot for toxicity of δ endotoxins of *Bt* isolate KG 12-0 on adult *P. truncatus* within 30 days following treatment.

Median Dose = 2.07 mg/ml
t for slope = 6.96 (148 df)
$P < 0.0001$

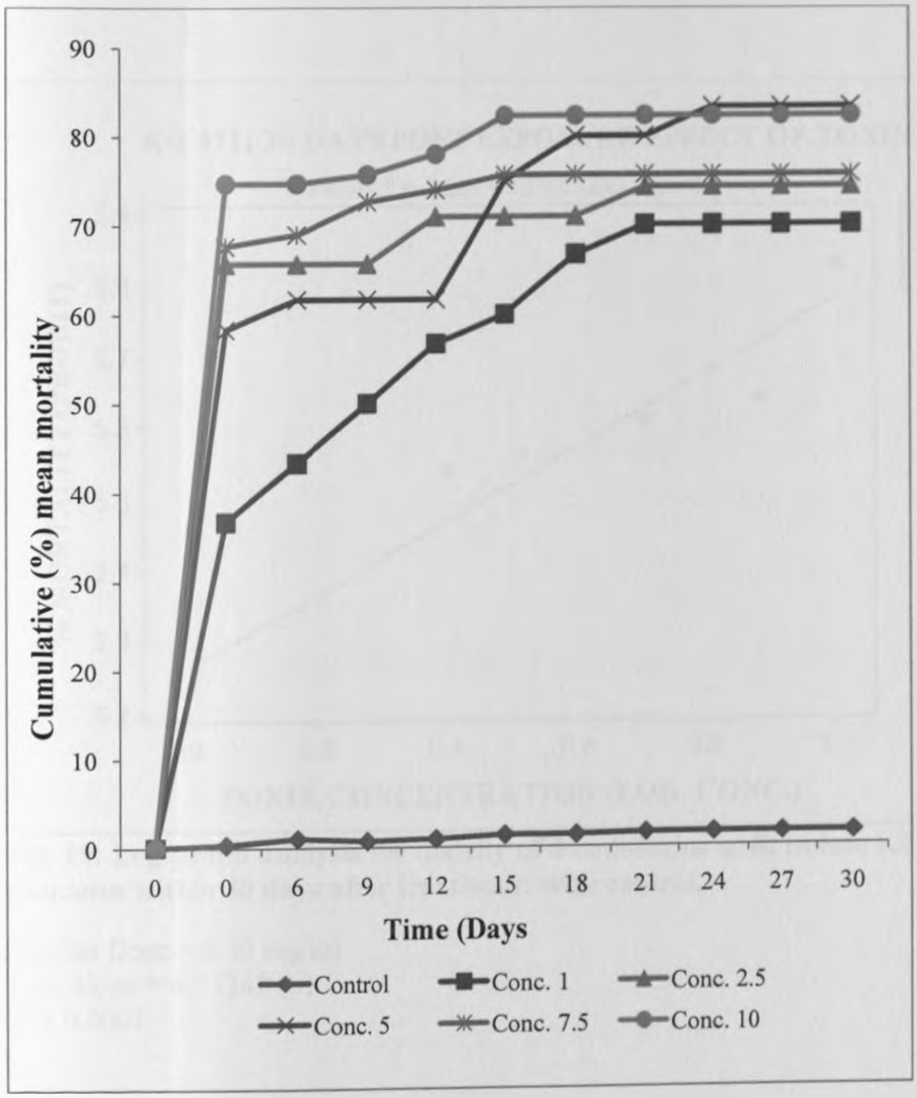


Fig. 18: Percentage mortality of adult *P. trancutus* exposed to different concentrations of δ-endotoxin of *Bt* isolate KG 411.

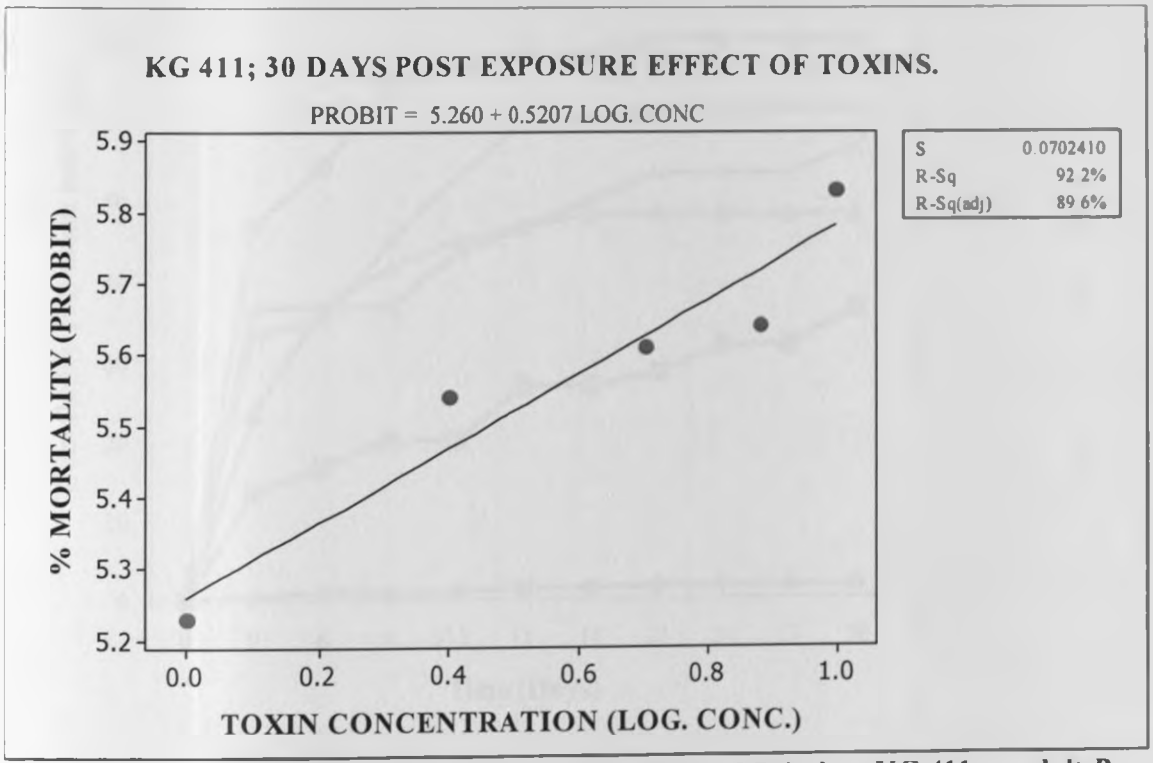


Fig. 19: Log probit analysis for toxicity of δ endotoxins of *Bt* isolate KG 411 on adult *P. truncatus* within 30 days after treatment; with control.

Median Dose = 0.30 mg/ml
t for slope = 4.5 (148 df)
P < 0.0001

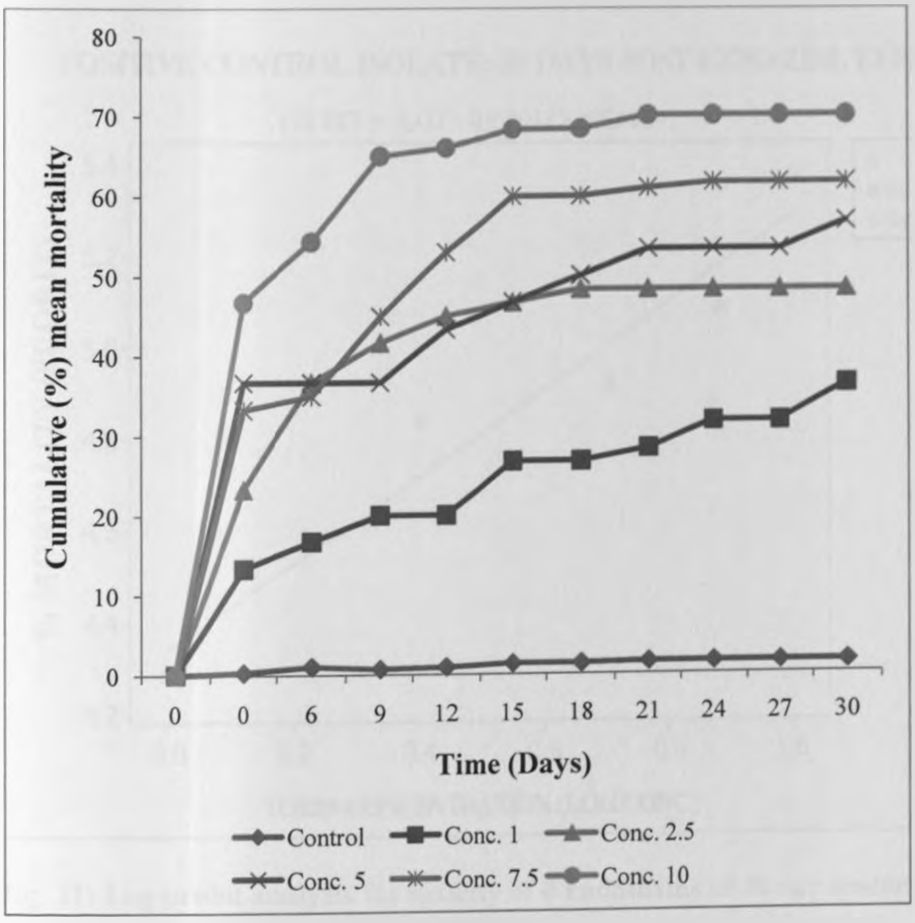


Fig. 20: Percentage mortality of adult *P. trancutus* exposed to different concentrations of δ-endotoxin of *Bt var tenebrionis*

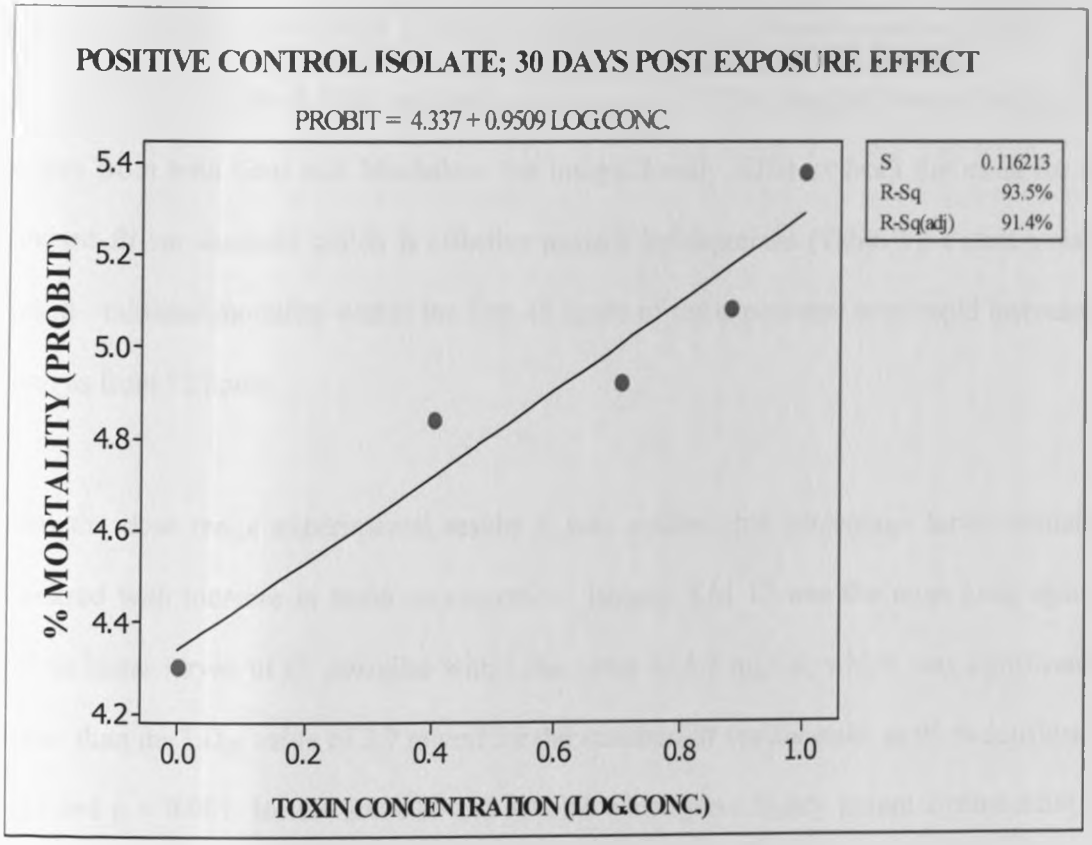


Fig. 21: Log probit analysis for toxicity of δ endotoxins of *Bt* var *tenebrionis* on adult *P. truncatus* within 30 days after treatment.

Median Dose = 4.9 mg/ml
t for slope = 9.79 (148 df)
$P < 0.0001$

4.6 Toxicity of *Bt* isolates against *C. partellus* second instar larvae

Results on effect of different *Bt* isolates on mortality of second instar larvae of *C. partellus* recorded after every 24 hrs illustrated that the isolates were averagely potent and had varying degree o f toxicity. Isolate KM 12 had the highest cumulative mean mortality of 5.05. This was significantly high at 95% confidence limit and $p < 0.001$ than the means for all other isolates from both Gazi and Machakos; but insignificantly different from the mean for the standard *Bt var kurstaki* which is effective against lepidopterans (Table 5). Potent isolates caused minimal mortality within the first 48 hours of the experiment with rapid increase in death as from 72 hours.

From the dose range experimental results it was evident that percentage larval mortality increased with increase in toxin concentration. Isolates KM 12 was the most toxic against second instar larvae of *C. partellus* with LD_{50} value of 4.7 mg/ml; which was significantly higher than the LD_{50} value of 3.7 mg/ml for the standard *Bt* var *kurstaki* at 95 % confidence limit and $p < 0.001$. Isolate KG 411 and KM 31 which were highly potent against adult *P. truncatus* were less potent against second instar larvae of *C. partellus* with LD_{50} of 6.7 mg/ml and 8.2 mg/ml respectively. Higher doses of the toxins were required against the larvae.

Table 5: Mean mortalities of second instar larvae of *C. partellus* due to effect of δ-endotoxins from the different *Bt* isolates.

ISOLATE CODE	TIME (HOURS) AND AVERAGE MEAN MORTALITY						
	24	48	72	96	120	MEAN	%MEAN
0.85% Saline H20	0	0	0.3	0.4	0.7	0.7	7
Bt tenebrionis	0.5	2.57	4.23	5.17	7.73	3.37	33.7
Bt var kurstaki	2.21	4.51	7.67	8.53	9.8	5.46	54.6
KM.31	1	3.85	5.27	6.01	7.85	4.01	40.1
KM.24	1.57	2.51	3.5	4.43	7.16	3.2	32
KG.12-0	0.97	2.92	4.03	5.91	8.18	3.67	36.7
KG. 20	1	2.8	4.71	7.01	7.29	3.8	38
KG. 411	2	3.6	5.21	6.24	8.21	4.21	42.1
KM.12	3.43	4.61	6.08	7.6	8.57	5.05	50.5
KM.23/14	0.5	1.95	4.87	6.08	7.55	3.49	34.9
GRAND MEAN	3.63						
	ISOLATE				HOURS	ISOLATE /HOURS	
L.s.d	0.56				0.44	1.38	
P	<0.001				<0.001	<0.001	
d.f	840				840	840	

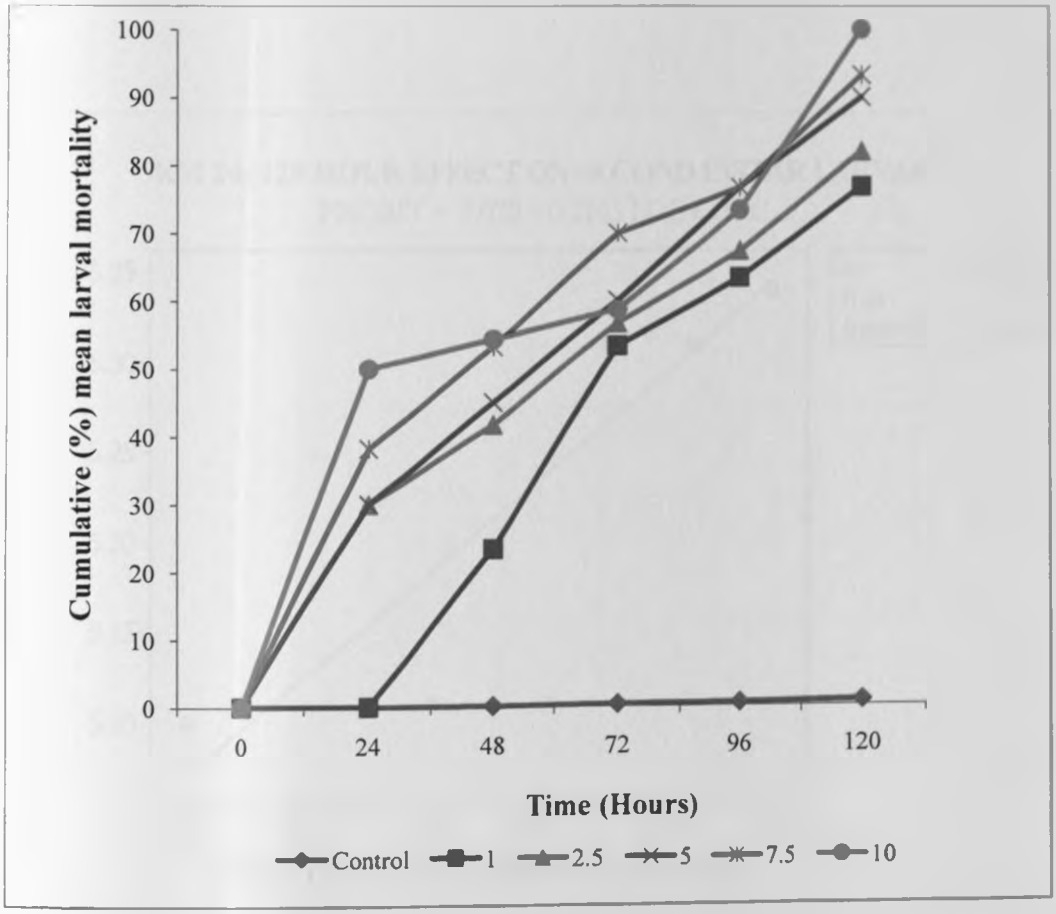


Fig. 22: Percentage mortality of second instar larvae of *C. partellus* at different concentrations of δ-endotoxin of *Bt* isolate KM 12

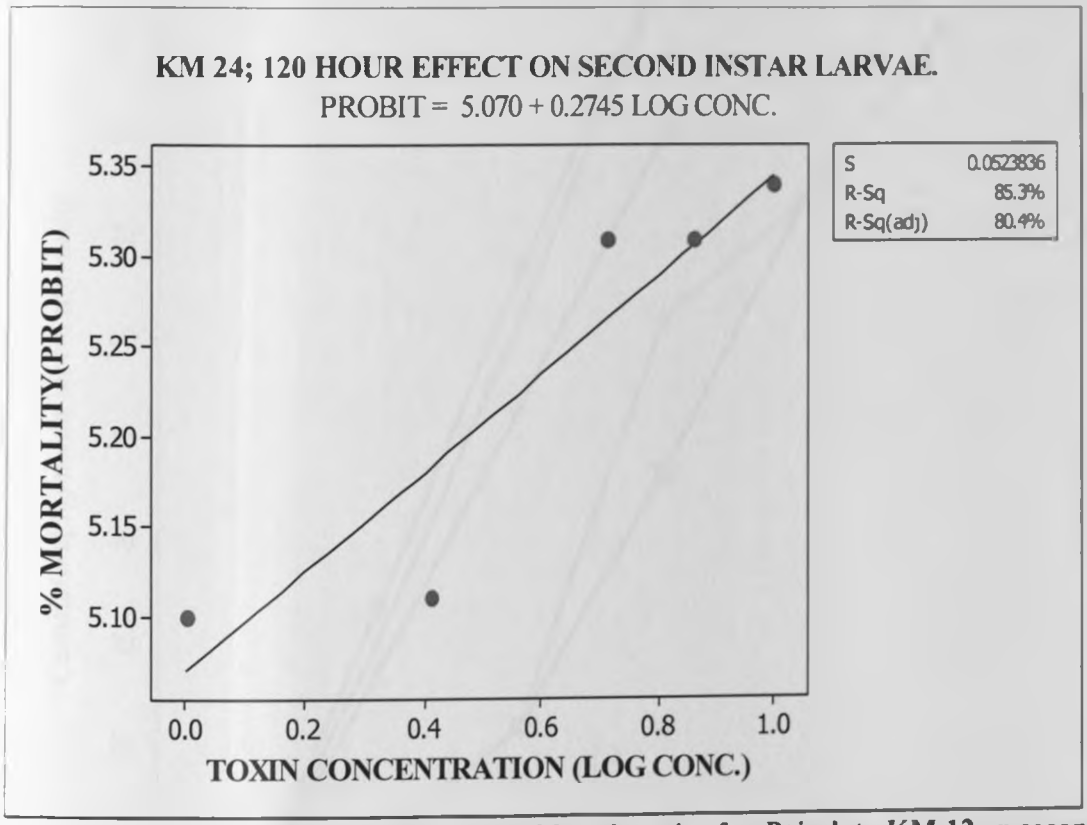


Fig. 23: Log probit analysis for toxicity of δ endotoxins for *Bt* isolate KM 12 on second instar larvae of *C. partellus* within 120 hours of exposure.

t for slope = 2.97 (73 df)
P = 0.02
Median Dose = 4.7 mg/ ml

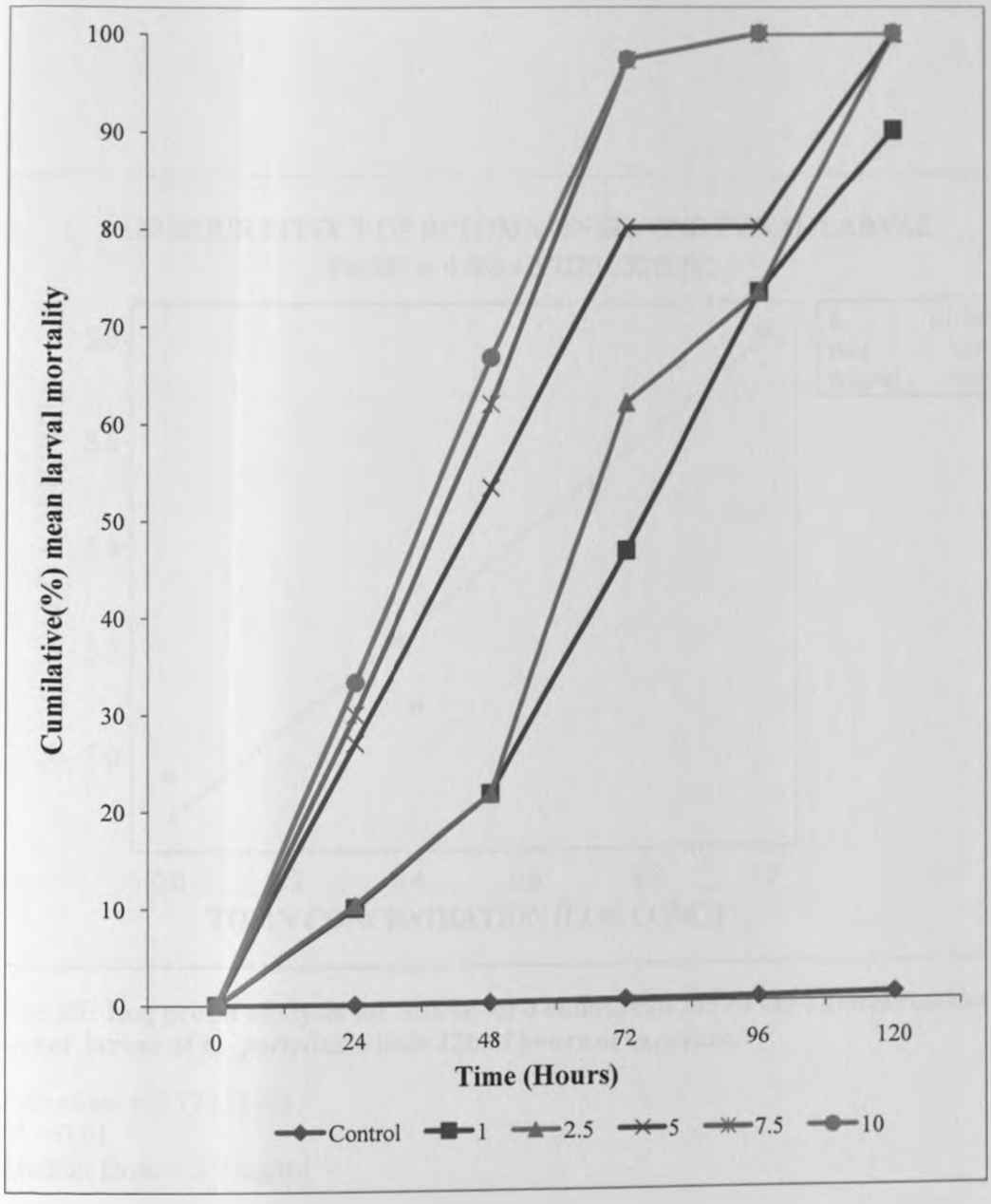


Fig. 24: Percentage mortality of 2nd instar larvae of *C. partellus* at different concentrations of δ-endotoxin of *Bt* var *kurstaki*

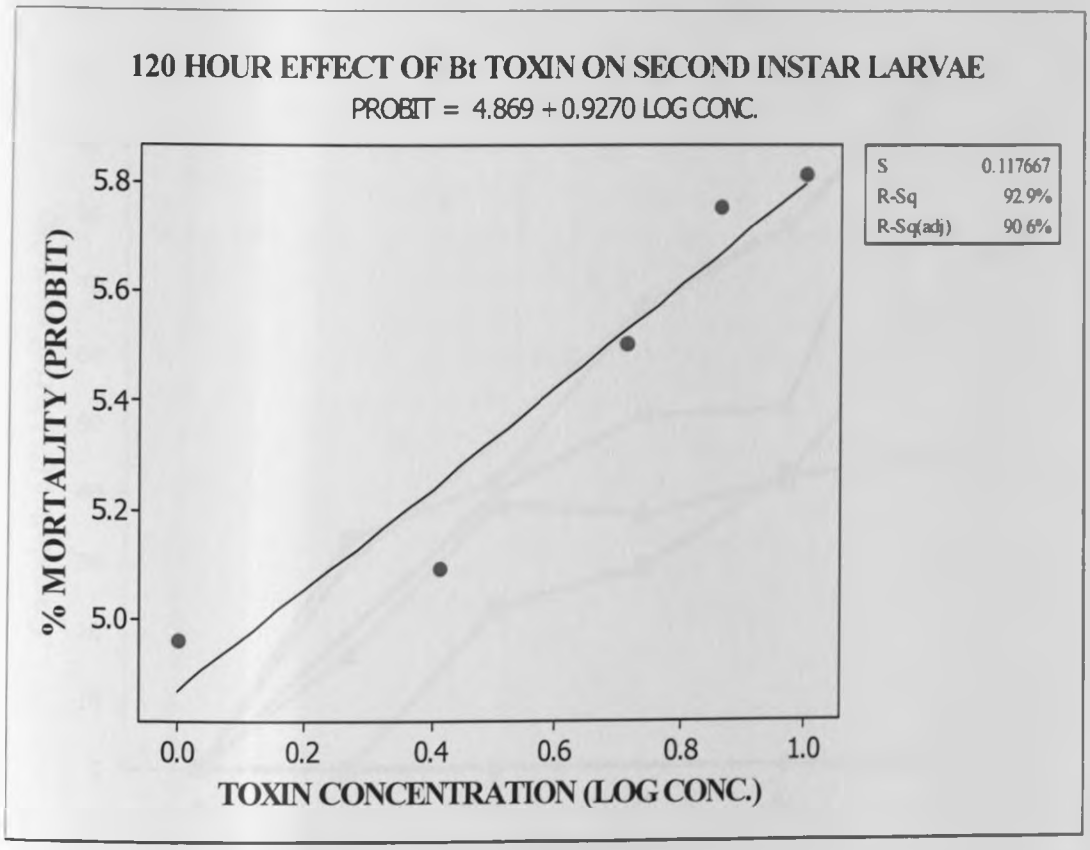


Fig. 25: Log probit analysis for toxicity of δ endotoxins for *Bt* var *kurstaki* on second instar larvae of *C. partellus* within 120 of hours of exposure.

t for slope = 2.77 (73 df)
P = 0.01
Median Dose = 3.7 mg/ml

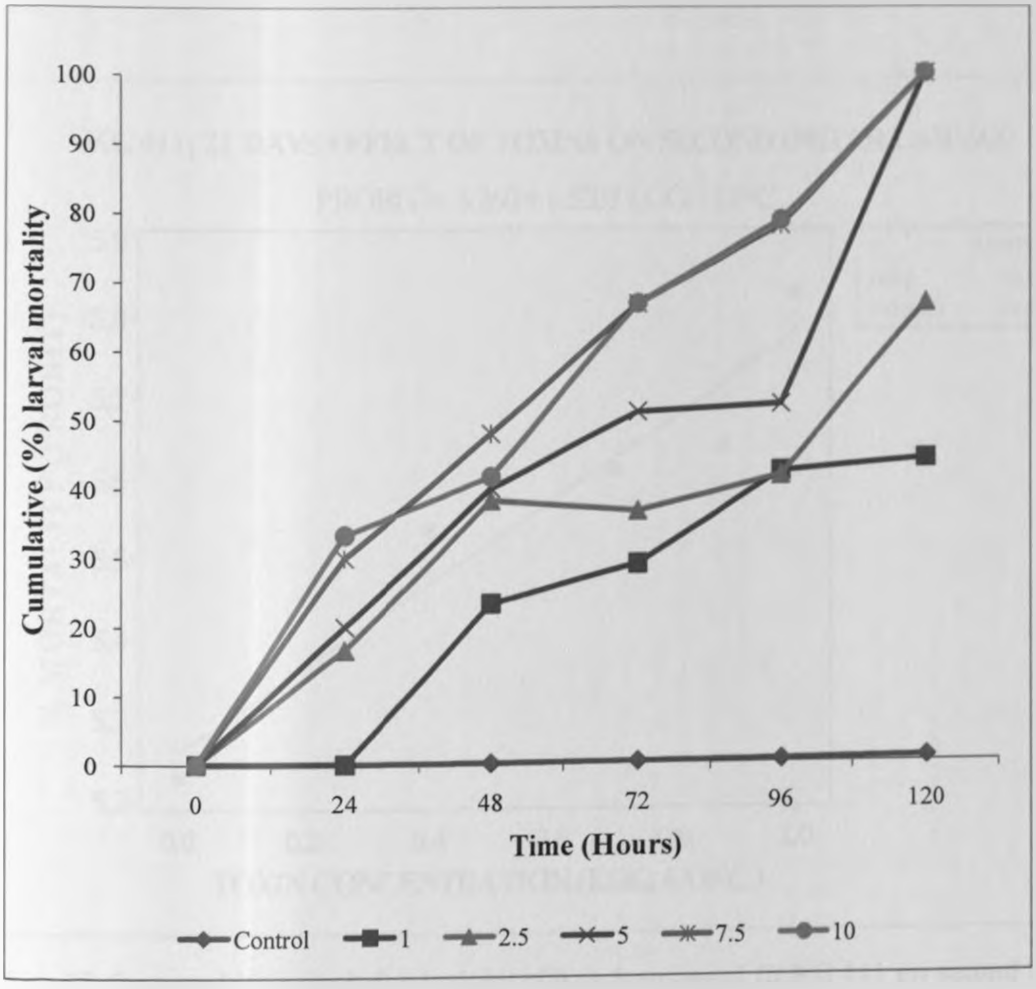


Fig. 26: Percentage mortality of second instar larvae of *C. partellus* at different concentrations of *Bt* δ-endotoxin of isolate KG 411.

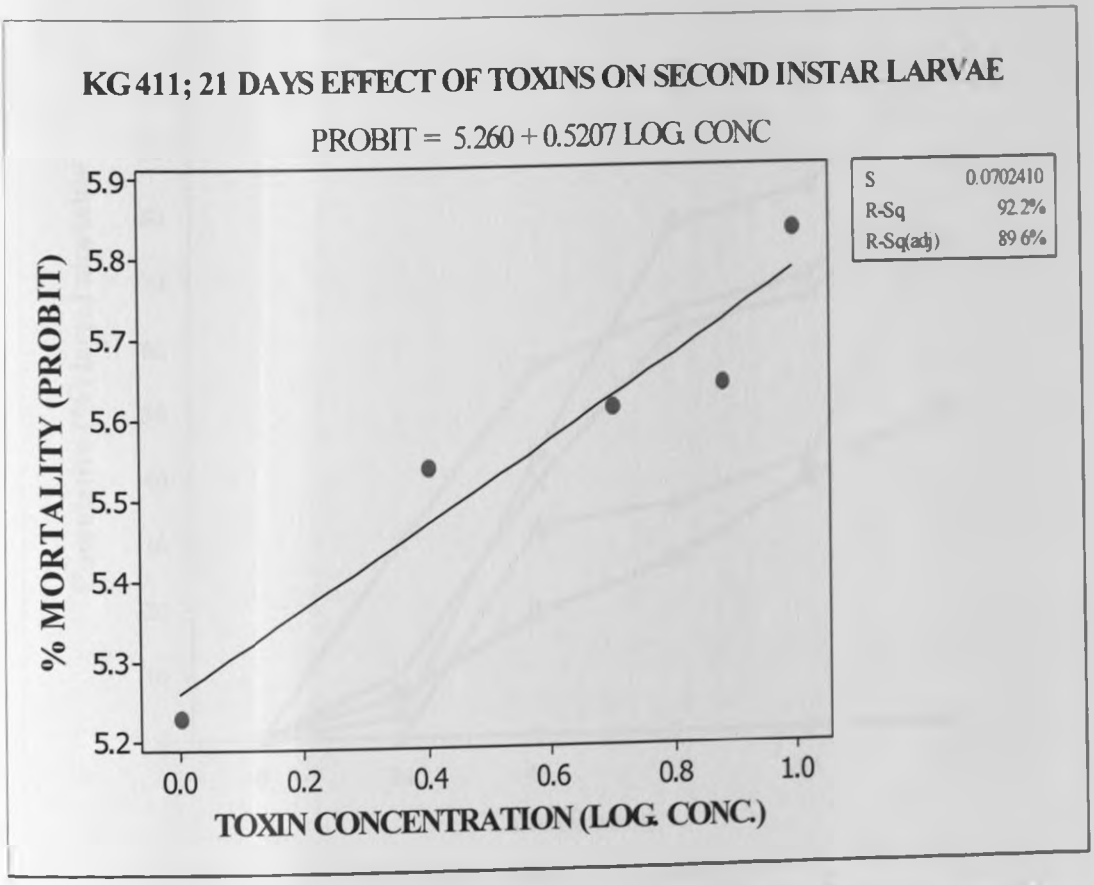


Fig. 27: Log probit analysis for toxicity of δ endotoxins of *Bt* KG 411 on second instar larvae of *C. partellus* within 120 hours of exposure.

t for slope = 3.44 (73 df)
P = 0.001;
LD50= 6.7 mg/ml

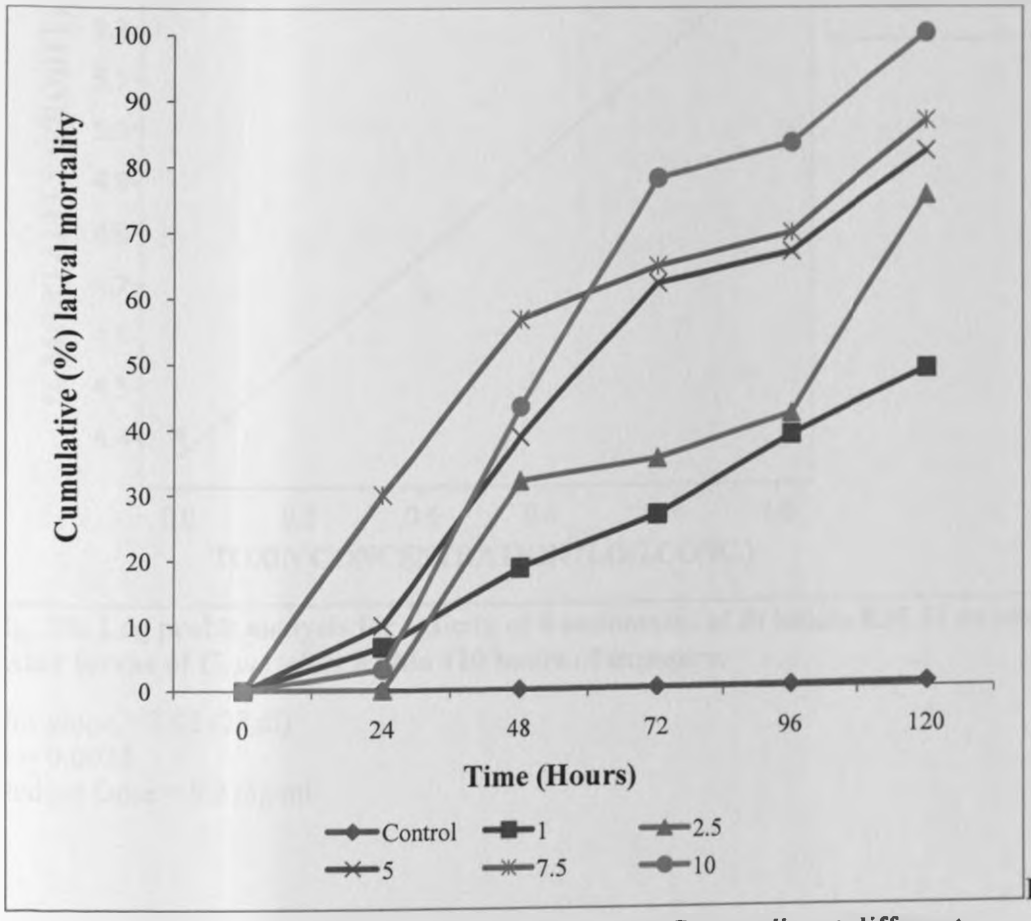


Fig. 28: Percentage mortality of second instar larvae of *C. partellus* at different concentrations of *Bt* δ-endotoxin of *Bt* isolate KM 31

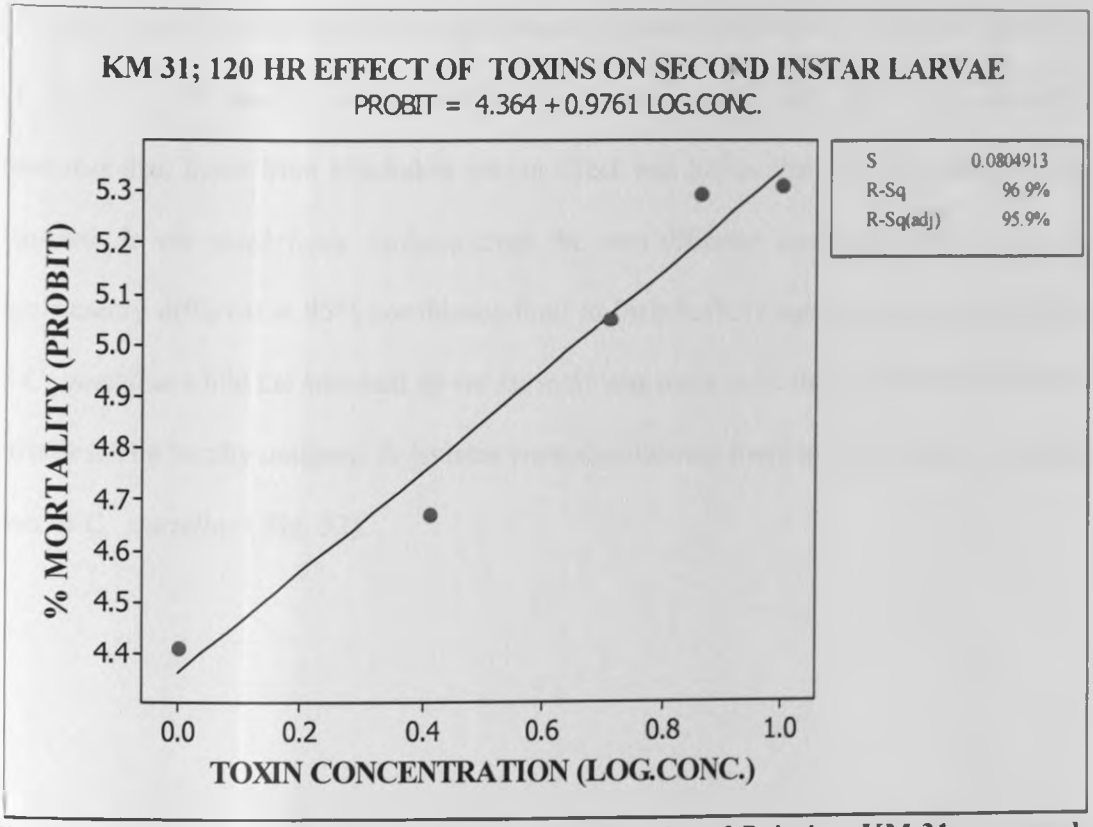


Fig. 29: Log probit analysis for toxicity of δ endotoxins of *Bt* isolate KM 31 on second instar larvae of *C. partellus* within 120 hours of exposure.

t for slope = 3.02 (73 df)
P = 0.0035
Median Dose = 8.2 mg/ml

A comparative analysis of locally obtained *Bt* isolates based on their ecological habitats and the target pests revealed significant differences in some case but not in others as shown in the figures 3o, 31 and 32. *Bt* isolates from Gazi were significantly more toxic to adult *P. truncatus* than those from Machakos whose effect was higher than for the positive control standard *Bt* var *tenebrionis*. Isolates from the two different ecological niches were not significantly different at 95% confidence limit in their toxicity against second instar larvae of *C. partellus* while the standard *Bt* var *kurstaki* was more toxic than the locally isolated *Bt* varieties. The locally obtained *Bt* isolates were significantly more toxic to adult *P. truncatus* than to C. *partellus* (Fig. 32).

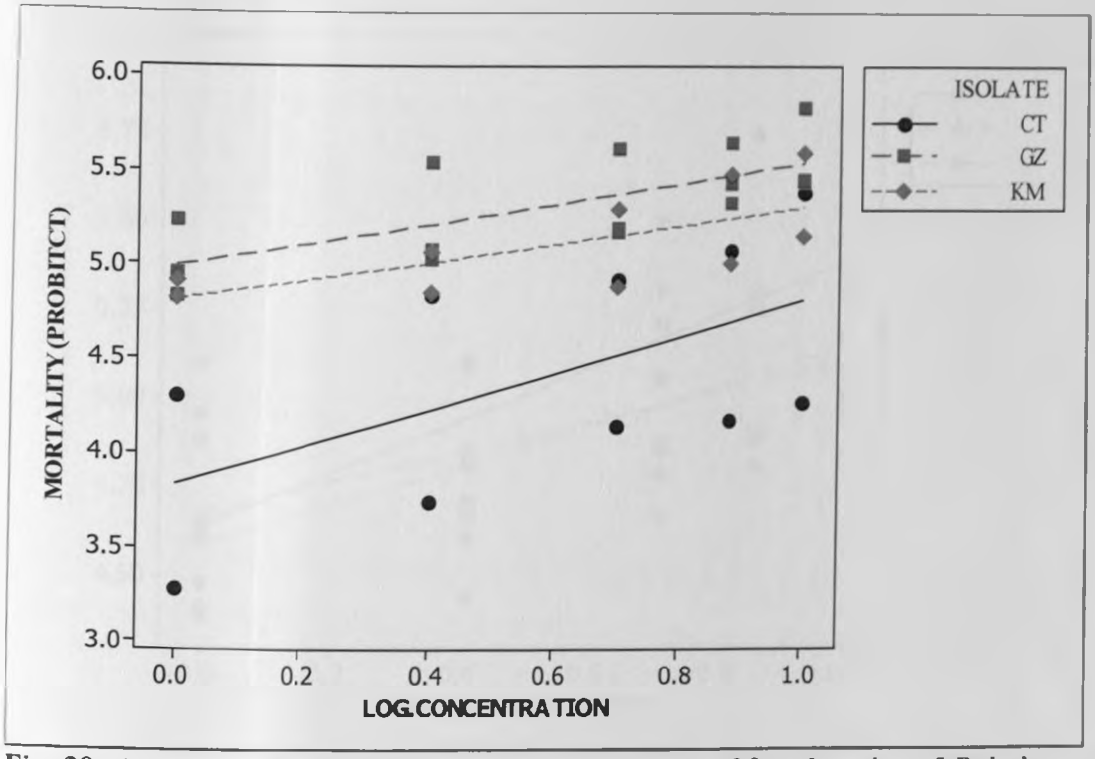


Fig. 30: A comparative log probit analysis for toxicity of δ endotoxins of *Bt* isolates on adult *P. truncatus* based on ecological area of isolation. (CT) Standard control *Bt* variety; (GZ) *Bt* isolates from Gazi; (KM) *Bt* isolates from Machakos.

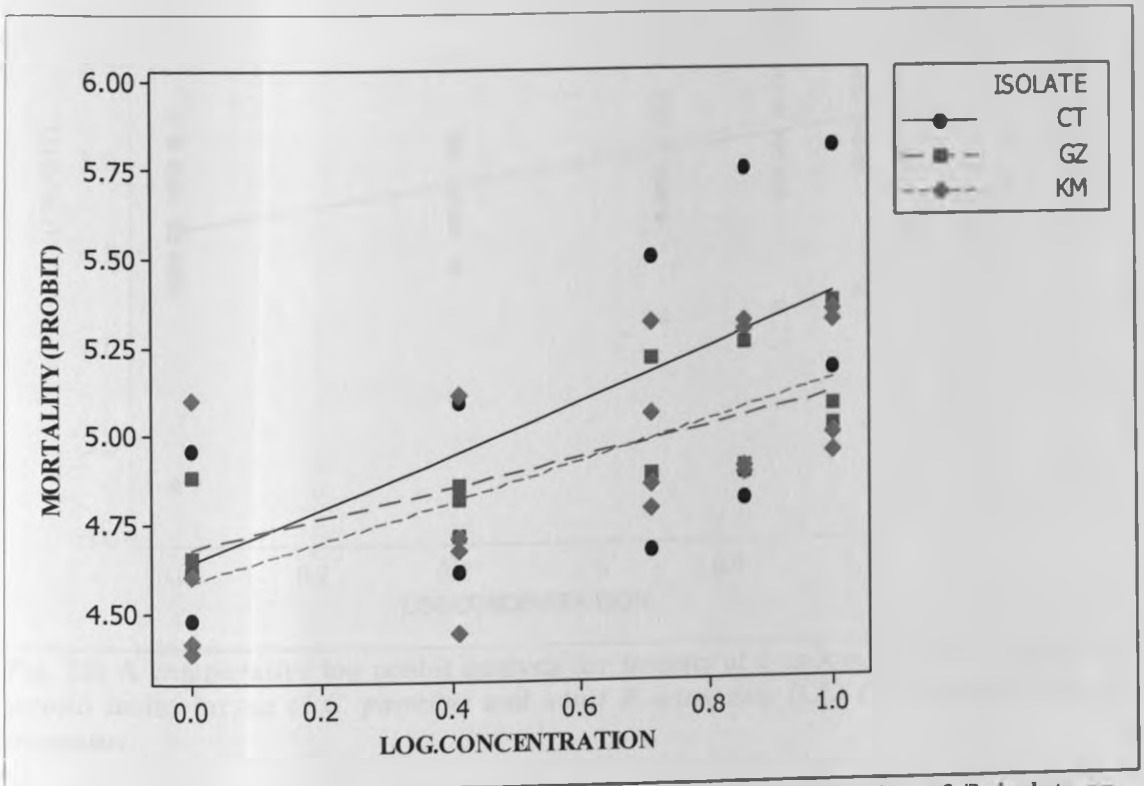


Fig. 31: A comparative log probit analysis for toxicity of δ endotoxins of *Bt* isolate on second instar larvae of *C. partellus* based on ecological area of isolation. (CT) Standard Control *Bt* variety; (GZ) *Bt* isolates from Gazi; (KM) *Bt* isolates from Machakos.

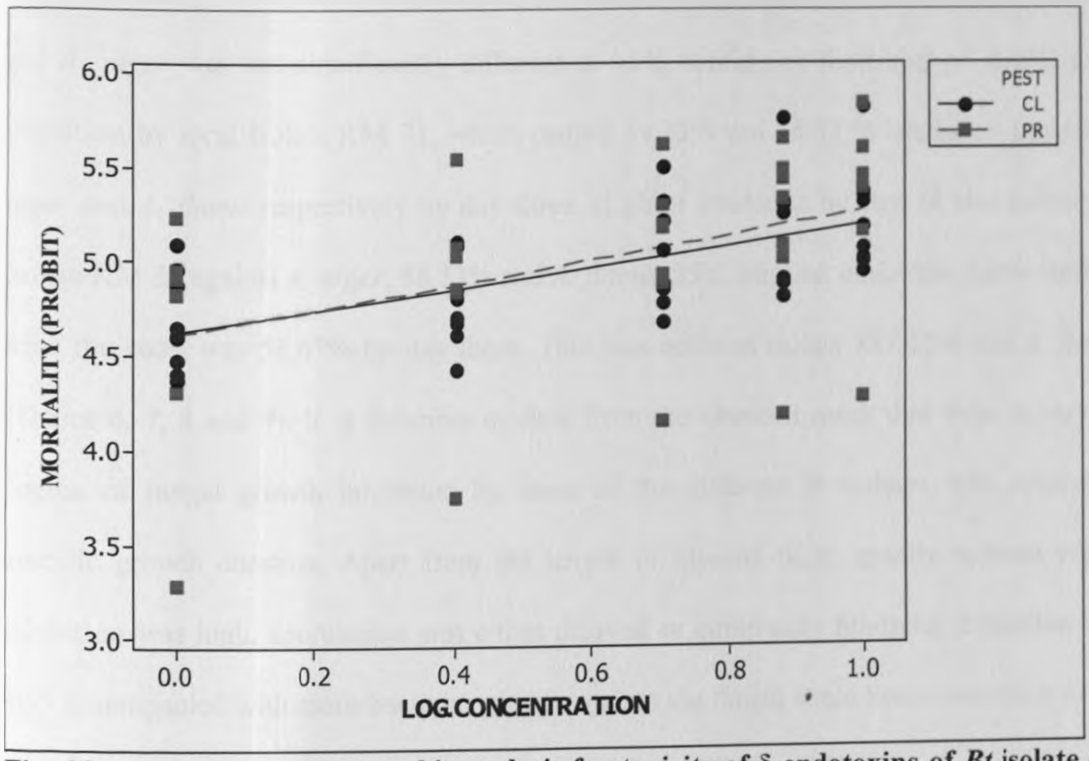


Fig. 32: A comparative log probit analysis for toxicity of δ endotoxins of *Bt* isolate on second instar larvae of *C. partellus* and adult *P. truncatus.* (CL) *C. partellus;* (PR) *P. trancatus*

4.7. Fungal growth inhibition test.

Inhibition of fungal growth by two of the *Bt* isolates was strongly evident between the fourth and seventh day of incubation. Inhibition by standard *Bt* var *israelensis* against both *A. flavus* and *A. niger* was not significantly different at 95% confidence limit and $p< 0.001$, from inhibition by local isolate KM 31, which caused 84.33% and 78.33 % inhibition against *A. niger* and *A. flavus* respectively by day three. Highest inhibition by day 14 was caused by isolate KM 31 against *A. niger*, 58.33% and *A. flavus*, 35%. Highest inhibition due to isolates from the coast was 51.67% by day three. This was between isolate KG 12-0 and *A. flavus* (Tables 6, 7, 8 and 9). It is therefore evident from the obtained result that there is varying degree of fungal growth inhibition by some of the different *Bt* isolates with respect to specific growth duration. Apart from the length of mycelia being greatly reduced where inhibition was high, sporulation was either delayed or completely hindered. Inhibition was also accompanied with more bacterial growth against the fungal strain hence reduction in the diameter of the affected fungal colonies (Plates: 1, 2, 3, 4, 7, 8, and 9).

Table 6: Means for growth inhibition effect of *Bt* isolates against *A. niger*

	Time (Days) & Means for inhibition zone				
Bt ISOLATE	0	3	4	7	14
No *Bt* (Control)	3	0.25	-0.2	-1.13	-3.58
Bt israelensis	3	2.55	2.45	1.2	-0.23
Bt tenebrionis	3	1.23	0.88	-0.33	-1.43
KG 411	3	1.23	0.75	-1.38	-1.43
KM 31	3	2.53	2.4	2.08	1.75
KG 12-0	3	0.88	0.63	0.35	-1.15
KG 20	3	1.4	1	0.13	0.75
Grand mean		0.66			
		Isolate		Time	
df		84		84	
LSD		0.47		0.36	
p		<0.001			

Table 7: % Means for growth inhibition effect of *Bt* isolates against *A. niger*

	Time (Days) and % mean inhibition			
Bt Isolate	3	4	7	14
No *Bt* (Control)	8.33	-6.67	-37.67	-119.33
Bt israelensis	85	81.67	40	-7.67
Bt tenebrionis	41	29	-11	-47.67
KG 411	41	25	-46	-47.67
KM 31	84.33	80	69.33	58.33
KG 12-0	29	21	11.67	-38.33
KG 20	46.67	33.33	-43	-25
Grand mean	0.47			
	Isolate		Time	
d.f	84		84	
L.S.d	0.47		0.36	
P	< 0.001			

Table 8: Means for growth inhibition effect of *Bt* isolates against *A. flavus*

	Time (Days) & Means for inhibition zone				
Bt ISOLATE	0	3	4	7	14
Control(No *Bt*)	3	0.25	-1.2	-0.45	-1.83
Bt israelensis	3	2.18	1.95	1.75	0.88
Bt tenebrionis	3	1.2	1.o8	0.23	-0.95
KG 411	3	0.85	0.48	-0.28	-0.03
KM 31	3	2.35	2.25	1.58	1.05
KG 12-0	3	1.55	1.25	0.2	-0.28
KG 20	3	1.35	1.25	0.45	-0.7
Grand mean		0.66			
		Isolate		Time	
df		84		84	
LSD		0.35		0.27	
p		<0.001			

Table 9: % Means for growth inhibition effect of *Bt* isolates against *A. flavus*

	Time(Days) and % mean inhibition			
Bt ISOLATE	3	4	7	14
Control (No *Bt)*	8.3	-4	-15	-61
Bt israelensis	72.67	65	58.33	29.33
Bt tenebrionis	4	36	7.67	-31.67
KG 411	28.33	16	9.3	-1
KM 31	78.33	75	52.67	35
KG12-0	51.67	41.67	6.67	-9.3
KG 20	45	41.67	15	-23
Grand Mean	0.66			
	Isolate		Time	
df	84		84	
LSD	0.35		0.27	P <0.001

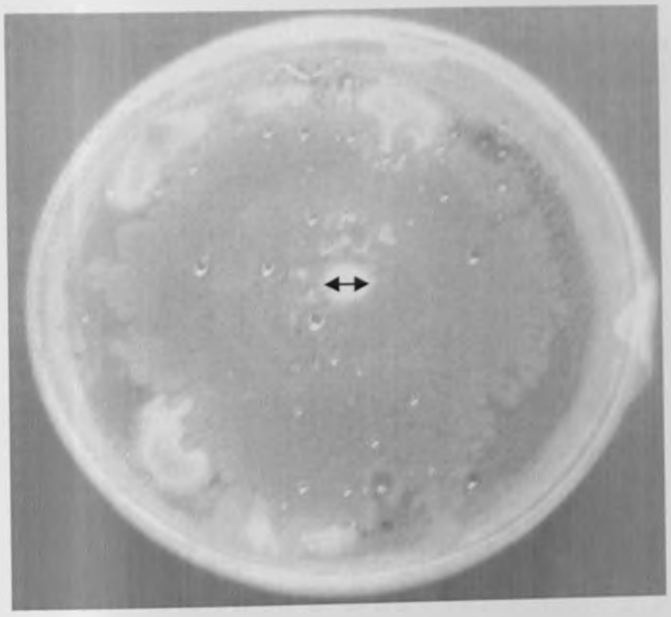


Plate 1: Inhibition test between *Bt* var *israelensis* and *A. niger*; day 7; (↔) Fungal diameter

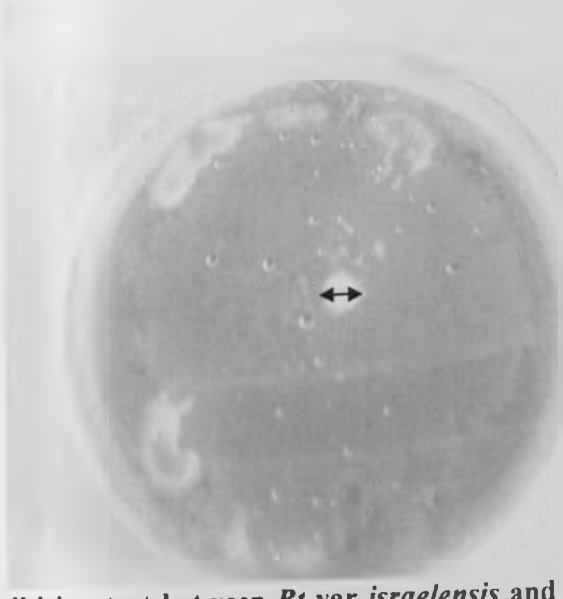


Plate 2: Inhibition test between *Bt* var *israelensis* and *A. flavus;* day 7; (↔) Fungal diameter

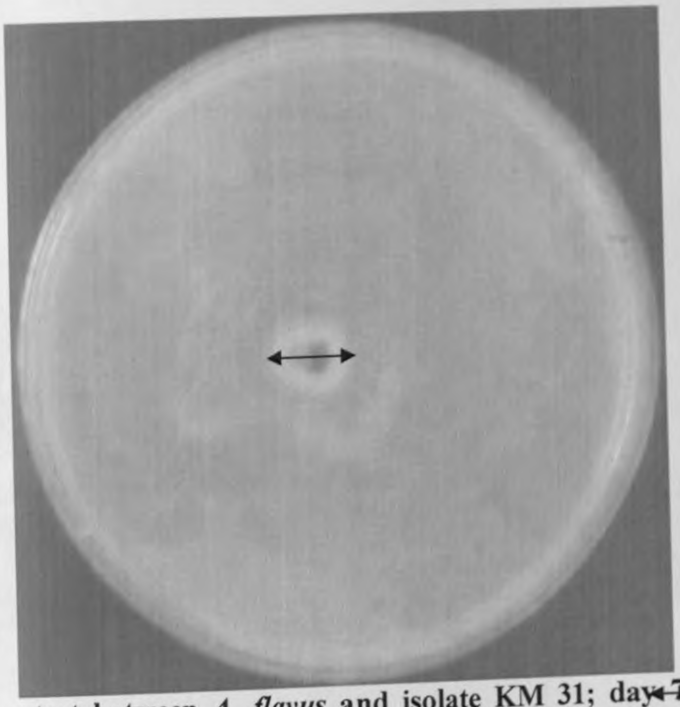


Plate 3: Inhibition test between *A. flavus* and isolate KM 31; day 7; ( ) Fungal diameter

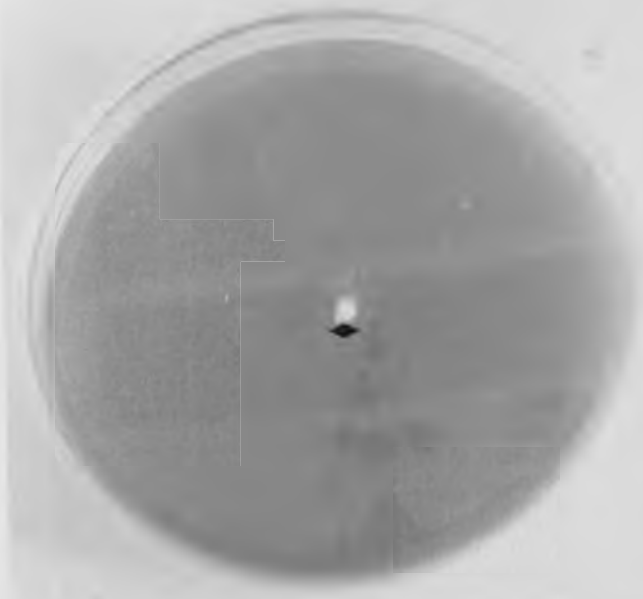


Plate 4: Inhibition test between isolate KM 31 and *A. niger*; day 7; (↔) Fungal diameter

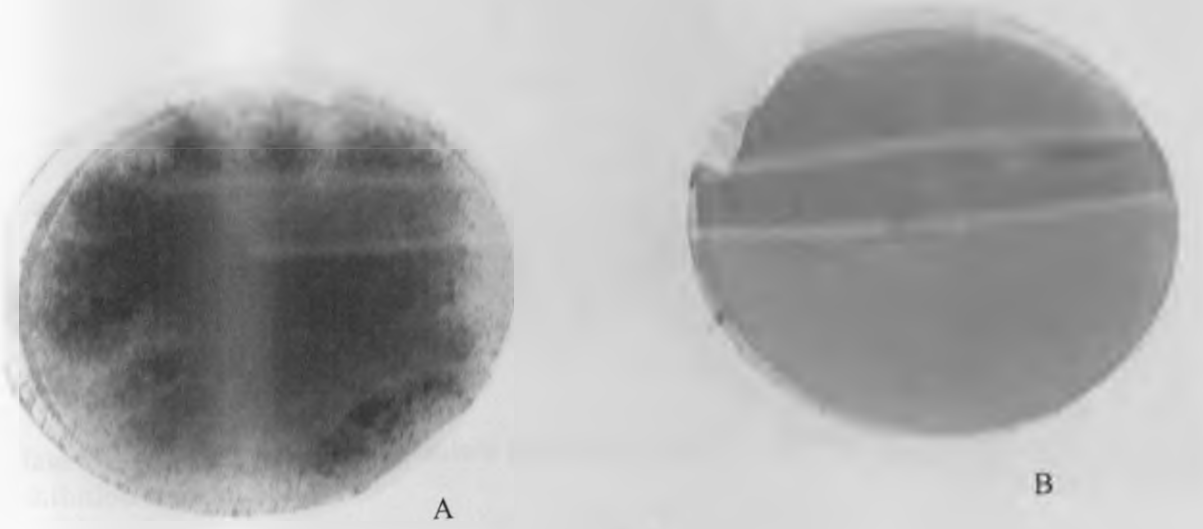


Plate 5: Controls; (A) *A. niger* (B) *A. flavus*; day 7.

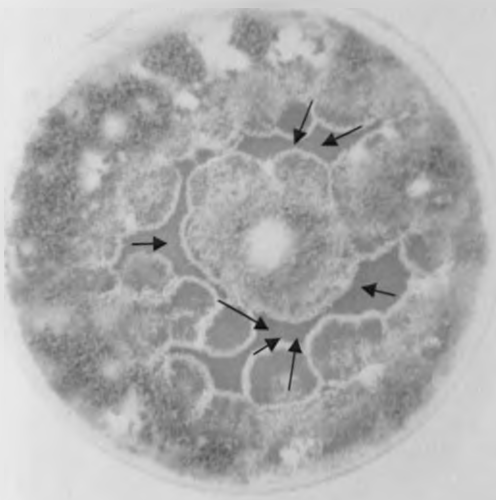


Plate 6: Inhibition test between isolate *Bt israelensis* and *A. flavus*; day 14 (→) Inhibition zone

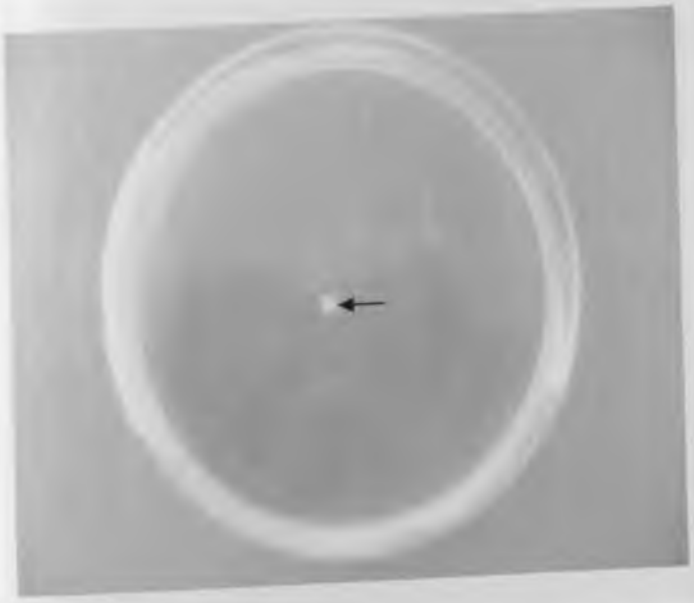


Plate 7: Inhibition test between isolate *Bt israelensis* and *A. niger*; day 14. (→) Fungal diameter

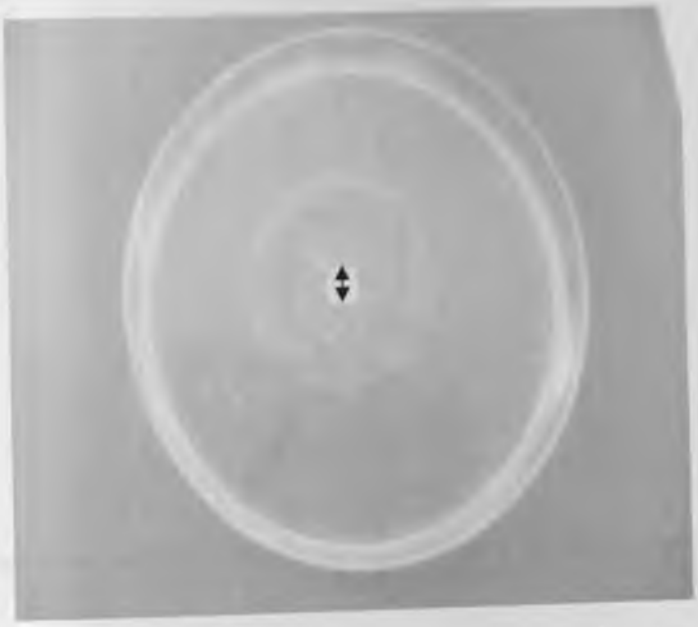


Plate 8: Inhibition test between isolate KM 31 and *A. flavus*; day 14. (↔) Fungal diameter

Plate 9: Inhibition test between isolate KM 31 and *A. niger*; day 14. (↔) Fungal diameter

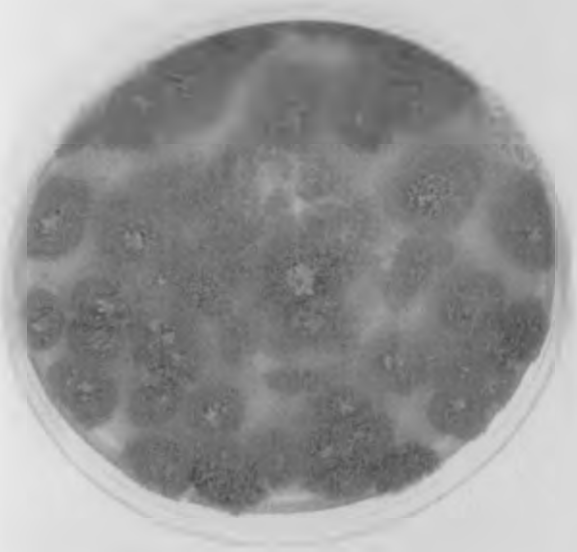


Plate 10: Inhibition test between isolate KG 411 and *A. flavus*; day 14

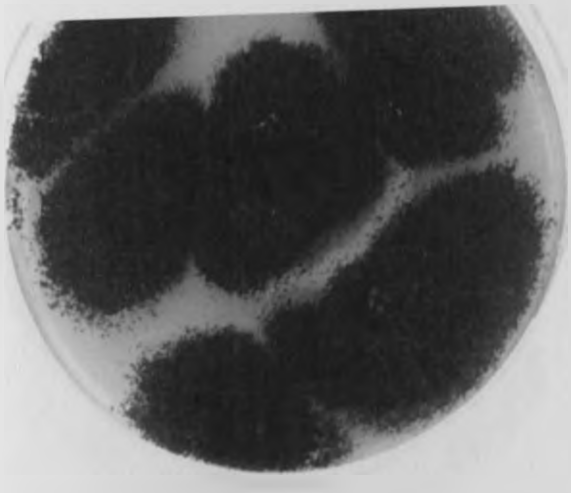


Plate 11: Inhibition test between *Bt* isolate KG 411 and *A. niger*; day 14.

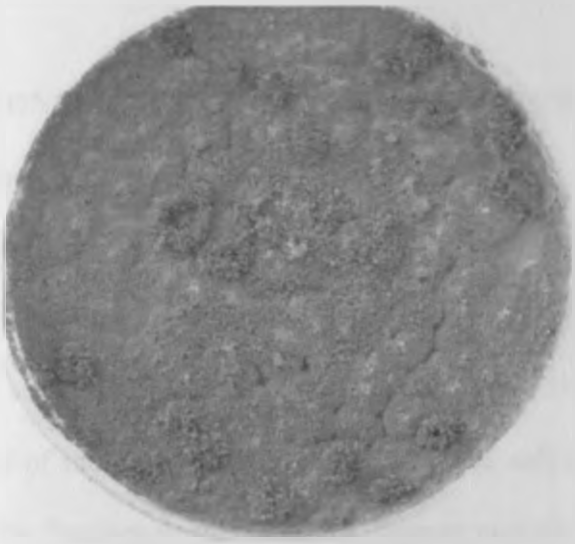


Plate 12: *A. flavus* for control

Plate 13: *A. niger* for control; day 14

CHAPTER FIVE

5.0 DISCUSSION CONCLUSIONS AND RECOMMENDATIONS

5.1 Isolation of *Bt* and characterization

The result of this study demonstrated that Kenya has a wide range of ecological zones ranging from terrestrial grasslands to aquatic mangroves for isolation of entomopathogenic as well as anti fungal strains of *Bt.* A total of 28 *Bt* isolates (Table 1) were obtained from the 63 soil samples. Half of these isolates were from mangrove sediments along the Kenyan coast. This supports the findings that *Bt* is also a common member in micro-flora of fresh water Iclimatsu *et al.,* (2000), Iriarte *et al.*, (2000) and; marine sediments Maeda *et al.*, (2001); and hence an abiquitous microorganism with a worldwide distribution, Martin and Travers, (1989). No *Bt* was isolated from *Sonneratia alba* (KG 3) mangroves. In agreement with the findings by Bull *et al.,* (1977); Fast, (1981), the obtained isolates were Gram positive, spore forming, rod shaped bacteria that produced proteinaceous parasporal inclusions some of which had insecticidal properties. Even though most of the isolates gotten produced crystal proteins with bipyramidal shapes, a typical shape of most crystals Fast, (1981); others produced squared, rhombus and round shaped crystals, a result that supports in part the findings by Mwathi, (2006). Result for characterization of the isolates based on biochemical tests confirmed that the isolates were *Bt* in agreement with the findings of Sergio *et al.,* (1992), and Hossain *et al.*, (1997). The obtained isolates did not vary a lot in their tolerance to changes in temperature and pH. However *Bt* isolate KG 411 from *R. mucronata* and *A. marina* mangroves along the banks of river Kidogoweni (KG 4) exhibited higher growth at pH value of 7.5, a characteristic that made it a little unique from other isolates.

5.2 Toxicity of *Bt* on *P. truncatus*

5.2.1 Determination of best form of maize diet for toxin delivery

Preliminary results demonstrated significant differences in mortalities due to differences in the type of diet for toxin delivery. The highest percentage mortalities were recorded where toxin delivery diet was in form of maize particles and; it was significantly different at 95% confidence limit from the other forms of toxin delivery diet. With whole maize grains a lot of toxins got absorbed into the maize core which is presumably attacked first because it offers less resistance than the relatively hard and slippery sheath hence the second best diet in toxin delivery. Particles had increased surface area for toxin absorption into the exposed endosperm and also provided rough surface which is easier to attack than undamaged maize hence consumption of more toxins resulting into more deaths. This finding is closely related to the report by Subramanyam *et al.*, (1988) which showed *that P. truncatus* individuals preferred artificially damaged versus undamaged kernels, and this was attributed to a combination of chemical and mechanical stimuli. In whole maize grain penetration of the toxins into the endosperm was minimal, implying that any entrance of the pest into the grain before intoxication offered the target pests protection. The smooth surfaces of the grains also do offer difficulties to the pest in attacking the grains. Artificial grains (maize flour pellets), quickly broke up into flour which reduces feeding of insects hence reduced intake of the toxins.

5.2.2 Toxicity of the obtained *Bt* solates to dult *P. truncutus*

The result of this research demonstrates the natural occurrence of native *Bt* strains (KM 31 and KG 411) with high potency to adult *P. truncatus* from both ecological sites of soil collection. The individual differences observed among all the isolates in their toxicity to

adult *P. truncatus* may be attributed to the specificity of δ endotoxins to target pest as well as the particular growth phase of the pest. For example *Bt* variety *tenebrionis* is very effective against the larval stage of coleopteran *Leptinortasa decemlineata* (colorado potato beetle-CPB), Krieg *et al.*, (1987) and not the adult stage while *Bt* variety *israelensis* is effective against mosquito larvae Goldberg and Margalit, (1977)., Sudarani and Balaraman, (1996). The intraspecic entomopathogenic activity observed by the various *Bt* isolates against *P. truncatus* can be likened to findings reported on coleopterans by other workers (Krieg *et al.*, (1983); Dovan *et al.*, (1988) and Mwathi, (2006). Although *Bt* toxins targeting larval stages of insects tend to be more effective than when adult stages of the same insect species is targeted, the result of *Bt* toxicity on adult *P. truncatus* in this study illustrates that some *Bt* strains are indeed lethal to adult insects. A similar finding was reported by Grove *et al.*, (2001) on effects of individual *Bt* insecticidal crystal proteins on adult *Heliothis virescens* and *Spodoptera exigua* (Hubner) lepidopterans. Stagnation and insignificant increase in mortalities of *P. truncatus* with increase in time as from day fifteen may be attributed to the antifeedant effects of the *Bt* toxins to the pest.

5.3 Toxicity of *Bt* isolates against *C. partellus* second instar larvae

Generally the obtained isolates caused minimal mortality within the first 48 hours of the experiment with rapid increase in death as from 72 hours; and 100 % mortality by day five (120 hrs) Other than being highly potent against *P. truncatus* isolates KG 411 and KM 31 caused over 50% mean mortality to the second instar larvae of *C. partellus* although higher concentration of the *Bt* toxins were used. This result is consistent with the findings by Kees, (2009), who reported that there is an increasing number of toxin families with cross order

activity with toxins tending to be much less toxic to taxa outside the family's specificity range.

The locally obtained toxic *Bt* isolates were significantly more toxic to adult *P.truncatus* than to *C. partellus* with increase in toxin concentration for higher mortality in *C. partellus*. Mortalities in C. *partellus* were minimal between 24 and 48 hours with different concentrations of *Bt* toxins after exposure; a phenomenon that may be linked to time for sporulation and toxin production by the *Bt* isolates.

5.4 Fungal growth inhibition test

The result obtained in this test exceptionally demonstrates the presence of native *Bt* isolate (KM 31) with over 74 % fungal growth inhibition against *A. flavus* and *A. niger*. Ramirez *et al*., (2004) found similar result in which antifungal activity of *Bt* var *israelensis* caused 55% to 82% inhibition in *A. flavus*, *A. niger* and other fungal strains. Inhibition was accompanied with reduced or total failure in fungal sporulation. This suppressed fungal colonization of other areas on the Petri dish. The general inhibition portrayed by isolate KM 31 is a clear indication that some strains of *Bt* may be considered for the bio - control of some mycotoxin producing fungi such as *A. flavus* either directly in form of biopesticides or indirectly in transgenic plant delivery.

5.5. Conclusions

The conclusions drawn from this study may be summarized as follows in view of the objectives of the study. Among the conclusions is that Kenyan Coastal aquatic mangroves are rich sites for isolation of entomopathogenic *Bt* with very high potency against adult *P. truncatus*. As much as isolate KM 31 was highly toxic to *P. truncatus* and also inhibited fungal growth, not all *Bt*

isolates toxic to *P. truncatus* and C. *partellus* can inhibit fungal growth. However, the result of KM 31 in this research is a positive indicator that some native *Bt* can be used to inhibit fungal growth including mycotoxin producing fungi of the genus *Aspergillus*. It is also worth noting that *Bt* strains with high potency against adult *P. truncatus* are not ecologically restricted to terrestrial habitats in Kenya but also to the coastal aquatic mangroves. There is therefore prospect in the use of novel native *Bt* strains for protection of maize from pre-harvest and post-harvest maize insect pests. For *Bt* toxicity bioassays against adult *P. truncatus*, maize grain particles offer the best diet for delivery of the *Bt* toxins.

5.6 Recommendations

P. truncatus is a pest of maize infesting both the stored crop Hodges *et al.*, (1985) and the standing crop Giles, (1975). Attack in the field may occur fairly early when the drying maize still has a moisture content of 40-50% Giles, (1975). Under such conditions, feeding on developing kernels by pests facilitate the infection of maize grains by mycotoxin producing fungi which continues to grow and spread further especially under poor storage conditions. *Bt* produces heterogeneous range of insectidal, nematocidal and acaricidal toxin, most notably the crystal (δ-endotoxin) proteins and the thuringiensin (β-exotoxin) whose host spectrum varies considerably (Drummond and Pinnock, 1994). Reyes-Ramirez *et al.*, (2004) reported that *Bt* chitinase may contribute to the biocontrol of *S. rolfsii* and other phytopathogenic fungi in Integrated Pest Management Programs, a finding pointing to its potential use against harmful fungi. With such a wide spectra in the use of *Bt* as a more reliable biocontrol agent and; in view of the findings of this study there is great need for:

1. Further screening of *Bt* isolates effective against both *P. truncatus* and mycotoxin producing fungi for use in transgenic plant delivery. This can be achieved either through genetic maize transformation or formulation of *Bt* biopesticides.

2. More isolation of *Bt* with higher potency against adult *P. truncatus* from mangrove habitats along the Kenyan coastal line to be done.

3. Other than growth inhibitory effect of *Bt* on the fungi in this study, further investigation underscoring its effect on production of fungal toxins, effect on sporulation and spore morphology to be done.

4. Since classification of *Bt* isolates used in this study was by appearance, presence of parasporal inclusions, growth and biochemical characteristics, further characterization based on molecular work would help to distinguish them from the already existing *Bt* strains as this could enhance their utilization as sources of new *Cry* genes for genetic transformation.

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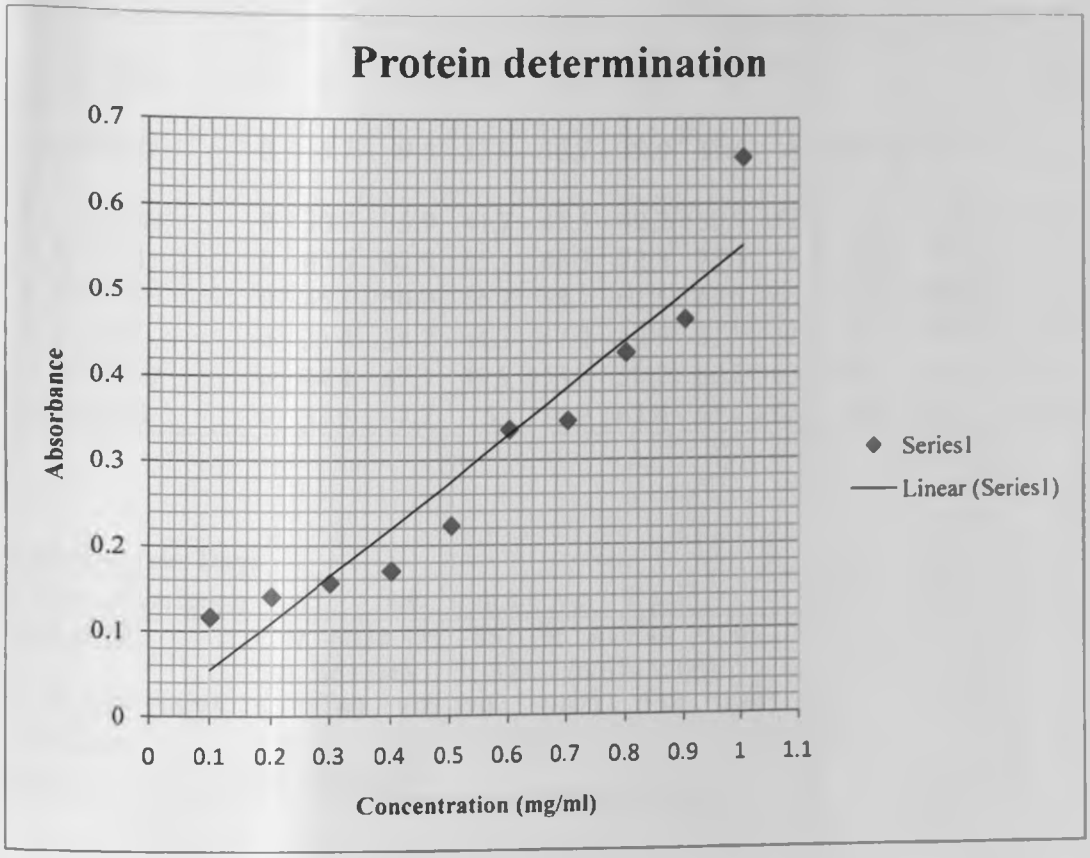
7.0 APPENDICES

APPENDIX I



Estimation of the *Bt* protein content

APPENDIX II

Mean percentage mortality of adult *P. truncutus* exposed to 10mg/ml for each *Bt* δ - endotoxins of different isolates. The mean is calculated from a mean of three replicates each with thirty insects.

Isolate	Days										
	0	3	6	9	12	15	18	21	24	27	30
Saline H20	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Bt Israelensis	0	5.7	9.8	13.9	15.7	15.9	17.6	18.3	19.4	19.4	20
Bt tenebrionis	0	30.7	35.9	41.7	45.5	49.7	50.7	52.2	53	53	55
Bt KG411	0	60.6	62.9	65.1	68.3	72.8	75.1	76.5	77.1	77.1	77
Bt KM31	0	46.1	50.9	55.2	58.7	62.5	65.5	65.9	65.9	65.9	66
Bt KM24	0	33.4	38.7	44.3	47.9	49.9	51.7	52.3	52.7	52.8	53
Bt KG12-0	0	44.4	47.1	49.9	53.7	58.2	62.2	63.1	63.5	63.5	64
Bt KG420	0	33.6	42.7	53	54.9	62.4	64.6	66.1	66.1	66.1	66

Isolate versus Days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ISOLATE	7	6028.371	861.196	447.46	<.001
DAYS	9	461.946	51.327	26.67	<.001
ISOLATE.DAYS	63	102.120	1.621	0.84	0.805
Residual	1120	2155.605	1.925		
Total	1199	8748.042			

Isolate and concentration

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ISOLATE	7	6028.371	861.196	509.25	<.001
CONC	4	597.035	149.259	88.26	<.001
ISOLATE.CONC	28	160.970	5.749	3.40	<.001
Residual	1160	1961.666	1.691		
Total	1199	8748.042			

APPENDIX III

Fig.4: Percentage mortality of adult *P. trancutus* exposed to different concentrations of different δ-endotoxin of *Bt* isolates.

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	0	0	1	2	3.3	2.3	2.3	10	10	12.3
Conc. 2.5	0	2.3	9	10.7	11.7	11.7	11.7	11.7	11.7	11.7	11.7
Conc. 5	0	8.6	12.3	18.3	18.3	24.3	24.3	25.3	25.3	25.3	25.3
Conc. 7.5	0	8.7	12.3	18.3	18.3	24.3	24.3	24.3	24.3	24.3	24.3
Conc. 10	0	14.3	17.6	17.6	24.3	24.3	24.3	27.6	27.6	27.6	27.6

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	13.3	16.7	20	20	26.7	26.7	28.3	31.7	31.7	36.3
Conc. 2.5	0	23.3	36.7	41.7	45	46.7	48.3	48.3	48.3	48.3	48.3
Conc. 5	0	36.7	36.7	36.7	43.3	46.7	50	53.3	53.3	53.3	56.7
Conc. 7.5	0	33.3	35	45	53	60	60	61	61.7	61.7	61.7
Conc. 10	0	46.7	54.3	65	66	68.3	68.3	70	70	70	70

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	36.7	43.3	50	56.7	60	66.7	70	70	70	70
Conc. 2.5	0	65.7	65.7	65.7	71	71	71	74.3	74.3	74.3	74.3
Conc. 5	0	58.3	61.7	61.7	61.7	75	80	80	83.3	83.3	83.3
Conc. 7.5	0	67.7	69	72.7	74	75.7	75.7	75.7	75.7	75.7	75.7
Conc. 10	0	74.7	74.7	75.7	78	82.3	82.3	82.3	82.3	82.3	82.3

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	38.3	38.3	40	40	45	55.3	55.3	55.3	55.3	55.3
Conc. 2.5	0	45	46	49	52.3	53.3	55.7	55.7	55.7	55.7	55.7
Conc. 5	0	51.7	51.7	54.3	63.3	64	64	66.3	66.3	66.3	66.3
Conc. 7.5	0	40	52.7	61.7	66.7	74.3	77.7	77.7	77.7	77.7	77.7
Conc. 10	0	55.7	66	71	71	75.7	76.7	76.7	76.7	76.7	76.7

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	35.3	36.7	43.3	44.3	44.3	45	45	45	45	45
Conc. 2.5	0	45	46	51	52.3	52.3	52.3	52.3	52.3	52.3	52.3
Conc. 5	0	23.3	26.7	31	40	43.3	50	60	60	60	60
Conc. 7.5	0	28.3	41.7	47.7	47.7	51	51	57.7	57.7	57.7	57.7
Conc. 10	0	35	42.3	48.3	50	58.3	60.7	63.3	65	65.7	65.7

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	26.7	30	30.7	41.7	50	51	51	51	51	51
Conc. 2.5	0	41.7	44	44	46.7	51.7	51.7	53.3	58	58	58
Conc. 5	0	46.7	47.7	56.7	61.7	66.7	67.3	67.3	67.3	67.3	67.3
Conc. 7.5	0	51	56	59.3	59.3	66.3	70	71.7	76.7	76.7	76.7
Conc. 10	0	56	57.7	59	59	61.3	71.7	71.7	76.7	76.7	76.7

Conc.	Days										
	0	0	6	9	12	15	18	21	24	27	30
Control	0	0.3	1	0.7	0.9	1.3	1.3	1.5	1.5	1.5	1.5
Conc. 1	0	26.7	26.7	45	45	53.3	53.3	57.7	57.7	57.7	57.7
Conc. 2.5	0	28	41.3	47.7	47.7	56.7	63.3	63.3	63.3	63.3	63.3
Conc. 5	0	33.3	36.7	46	49.3	66.7	68.7	69	69	69	69
Conc. 7.5	0	26.7	50	66.7	67.7	67.7	68.7	69.3	69.3	69.3	69.3
Conc. 10	0	53.3	58.7	62.7	67.6	71	71	71	71	71	71

APPENDIX IV

ANOVA *C. partellus* Isolate and concentration

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ISOLATE	9	2002.788	222.532	24.77	<.001
CONC	4	384.809	96.202	10.71	<.001
ISOLATE.CONC	36	170.014	4.723	0.53	0.991
Residual	700	6289.596	8.985		
Total	749	8847.208			

ANOVA *C. partellus* Isolate and Hours

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
ISOLATE	9	2002.788	222.532	50.63	<.001
H0URS	4	3278.630	819.658	186.48	<.001
ISOLATE.H0URS	36	488.929	13.581	3.09	<.001
Residual	700	3076.860	4.396		
Total	749	8847.208			

APPENDIX V

Table for transformation of concentrations to Log concentrations and means to percentage mortalities for *P.truncatus* to probits.

ISOLATE	CONC.Mg/ml	MORTALITY (%)	LOG.CONC	PROBIT
WATER	1	0	0	
	2.5	0	0.4	
	5	0.8	0.7	2.59
	7.5	2.1	0.88	2.97
	10	2.9	1	3.1
Bt var israelensis	1	4.3	0	3.28
	2.5	10.4	0.4	3.74
	5	19.4	0.7	4.14
	7.5	20.4	0.88	4.17
	10	23	1	4.26
CONTROL *Bt*	1	25.1	0	4.3
	2.5	43.5	0.4	4.84
	5	46.7	0.7	4.92
	7.5	53.3	0.88	5.08
	10	64.9	1	5.38
KG 411	1	59.3	0	5.23
	2.5	70.7	0.4	5.54
	5	73	0.7	5.61
	7.5	74	0.88	5.64
	10	79.7	1	5.83
KM 31	1	46.8	0	4.91
	2.5	52.4	0.4	5.06
	5	61.4	0.7	5.29
	7.5	68.4	0.88	5.48
	10	72.3	1	5.59
KM 24	1	42.8	0	4.82
	2.5	44.2	0.4	4.85
	5	45.4	0.7	4.88
	7.5	49.8	0.88	5
	10	56	1	5.15
KG 12-0	1	43.4	0	4.83
	2.5	50.7	0.4	5.02
	5	56.8	0.7	5.17
	7.5	66.3	0.88	5.42
	10	67.2	1	5.45
KG 20	1	48.1	0	4.95
	2.5	53.2		5.08
	5	57.5	0.7	5.19

	7.5	62.5	0.88	5.32
	10	66.5	1	5.43

APPENDIX VI

Anova for *Bt* isolates against *A. niger*

SOURCE OF VARIATION	d.f	s.s.	m.s.	v.r.	F pr.
ISOLATE	6	113.26	18.88	42.11	< .001
TIME	3	97	32.33	72.13	< .001
ISOLATE. TIME	18	14.62	0.81	1.81	< .037
RESIDUAL	84	37.65	0.45		
TOTAL	111	262.53			

APPENDIX VII

Anova for *Bt* isolates againt *A. flavus*

SOURCE OF VARIATION	d.f	s.s.	m.s.	v.r.	F pr.
ISOLATE	6	76.2	12.7	51.2	< .001
TIME	3	48.02	16.06	64.53	< .001
ISOLATE. TIME	18	4.6	0.26	1.03	< .44
RESIDUAL	84	20.84	0.25		
TOTAL	111	149.65			