ISOLATION AND PARTIAL CHARACTERIZATION OF *Bacillus thuringiensis* FROM KAKAMEGA AND MACHAKOS DISTRICTS IN KENYA.

By

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Botany (Microbiology) in the University of Nairobi

2001

DECLARATION

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

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ACKNOWLEDGEMENTS

First I wish to express my sincere appreciation and gratitude to the University of Nairobi for awarding me a scholarship for this program. I am greatly indebted to my supervisors, Dr. J. H. P. Kahindi and Prof. J. O. Ochanda for their unfailing guidance and support during the entire study period. I also wish to express my sincere gratitude to Biotechnology Trust Africa (BTA) and the Netherlands Government Special Programme for Biotechnology for providing finances for this work.

I sincerely thank farmers from Machakos and Kakamega Districts for willingly participating in the project and for providing soil samples from which *B. t* isolates were obtained. I also wish to thank the Masii Location extension officer, Mr. David Mutua for coordinating field trips to farms in Machakos.

My gratitude also goes to the members of staff (Academic and Technical) in the Departments Botany and Biochemistry for their kind support and enabling environment during the study. My sincere appreciation also goes to Mr. Francis Onyango and the entire staff of ICIPE's Insect Rearing and Quarantine Unit (*Chilo partellus* and *Busseola fusca*) section for their efficiency in supplying the Larvae for the study. The support of my colleagues, Esther Kanduma, John Muchiri, Dolphine Achieng, Elizabeth Ouna, Jacinta Nyaga, Ken Kamwere and Beth Ndeeri during the study cannot go unnoticed and to all of you I say, thank you.

DEDICATION

I wish to dedicate this thesis to my Dad, Mr. Joseph Wang'ondu and my lovely Mum, Mrs. Mary Nyambura for supporting my education, to my family members, lovely husband, Mr. Josphat Githaiga..and children, daughter Mary Nyambura and son, Sammy Kanyi for their love, patience, endurance and understanding throughout the period of study and lastly to my late grandmother, Mrs. Monicah Wangui Nduhiu, may the almighty God rest her soul in Eternal peace.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
δ	delta
AF	acriflavin
AMC	acetylmethylcarbinol
BSA	Bovine Serum Albumin
LD_{50}	lethal dose
NaOH	sodium hydroxide
NH_4OH	ammonium hydroxide
OD	optical density
PAGE	polyacrylamide gel elctrophoresis
SDS	sodium dodecyl sulphate
Tris-HCl	Tris-hydrochrolic acid
UV	ultra violet
VP	Voges-Proskauer

UNIT ABBREVIATIONS

%	Percentage
µg	microgram
µl	micro litre
µm	micro metre
c.f.u./g	Colony forming units per gram
g	gram
hr	hour
kDA	Kilo dalton
L	Litre
M	molar
mg/ml	milli gram/ milli litre
min	minute
mA	milli Amperes
ml	milli litre
mM	milli Molar
ng	nano gram
nm	nano metre
°C	degrees centigrade
r.p.m.	revolutions per minute
sec	Second
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

ABSTRACT

This study addressed the need for isolating local *B.t* isolates and screening them for their toxicity against three maize stalk borers, *Chilo partellus, Sesamia calamistis* and *Busseola fusca.* Twelve (*B.t)* strains were locally isolated from soils from Kakamega (12F-K, K10-2 and K13-1) and Machakos (1M, VM-10, V24-M, 44M, 45M, V15-M, 34M, 35M and V14-M) districts. In their morphological characterization, the *B.t* strains were different in regard to rod length, but the crystal morphology (bipyramidal) was similar in all the isolates.

Preliminary studies produced seven highly toxic *B.t* isolates that were selected for bioassays, namely 1M, VM-10, V24-M, 44 M, V14-M, 12F-K and K10-2. The isolates were screened for activity against 1st and 2nd instar larvae of *C. partellus* and 2nd instar for *B. fusca* and 1st instar larvae of *S. calamistis*. Differences in the toxicity of different isolates to *C. partellus* were observed in the bioassays. Isolates 1M, VM-10 and 44M (from Machakos district) were found to be the most potent against *C. partellus*. The isolates achieved more than 80% mortality within a period of 72 hrs at a concentration of 0.5 mg/ml and 0.86 mg/ml respectively, whereas isolate 44M had 100% larval mortality in 72 hrs at a concentration of 3.4 mg/ml. Their LD_{50} values were 0.004, 0.04, and 0.03 mg/ml respectively.

When the 7 isolates were tested at the same concentration of 8.6 mg/ml against 1st and 2nd instar larval stages of *C. partellus* the above three isolates also proved to be the most toxic, with VM-10 and 1M achieving 100 % mortality within 72 hrs for 1st instar and V14-M for 2nd instar. Isolates from Kakamega (K10-2 and 12F-K) used for the

bioassays were potent, though they caused lower percentage larval mortalities than those from Machakos.

At a concentration of 6.7 mg/ml, 12F-K achieved 59% mortality by 72 hrs whereas isolate K10-2 attained 62% mortality at a concentration of 4.2 mg/ml within the same time. At the same concentration with the other isolates (8.6 mg/ml), 12F-K and K10-2 attained 100% mortality at 96 and 120 hrs respectively, for 1st instar larvae and 93 % and 100% mortality within 96 hrs and 120 hrs for 2nd instar larvae respectively. High larval mortality rates were observed with leaf disk assays than with the artificial diet, thus suggesting the larval preference for natural diet. The *B.t* isolates caused 100% larval mortality in 72 hrs.

The *B.t* isolates were also tested for their toxicity against *S. calamistis* At a concentration of 0.86 mg/ml isolate 44M was the most toxic to the larvae with a larval mortality of 77% after 4 days. Isolates 1M and V14-M followed respectively with larval mortalities of 61% and 55% within the same duration. Isolate 12F-K was the least toxic with larval mortality of 27%. Increase in toxin concentration resulted in an increase in larval mortality. However, at a higher concentration, increase in time from 72 to 96 hrs did not result in any increase in mortality for *S. calamistis*. Bioassays with *B.t* toxin against *B. fusca* showed that the isolates were less toxic to the 2nd instar larvae. Isolate 44M was the most toxic with a larval mortality of 51% in 6 days.

Polyacrylamide gel electrophoresis of the proteins of the different *B.t* isolates revealed major protein bands of molecular weight ~28, ~ 65 and ~130 kDA, for most of the isolates except isolate 1M which did not express the ~130 kDA protein band but expressed the other two protein bands.

The study concluded that local *B. t* isolates could be used in pest control pragrammes as they were highly toxic to the target insect species, *C. partellus*, *S. calamistis* and *B. fusca* and elaborate analysis needs to be done to find out whether the isolates are toxic to other insects pests of other orders such as Coleoptera and Diptera.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction

Africa's food deficit is largely due to pre-and post-harvest crop losses, especially for graminaceous crops such as maize, rice, wheat and sorghum (Brownbridge, 1991). In addition to drought and land degradation, a major threat to increased food production is damage to crops by insect pests both in the field and during storage. These are the major constraints to efficient cereal production in developing countries.

Among lepidopterous pests, the spotted stem borer (*Chilo partellus)* (Swinhoe) and the maize stem borer *(Busseola fusca*) rank as the most important stem borer pests of sorghum and maize in Africa (Sithole, 1989; Seshu Reddy, 1989). They are predominant borer species of economic importance in Eastern Africa and Southeast Asia (Brownbridge, 1991). They feed exclusively on graminaceous plants whereby heavy infestations may result in severe crop losses and hunger, given that maize and sorghum are staple foods in the East Africa region and mostly grown by subsistence farming.

Maize and sorghum are the major host plants of *Chilo partellus*, but it has also been observed damaging pearl millet, finger millet, rice wheat and sugarcane in the field (Sithole, 1990). Wild host plants include such grasses as *Andropogon spp*, *Sorghum halepense, S. verticilliflorum* and *Panicum maximum.* Larvae of *C. partellus* cause more or less similar damage symptoms on maize and sorghum plants (Sithole, 1990). They migrate upwards in the direction of the whorls soon after hatching. Once in the whorls, they feed on the leaves for a while and cause a characteristic pattern of small holes and scarification of leaf epidermis. Later they penerate into the growing points and the

symptom that develops is the "dead heart" characterised by dead central leaves in young host plants. Later the larvae penetrate into the stem and feed whereby they create tunnels. This reduces plant vitality, the grain filling process and promotes lodging of plants they mature (Sithole, 1990). Infestation levels and yield losses caused by *C. partellus* have not been quantified in most countries in the subregion. Estimates reported in publications have been based on visual evaluations of the infestations. (Sithole, 1989a).

Busseola fusca is also a major pest of maize and sorghum in many countries of tropical Africa (Harris, 1989). It occurs in the cooler high lying inland areas. Young larvae cause damage to the leaves while older larvae bore into the main stem and cobs. In severe attacks the central shoot withers, causing a typical "dead heart" syndrome, which results in reduced yields (Swaine, 1957). Research on this important pest has been hampered by the occurrence of a six- month long diapause by the second-generation larvae in the last larval stage (Smithers, 1969; Harris, 1989).

S. calamistis occurs in the warmer coastal regions (Harris, 1989). Larvae bore into the stems under the leaf sheath on plants. Their stem boring may kill young plants, make older plants more susceptible to lodging and seriously reduce yield. Later generations of *S. calamistis* develop on maize cobs (Dabrowski *et al.*, 1984).

Control measures have largely been based on chemical pesticides. These broad-spectrum chemicals have been used abundantly in the containment or eradication of pests. Important problems have however confronted the use of chemical pesticides. Non - degradable chemical residues accumulate to high levels in the environment. These chemicals find their way into the rivers therefore endangering the fauna and flora in these environments. Chemical pesticides have also disrupted resident natural enemies and

allowed the development of secondary pests. Chemical control of the American bollworm (*Heliothis armigera*) on cotton on the Sudan led to an outbreak of the whitefly (*Bemisia tabacci*), as a consequence of the elimination of natural agents (Hussey, 1980).

Increased use of chemical pesticides also results in resistance of the target insects to chemical pesticides. They have also created an ecological imbalance through the destruction of beneficial non-target insects, and even most of them if not all are highly toxic to man. Furthermore, chemical pesticides have to be imported, hence expensive and are often unavailable to the small-scale farmers who form the bulk of the food producers in Africa.

Alternatives have been sought, and the search is on for sociologically acceptable, sustainable and enviromentally safe Integrated Pest Management (IPM) strategies for these key crops. Integrated pest management systems have been developed whereby, all control strategies are considered including cultural control, chemicals, and biological control in order to come up with a more economic, sustainable and ecologically sound approach to pest management. Recent promising progress in agricultural entomology and microbiology demonstrates that there is a considerable unexploited potential. The microbial pesticides based on *Bacillus thuringiensis (B.t)* can in an integrated control programme be used rapidly and efficiently to destroy insect pests (Dulmage, 1993).

1.2 Microbial control of insects

Many species of entomopathogenic microorganisms (inducing fatal or debilitating diseases in insects) have been suggested for development as microbial insecticides. Today many species of entomopathogenic bacteria, fungi, protozoa and viruses have been formulated into over 40 commercial or experimental microbial insecticides.

Such agents include:

Fungi: *Metarrhizium spp, Beauveria spp.*

Protozoa: *Nosema spp; Thelohania spp; Martessia spp.*

Viruses: Baculoviridae; Reoviridae.

Bacteria: *Bacillus popilliae, B. thuringiensis, B. sphaericus, B. cereus.*

However, some of these entomopathogens lack some of the attributes that would make them desirable as microbial control agents.

An entomopathogen should:

- Have high pathogenicity
- Be able to persist in the environment of the host
- Have high transmission efficiency
- Be easy to produce
- Be safe to non-target fauna and flora.

In the case of entomopathogenic fungi, several are being tested and commercial products have appeared in the market. Nevertheless, technological problems relating to production, formulation, product stability, fate in the environment and performance are serious (McCoy, 1990). It is not surprising therefore that products based on *B. thuringiensis* are today the most successful of all living insecticides (Burges, 1982).

1.3 Bacteria as microbial insecticides.

Nearly all entomopathogenic bacteria belong to the class Schizomycetes in the order Eubacteriales. Both sporulating and non-sporulating bacteria are potential candidates, but more success has been achieved with spore formers of the genus *Bacillus*; family Bacillaceae. They are aerobic, gram positive, rod shaped bacteria that form spores

during a stage in their life cycle. Most species of insect pests including caterpillars, beetles, flies, grasshoppers and termites are susceptible in varying degrees to the vegetative cell, endotoxins or exotoxins produced by more than 30 varieties of *B. thuringiensis*.

Other spore-formers that have been evaluated include *B. sphaericus* that is effective against gnats and mosquitoes. The list of appropriate bacterial species, although short contains the most promising microbial control agents (Burges, 1982). The range of pest species susceptible to entomopathogenic bacteria is considerable, and contains some of the most serious insect pests in agriculture and medical entomology (Table 1).

Table 1: Examples of insect pests of agricultural and medical importance that are controlled by bacteria (Osir and Vundla, 1999; Brownbridge and Onyango, 1992; Kariuki, 1987; Orduz *et a.l,* 1992; Kahindi, 1987). The table was personally developed.

	Pest		Crop attacked	Bacteria used
	Common name	**Scientific name**		
Agriculture	Cotton bollworm	*Helicoverpa armigera*	Cotton	*B. thuringiensis*
	Maize stalk borer	*Busseola fusca*	Maize, sorghum	*B. thuringiensis*
	Spotted stalk borer	*Chilo partellus*	Maize, sorghum	*B. thuringiensis*
	Pest		**Host animal**	**Bacteria used**
Medical Entomology	**Common name**	**Scientific name**		
	Tsetse fly	*Glossina morsitans*	Human beings, animals	*B. thuringiensis*
	Mosquitoes	*Aedes aegypti, Culex pipiens, Culex quinquefasciatus*	Human beings	*B. thuringiensis B.sphaericus*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History on the development of insect pathology

From early times man has been aware that insects suffer from diseases. This awareness has developed at an ever-accelerating rate into the science of insect pathology. Early interest in insect diseases was focussed on beneficial insects such as the honeybee and the silk worm. In Europe, Aristotle was the first to mention that bees suffered; and in 1835 Agostino Bassi showed that animal disease could be caused by a microorganism, when he found that the fungus *Beauveria bassiana* causes muscardine (infectious disease) of silk worms (Burges, 1982).

These studies focusing on the beneficial insects (the honeybee and silk worm), became the foundation of fundamental work on the nature of insect disease and its role in the ecology of insects. Gradually these studies were extended to pest species too and the concept of utilizing disease to control these insects was born. Insecticidal preparations based upon various strains of *B.thuringiensis* currently account for 80-90% of all biological pest control agents sold worldwide, making it the most successful type of bioinsecticide (Bernhard *et al.*, 1997).

2.2 *Bacillus thuringiensis (B.t.)*

B. thuringiensis is an aerobic, spore-forming, gram-positive bacterium. *B.thuringiensis* is a ubiquitous microorganism with a worldwide distribution and abundance (Martin and Travers, 1989). *B. thuringiensis* was first discovered by Ishiwata who found it in a colony of sick or dying silkworms in Japan in 1901. Although his

description was good enough to let us recognize that he had isolated *B.t.*, his nomenclature was improper and it was left to Berliner in 1911 to name his isolate, *B. thuringiensis* after the province of Thuringen in Germany where he had found it (Dulmage, 1983). Most known isolates cause diseases in insect larvae initially described as *sotto* (Ishiwata, 1901) or Schlaffsucht disease (Berliner, 1915; Bernhard *et al*, 1997).

B.thuringiensis is indistinguishable from the more common soil bacterium *Bacillus cereus* by most phenotypic characteristics (Bauman *et al*, 1984). It can be distinguished by production of one or more proteinaceous parasporal crystal (delta-endotoxin) during sporulation. The parasporal crystals predominantly consist of delta-endotoxin, of molecular masses ranging between 25 and 140 kDa (Bernhard *et al.*, 1997). Bernhard and his collaborators found that the proportion with bipyramidal shaped crystals was 46%, while among the range of other shapes, 14% were spherical and 4% were rectangular.

These proteins are specifically toxic to insect larvae and are widely used as bioinsecticides against, Lepidoptera (Caterpillars), Diptera (mosquitoes and black flies), and Coleoptera (beetles) pests. However, not all strains of *B.t.* are toxic to either of the above group of insect pests. Martin and Travers, (1989), found out that 40% of the crystal formers they isolated were not toxic to any of the insects that were tested. Chilcott and Wigley, (1993), also found that some of their isolates were not toxic to any of the insects tested. Little attention has been paid to non-toxic isolates of *B.t.*, despite the fact that their distribution in natural environments is wider than that of the toxic ones (Ohba and Aizawa, 1986).

When *B.t* is growing in or on artificial media, a period of rapid vegetative growth is followed by sporulation, (the formation of an environmentally resistant endospore). Sporulation is usually accompanied by crystal formation (Fast, 1981). The vegetative cells of *B.t.* are peritrichously flagellated rods, which often occur as filaments of four or more cells (Luthy *et al,* 1982). Since its discovery *B.t.* has received considerable attention in the research community.

2.3 Nutritional Requirements of *B.t*

The most basic media used for the production of *B.t* were chemically defined containing nitrogenous sources, such as ammonium phosphate, and energy -producing carbohydrate sources, such as dextrose, which are easily assimilated by bacteria (Morris *et al.*, 1997). *B.t* also requires for optimal growth a variety of organic sources which function physiologically as protein, nucleic acid, and co-enzyme constituents and as co-factors of numerous enzyme reactions and bacteria cytochromes (Morris *et al.*, 1997).

2.4 Classification of *B.t*

Classification of *B.t* is based on the antigenic reactions of the flagellae associated with young actively, motile cells of the *Bacillus.* This was proposed in 1958, by Bonnefoi and de Barjac (Dulmage, 1993). Depending on antigenic characteristics, isolates are either assigned an " H-number" (also called serotype or subspecies) in consecutive order or placed in a group of other isolates (Dulmage, 1993). For example

H-number	**strain**
H-3a, 3b	*kurstaki* HD- 1
H-7	*aizawai*
H-14	*israelensis*

Serotyping *B.t.* flagella by agglutination proved to be the most sensitive, specific reliable and rapid method of identification (de Barjac, 1981). Currently, the number of serotypes exceeds 40.

Crystal protein antibodies have also been used to differentiate among the numerous *B. thuringiensis* isolates in an attempt to categorize variants with similar characteristics. The largest effort to compare the serological differences of *B.t* crystals was reported by Krywienczyk et al., 1978. They suggested that 14 crystal types identified using double immunodiffusion are distinctively different from one another. Those exceptions are the similarity between two variants of *kurstaki* k-1 and k-73 (Krywienczyk *et al.*, 1978). However there was a recommendation of reducing the 14 different crystals types to five groups based upon data resulting from use of an immunodiffusion technique involving microcomplement fixation (Lynch and Bauman, 1985).

Use of crystal serology for differentiating *B.t.* isolates on the basis of toxicity toward insects appears useful in detecting specificity among orders but not within genera (Smith, 1987). Currently there are more than 40 *B.t* serotypes or subspecies. The typical shape of most crystals is bipyramidal (Fast, 1981). However, other shapes such as irregular, amorphous, cubical and parallelogramic have also been reported (Mikkola *et al*, 1982; Abel Hamed *et al.*, 1990).

2.5 Insecticidal toxins produced by *B.t*

B.thuringiensis produces several types of toxins. These are α -exotoxin (heat-labile exotoxin), β – exotoxin (heat-stable exotoxin, fly-factor, an adenine containing compound), δ-endotoxin (crystal protein) and the louse factor (Dulmage *et al*, 1981).

The α -exotoxin and the β-exotoxin are non-proteinacious and are secreted by the vegetative cells. The α-exotoxin, identified as lecithinase c, is water soluble, heat- labile and toxic to insects. β-exotoxin is heat stable, highly toxic to larvae of several species of flies. It is commonly referred to as " thuringiensin", and has a broad spectrum of activity. δ-endotoxin (crystal protein) has a more limited host range i.e. they are specific in their action and are only toxic to insect pests of particular orders. For example a δ -endotoxin that is toxic to insects of the order Lepidoptera may not be toxic to those of the order Diptera or Coleoptera and vice versa.

Gonzalez *et al.*, (1982) demonstrated that most δ - endotoxin genes are located on large plasmids, which either are self transmissible or can be co- transferred from a donor to a receptor strain in a conjugation - like process. Crystal proteins from numerous strains of *B.t* have been classified according to the similarity of their amino acid sequences and their insecticidal specificity (Hofte and Whitely, 1989).

They classified crystal genes into four major classes. The classes were CryI (Lepidoptera specific); CryII (Lepidoptera and Diptera- specific); CryIII (Coleoptera-specific) and CryIV (Diptera-specific). Since then, two classes of nematode active toxins have been described, namely; CryV and CryVI. However, (Zhong *et al*, 2000), reported the first *B.t*, toxin which is toxic to insects from three insect orders (Diptera, Coleoptera, and Lepidoptera). They showed that Cry1 Ba1 from YBT-226 is additionally toxic to selected dipteran insects and can be classified as a lepidopteran-coleopteran-dipteran triple toxin.

2.6 Mode of action of *B.thuringiensis*

The mode of action of *B.t.* entomopathogenic toxins has been extensively studied but it has not yet been totally clarified (Rajamohan *et al.*, 1998). The ultimate cause of the insecticidal effect of *B.t* toxins is an extensive damage of the midgut epithelial cells (Rausell *et al.*, 2000). The crystal endotoxin is a protoxin that requires the high pH and proteases characteristic of the larval insect midgut for activity (Lecadet and Mortouret, 1965; Haider *et al.*, 1986). Toxins of the CryI class, which are active against lepidopteran insect larvae, are formed as 120 to 130 kDA protoxins, which are solubilized in the alkaline environment of the lepidopteran midgut. After solubilization and processing by the midgut proteases, the protoxins results in a relatively stable mature 60 to 65 kDA toxin. In susceptible insects, the mature toxin binds to the midgut epithelium and forms membrane pores, which results in lysis of the epithelial cells and eventually death of the insect (Hofte and Whitely, 1989 and Knowles *et al.*, 1993).

Although the primary action *B.t.* toxin is in the midgut epithelium of sensitive insects, investigators have only recently demonstrated that the toxin binds specifically to the Brush Border Membrane Vesicles (BBMV) prepared from the midgut (Gill *et al.*, 1992). The model for the mode of action of *B. thuringiensis* toxin proposes that toxicity is related to a chain of events: toxin solubilization, activation, binding, pore formation, and osmotic swelling of the cells (Rausell, 2000). Their work gave support to the correlation of post activation events in Nun Moth (*Lymantaria monacha*), validating this model.

2.7 Persistence of *B.t* in the environment

The short persistence of *B.t* on crops following exposure to direct sunlight has been reported (Beegle *et al.*, 1981). The sunlight mediated inactivation of *B.t* preparations in the field is a problem affecting the efficacy and economics of this bioinsecticide (Beegle *et al.*,1981). Residual activity on crops appears to be related to more than just ultraviolet radiation. Other factors such as heat, desiccation, or pH may also play some role (Leong *et al.*, 1980).

Morris and Moore (1975), for example reported that *B.t* applied to spruce trees lost 50% of their insecticidal activity in eight days in shade, compared with 50% loss in 2 days in sunlight. *B.t* exposed to irradiation loses its toxicity toward larvae of *Heliothis armigera* (Cohen *et al.*, 1991). Even 3 hours of irradiation resulted in an appreciable decrease in *B.t.* toxicity as 20% of insects survived. Longer exposure times resulted in a lower mortality, as 70 and 95% of the insects were alive after 6 and 12 hr of irradiation, respectively. Amino acid tryptophan was destroyed in sunlight-mediated inactivation (Pozsgay *et al.* 1987). Exposure to either natural sunlight or to light from the solar simulator brings about a loss of toxicity in purified *B.t* var *kurstaki* HD1 crystals.

The most recent study by Kaplan *et al.*, (as reported by Morris in 1993) found the 300-350nm solar spectrum to be largely responsible for crystal damage and loss of toxicity and that the absorption of the chromophores (light absorbing molecules) by the crystals exposed to the fermented liquor made the crystals photosensitive. The effectiveness of several UV-absorbing materials to improve the performance of *B.t* was investigated (Morris, 1989). However, some of the UV photostabilizers may introduce ecological problems related to soil and water pollution. A new approach to extend and

maintain the biological activity of photolabile pest control has been recently suggested (Margulies *et al.*, 1985). This approach involves a specifically intimate alignment between an organic pesticide and a selected organic chromophore. Such spatial arrangement facilitates transfer of energy or electrons between the excited molecule and the chromophore. In this process a probable photochemical reaction is fully or partially prevented.

In their study Cohen *et al.*, (1991) observed that photoprotection of the toxic component was obtained by adsorption of cationic chromophores such as acriflavin (AF), methyl green, and rhodamine B to *B. thuringiensis*. AF gave the best photoprotection and a level of 0.42 mmol/g dye absorbed per gram of *B.t.* was highly toxic after 12 hr of ultraviolet (UV) irradiation.

2.8 Advantages of *B.t* as a Biopesticide

At present *B.t* accounts for perhaps 80-90% of the total world microbial insecticide market (Carlton *et al,* 1990). The following advantages are in support of *B.t.* (Luthy *et al,* 1982):

- δ-endotoxin as well as the spores incorporated in the products have no mammalian toxicity.
- They do not harm predators and non-target insect populations.
- *B.t* has no phytotoxicity.

Because of the safety demonstrated with *B.t* these preparations are exempt from restrictions. Their use is permitted up to the date of harvest, which is important for plant protection in vegetable crops such as tomatoes, cabbages etc.

2.9 Isolation and screening of *B.t.* in Kenya

Several crystal forming *B.t* strains have been isolated from soils collected from various geographical regions in Kenya (Brownbridge, 1991). These isolations were done at the International Insect Physiology and Ecology, where more than 150 *B.t.* strains were isolated. Studies to screen isolates of *B.t* to elucidate virulent strains, which could be of use in the field were also undertaken with emphasis on the isolation of native *B.t* strains, due to possible objections to the wider scale introduction and usage of exotic bacterial strains. Many of the identified exotic strains have been isolated from temperate zones, and may not be so well suited for use in a tropical environment.

Local isolates could potentially posses' better characteristics such as greater field persistence and toxicity at a higher temperature range (Brownbridge, 1991). A range of materials was collected as being likely sources of entomopathogenic bacteria, such as, insect frass, dead insect material and soils. They were sampled from a number of different sites and ecological zones, which included Njoro, Lambwe Valley, Mombasa area, Busia and Mfangano Island.

From another study carried out by Brownbridge and Onyango in 1992, whereby they screened exotic and locally isolated *B.t.* strains for toxicity to the *C. partellus* (Swinehoe) at International Insect Physiology and Ecology laboratories, some local isolates were apparently more toxic than any of the exotic strains obtained and tested. The results further highlighted the differences in the activity of different *B.t.* subspecies, and showed that there was variation in the potency of strains within the same subspecies. Thus the origin of the strain appears to affect its toxicity to a particular target species

(Brownbridge and Onyango, 1992). Isolate code, source and isolation materials for some of the *B.t.* Strains are shown in Table2.

Although these *B.t* isolates from Kenya have not been classified (to strains or variety), some of the isolates have been found to be active against *Aedes aegypti*, *Glossina morsitans, Spodoptera exempta* and *Chilo partellus* (Brownbridge, 1989). Isolates A-3, B1-1, A-C-2 and L1-6, recovered from dead *Heliothis armigera* larvae and insect frass, were the most potent strains recovered, (Brownbridge, 1992).

No isolations were done from Kakamega and Machakos districts, yet these are highly potential agricultural areas. Isolation of *B.t* isolates from these areas may come up with potent *B.t* strains that can be used in the control of stem borers that are rampant and cause serious damage to maize, which is the main crop under cultivation in these areas.

Table 2: Toxic strains isolated from Kenya (Brownbridge, 1991)

Isolate code	Isolation material	Site
A -3, B1-1, A-C-2	*Heliothis armigera* larvae	Njoro, Rift Valley Province.
L1-5, L1-6	Armyworm frass	Lambwe Valley, Nyanza Province
M44-7	Soil sample	Mombasa area, Coastal province
BUS – 3/7	Soil sample	Busia ,Western Province
MF–3A-1	Soil sample	Mfangano Island, Nyanza Province

2.10 Cases of *B.t.* biopesticide use in Kenya.

In Kenya preliminary experiments using *B.t* based biopesticide have shown that the cereal stalk borer *Busseola fusca* and *Chilo partellus* and also *Eldana saccharina*, *Sesamia calamistis* and the legume pod borer *Maruca testulalis* can be controlled effectively (Kariuki, 1987). *B.thuringiensis* has also been used on coffee in Kenya to control *Ascotis selenaria* (Waikwa and Mathenge, 1977).

2.11 Resistance to *B.t.* Toxin

Laboratory-selected resistance to *B.t* toxin has been documented in a number of pest species and field-evolved resistance has been detected in *Plutella xylostella* (Diamondback moth). *P. xylostella* is renowned as the only insect to have evolved significant resistance to *B.t* subspecies *kurstaki* from field exposure alone in the Hawaii (Tabashnik *et al.*, 1990) and Florida (Shelaton *et al.*, 1993).

Currently, there are two strains of *B.t.* used commercially on *P.xylostella. B.t* subspecies *kurstaki* (*Btk)* and *B.t* subspecies *aizawai (Bta).* Unfortunately, prolonged use of *Btk* has led to multiple cases of resistance worldwide for this damaging pest of crucifers. In those populations examined, the primary mechanism appears to be reduced binding of the Cry1A toxins, Cry1A (a), and Cry1A(c), to its gut membrane binding site with relatively little cross resistance to toxins from other families, eg. CryIB, CryIC, CryID, CryIE, CryIIA, etc. (Tabashnik *et al.*, 1994; Tang *et al.*, 1994).

Resistance to *Btk* spore has also been observed, but so far no mechanism other than reduced CryIA binding has found to account for the spore resistance (Tang *et al*, 1994). *Btk* can produce CryIA (a), CryIA (b), CryIA(c), CryIIA and CryIIB protein, and *Bta* can produce CryIA (a), CryIA (b) or CryIA(c), depending upon isolate, and CryIC

and CryID proteins (Koziel *et al*, 1993). Since resistance to *Btk* in certain regions of Florida has dramatically reduced its efficacy (Shelaton *et al*, 1993), use of *Bta* to reduce pest populations of Diamondback moth has become more popular. As a consequence, the development of resistance is a major concern for the continued success of *B.t* bioinsecticides.

2.12 Justification of the present study

Maize (*Zea mays*) was introduced into East Africa at the end of the 19th Century. Maize is now the most widely grown cereal in Kenya and many other parts of East Africa. Kakamega District is one of the highly agricultural productive regions in our country, maize being the main crop under cultivation. Machakos District is also an area with a high potential for agriculture. This important coarse-grain crop together with sorghum (*Sorghum bicolor*) is attacked by a complex of stem borers, three of which are the target pests of this study: *C. partellus, S. calamistis* and *B. fusca.*

The introduction and widespread cultivation of maize has undoubtedly led to an increase in abundance of stem borers by providing a highly nutritious and readily available food source with little natural resistance to borer attack. The cost of the control of the borers using chemical pesticides have become increasingly expensive for the resource poor farmers and therefore the search is on for use of alternative control mechanisms such as the use of *B.t* based biopesticides which if based on local *B.t* isolates are cheaper and are also environmentally friendly.

Many of the well-characterised *B.t* strains have been isolated in temperate zones and may not be as well suited for use in pest management programmes in the tropics as local isolates, which may possess useful attributes such as greater field persistence at high

temperature. Therefore this study seeks to isolate *B.t* from these areas and screen them for potency against the target pests.

2.13 Specific Objectives

It's the purpose of the study to:

(a) Isolate and partially characterize *B.t.* from soil samples collected from specified sites in Kakamega and Machakos districts.

(b) Compare the potential toxicity of the *B.t* isolates to *C. partellus, S. calamistis* and *B. fusca.*

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Insects and Artificial Diet

Insects and their artificial diet were purchased from the Department of Insect Rearing and Quarantine Unit at the International Centre of Insect Physiology and Ecology (see Tables 3 and 4). Insects were reared on a lepidopteran artificial diet. Unless otherwise stated neonate larvae (1^{st} instar) and 2^{nd} instar larvae of *C. partellus*, 2^{nd} instar larvae of *B. fusca* and 1^{st} instar of *S. calamistis* were used for the bioassays.

3.2 Soil sample collection

About ten grams of surface soil samples scraped to a 2-inch depth with a jembe were collected randomly from agricultural lands in 14 different farms of Kakamega District and 9 of Machakos District. The samples were placed in sterile plastic bags and stored at ambient temperature. The fields from which these samples were taken are usually cultivated for crops such as maize, beans and cowpeas. However it was the dry season in Machakos at the time of collection of the samples, and no crop was on the ground. These soils had never been treated with or exposed to *B.t.* or its products before.

3.3 Media Preparation

3.3.1 Nutrient Agar Plates

The methods used in media preparation (nutrient agar plates) are as described by Poinar and Thomas (1978) and by the manufacturer's instructions. Twenty-eight grams of nutrient agar was weighed and put into a two litre conical flask. One litre of distilled water was added and to boiled to dissolve the nutrient agar completely. The mouth of the conical flask was firmly secured with cotton wool rolled into a ball and covered with

aluminium foil. The media was then autoclaved for 15 min at 121° C at a pressure of 15lb/sq.in. The media was then allowed to cool down to 45° C and about 20 ml poured aseptically into each sterile plastic (disposable) petri dishes. The plates were stored in the refrigerator at 5° C.

3.3.2 Nutrient Broth

Nutrient broth (NB) was prepared according to the manufacturer's instructions. Thirteen grams of NB was weighed and put in a one litre conical flask. It was mixed thoroughly and dispensed into 250-ml conical flasks, which were then covered with cotton wool and aluminium foil. These were autoclaved at 121° C, 15lb/sq.in. for 15 min.

3.3.3 Inoculation of the medium

The method used was the streak plate method as outlined by Poinar and Thomas (1978) and Baker and Silverton (1978). The inoculating loop was flamed along its entire length until red-hot, and then the holder was passed quickly through the flame a few times. The loop was dipped into the inoculum and used to streak on the agar in the petri dish, and spread at several angles. The plates were then incubated at 30° C for 24 hrs. Unless otherwise stated the same protocol outlined above was used in all the subculturing exercises of this study

Table 3. Artificial diet composition for *Chilo partellus* (Onyango and Bungu, 1994).

Ingredients	%	Quantity for 10 Litre Diet
Fraction A		
1. Distilled water (ml)	46.52	4652
2. Benlate (g)	0.02	2
3. Grabacin (g)	0.003	0.3
Fraction B		
4. Bean powder (g)	10.2	1020
5. Sorghum leaf powder (g)	3.72	372
6.Brewer's Yeast (g)	0.74	74
7. Ascorbic Acid (g)	0.17	17
8. Vitamin E (g)	0.1	10
9. Sorbic Acid (g)	0.09	9
10. Methyl Paraben (g) (Dissolved in 62.5 ml ethanol)	0.15	15
11. Formaldehyde 40% (ml)	0.19	19
Fraction C		
12. Agar Tech. No.3	0.88	88
13. Distilled Water (ml)	37.22	3722
TOTAL	100	10L

Table 4. Diet Composition for *B. fusca* and *S. calamistis* (Ochieng' Odero *et al.*, 1991)

Ingredient	Quantity for 1.2 L diet
Bean (*Phaseolus vulgaris*) powder	80.0 g
Brewer's Yeast	34.0 g
Sorbic Acid	1.2 g
Ascorbic Acid	3.2 g
Methyl *p*-hydroxybenzoate	2.0 g
Benlate	1.0 g
Distilled water for blending	500.0 ml
Sorghum stem powder*	40.0 g
Agar -agar	20.0 g
Distilled water for Agar -agar	800.0 ml
Formaldehyde 40%	2.0 ml
Vitamin suspension **	10.0 ml

*4-8 weeks old stems

** Vitamin suspension was prepared by dissolving 2.6 g Vanderzant vitamin mixture in 100 ml-distilled water.

3.4 Isolation of Bacilli containing crystalline structures

The isolation of the spore forming bacteria was performed according to the pasteurization method described by Ohba and Aizawa (1989). One gram of each sample was suspended in 10 ml of sterile distilled water and the preparations mixed vigorously by vortexing for 1 min. The solid matter was then allowed to settle for 3 min and then 1 ml of the supernatant was pasteurized at 80° C for 3 min in pre-warmed 20-ml glass universal bottles. This was to ensure that the vegetative forms of both spore and non-spore forming bacteria were killed.

Samples were then plated at two concentrations (undiluted and 10^{-1} dilution) onto nutrient agar plates. The plates were incubated at 30° C for 48 hrs and examined for colonies having *Bacillus cereus* / *B. thuringiensis* morphology. They were further sub-cultured on nutrient agar plates and incubated at 30° C for 5 days and then stained with the Smirnoff stain for observation of parasporal bodies.

3.5 Selective growth of *B. t*

The method described by Travers *et al.*, (1987) was followed. Bacteria from colonies described above (section 3.4) were shaken in liquid broth buffered with 0.25M Sodium Acetate for 4 hrs at 200 rotations per minute (r. p. m) at 30°C. A small sample of this was heated and then streaked on nutrient agar plates. The new colonies formed after overnight incubation at 30°C were observed for the presence of crystal inclusion.

3.6 Identification

3.6.1 Microscopy and staining procedures

3.6.1.1 Gram staining

Gram staining was done according to Poinar and Thompson (1978) to ascertain the Gram reaction of the culture. This was done at 18 hrs, when the culture was still in the exponential growth phase using the high power objective. Observations were also done using the phase contrast microscope but this required no staining. The gram-positive bacteria appeared dark violet whereas gram-negative bacteria were coloured pink.

3.6.1.2 Smirnoff staining

This was done for the parasporal crystal bodies, as outlined by Smirnoff (1962). A few drops of solution A (1.5 g Amido black dissolved in 50 parts 90 % methanol, 40 parts distilled water, and 40 parts acetic acid was added to the heat fixed slides of single colony isolates and washed off after 70 sec. Solution B (1g of basic fuchsin dissolved in 10 ml of 95 % ethanol and with 5g of phenol dissolved in 90 ml of distilled water) was then added to the slide and left for 20 sec before washing in cold tap water and dried on a filter paper. Examination was by means of a standard light microscope with an oil immersion objective, a standard filter being used. All crystals in the smear stained in a black luster with lilac blue tint. The spores stain pink with clear lilac margin marking their contours. Bacterial cells and their fragments assume a light lilac taint when.

3.7 Biochemical Tests

3.7.1 Catalase Test

Isolates selected as having *B.t* or *B. cereus* morphology (rod shaped cells, gram positive) were subjected to a catalase test for the presence of catalase enzyme. Individual

colonies were scrapped from a plate with a non-metallic instrument and suspended in a drop of 3 % hydrogen peroxide (three ml in a 100 ml of sterile distilled water) on a slide. It was examined immediately and then after 5 min for bubbles. The appearance of oxygen bubbles showed the presence of catalase. Inside the bacteria this enzyme catalyses the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase enzyme destroys the H_2O_2 molecules produced during aerobic respiration.

3.7.2 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 butylene glycolic fermentation). Detection of this substance is a useful phenotypic test. MRVP medium (polypeptone 5g, Glucose 5g, Sodium chloride 5g, and distilled water one litre) was used. The media was dissolved gently by heating, and adjusted to pH 7. The media was dispatched into 5-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 *B.t* isolates and incubated at 30° C for 48 hrs. One ml of culture was mixed with 0.6ml VP reagent A (Potassium hydroxide 40g, Distilled water 100ml) and 0.2ml VP reagent B (Alpha-napthol 6g, Absolute ethanol 100ml). The tubes were placed open on a slant to increase contact with air. The surface changed to a pink color within 10-30 min indicating that the test was positive.

3.8: Morphological characterization of the *B.t* isolates

Morphological characteristics (rod length, rod width, spore shape, spore location, shape of the crystal) of the isolates were determined using the ocular micrometer. The measurements are a mean of 10 cells.

3.9 Growth of Isolates for Bioassays

Sporulated cultures of *B.t* isolates were used to inoculate 50 ml of Liquid Broth (Lab-lemco powder 1.0 g; Yeast Extract 2.0 g; Sodium Chloride 5.0 g per liter) in 250 ml fluted Elrenmeyer flask. The inoculated flasks were incubated on a rotary shaker for 96 hr at 30° C at 200 r.p.m. At the end of the incubation period. the spores and endotoxin were harvested by centrifugation at 4000 r.p.m. for 10 min. The supernatant was discarded and the pellet washed thrice by centrifugation in sterile 0.85 % saline and suspended in 5-ml saline and stored at –20° C.

3.10 Protein Determination

The UV protein determination method was used whereby Bovine Serum Albumin (BSA) was used as a standard at a concentration of one mg/ml. Absorbance was measured at 280 nm for dilution ranging 0.1 to 1mg/ml. A standard curve was obtained, which was used to determine the protein concentration of *B.t* isolates used for bioassays.

3.11 Determination of pH tolerance of *B.t* isolates

The different isolates of *B.t* obtained were tested for their pH tolerance. They were grown on nutrient broth at different pH values (5.0, 6.0, 6.5, 7.6 and 8.0) in screw cap tubes and incubated for 48 hrs at 30° C. Their optical density was then measured using a light spectrophotometer at 280 nm. The optical density of a blank containing nutrient broth at the same pH value but not inoculated with the *B.t* isolate was also taken.

The optical density of the isolate in the different pH was then subtracted from the optical density of the blank (broth at a particular pH without the bacterial innoculum) so as to determine the protein concentration of the isolate in the different pH values. The growth was then expressed as a percentage.

3.12 Screening assays

All screening and Bioassays were carried out using neonate (1^{st} instar) and 2^{nd} instar larvae of *C. partellus* and *B. fusca.* For the bioassays using *S. calamistis* only 1^{st} instar larvae were used. Two ml of the suspended *B.t* toxin was thoroughly mixed with 100 ml of agar based artificial diet at 50° C since the toxin is heat sensitive. Two ml of the diet was then dispensed into 20 ml tubes and allowed to cool to room temperature (25°C). One larva was then introduced per tube. The tube was then tightly closed with cotton wool and wrapped with gauze to prevent the larvae from escaping. Each set of experiment had 15 larvae, and was replicated 3 times on separate days. A control was also set up that consisted of 20 larvae. The larvae were also introduced singly into tubes containing untreated diet (without toxin). The larvae were incubated at 28° C. Larval mortality was recorded after every 24 hr. Isolates that caused 80 % larval mortality were retained for bioassay.

3.13 Bioassays

The protein content of each isolate was determined using the method described in section 3.8. By appropriate dilution of the stock suspension in sterile saline solution (0.85%) five concentrations were prepared for bioassays. A control treatment, which consisted of the artificial diet only, was also prepared. For each concentration, 1 ml of the *B t* suspension was mixed with 100 ml of the artificial diet, thereby adding a further 100-

fold dilution of the pathogen. Each concentration also had 15 larvae, (1per tube) replicated 3 times on separate days. Larval mortality was recorded after every 24 hr.

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

$$\text{Adjusted \% mortality} = \frac{\text{observed \% mortality} - \text{average contol \% mortality}}{100\ \% - \text{average control mortality}}$$

This process was followed for each of the *B.t.* strains that were used for bioassay. The data obtained was used to calculate LD_{50} values. Bioassays were also done with toxin from a *B. t* commercial product "Thuricide®" which is based on *B. t* subspecies *kurstaki* and an isolate from ICIPE, "MJ992" using 1st instar of *C. partellus.*

3.14 Leaf disc bioassays

In this bioassay three excised maize leaves (3cm each) were immersed in 50 ml of *B.t* suspension for 3 min making sure all the surfaces of the leaf are covered and then allowed to dry. They were then were placed in a petri dish. A piece of wet filter paper (about $1cm^2$ was dipped in distilled water) and placed in the dish to provide moisture. Leaf disc bioassays were carried out with all the *B.t* isolates, at a concentration of 8.6mg/ml. Fifteen larvae were introduced per dish. In the control dish untreated leaf discs were used. The experiment was replicated three times and on three separate days to cater for variation. The dishes were then sealed with two straps of stretched parafilm and incubated at 25 ± 2 ° C. Larval mortality was recorded after 24 hrs.

3.15 Characterization

3.15.1 Polyacrylamide gel electrophoresis (PAGE)

3.15.1.1 Analysis of toxin by electrophoresis

Electrophoresis on 12 % SDS-polyacrylamide gels was carried out under reducing conditions on a vertical electrophoresis unit (BIO-RAD), using 25mM Tris-glycine buffer, pH 8.3. Samples were diluted 1:1 with Laemmli sample buffer (Laemmli, 1970) containing 2 % (w/v) SDS, 6 % (v/v) β-mecarptolethanol, 10 % (v/v) glycerol, 0.002 % (w/v) Bromophenol Blue dye in 0.125M Tris-HCl buffer pH 6.8. Samples were boiled for 5 minutes before loading 25µl per well alongside standard molecular weight markers. Electrophoresis was carried out at 30mA until the tracker dye (Bromophenol blue) reached the bottom of the gel. Gels were stained by the Coomassie brilliant blue method described below.

3.15.1.2Coomassie brilliant blue staining procedure

Staining solution was made by dissolving 0.1% (w/v) Coomassie brilliant blue in 40% (v/v) methanol, 10% (v/v) acetic acid, and then filtered after the dye had dissolved. Gels were immersed in the staining solution for 30 min at room temperature. Destaining was done in excess 40% (v/v) methanol, 10% (v/v) acetic acid with several changes of the destaining solution until a clear background was obtained.

3.15.1.3 Estimation of molecular weight of toxin by SDS-PAGE

The molecular weights of proteins (spore /crystal complex) of the various *B.t* isolates were estimated by measuring the relative movement of the standard markers and constructing a standard curve. The relative movement was calculated by the formula;

$$RF = \frac{\text{Distance moved by each protein}}{\text{Distance moved by tracker dye}}$$

Where RF= relative movement of the standard markers.

The relative movement of the different toxins was similarly determined.

3.16 Statistical analysis

Statistical analysis was done using the SPSS program (version 7.0). This was for both significance tests (t-test), and probit analysis (LD_{50}) determinations.

CHAPTER FOUR

4.0 RESULTS

4.1: Soil analysis

Analysis of 14 soil samples from Kakamega and 9 soil samples from Machakos revealed that the soil pH values were different. The soil from Kakamega has a pH range of 4.28 -5.65, while that of Machakos has a pH range of 5.42 - 7.32. Thus the soil from Kakamega was found to be slightly acidic compared to that from Machakos.

4.2: Growth of *B.t* on Nutrient Agar plates.

B thuringiensis grows very fast. At 30° C it produces smooth, creamish white colonies, which are slightly raised from the nutrient agar after overnight incubation (Fig.1). In total twelve *B.t* isolates were isolated from the soil samples, of which 3 were from Kakamega (12F-K, K13-1 and K10-2) and nine from Machakos soil samples, 1M, VM-10, V14-M, 44M, 34M, 35M, 45M, V24-M and V15-M.

4.3: Selective growth of *B. t*

This method of selection for *B.t* did not select for *B.t* only, but colonies of other spore formers were also selected. Therefore this method was abandoned and pasteurization used instead.

4.4: Gram staining

B thuringiensis isolates were gram positive after staining at 24 and 48 hrs (Fig. 2). They had a dark violet coloration. At 48 hrs when the cells had sporulated, the spore, which is terminal, did not take up the stain and appeared colourless from the background of the cell (Fig. 3).

4.5: Smirnoff staining

B.thuringiensis isolates were stained at 72 hrs for the presence of parasporal bodies (crystals) (Fig.4). All crystals stained with an almost black luster with a lilac blue stint. The spores stain pink, whereas bacterial cells and their fragments assume a light lilac tint.

4.6: Morphological characteristics of the *B.t* isolates

Further morphological analysis of the isolates revealed differences only in the rod length. Seven of the isolates V24-M, V14-M, 12F-K, K10-2, K13-1, 1M and V35-M had a rod length of 3μm. Two isolates, 44M and V15-M had a rod length of 2.5μm, whereas three isolates, 45M, VM-10 and V35-M had a rod length measuring 2μm (Table 5). The spore shape was ellipsoidal and terminally placed in all the *B.t* isolates. The parasporal inclusions (crystal) of all the *B.t* isolates had a bipyramidal shape.

4.7: Biochemical Characteristics

All the twelve *B.t* isolates obtained tested positive for Gram stain, catalase and Voges-Proskauer tests (Table 6).

Fig. 1: Colonies of *B.t* on Nutrient Agar media .The colonies have a creamish white appearance with rough edges.

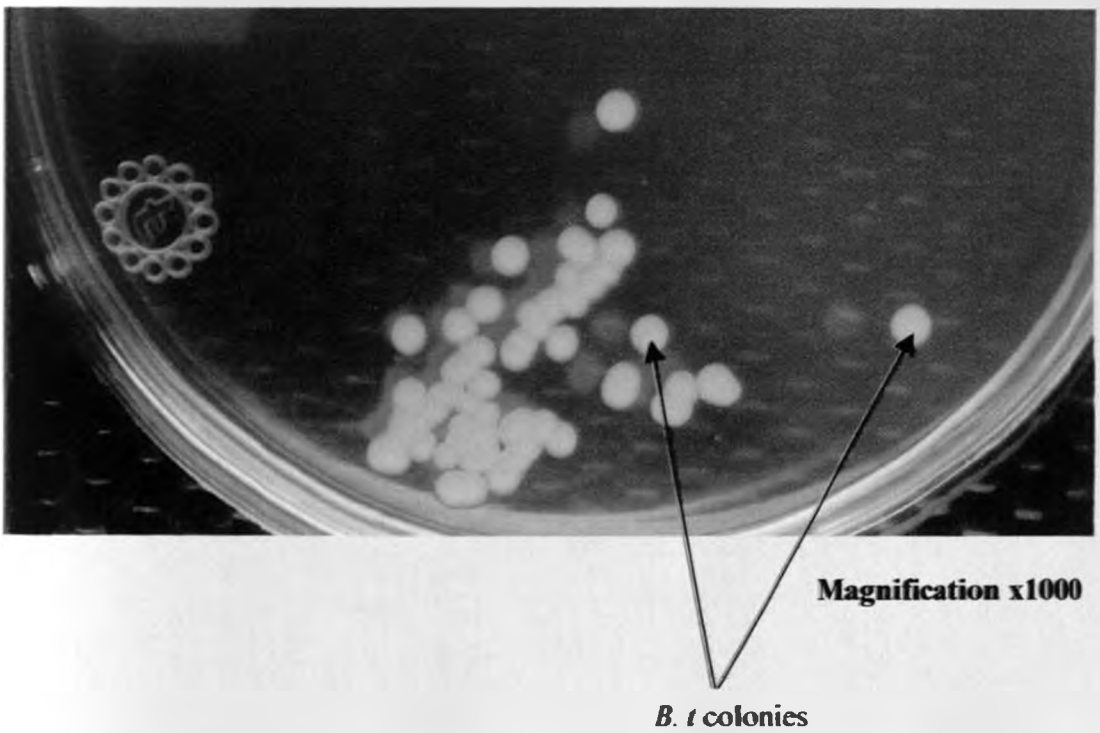


Fig. 2: A gram stain reaction of vegetative cells of *B.t* isolate V24-M after 24 hrs. The vegetative cells are rod shaped and gram positive. They are either single or chains of two, three or more rods.

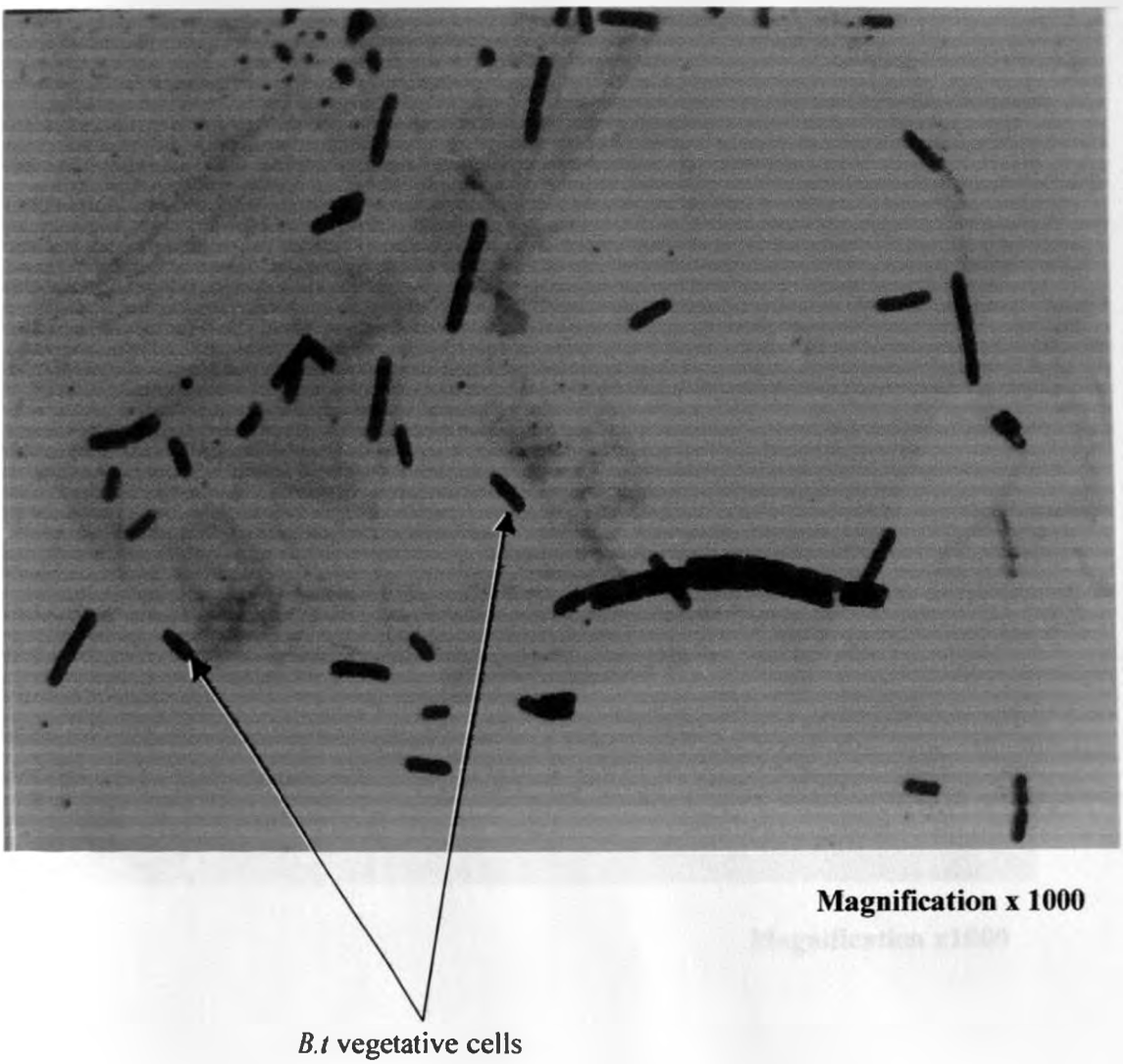
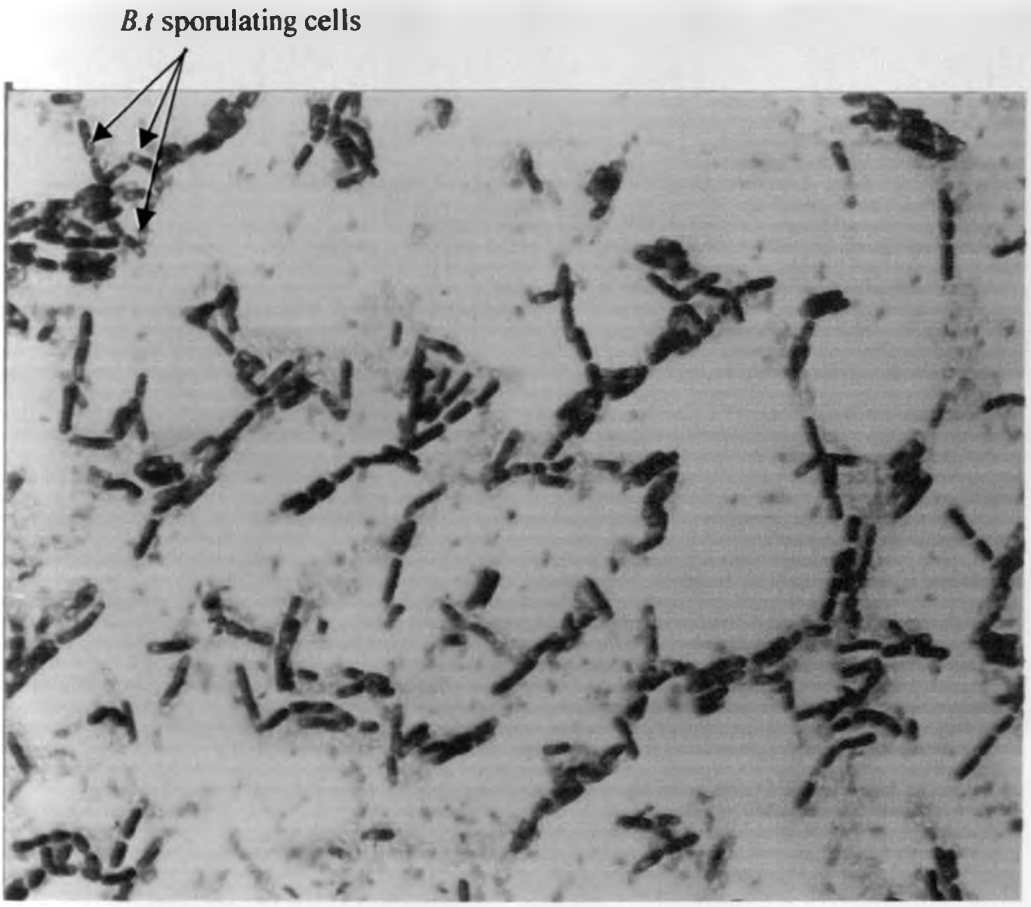
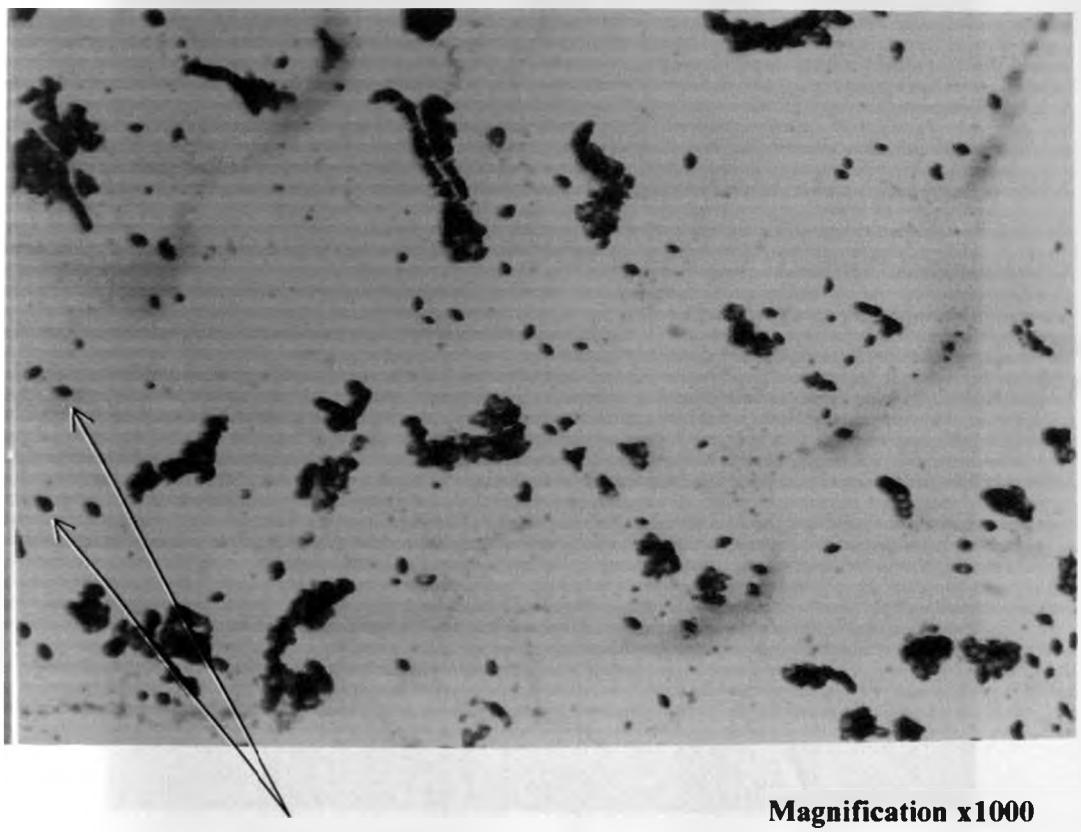


Fig. 3: A Gram stain reaction of sporulating cells of *B. t* isolate V24-M after 48 hrs. The spores, which appear as clear in the cell, did not take up the stain.



Magnification x1000

Fig. 4: Smirnoff staining of *B.t* isolate V24-M, parasporal bodies (crystals) at 72 hrs. The shape of the crystal is bipyramidal.



Magnification x1000

B.t bipyramidal crystals

Fig. 5: Re-isolation of *B.t* from dead larvae of *C. Partellus*. The creamish white colonies of *B.t* grew around the dead larvae.

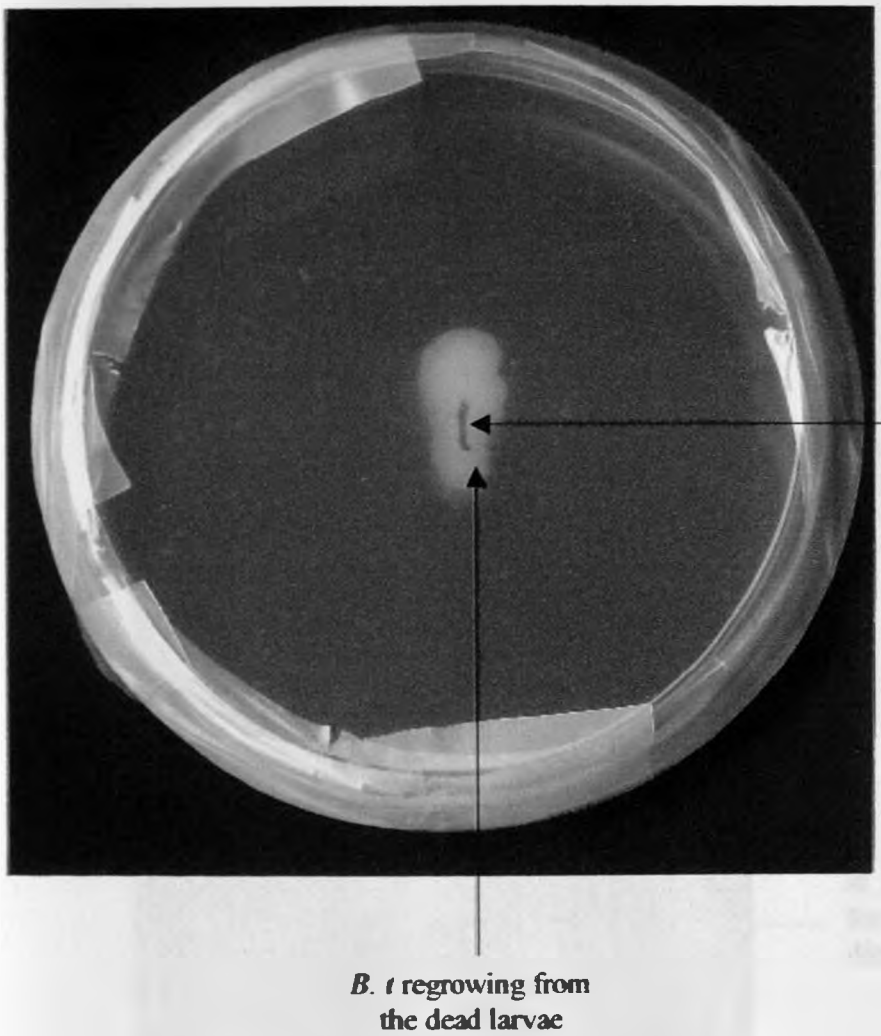


Fig. 6: Larvae (3rd instar) of *Busseola fusca* (control) feeding on the artificial diet in a specimen tube. The larva feeds on the sides of the tube in the early stages and burrows into the diet in the later stages.

Table 5: Morphological characteristics of different isolates of *B.t.* Rod length, crystal shape, spore shape, spore position and width of the rod.

Isolate No.	Length of rod	Width of rod	Spore shape	Spore position	Crystal shape
V24-M	3µm[a]	1µm	E	T	BP
V14-M	3µm	1µm	E	T	BP
12F-K	3µm	1µm	E	T	BP
K10-2	3µm	1µm	E	T	BP
K13-1	3µm	1µm	E	T	BP
1M	3µm	1µm	E	T	BP
44M	2.5µm	1µm	E	T	BP
45M	2µm	1µm	E	T	BP
VM-10	2µm	1µm	E	T	BP
V34-M	2µm	1µm	E	T	BP
V15-M	2.5µm	1µm	E	T	BP
V35-M	3µm	1µm	E	T	BP

a-Measurements are a mean of readings from 10 cells

T, terminal

E, Ellipsoidal

BP, Bipyramidal

Table 6: Biochemical characteristics of the different *B.t* isolates.

Isolates	Parameters		
	Gram stain	**Catalase**	**Voges-Proskauer**
V24-M	+	+	+
1M	+	+	+
VM-10	+	+	+
V14-M	+	+	+
45M	+	+	+
44M	+	+	+
K13-1	+	+	+
35M	+	+	+
K10-2	+	+	+
34M	+	+	+
12F-K	+	+	+
V15-M	+	+	+

+ ▶ Positive reaction

4.8: Growth of *B.t* on different pH media

Analysis of growth parameters revealed that the *B.t* isolates obtained were different in their pH sensitivity. The pH values tested were pH 5, 6, 6.5, 7.6 and 8. The Nutrient broth on which the isolates were grown is usually at a pH of 7.4 ± 0.2. The percentage growth of the different isolates from the different localities are shown on Table 7 (Kakamega) and Table 8 (Machakos). The isolates of *B.t* showed varying sensitivity to different pH ranges. All the strains showed maximum growth at pH 7.6 which is the normal pH of the nutrient broth media used, except for isolate V15-M which showed maximum growth at pH 8.0. The isolates were grouped into three categories depending on their response to the various pH ranges.

(a) Isolates, VM-10, V15-M, 34M and K13-1 related closely in their pH tolerance in that no growth was observed at the pH range below 7.6 (Fig. 7).

(b) Isolates 45M, 12F-K, 1M.and V24-M could not grow at a pH below 6.5. (Fig. 8)

(c) Isolates V14-M, 44M, K10-2, and 35M, could not grow at a pH below 6.0. (Fig. 9)

None of the *B.t.* isolates had growth at pH 5.0 suggesting their sensitivity to low pH. Isolates 44M, 35M, V14-M, 45M and V24-M showed no growth at pH 8.0. However even the isolates that showed growth at pH 8.0, their percentage growth was lower than that at pH 7.6. It was therefore concluded that *B.thuringiensis* grows in the range 6.0 - 8.0 with optimal growth at pH 7.6.

Table 7: pH tolerance of *B.t* isolates from Kakamega district. (-) = No growth: (+) = Minimal growth: (+++) = Maximum growth.

Isolates	pH Range Tested				
	5.0	**6.0**	**6.5**	**7.6**	**8.0**
12F-K	–	–	+	+++	+
K10-2	–	+	+	+++	+
K13-1	–	–	–	+++	+

Table 8: pH tolerance of *B. t* isolates from Machakos District. (-) = No growth: (+) = Minimal growth: (+++) = Maximum growth.

B.t isolates	pH range tested				
	5.0	**6.0**	**6.5**	**7.6**	**8.0**
1M	–	–	+	+++	+
VM-10	–	–	–	+++	+
V24-M	–	–	+	+++	–
V14-M	–	+	+	+++	–
44M	–	+	+	+++	–
45M	–	–	+	+++	–
34M	–	–	–	+++	–
35M	–	+	+	+++	–
V15-M	–	–	–	+++	–

Fig.7: pH tolerance of *B.t* isolates VM-10, V15-M, 34M and K13-1, after 48 hrs of incubation. None of the isolates grew below pH 7.6.

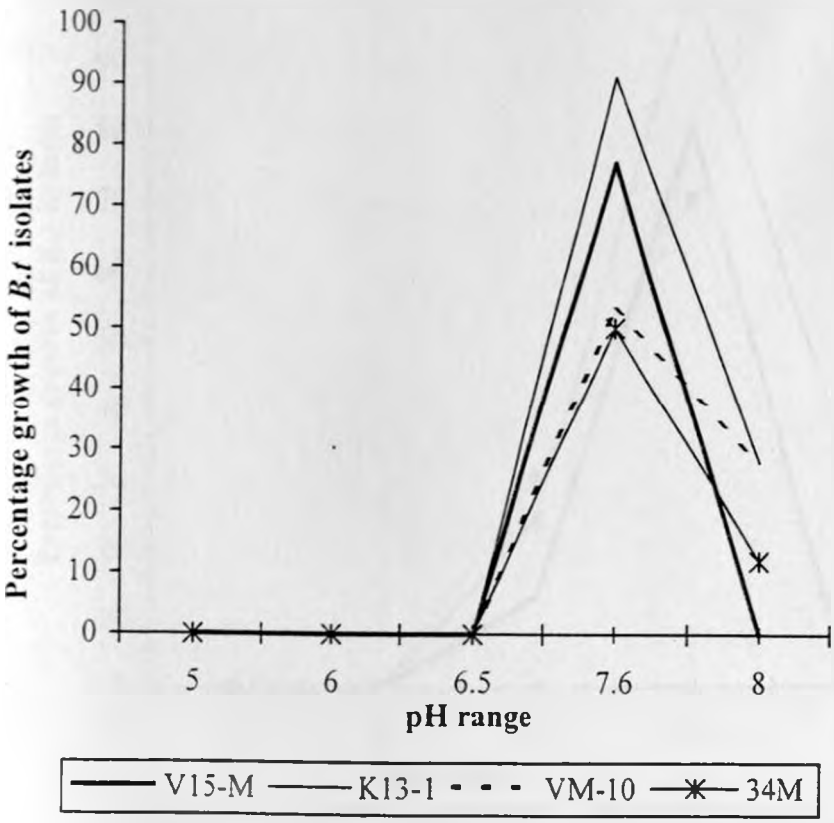


Fig.8: pH tolerance of *B.t* isolates 45M, 12F-K, 34 M, 1M,and V24-M after 48 hrs of incubation at 30° C. These isolates could not grow below pH 6.5.

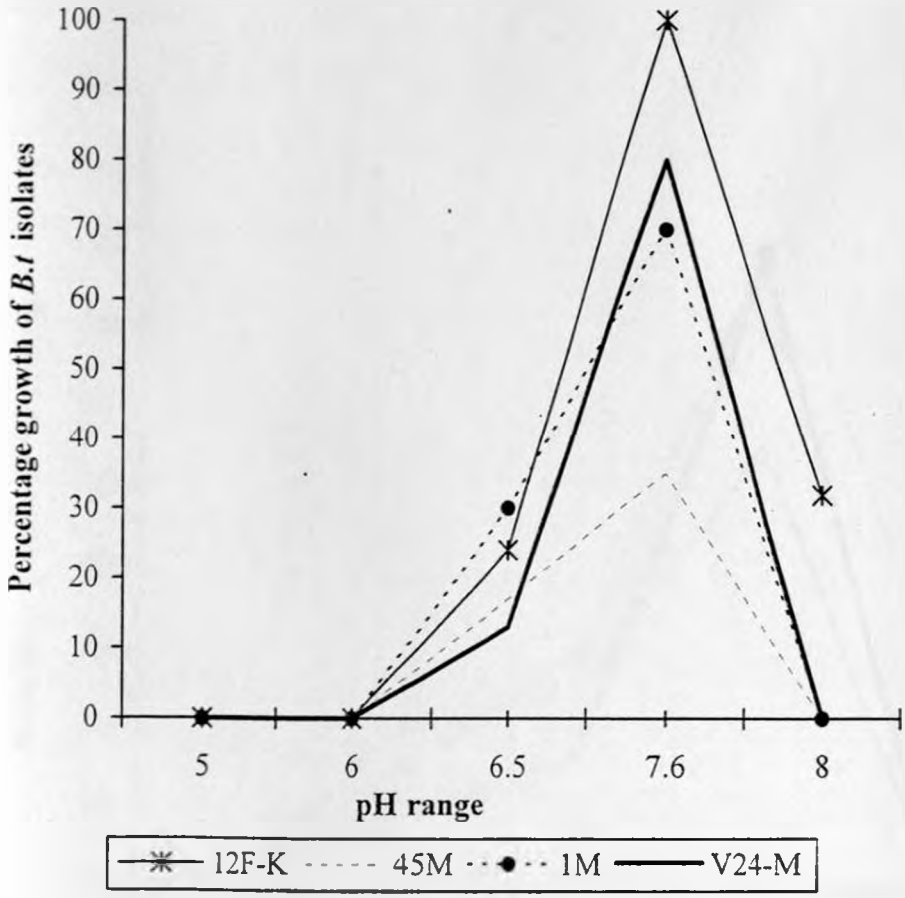
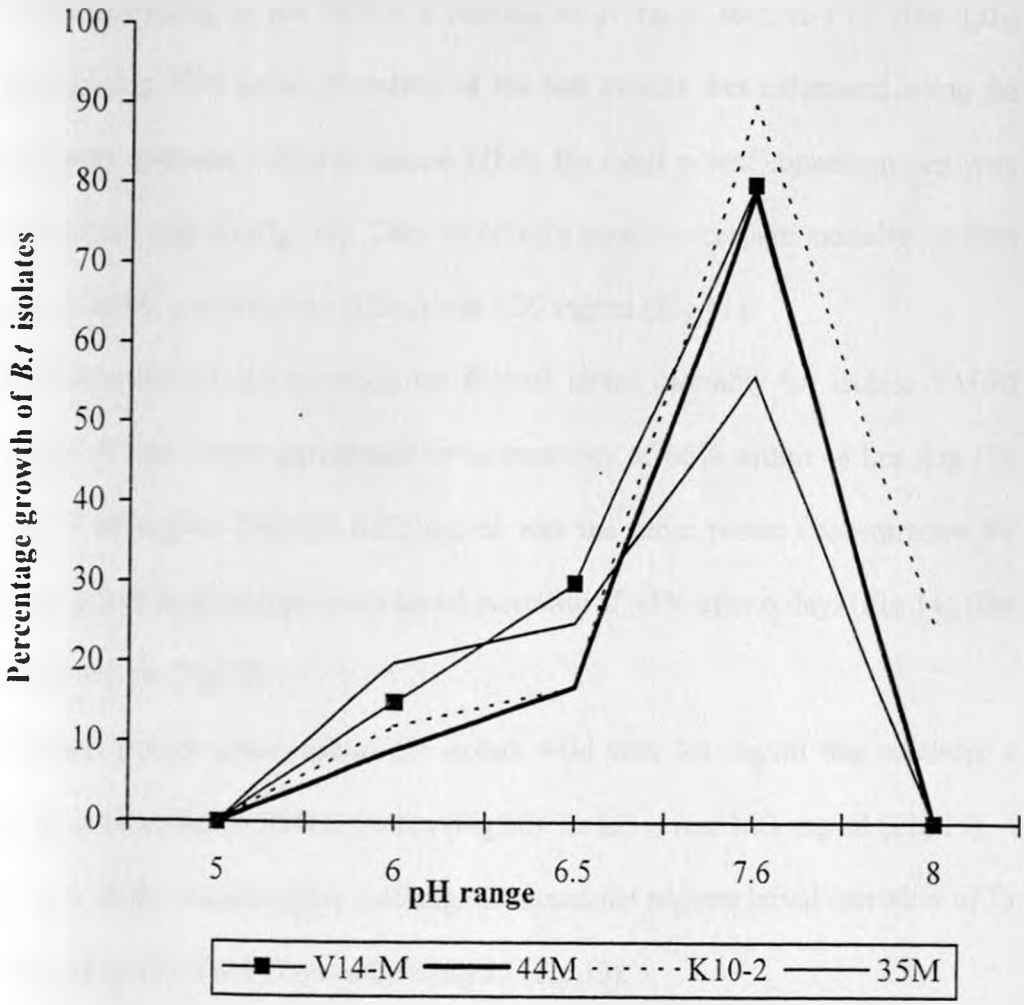


Fig.9: pH tolerance of *B.t* isolates V14-M, 44M, K10-2, and 35M after 48 hrs of incubation. None of the isolates grew at pH 6.0.



4.9: Bioassays on *C. partellus*

The different *B.t* isolates were tested for their toxicity to 1st instar larvae of C. *partellus* at various concentrations. Mortality was recorded after every 24 hrs and the percentage mortality recorded. Where control mortality exceeded 5%, adjusted mortality was calculated according to the Abbott's formula as given in section 3.13. The LD_{50} (lethal dose causing 50% larval mortality) of the test insects was calculated using the Statistica program (version 7.0). For isolate 12F-K the most potent concentrations were 0.67 mg/ml and 6.7 mg/ ml (fig. 10). They recorded a mean percentage mortality of 90% after 6 days. Its lethal concentration (LD_{50}) was 0.39 mg/ml (Fig.11).

The concentration that recorded the highest larval mortality for isolate VM-10 was 0.5 mg/ml. It had a mean percentage larval mortality of 66% within 48 hrs (Fig.12). Its LD_{50} was 0.04 mg/ml (Fig.13). 4.22 mg/ml was the most potent concentration for isolate K10-2. It had a percentage mean larval mortality of 93% after 6 days (Fig.14). The LD_{50} was 0.44 mg/ml (Fig.15).

The most potent concentration for isolate 44M was 3.4 mg/ml that recorded a percentage mean mortality of 58% in 24 hrs (Fig.16). Its LD_{50} was 0.03 mg/ml (Fig.17). For isolate V24-M the concentration 2.69 mg/ml caused the highest larval mortality of 75 % after 6 days (Fig.18). The LD_{50} was 0.76 mg/ml (Fig.19).

1.05 mg/ml was the most potent concentration for isolate V14-M. The mean percentage mortality was 73% in 72 hrs (Fig.20). The LD_{50} was 0.15 mg/ml (Fig.21).

For isolate 1M the concentrations that were most potent were 8.6 mg/ml and 0.86 mg/ml. They caused a mean percentage larval mortality of 100% by 72 hrs (Fig.22). The LD_{50} was 0.004 mg/ml (Fig.23).

From the dose range experimental results it was evident that percentage larval mortality increased with increase in toxin concentration. The study indicated that the lower the toxin concentration the lower the percentage larval mortality. Isolates 1M, 44M and VM-10 were the most potent against *C. partellus* with LD_{50} values of 0.004, 0.03 and 0.04 mg/ml respectively.

Fig.10: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B. t* δ-endotoxin of isolate 12F-K.

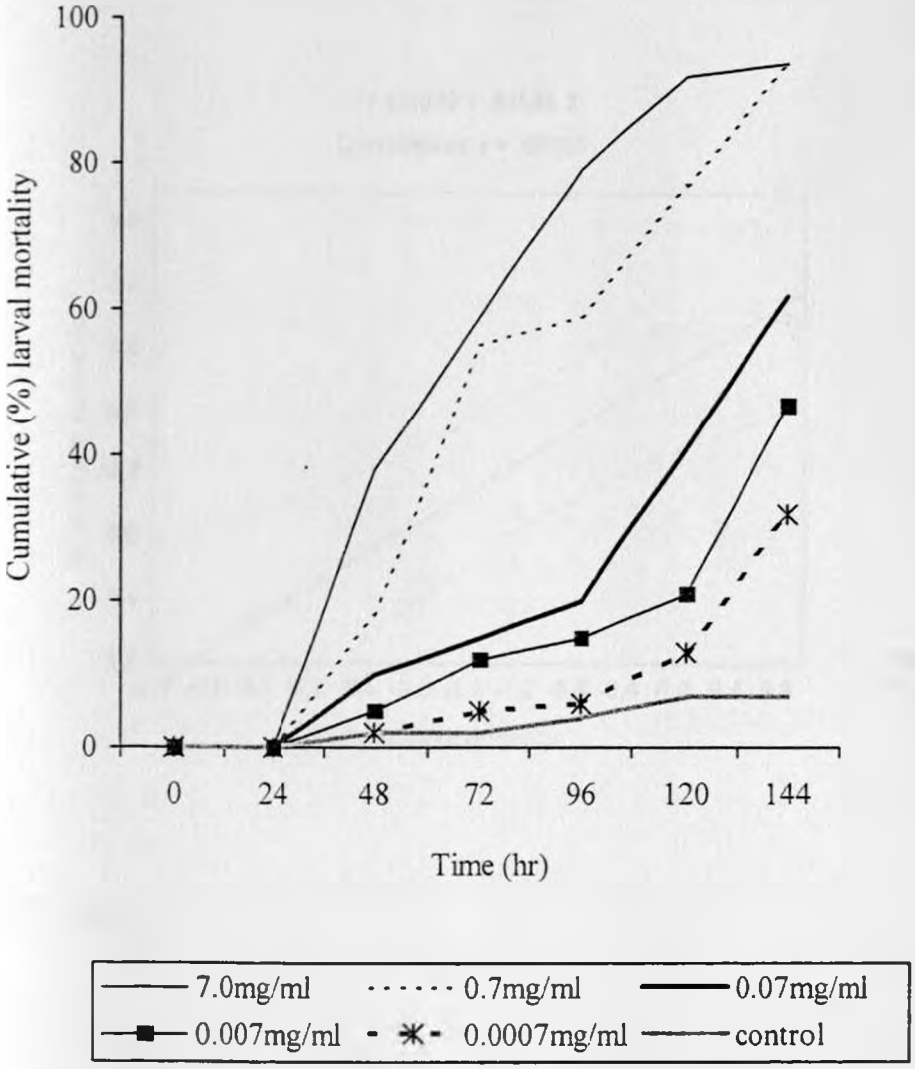
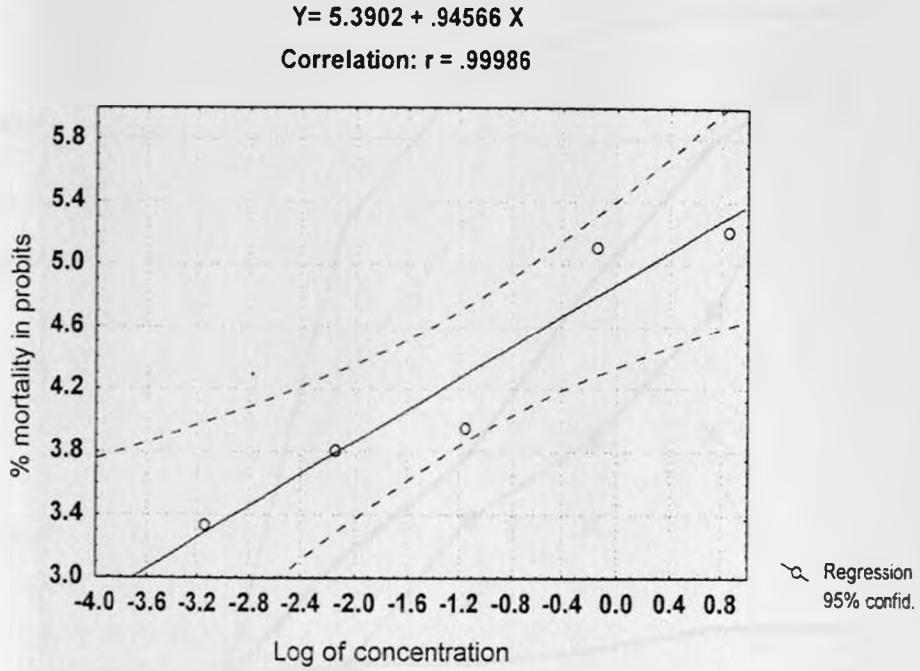


Fig.11: Effect of different *B t* toxin concentrations of isolate 12F-K on 1st instar larvae of *C. partellus* after 72 hrs.



LD_{50} = 0.39 mg/ml

Fig.12: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate VM-10. Control mortality was 7%; therefore, corrected mortality was calculated using the method described in materials and methods.

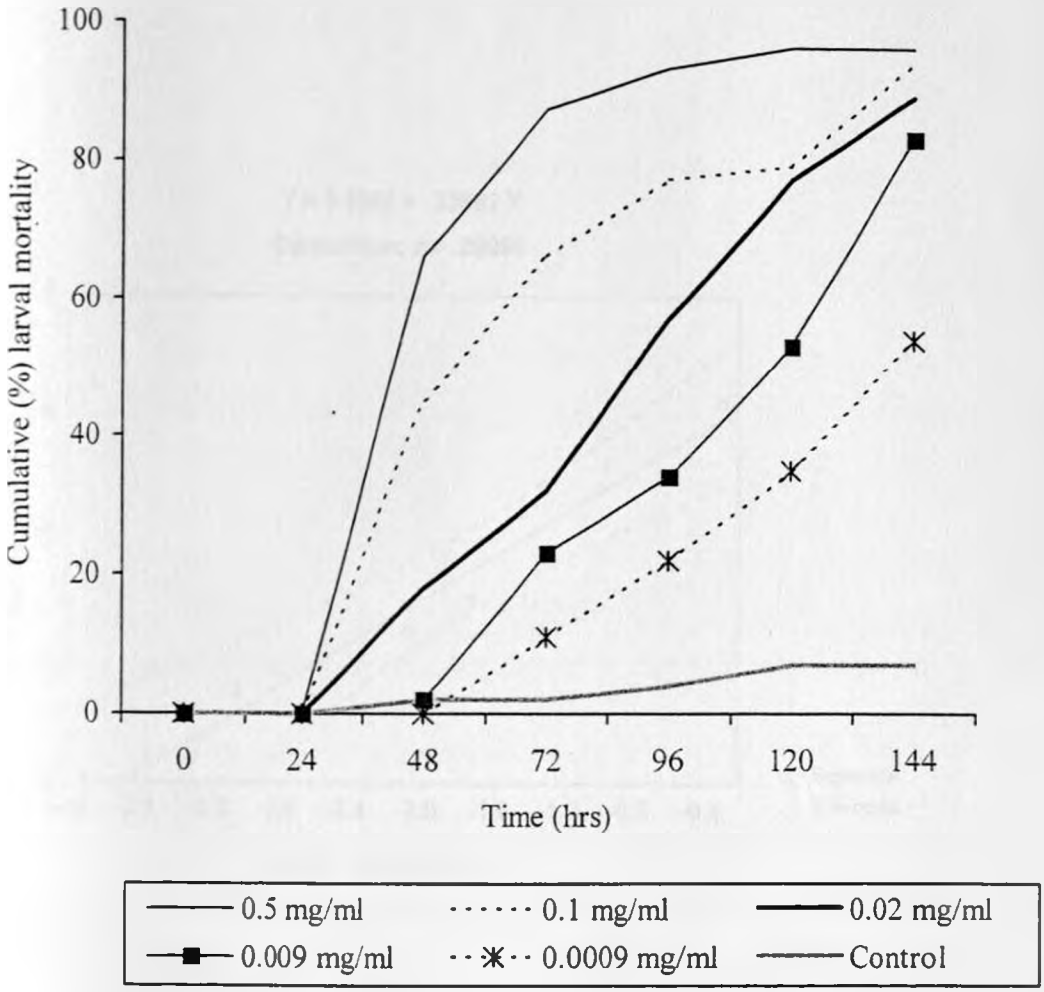
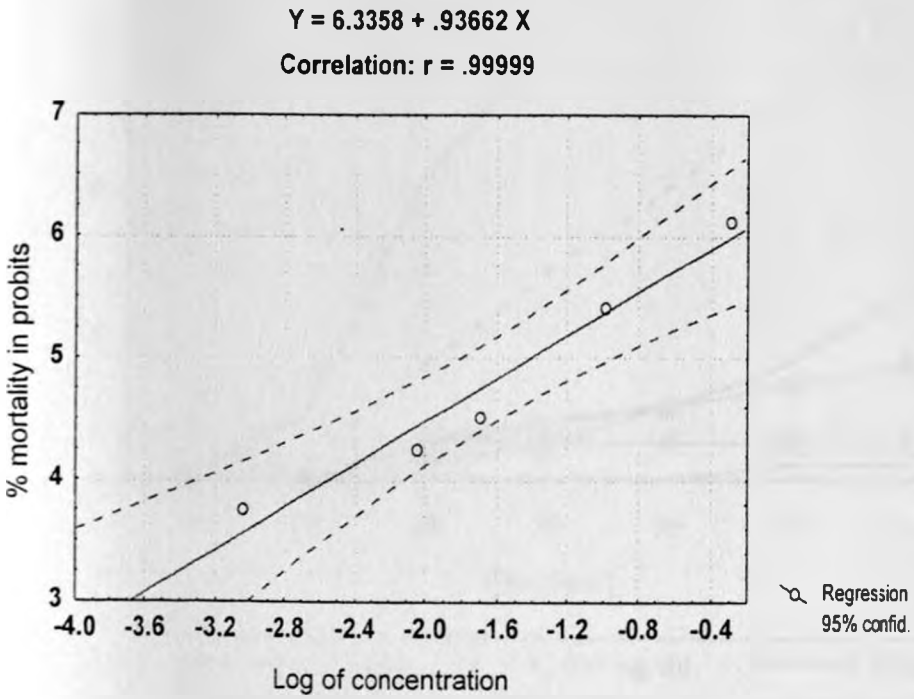


Fig.13: Effect of different *B.t* toxin concentrations of isolate VM-10 on 1st instar larvae of *C partellus* after 72 hrs.



LD_{50} = 0.04mg/ml

Fig.14: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate K10-2. Control mortality was 2 %.

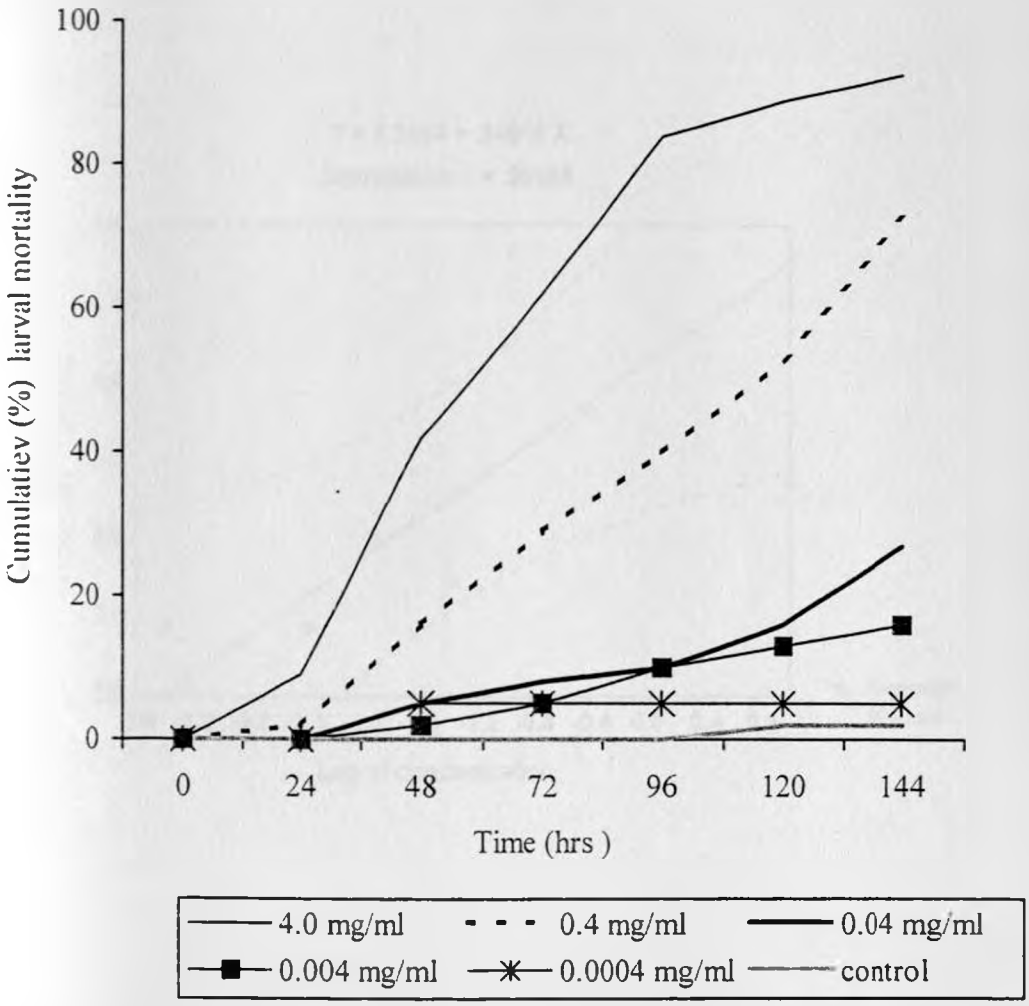
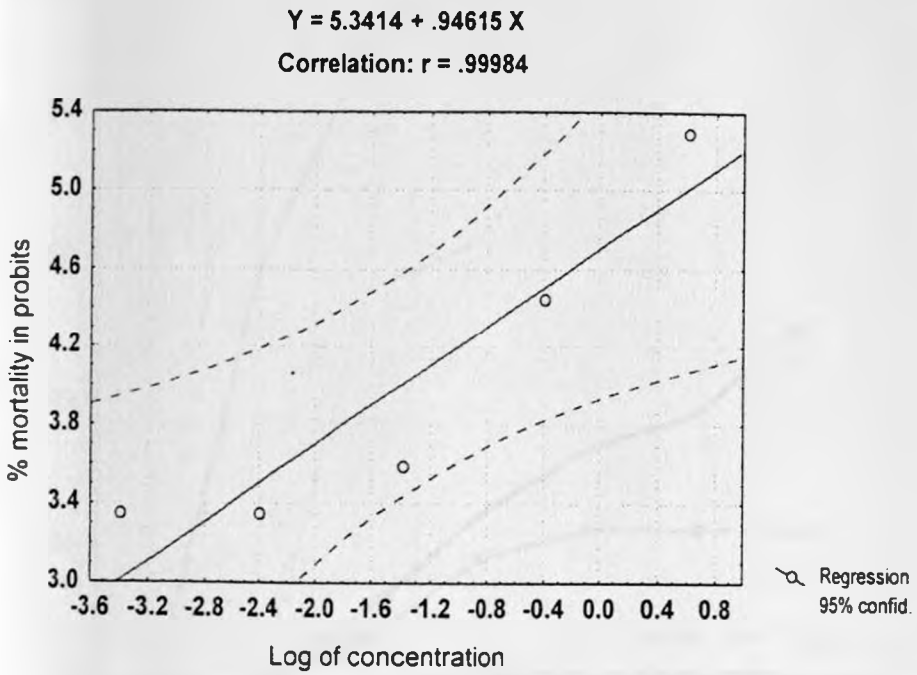


Fig.15: Effect of different *B.t* toxin concentrations of isolate K10-2 on 1st instar larvae of *C partellus* after 72 hrs.



LD_{50} = 0.44mg/ml

Fig.16: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate 44M. Control mortality was 2 %.

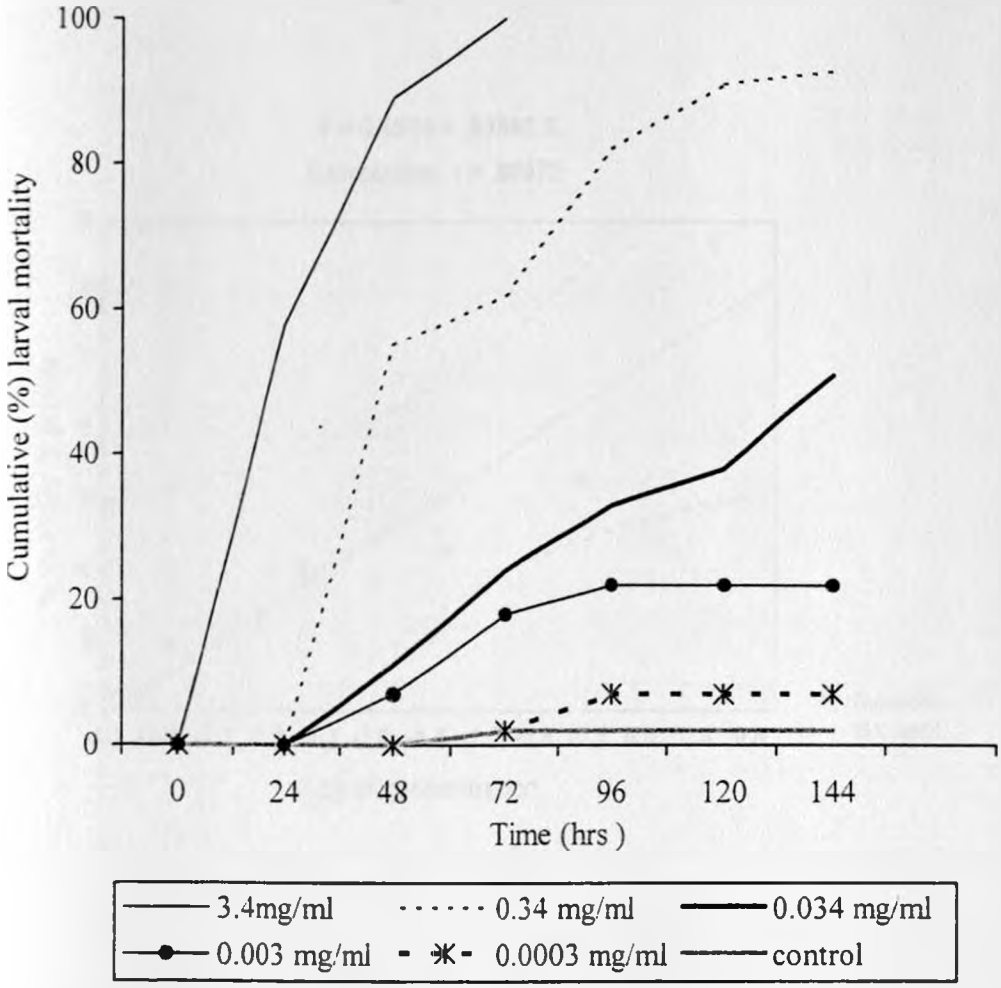
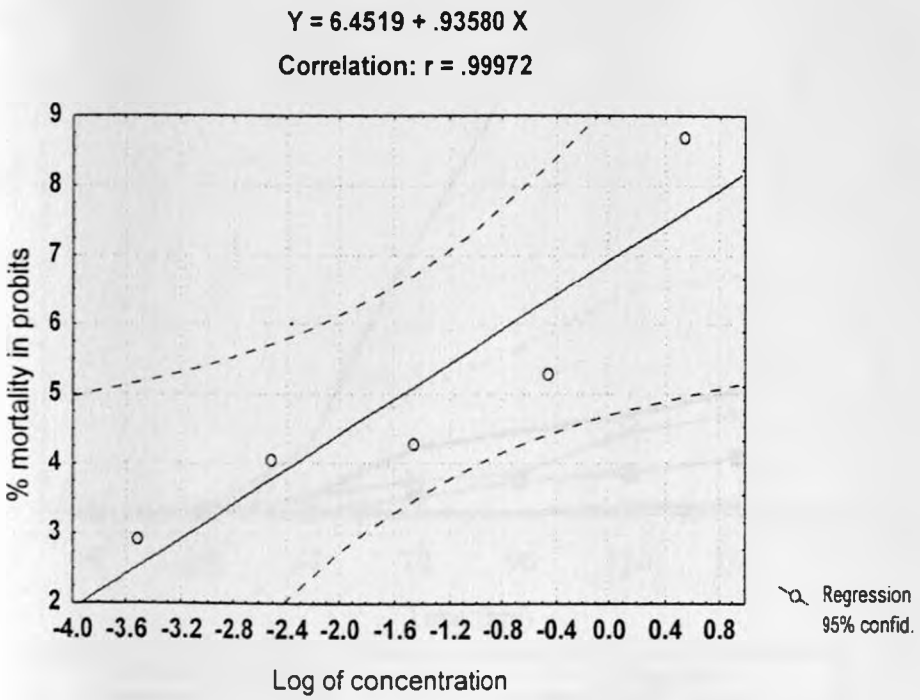


Fig. 17: Effect of different *B.t* concentrations of isolate 44M on 1st instar larvae of *C. partellus* after72 hrs.



LD_{50} = 0.03 mg/ml

Fig.18: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate V24-M. Control mortality was 2 %.

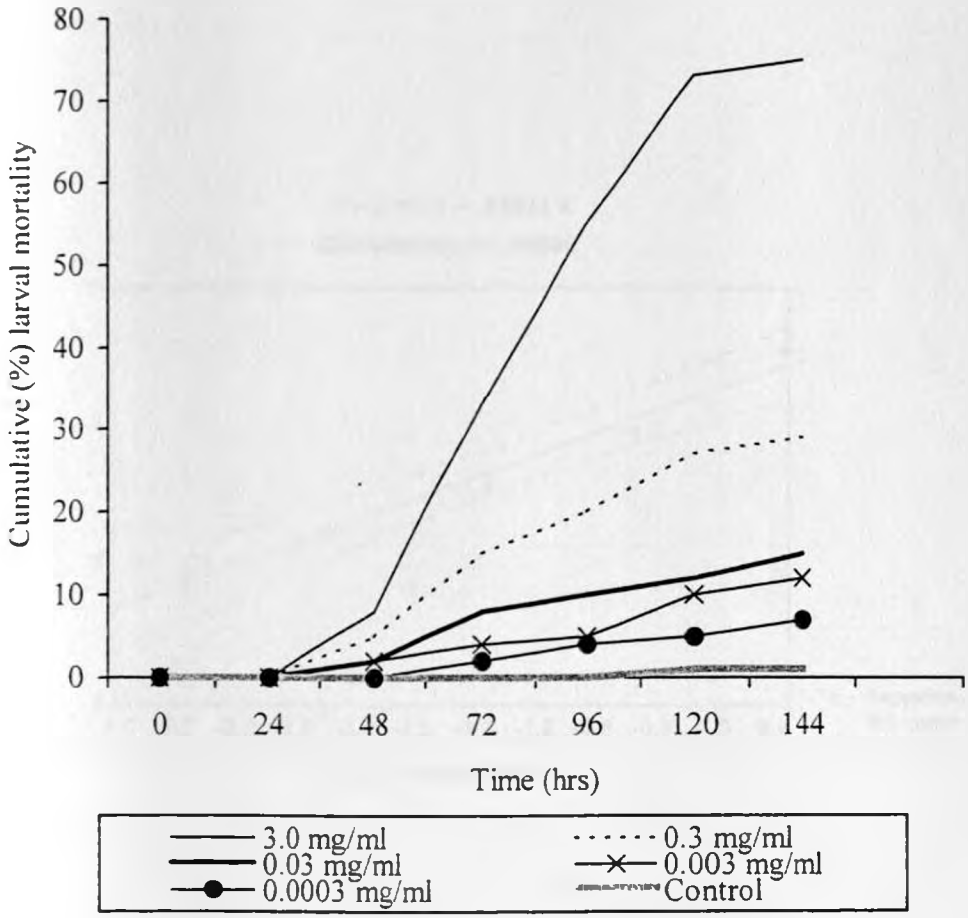
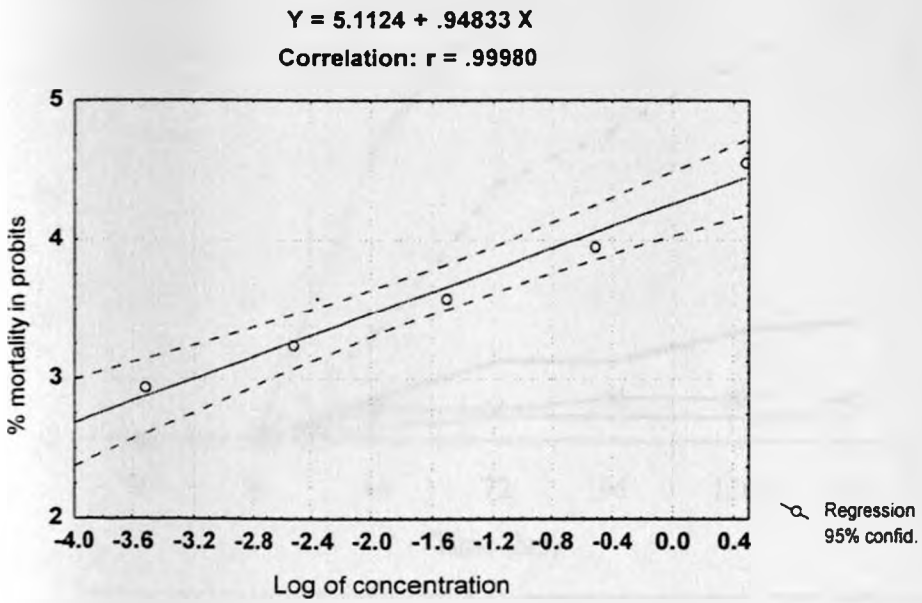


Fig. 19: Effect of different *B.t* toxin concentrations of isolateV24-M on 1[st] instar *C. partellus* after 72 hrs.



LD_{50} = 0.76 mg/ml

Fig.20: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate V14-M. Control mortality was 4 %.

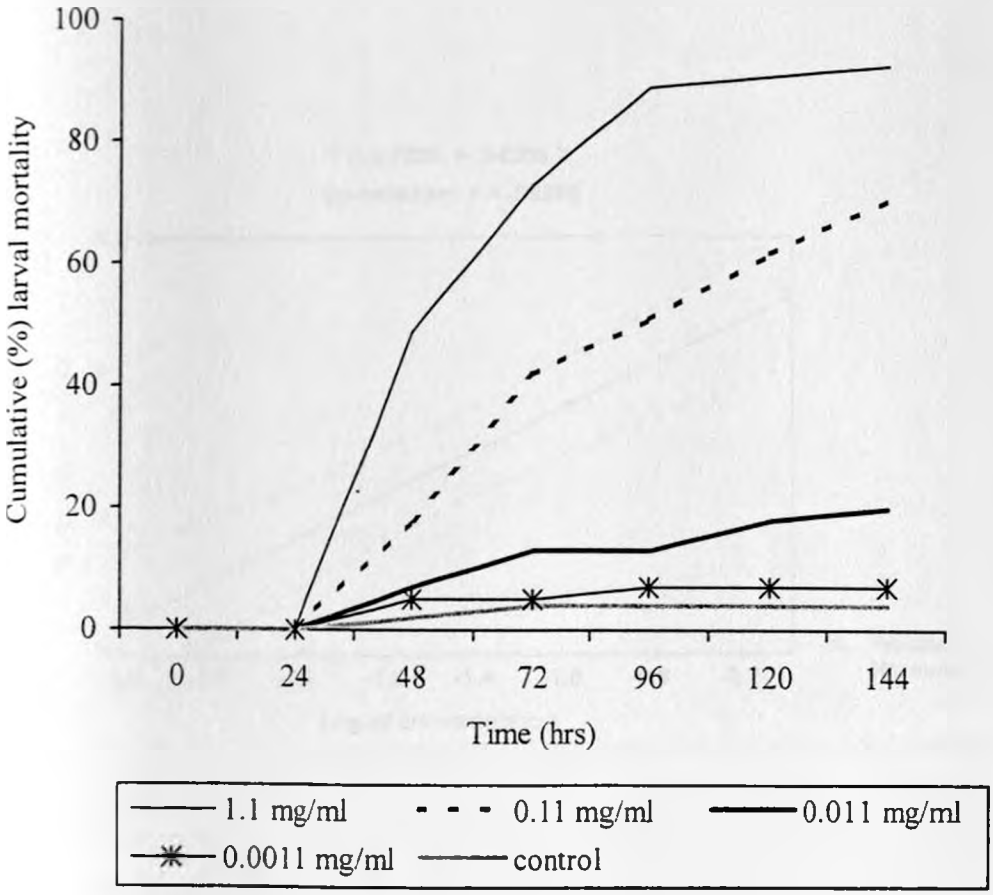
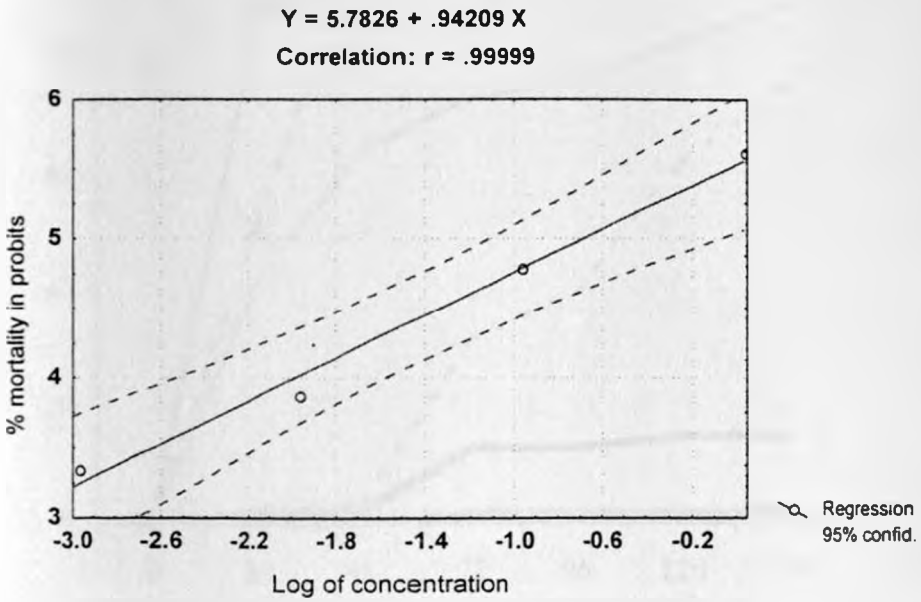


Fig. 21: Effect of different of *B t* toxin concentrations of isolate V14-M on 1st instar larvae of *C. partellus* after 72 hrs.



LD_{50} = 0.15 mg/ml

Fig. 22: Percentage mortality of 1st instar larvae of *C. partellus* exposed to different concentrations of *B.t* δ-endotoxin of isolate 1M.

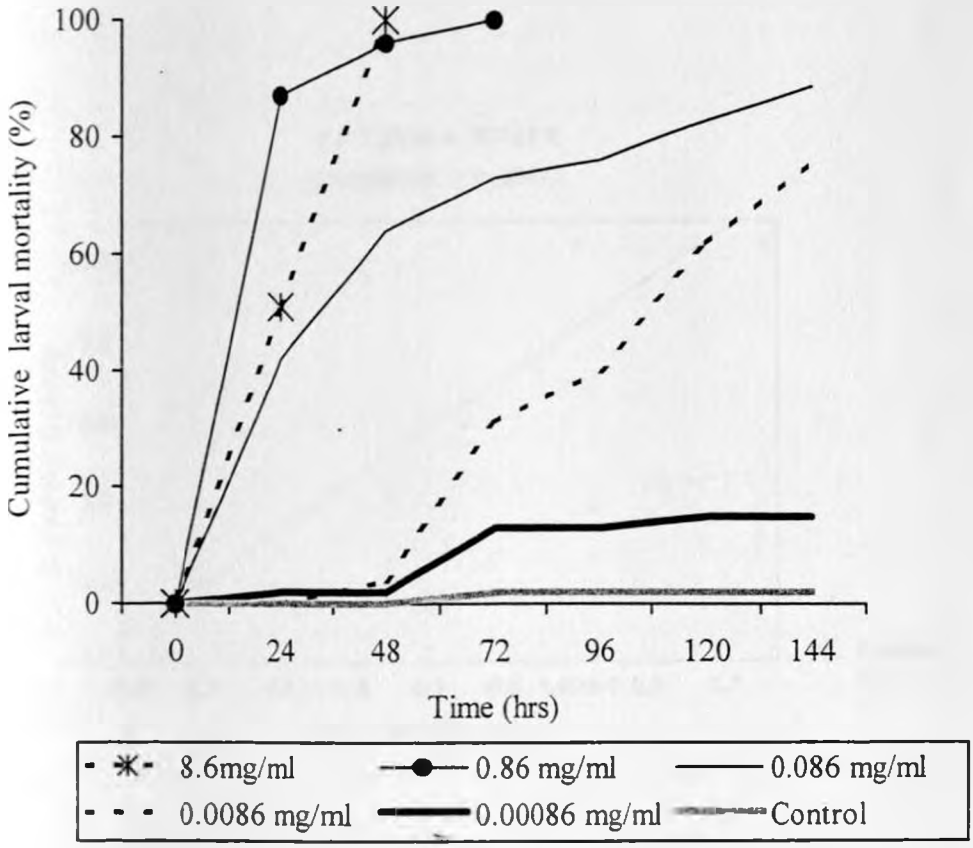
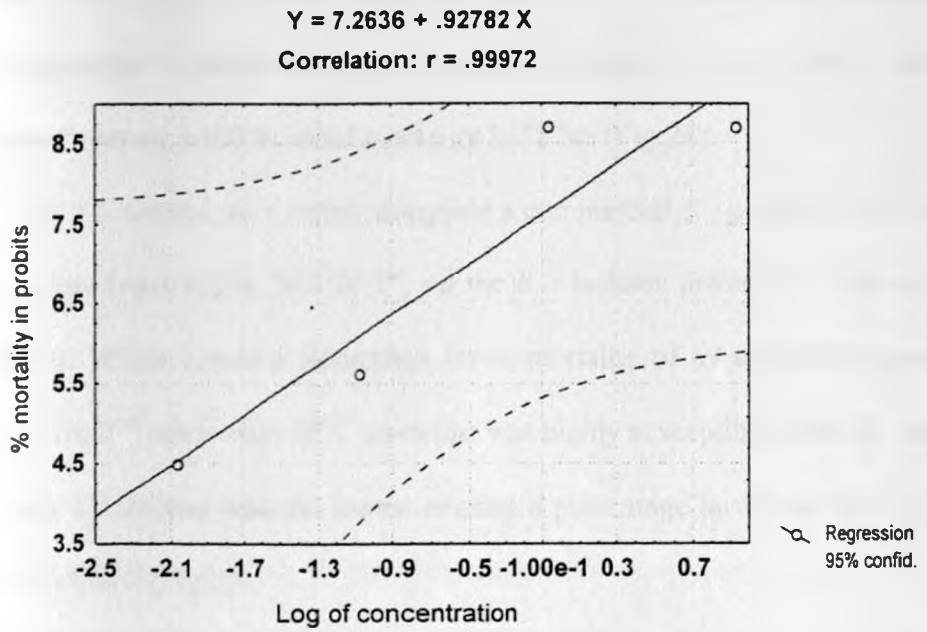


Fig.23: Effect of different *B.t* toxin concentrations of isolate 1M on 1st instar larvae of *C. partellus* after 72 hrs.



LD_{50} = 0.004 mg/ml

4.10: Effect of *B.t* isolates on different larval stages of *C. partellus*

The different *B. t* isolates were tested against 1st and 2nd larval stages of *C. partellus* to ascertain their susceptibility to the *B.t* toxin. The concentration tested was 8.6 mg/ml. The *B. t* isolates were highly toxic to the 1st instar larval stage of *C. partellus* with the least recording a percentage larval mortality of 77 % after 72 hrs, by isolate V24-M (Fig.24). After testing the isolates at the same concentrations of 8.6 mg/ml against the 1st instar larvae of *C. partellus* isolates 1M and VM-10 were the most potent causing a 100 % larval mortality in 72 hrs (Fig.24).

The *B.t* isolates were tested alongside a commercial *B.t* product "Thuricide®" and an isolate from ICIPE "MJ 99 2", all the *B. t* isolates proved to be more potent than the two which caused a percentage larval mortality of 69 and 58% respectively (Fig.24). The 2nd instar stage of *C. partellus* was highly susceptible to the *B. t* toxin of the various *B.*t isolates with the lowest causing a percentage larval mortality of 77 % by isolate VM-10 (Fig.25).

Different *B.t* isolates caused varying larval mortalities to the two larval stages, in that some isolates killed the 1st instar larvae faster than the 2nd while others killed the 1st instar larvae faster. Isolate 12F-K was equally toxic to the two larval stages at 72 hrs. recording a percentage mean larval mortality of 93 % for the two instars. The students t-test however showed that there was no statistically significant difference, ($t= 3.564$, $p= 0.14$). Isolate VM-10 was highly toxic to the 1st instar than the 2nd as it caused a 100% and 95% mean larval mortality at 72 and 144 hrs respectively ($t = 6.5$, $p= 0.194$), (Fig. 27).

Isolate V14-M was highly toxic to the 2nd instar larval stage than the 1st as it recorded a 100% larval mortality in 72 and 120 hrs respectively ($t = 9.0$, $p = 0.14$)

(Fig.28). Isolate V24-M was equally toxic to the two larval stages with a larval mortality of 98 and 100% for the 1st and 2nd instar larval stages respectively (t = 27, p = 0.048). (Fig.29). The 1st and 2nd larval stages were equally susceptible to the *B.t* toxin of isolate K10-2 with a larval mortality of 100% at 120 hrs for the two stages (t = 8.0, p = 0.158), (Fig.30).

Isolate 44M, expressed equal toxicity for the two larval stages with a larval mortality of 95% and 100% for the 1st and 2nd larval stages respectively (t =19.0, p =0.066), (Fig.31). Isolate 1M was more toxic to the 1st instar larval stage than the 2nd as it had a larval mortality of 100% at 72 hrs and 144 hrs respectively (t =14.0, p = 0.09) (Fig.32). Therefore, from the study it was clear that the two larval stages of *C. partellus* are equally highly susceptible to *B. t* toxin of all the *B. t* isolates tested.

Fig.24: Percentage mortality of 1st instar larvae of *C. partellus* exposed to 8.6 mg/ml *B t* δ-endotoxin of different isolates. There were no deaths observed with the control insects.

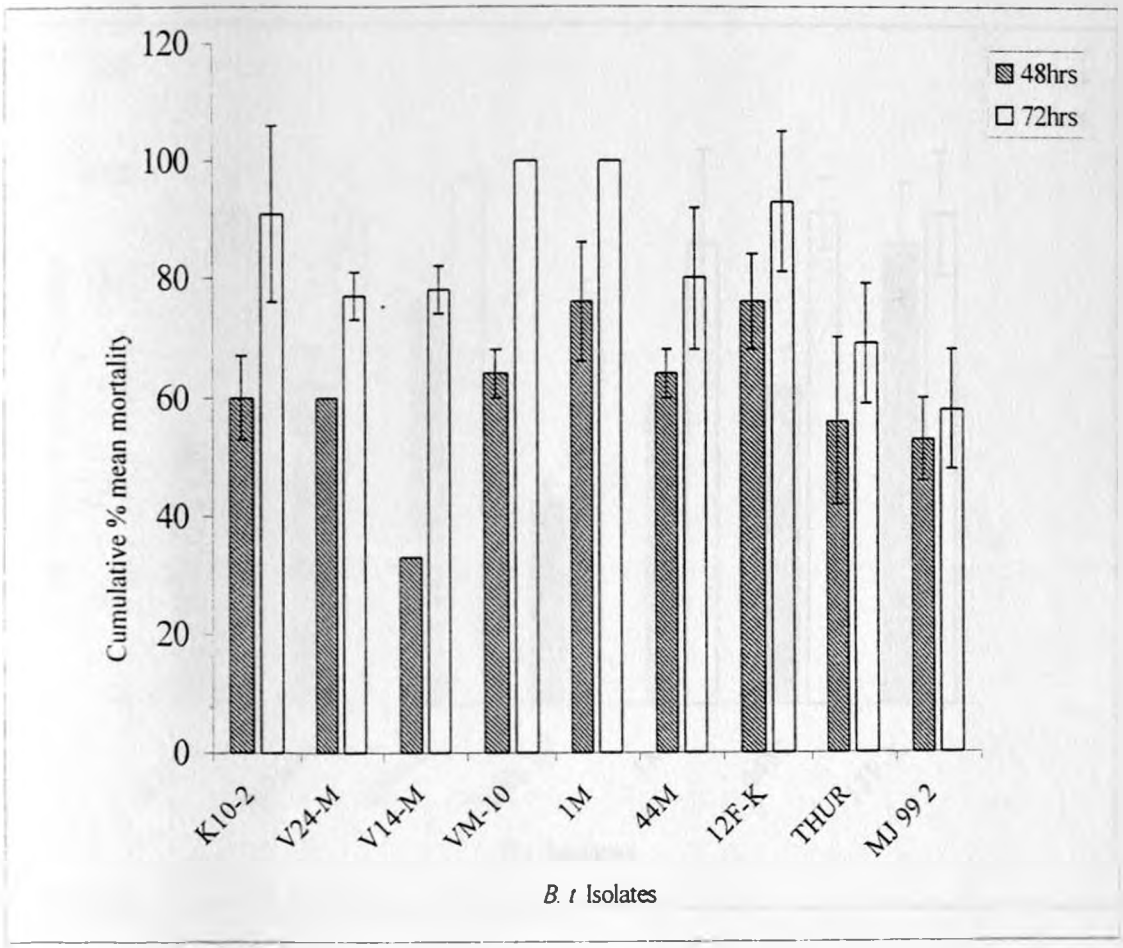


Fig.25: Percentage mortality of 2nd instar larvae of *C. partellus* exposed to 8.6 mg/ml *B.t* δ-endotoxin of different isolates. There were no larval mortalities among the controls.

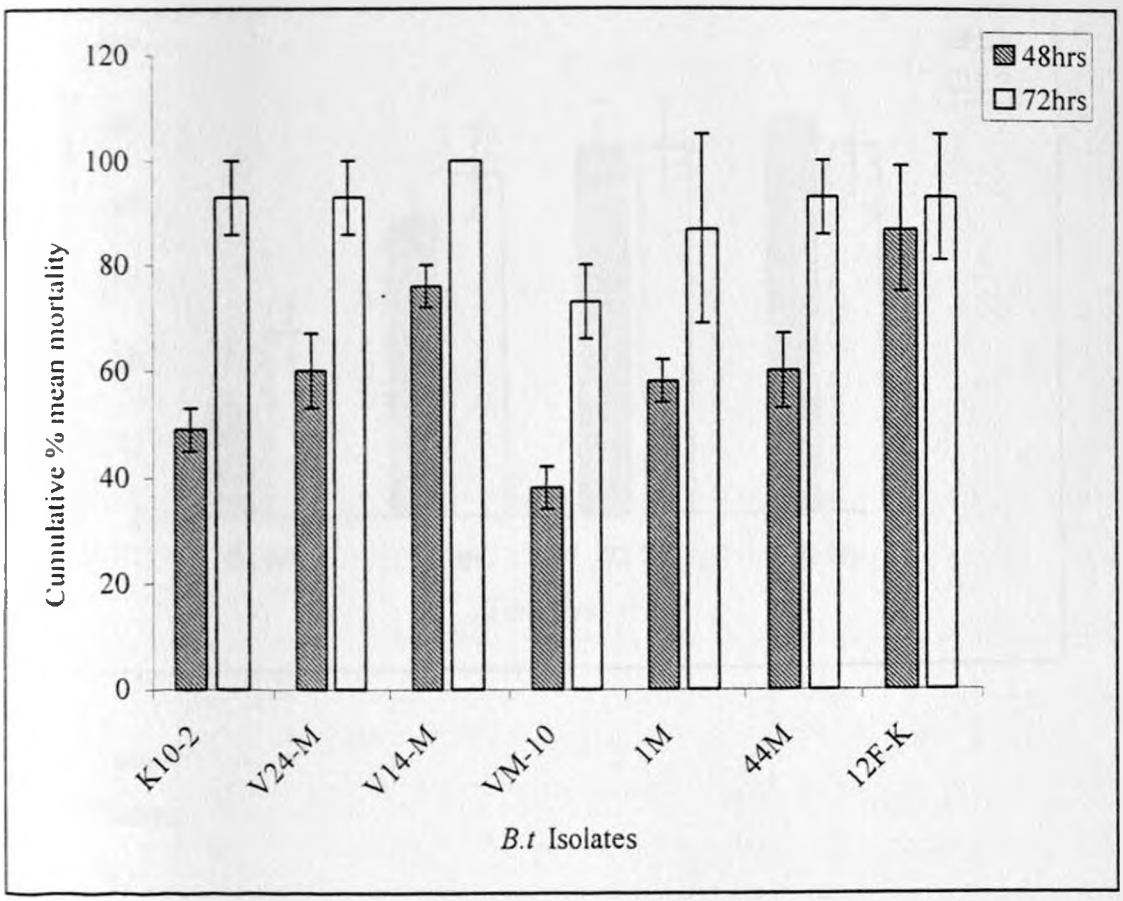
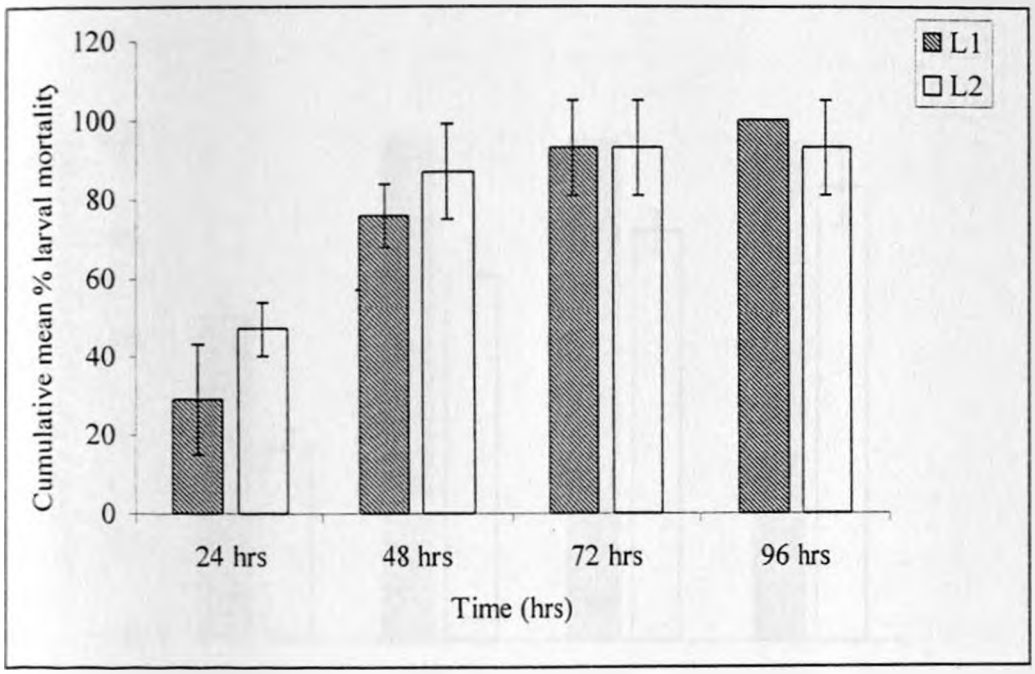


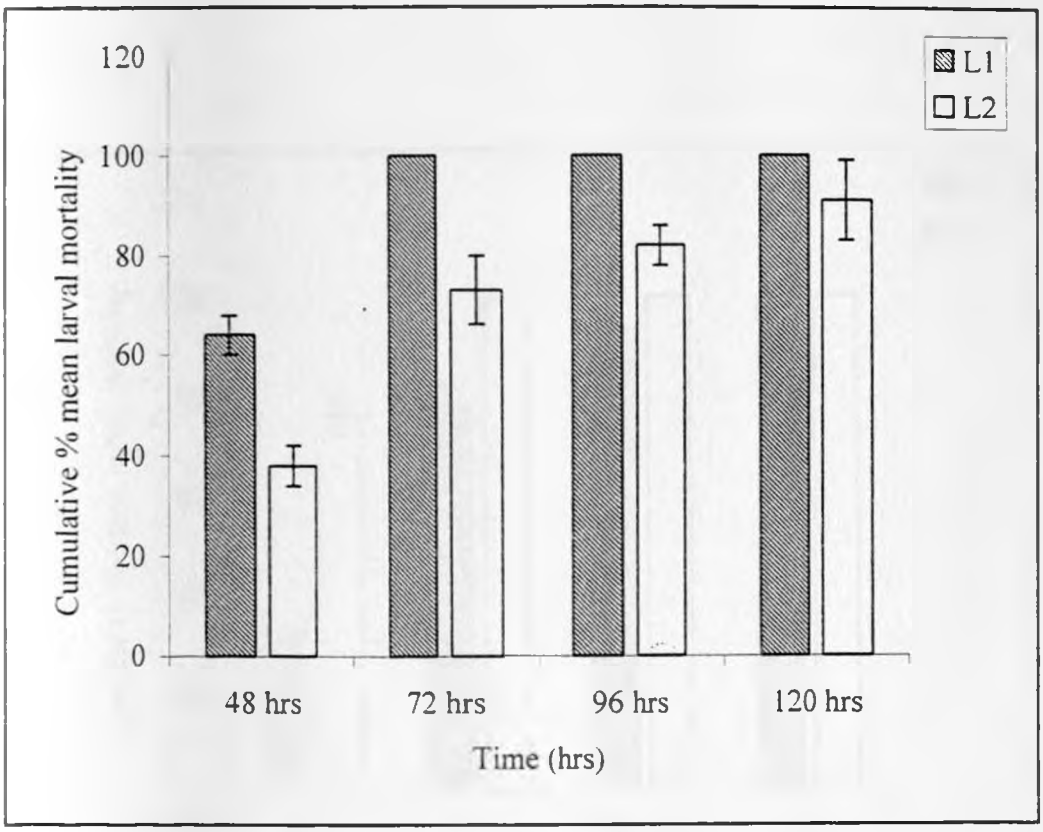
Fig.26: Percentage mortality of 1st and 2nd instar larval stages of *C. partellus* exposed to 8.6 mg/ml *B.t* endotoxin of isolate 12F-K. Control mortality was 5 %. Students *t*-test for the difference was $t = 3.564$, $p = 0.14$ at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

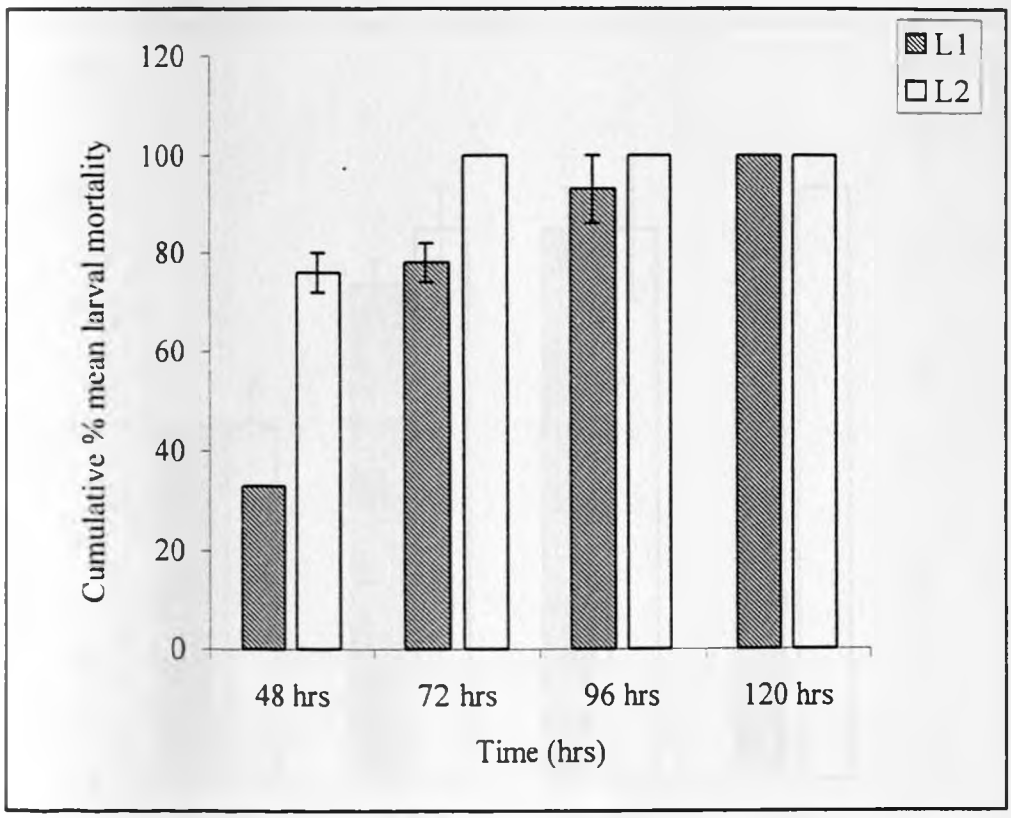
Fig.27: Percentage mortality of 1st and 2nd instar larval stages of *C. partellus* exposed to 8.6 mg/ml *B.t* δ-endotoxin of isolate VM-10. There were no larval mortalities in the control. Students *t*-test for the difference was t = 6.5, p= 0.194 at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

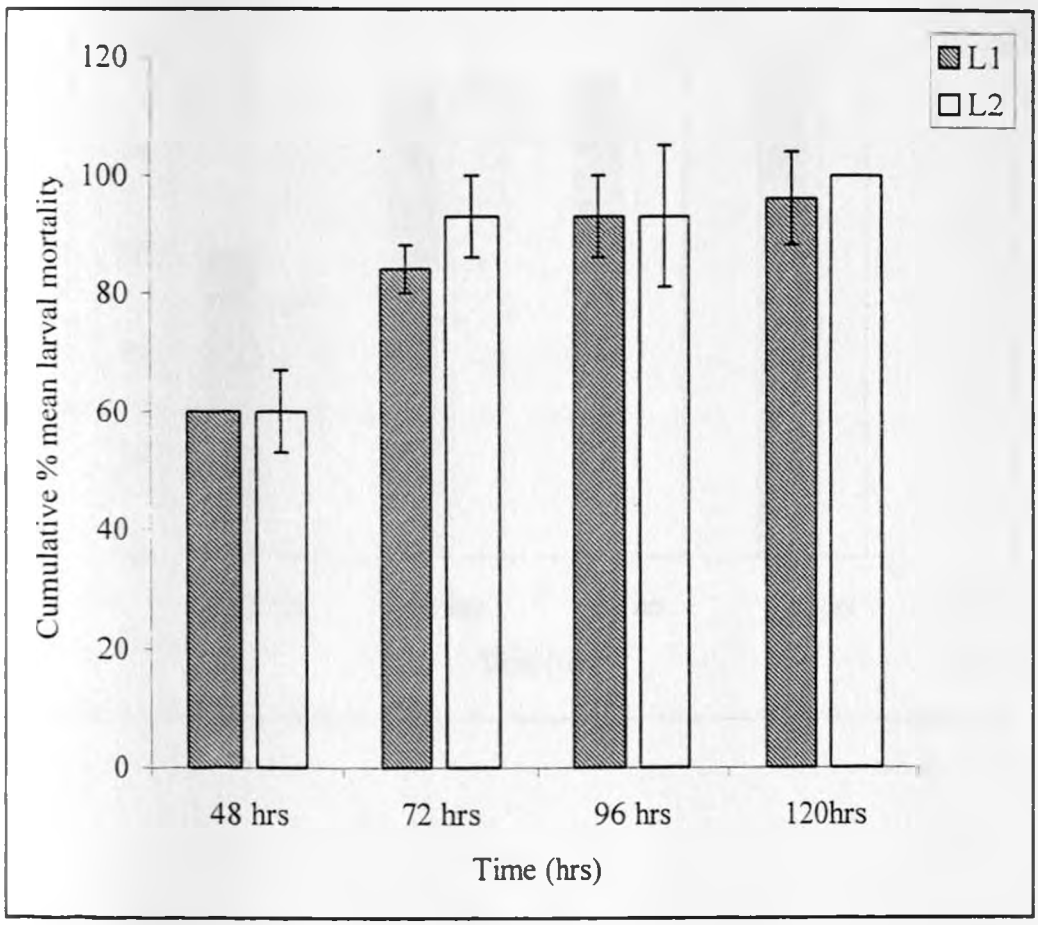
Fig.28: Percentage mortality of 1st and 2nd instar larval stages of *C. partellus* exposed to 8.6 mg/ml *B.t* δ-endotoxin of isolate V14-M. There was no larval mortality in the control. Students' *t*-test for the difference was; $t = 9.0$, $p = 0.14$ at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

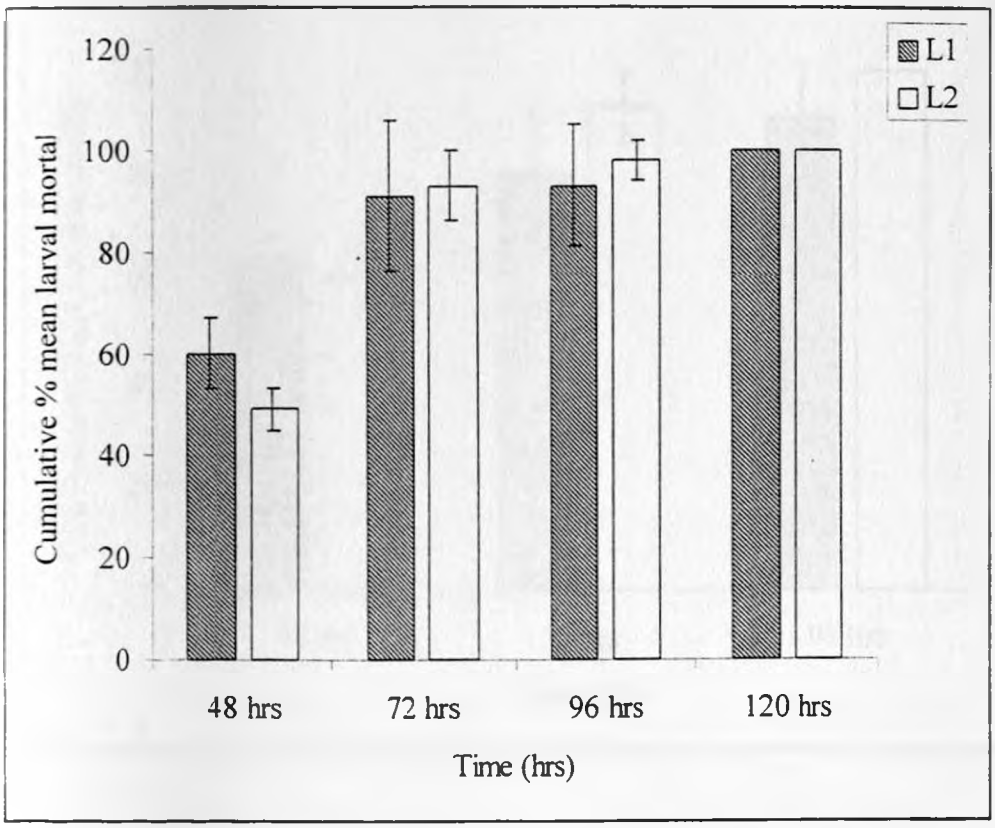
Fig.29: Percentage larval mortality1st and 2nd instar larval stages of *C. partellus* exposed to 8.6 mg/ml *B.t* δ-endotoxin of isolate V24-M. There was no larval mortality in the control. Students *t*- test for the difference was $t = 27$, $p = 0.048$ at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

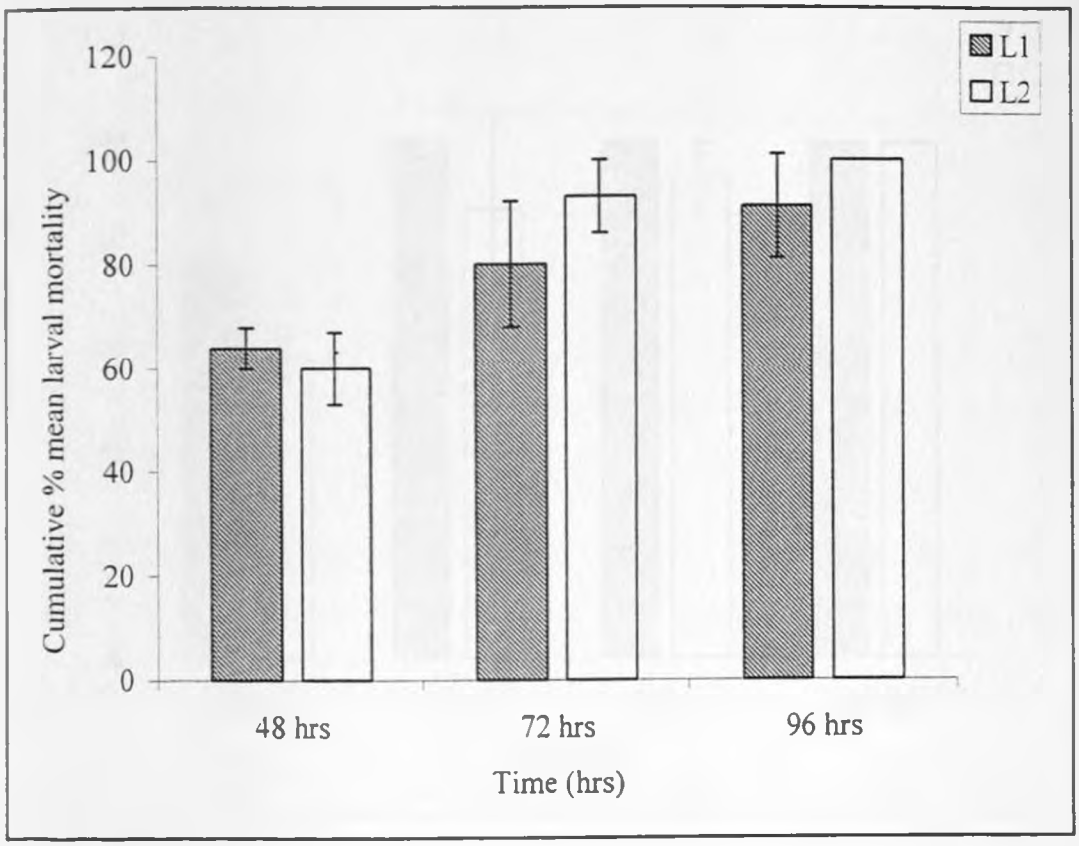
Fig.30: Percentage mortality of 1st and 2nd larval stages of *C. partellus* when exposed to 8.6 mg/ml of *B.t* δ-endotoxin of isolate K10-2. There was no larval mortality in the control. Students *t*- test for the difference was $t = 8.0$, $p = 0.158$ at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

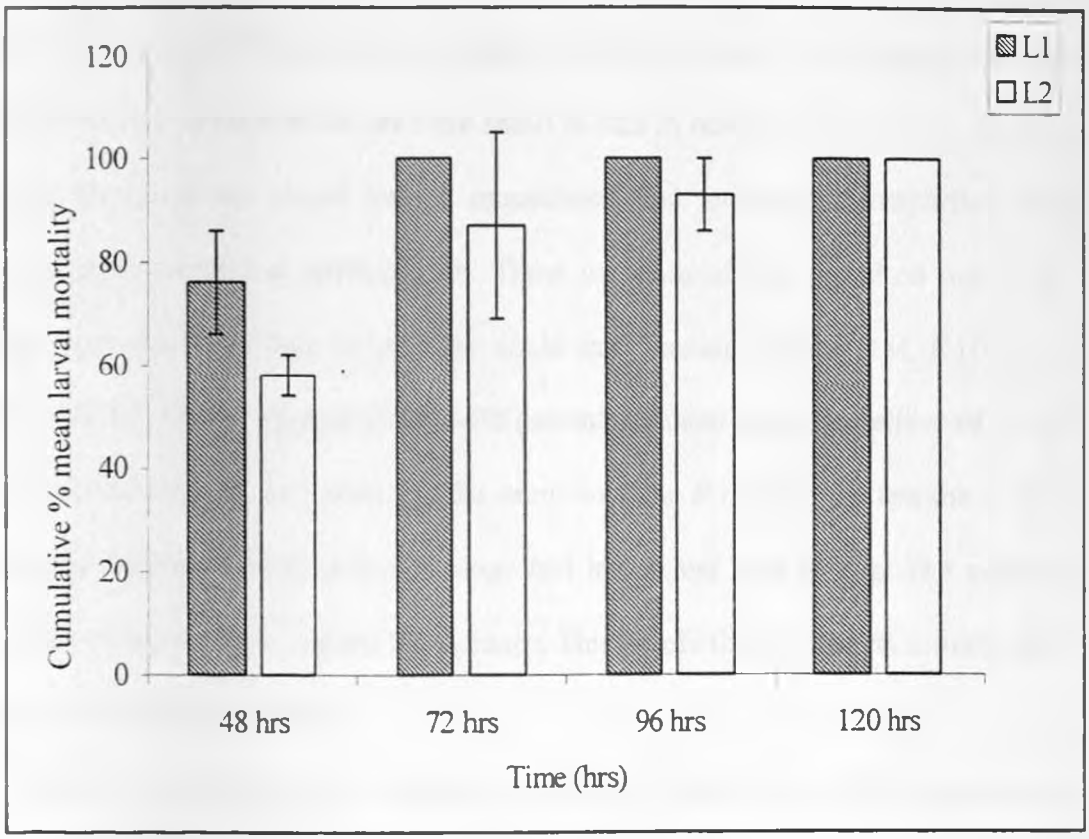
Fig.31: Percentage l mortality of 1st and 2nd larval stages of *C. partellus* exposed to 8.6 mg/ml of *B.t* δ-endotoxin of isolate 44M. Control mortality was 5 %. Students *t*-test for the difference was t =19.0, p =0.066 at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

Fig.32: Percentage mortality of 1st and 2nd larval stages of *C. partellus* exposed to 8.6 mg/ml of *B.t* endotoxin of isolate 1M. There was no larval mortality in the control. Students *t*-test for the difference was $t = 14.0$, $p = 0.09$ at 0.05 confidence level.



L1 = 1st instar

L2 = 2nd instar

4.11: Leaf disk bioassays on *C. partellus*

Leaf disk bioassays was carried out with all the seven isolates at a concentration of 8.6 mg/ml. All the larvae had died within 72 hrs. The controls were actively feeding and started immediately after introduction into the experimental tubes. Larvae in treated leaves were feeding minimally and death was observed within 24 hrs. Dead larvae turned darkish, whereas the live larvae were creamish with black heads. Those larvae in treated leaves that had not died after 24 hrs were small in size in comparison with the controls. Larvae in control leaves started feeding immediately and actively in comparison with their counterparts reared in artificial diet. Those on artificial diet stayed on top of the specimen tube for some time before they could start feeding. Isolates 1M, K10-2 and V14-M recorded a faster mortality rate, with percentage mean larval mortalities of 71, 67 and 60% respectively. It was noted that the more toxic the *B.t* isolate the less the area of leaf damage. Isolates which were less toxic had larger leaf area damage.The controls which had no toxin had the highest leaf damage. This proofs that the insects actually died as a result of toxin consumption.

There was a strong positive Pearson correlation (r^2=0.883, sig. 0.000) between the mortality rate (number of deaths) of different isolates with time. Correlation is significant at 0.01 level (2-tailed).

4.12: Bioassays on *S. calamistis*

Bioassays were done with 1st instar of *S. calamistis*. The larvae unlike that of *C. partellus* indicated a different behavioral pattern once introduced into the tubes containing artificial diet. On introduction they moved away from the diet to the top of the tube (the gauze) for about 12 hrs. However, *C. partellus* larvae stayed on the diet.

Concentrations of *B.t* δ -endotoxin that were tested were 0.86 mg/ml and 8.6 mg/ml. At a concentration 0.86 mg/ml isolate 44M was the most toxic to the larvae with a larval mortality of 54% after 72 hrs. Isolate 1M followed with larval mortality of 47% within the same duration. Isolate 12F-K was the least toxic with larval mortality of 18%. However none of the isolates achieved a larval mortality of 100% within the time of the bioassay (Fig.34). At a concentration 8.6 mg/ml of *B.t* toxin, isolate 44M was still the most potent, with a larval mortality of 73% after 72 hrs.

At 72-hrs, increase in toxin concentration resulted in an increase in larval mortality. All the *B.t* isolates responded to increase in toxin concentration (from 0.86 to 8.6 mg/ml) except isolates K10-2 and V14-M, whose increase in toxin concentration did not result in an increase in larval mortality (Fig.36). At 96-hrs, increase in concentration did not result in an increase in larval mortality for all the *B.t* isolates, but for isolates K10-2 and 44M increase in toxin concentration resulted in a drop in larval mortality (Fig.37). There was a weak significant positive Pearson correlation (r^2=0.249,sig.= 0.022) between *B.t* toxin concentration and mortality rate and also exposure time and mortality (r^2= 0.257, sig=0.018). Correlation is significant at the 0.05 level (2-tailed).

4.13: Bioassays on *B. fusca*

Results for *B. fusca* bioassays showed that the *B.t* isolates were not toxic to 2nd instar larvae of *B. fusca*. Isolate 44M had the highest larval mortality of 51% after 7 days. The lowest larval mortality was that of isolate 12F-K, which was 20% (Fig 38).

Fig.33: Percentage mortality of 1st instar *C. partellus* larvae by leaf disc bioassay

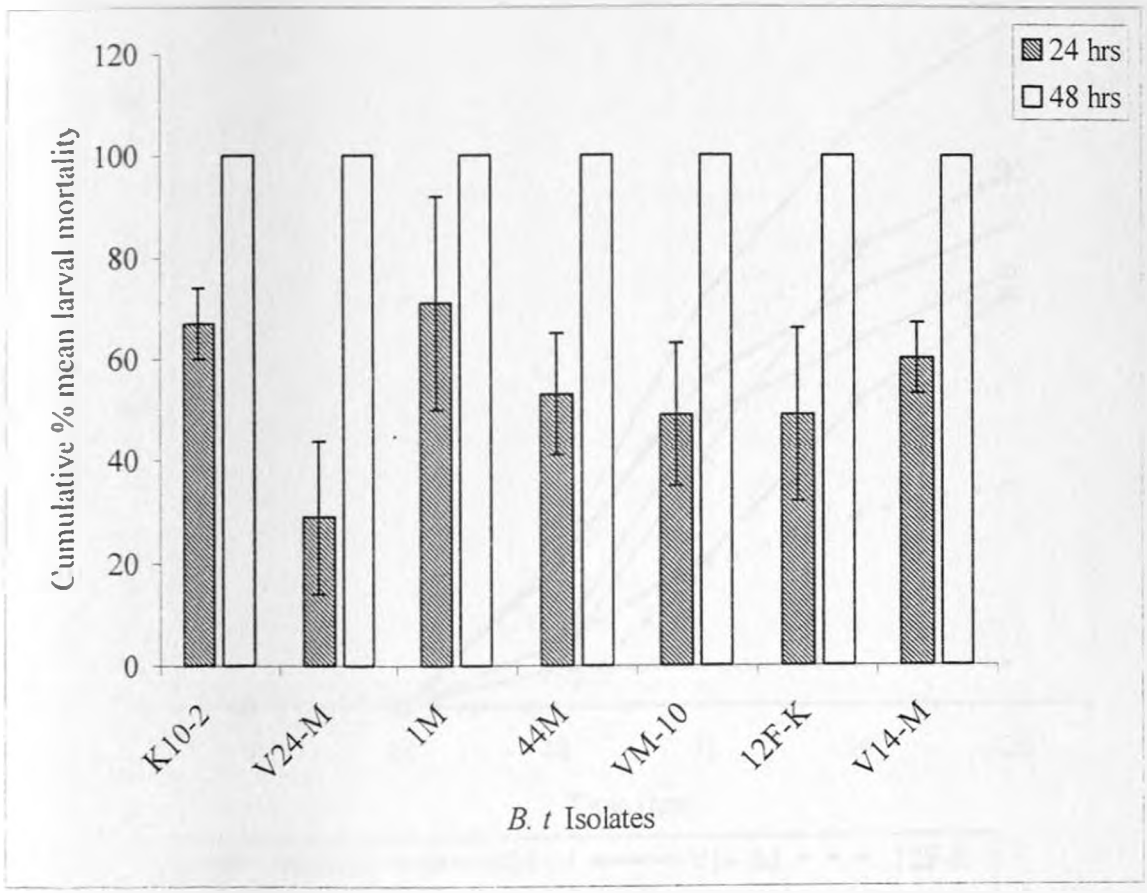


Fig.34: Percentage mortality of 1st instar larvae of *Sesamia calamistis* exposed to 0.86 mg/ml delta- endotoxin of different *B.t* isolates. Control mortality was 5 %.

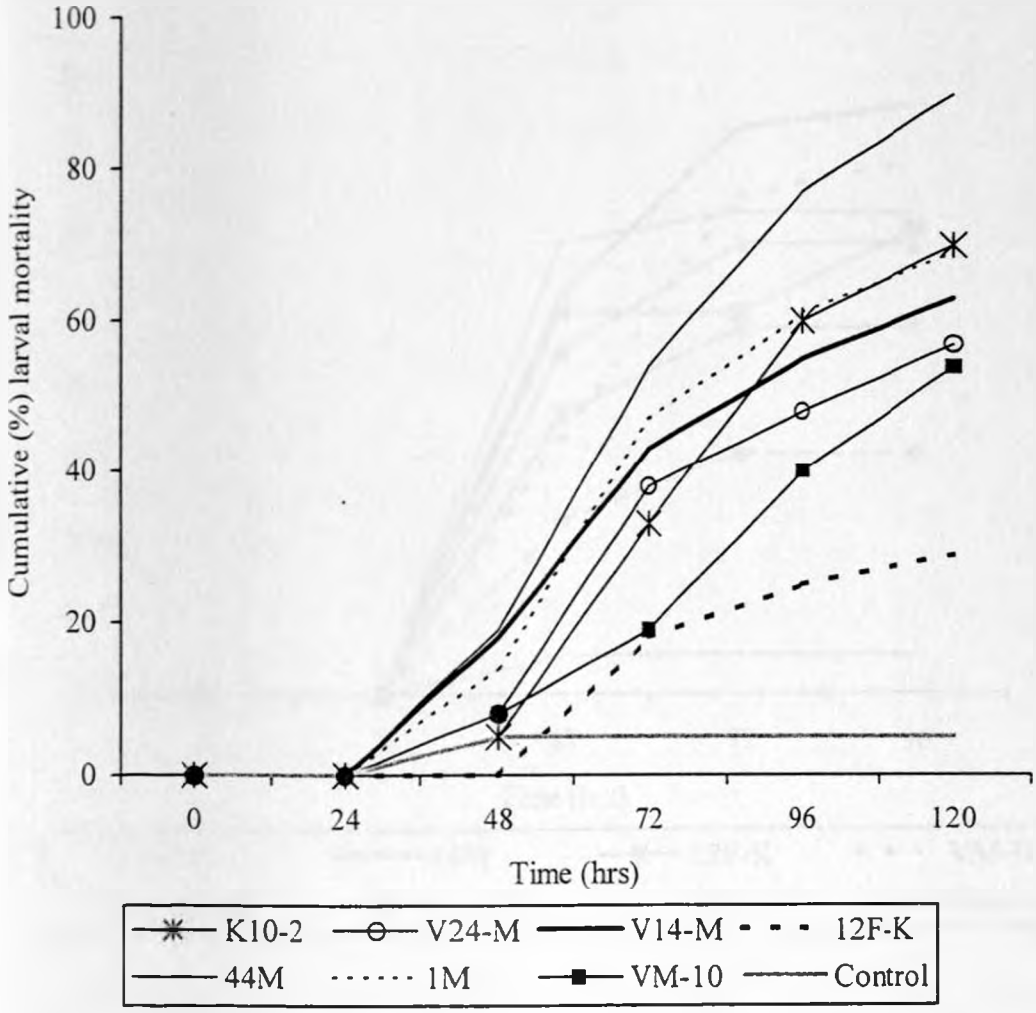


Fig. 35: Percentage mortality of 1st instar larvae of *S. calamistis* after exposure to 8.6-mg/ml δ-endotoxin of different *B.t* isolates.

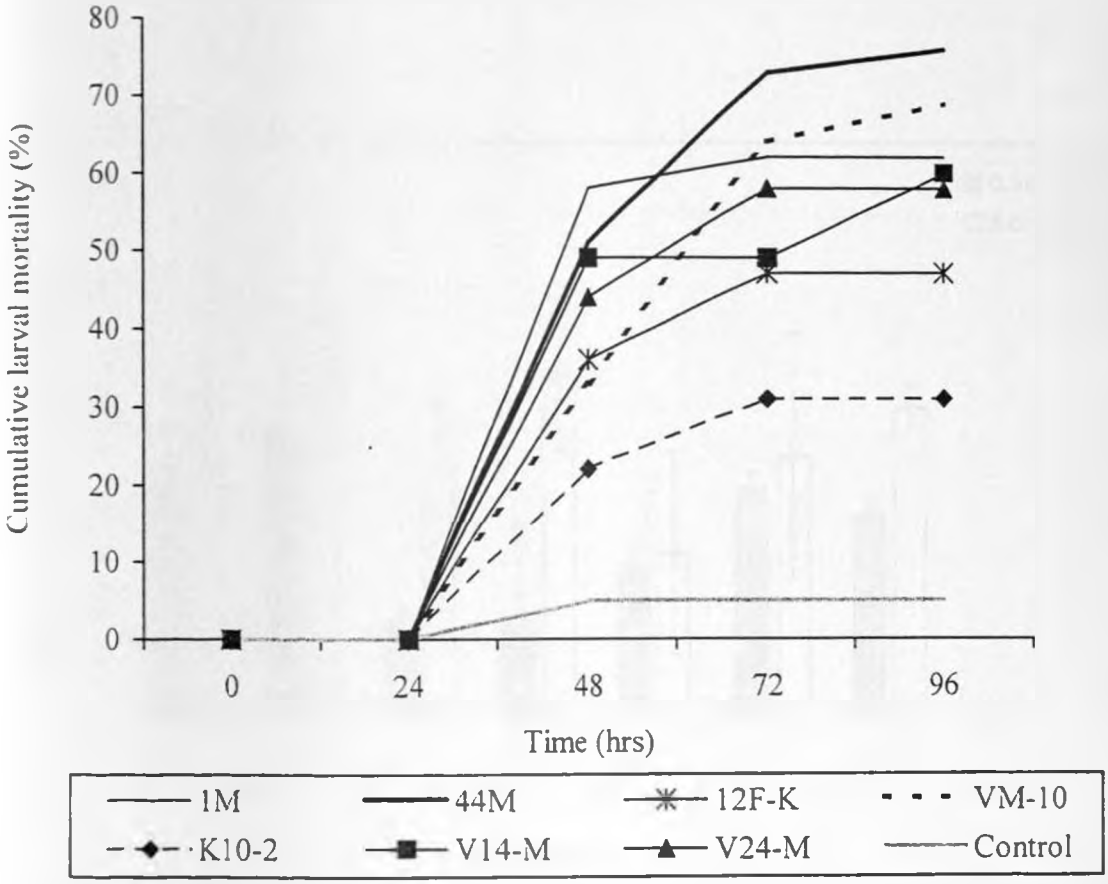


Fig. 36: Percentage mortality of *Sesamia calamistis* 1st instar larvae after 72 hrs of incubation at 0.86 and 8.6 mg/ml.

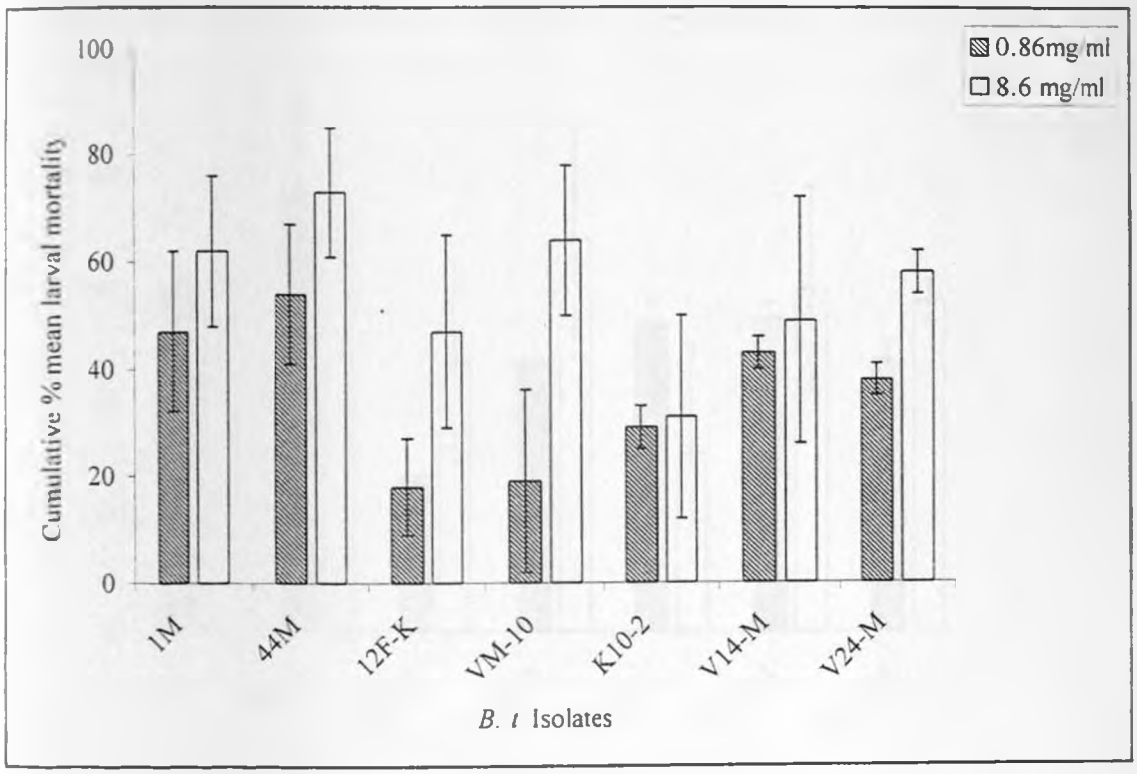


Fig. 37: Comparison of mortalities of 1st instar larvae of *S. calamistis* caused by different *B.t* isolates at 0.86 mg/ml and 8.6 mg/ml. There were no larval mortalities in the controls.

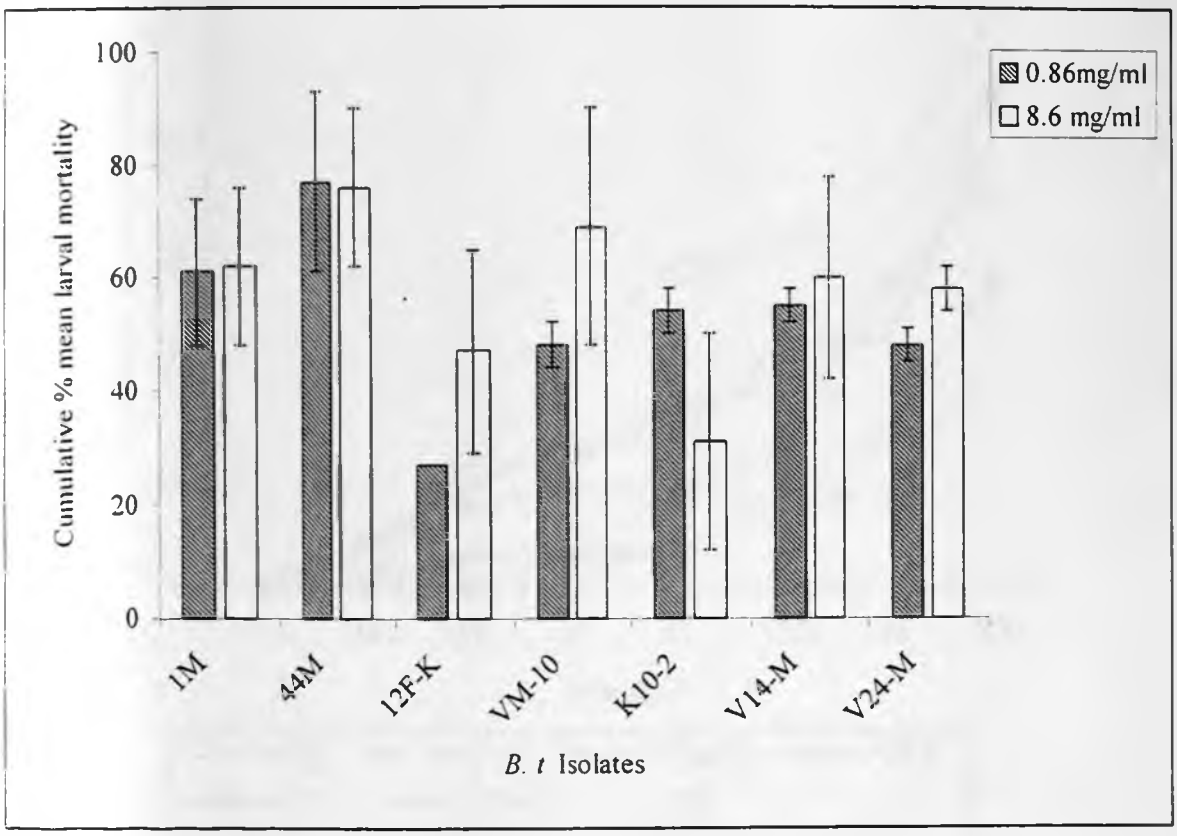
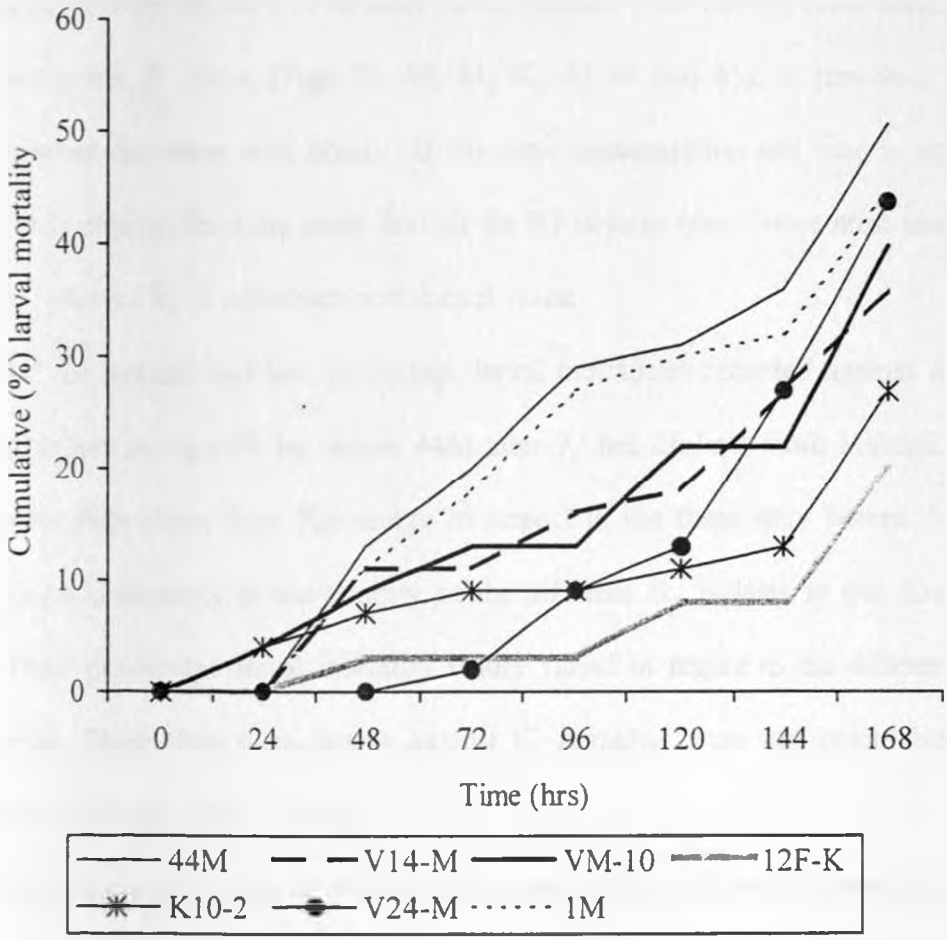


Fig. 38: Percentage mortality of 2nd instar larval stage of *Busseola fusca* exposed to 8.6 mg/ml of *B.t* δ-endotoxin of different isolates. There were no larval mortalites among the controls.



4.14: Evaluation of the different *B.t* isolates for their toxicity to the three stem borers.

The activity of the different *B. t* isolates against the three stem-borers was also evaluated. All the isolates were highly potent against *C. partellus* with the lowest larval mortality of 77% by isolate V24-M after 72 hrs. Isolate 44M was the most toxic against *S. calamistis* and *B. fusca*. (Figs 39, 40, 41, 42, 43 44 and 45). A summary of their toxicity against the three stem borers at the same concentration and time is shown on Table 9. It is evident from the study that all the *B.t* isolates tested were most toxic to *C. partellus*, followed by *S. calamistis* and then *B. fusca*.

All the isolates had low percentage larval mortalities recorded against *B. fusca*, with the highest being 55% by isolate 44M after 72 hrs. Isolates from Machakos were more potent than those from Kakamega in respect to the three stem borers. However there was no uniformity in the toxicity of the different *B.t* isolates to the three stem-borers. Their percentage larval mortality values varied in regard to the different target insect pests. Those that were potent against *C. partellus* were not potent against *S. calamistis* or *B. fusca* and vice versa.

The most potent isolates from Kakamega and Machakos were compared for their toxicity against the three stem-borers. Isolates 1M, VM-10 and 44M (Fig.46) and isolate 1M against isolates 12F-K and K10-2 (from Kakamega) (Fig.47).

Fig. 39: Comparison of toxicity levels of δ-endotoxin from isolate 1M to 1st instar larvae of *C. partellus*. *B. fusca* and *S. calamistis*. The toxin concentration was 8.6 mg/ml.

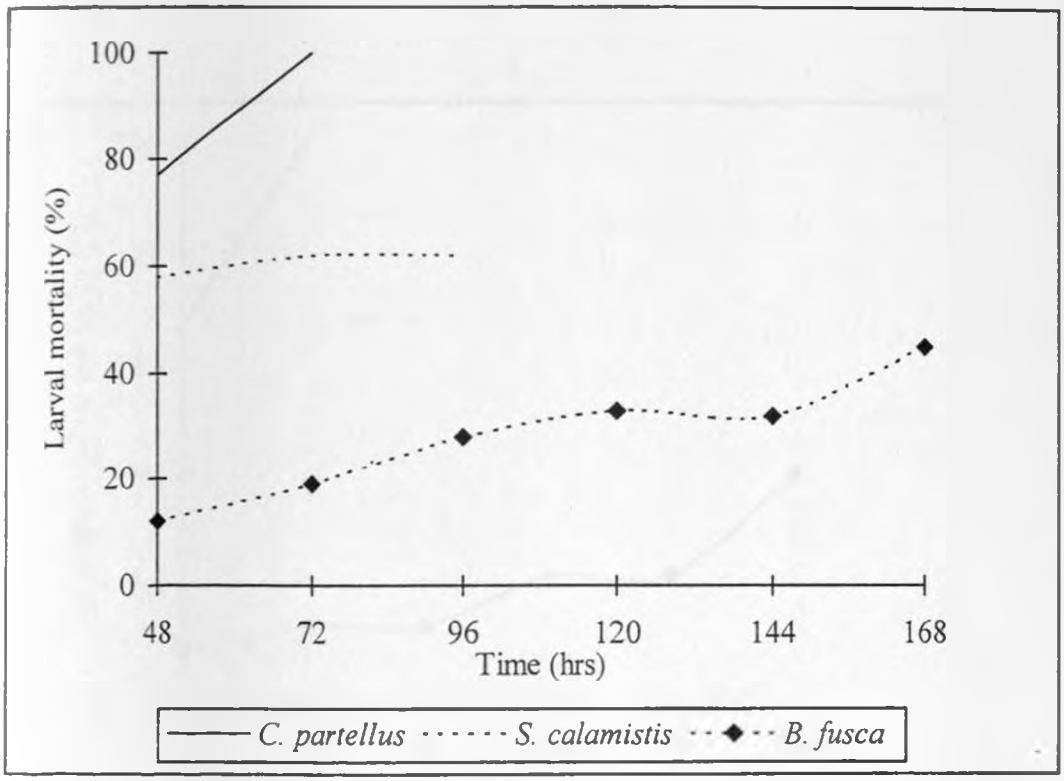


Fig. 40: Comparison of toxicity levels of δ- endotoxin from isolate VM-10 to 1st instar larvae of *C. partellus*, *B. fusca* and *S. calamistis*. The concentration of the toxin was 8.6 mg/ml.

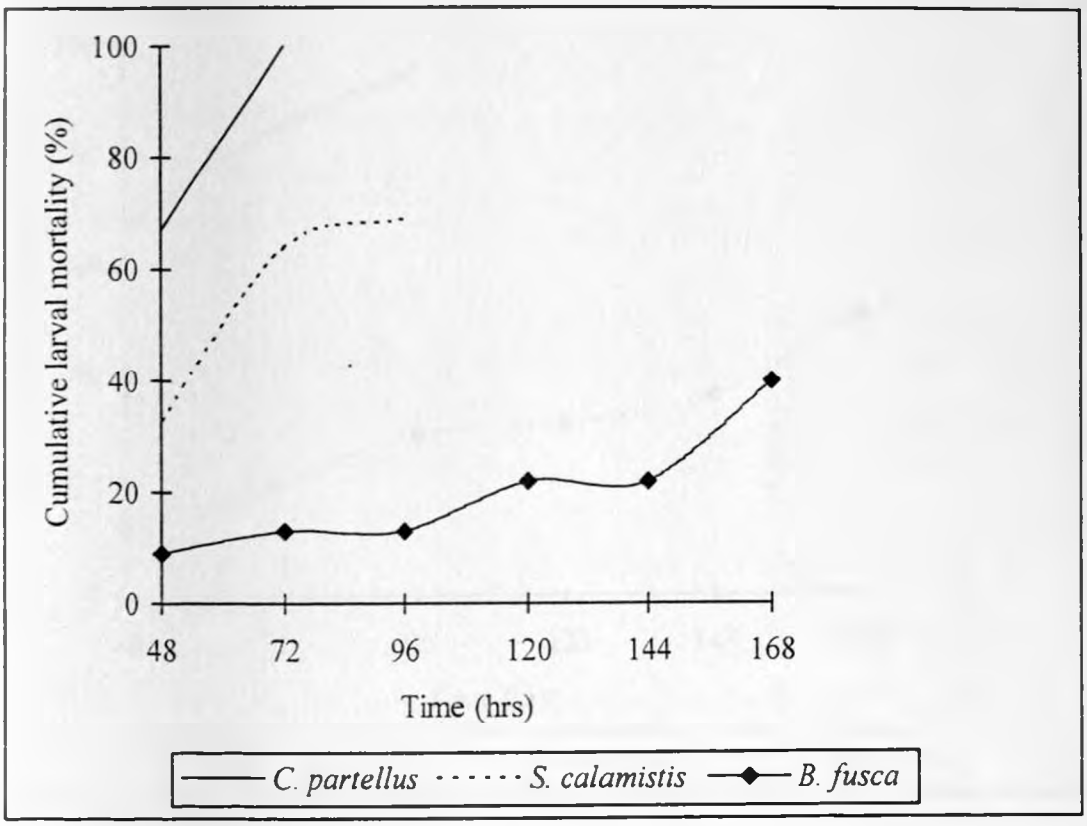


Fig. 41: Comparison of toxicity levels of δ- endotoxin from isolate 44M to 1st instar larvae of *C. partellus*, *B. fusca* and *S.calamistis*. The concentration of the toxin was 8.6 mg/ml.

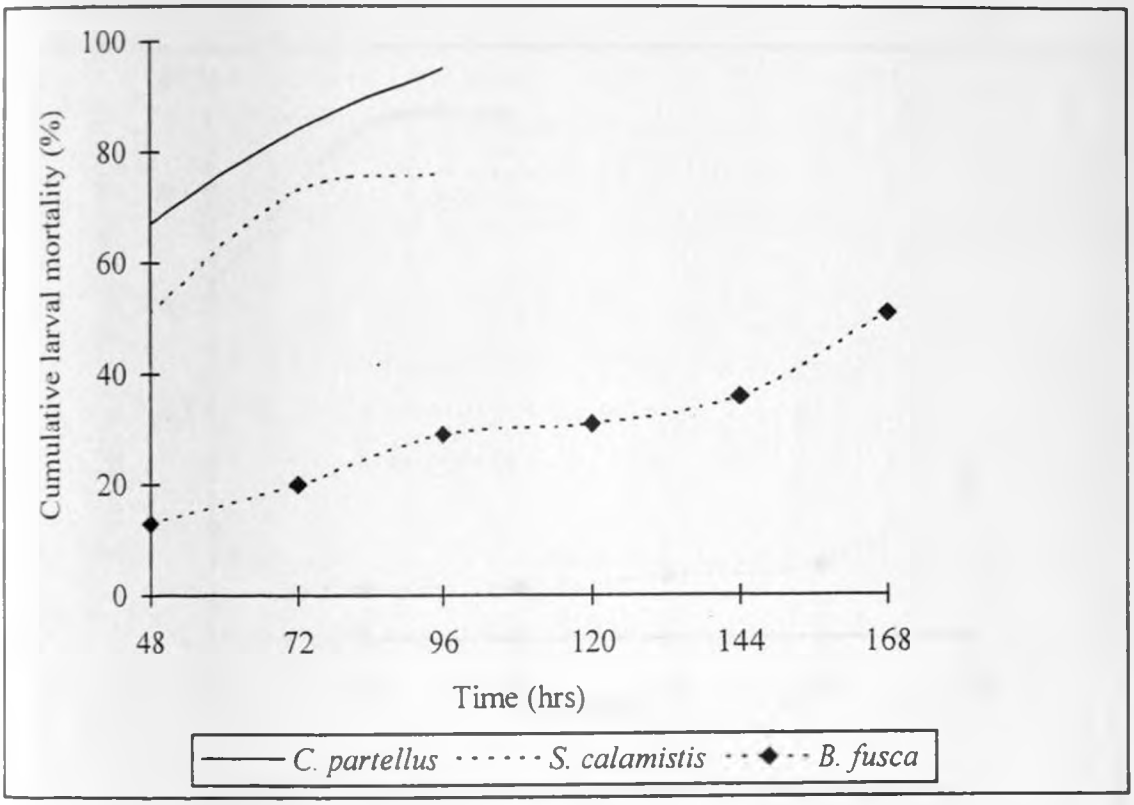


Fig. 42: Comparison of toxicity levels of δ-endotoxin from isolate K10-2 to 1st instar larvae of *C. partellus*, *B. fusca* and *S.calamistis*. The toxin concentration was 8.6 mg/ml.

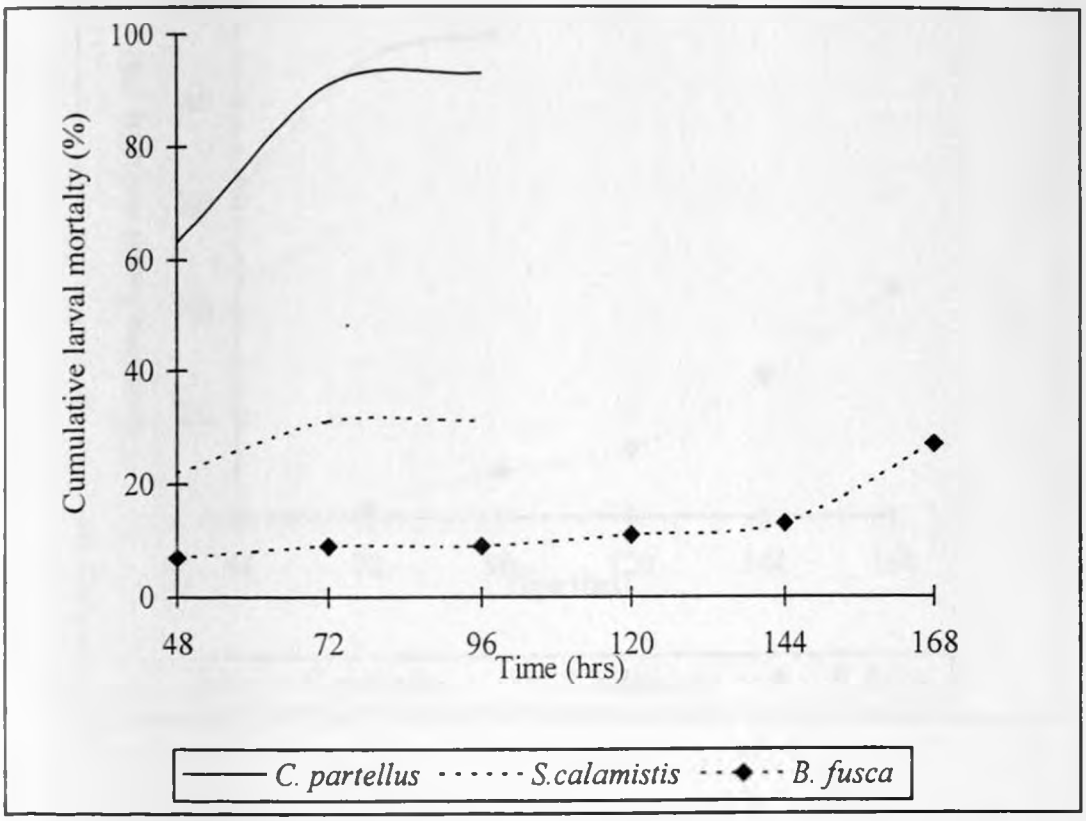


Fig. 43: Comparison of toxicity levels of δ-endotoxin from isolate V24-M to 1st instar larvae of *C. partellus*, *B. fusca* and *S. calamistis*. The toxin concentration was 8.6 mg/ml.

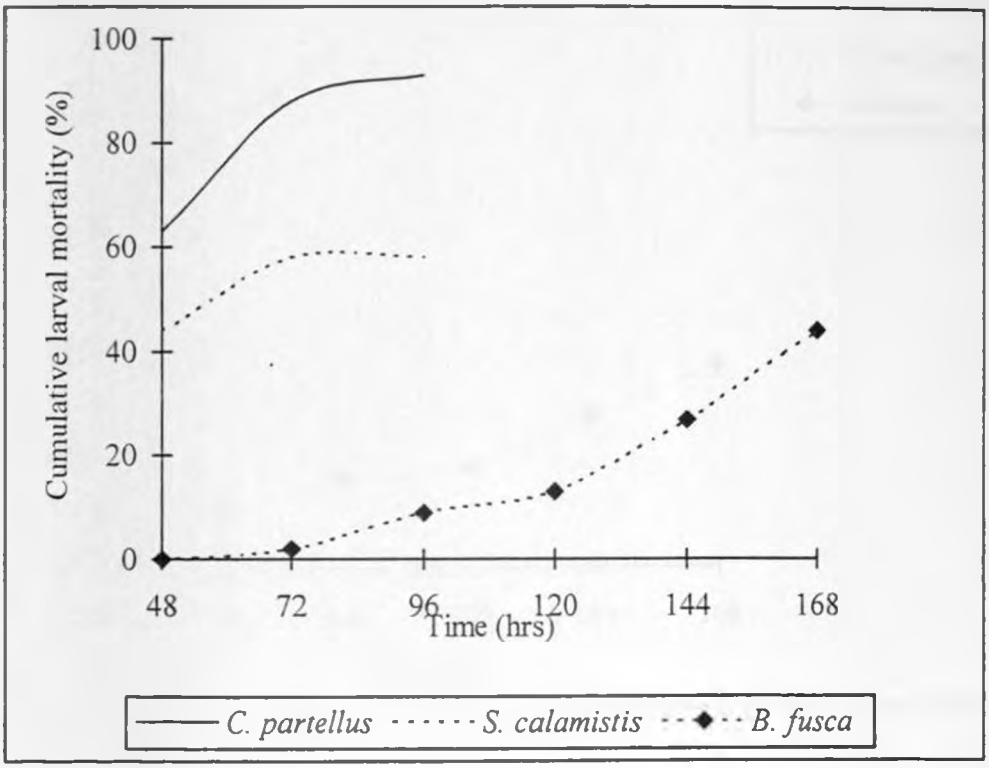


Fig. 44: Comparison of toxicity levels of δ-endotoxin from isolate V14-M to 1st instar larvae of *C. partellus*, *B. fusca* and *S. calamistis*. The toxin concentration was 8.6 mg/ml.

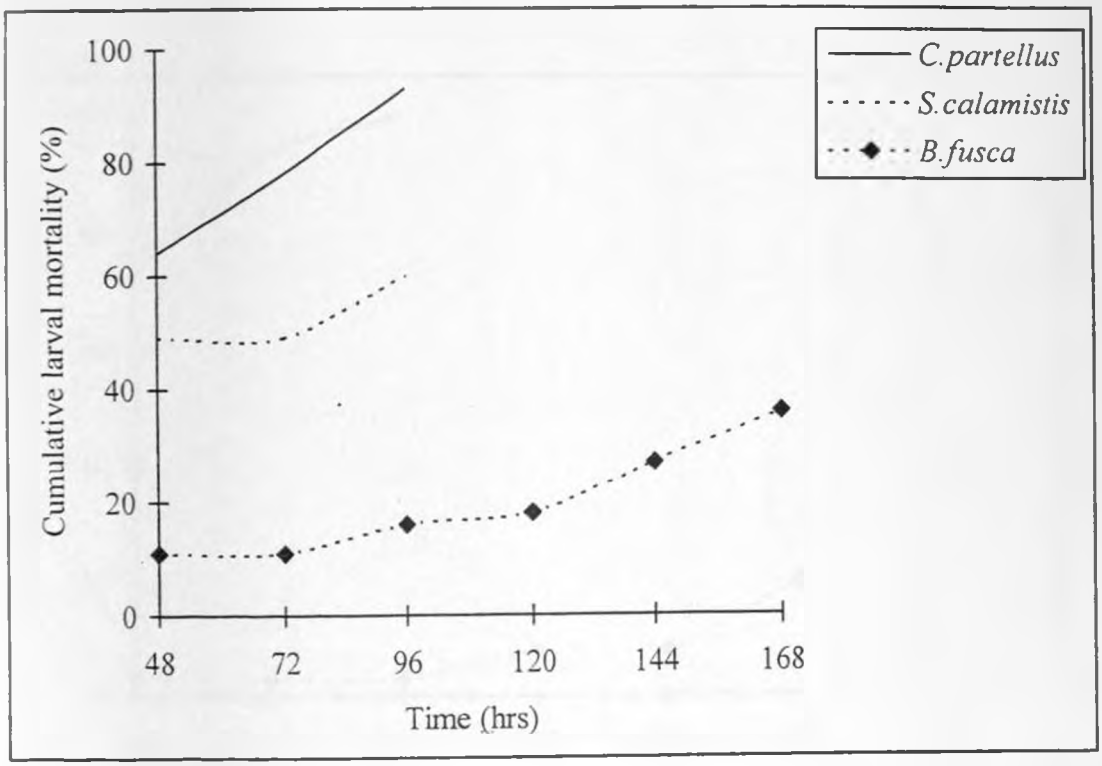


Fig. 45: Comparison of toxicity levels of δ-endotoxin from isolate 12F-K to 1st instar larvae *of C. partellus, B. fusca* and *S. calamistis*. The toxin concentration was 8.6 mg/ml.

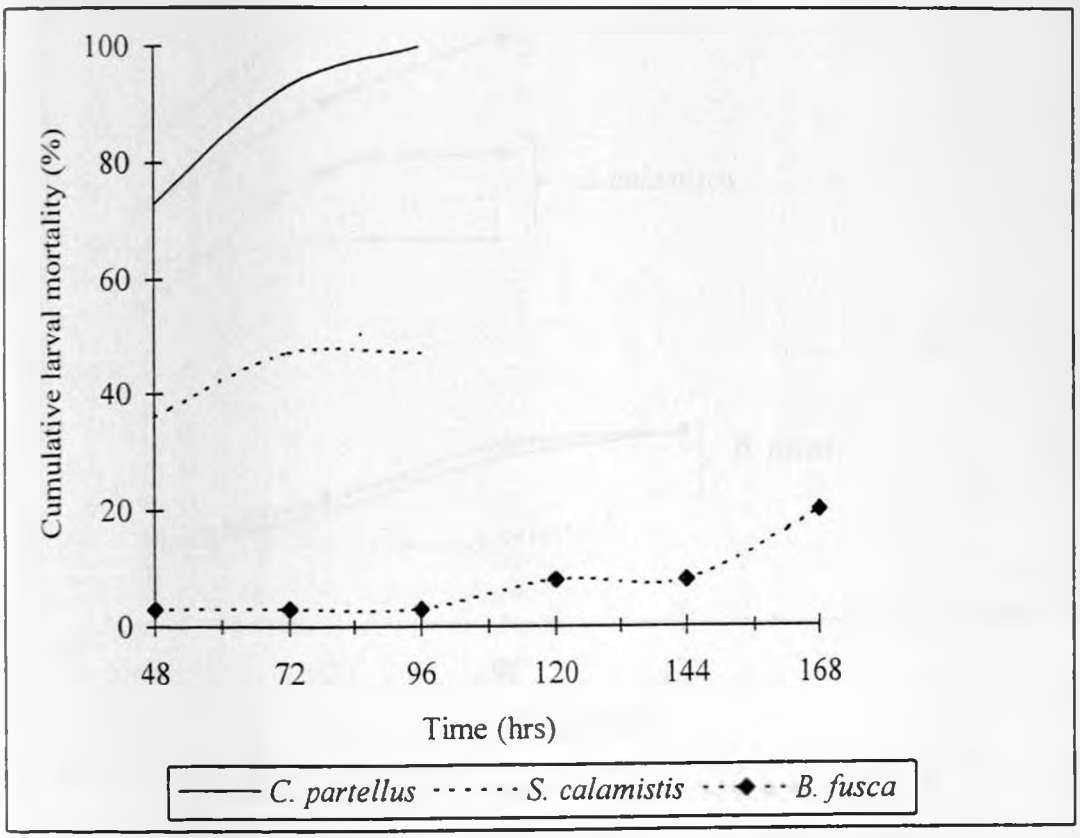


Fig. 46: Comparison of the most potent isolates: 1M, VM-10 and 44 M for their activity against the stem borers: *C. partellus* (*C. p*), *B. fusca* (*B. f*) and *S. calamistis* (*S. c*).

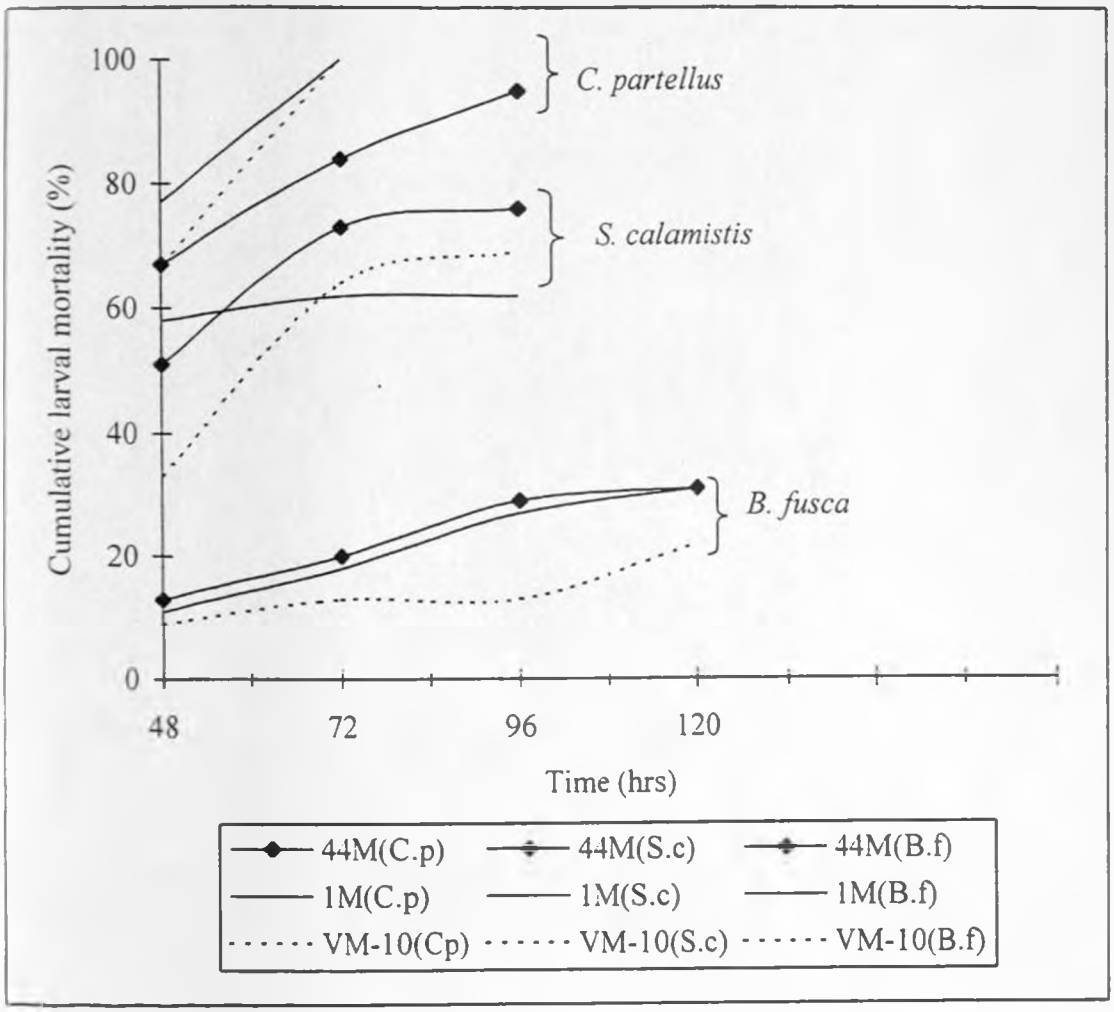


Fig. 47: Comparison of *B. t* Isolates from Kakamega (K10-2 and 12F-K) with isolate 1M from Machakos for their toxicity against 1st instar larvae of *C. partellus* (C. p), *B. fusca* (B. f) and *S. calamistis* (S. c). The concentration tested was 8.6 mg/ml.

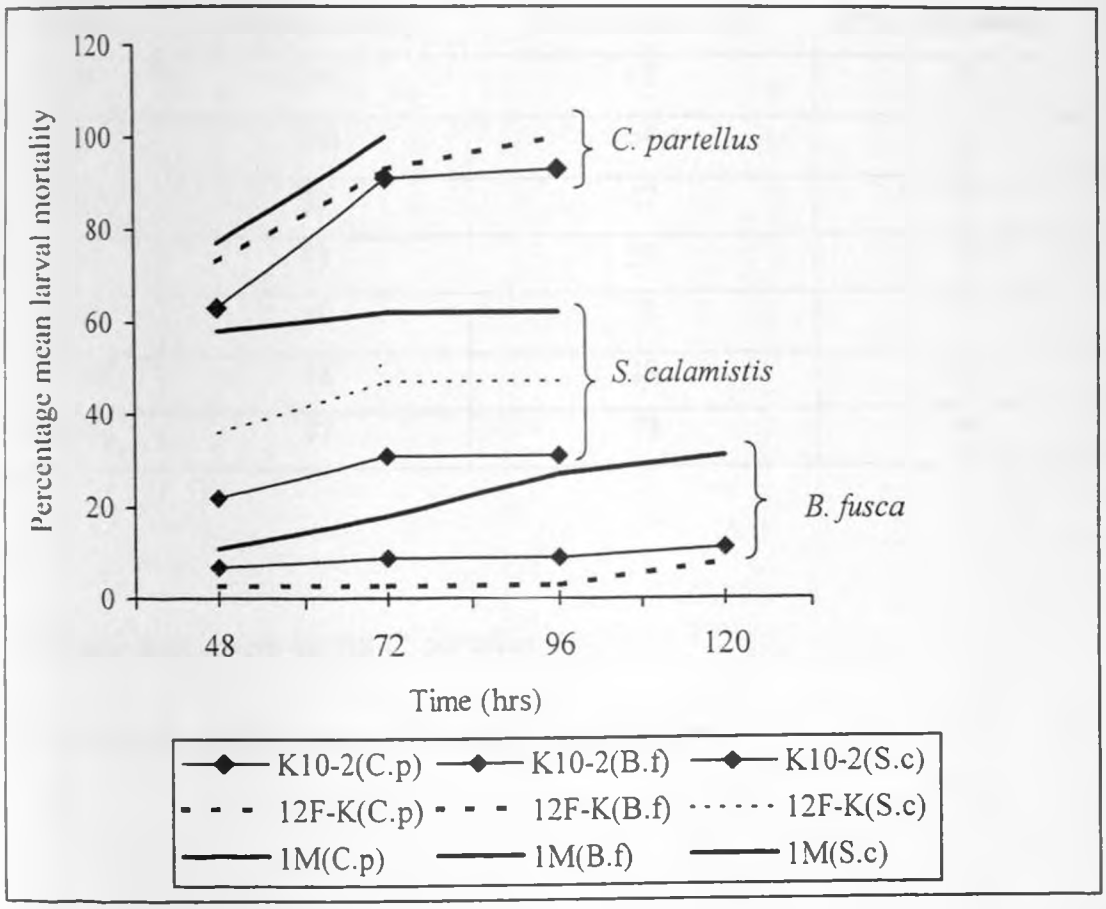


Table 9 : A summary of the toxicity of the different *B.t* isolates to different corn borers by the artificial diet bioassays. Larval stage : 1st instar: Concentration : 8.6 mg/ml: Time: 72 hrs for ***C. partellus*** and ***S. calamistis*** and 168 hrs for ***B. fusca.***

***B.t* isolate**	***C. partellus* % mean larval moratlity**	***S. calamistis* % mean larval mortality**	***B. fusca* % mean larval mortality**
1M*	100	62	45
VM-10*	100	64	40
12F-K	93	47	20
K10-2	91	31	27
44M**	80	73	51
V14-M	78	49	36
V24-M	77	58	44

*- Isolates most potent against *C. partellus*

** - Isolate most potent against *S. calamistis* and *B. fusca*

4.15: Estimation of molecular weights by polyacrylamide gel electrophoresis.

B.t δ- endotoxin complex of the different isolates were analysed for molecular weight by electrophoresis on a 12% SDS polyacrylamide gel (Fig. 48). The gel was stained for proteins using Coomassie Brilliant Blue. The molecular weights of the proteins were determined from plots of log. molecular weight versus relative migration of the molecular weight markers (Fig. 49). Electrophoretic analysis of crystals of the different *B.t* isolates revealed three major protein subunits of molecular weight ~28 kDA (A) ,~ 65 kDA (B) and ~130 kDA (C) for all the isolates except for isolate 1M (Lane 8) which did not have the 130 kDA protein band. It showed only the 28 kDA and 65 kDA protein bands. (Fig. 48).

Fig. 48: Estimation of molecular weights of δ-endotoxin of different *B.t* isolates by SDS-PAGE. Lane 1: Molecular weight markers; Lane 2: Isolate V14-M; Lane 3: Isolate 44M; Lane 4: Isolate K10-2; Lane 5: Isolate 12F-K; Lane 6: Isolate VM-10; Lane 7: Isolate V24-M; Lane 8: Isolate 1M.

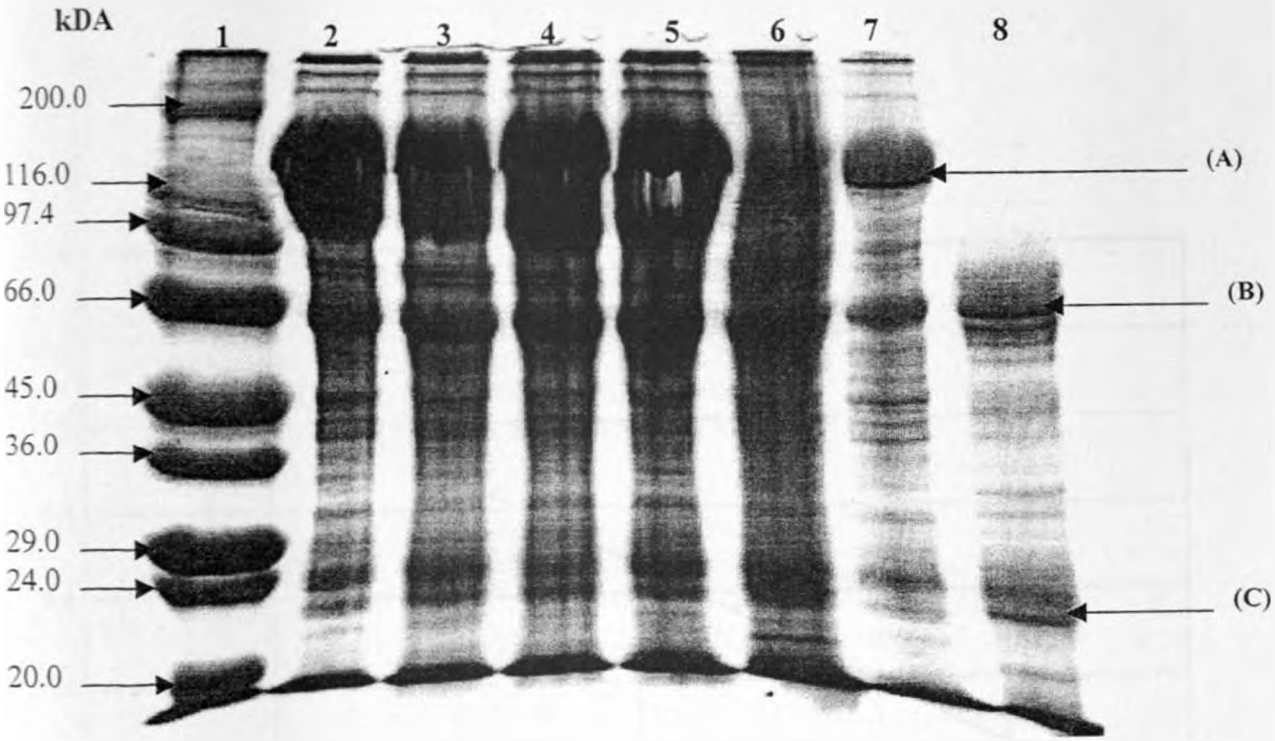
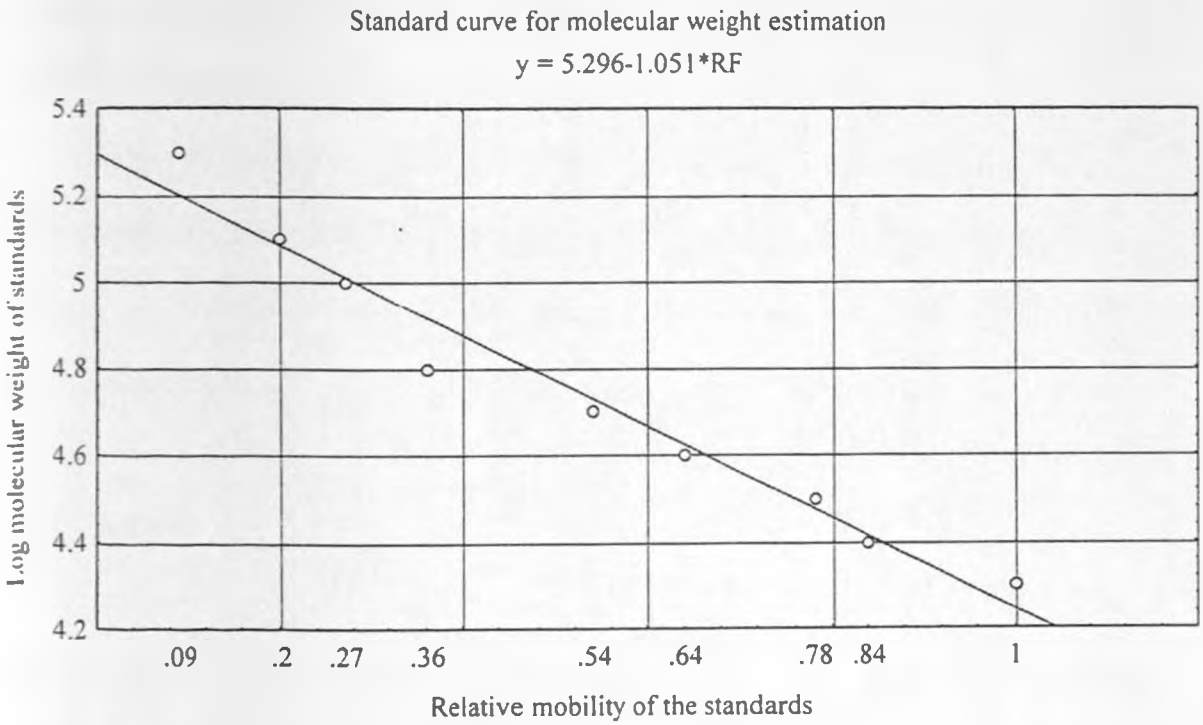


Fig. 49: Standard curve for estimation of molecular weights SDS-PAGE

The standard curve was constructed by measuring the relative movement of molecular weight markers on a 12 % SDS polyacrylamide gel electrophoresis and plotting against log molecular weights of the standards.



CHAPTER FIVE

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Maize is the principal staple food in Kenya. It is the most important food crop grown and consumed in Kenya. It dominates the diet of rural and urban poor. It ranks first in Kenya for yield per hectare. It is grown almost exclusively under rain fed conditions from sea level to 2,400 meters and although producers technology varies greatly, it is largely traditional resulting in low productivity in most zones except the transitional highlands.

Given the large area planted to maize and the number of farmers involved in maize production, the development and adoption of improved technology has significant potential to elevate income and make the country sufficient in basic grains. The devastating effects of pests and diseases in Kenyan agriculture are reflected in the amount of resources spent by farmers on their control. Use of chemical pesticides, is not affordable to the resource poor farmers who form the bulk of maize production in Kenya.

Bacillus thuringiensis is a ubiquitous soil microorganism that has been known for over 30 years for its potential as a biopesticide. However, its use in Kenya has been limited by the fact that the commercial products of *Bacillus thuringiensis (B. t)* in the market are imported and thus are expensive. They also originate from temperate regions thus may not be well suited for insect pest control in the tropics due to the different climatic conditions. Due to the advantages associated with its use as a biopesticide, it is increasingly becoming a most preferred method of insect pests' control that have become a major cause of pre-harvest crop losses.

The findings on the toxicity of *B.t* isolates as a microbial control agent of target lepidopteran species in Kenya adds to the pool of already existing information available on the possibility of using this microorganism for pest larval control purposes.

The pest larval stage studied is the most destructive stage and finding a way of controlling it would go a long way in enhancing food production thus improving our country's economy, which largely relies on agriculture.

5.1: Isolation of *B.t* from soil samples

Isolation of *B. t* from soil samples from Kakamega and Machakos Districts proved that *B.t* is an ubiquitous microorganism and has a worldwide distribution as previously reported by Martin and Travers (1989) working on soil samples from different regions of the world. Twelve isolates of *B.t* containing parasporal inclusion bodies (crystals) were obtained, of which three were from Kakamega (12F-K, K10-2 and K13-1) and nine from Machakos (1M, VM-10, V14-M, V24-M, V15-M, 34M, 35M, 44M and 45M). Therefore the study found out that Machakos soil samples were more productive than those from Kakamega in the search for *B.t*. This was also similar to the findings of Martin and Travers (1989).

In the current study, soil samples from Kakamega were more acidic than those from Machakos. The pH range of soil samples from Kakamega was 4.28 - 5.65 in comparison to a pH range of 5.42 -7.32 from Machakos. The high acidity levels in Kakamega soil samples could have prevented the survival of *B.t*.

5.2: Morphological and biochemical characteristics of the *B.t* isolates

The morphological and colony characteristics of the *B.t* isolates (rod shape, spore shape and form, gram-reaction, whitish creamish colonies on Nutrient Agar with

rough edges) were studied. Also confirmed was the fact that *B.t* produces a parasporal body. Measurements of the vegetative cells of the different *B.t* isolates revealed some differences in rod length, however, the rod width was similar for all the isolates. Characteristics such as location of the spore, and shape of the crystals were similar for all the isolates. The location of the spore was terminal, whereas the shape of the crystal was pyramidal for all the isolates.

Biochemical characteristics of the isolates were investigated in this study. These were Gram reaction, catalase and Voges- Proskauer tests. It was found that all the isolates were Gram positive and can break down hydrogen peroxide into water and oxygen indicating the presence of catalase enzyme. Presence of acetylmethylcarbinol (AMC) produced from pyruvic acid in the course of butylen-glycolic fermentation was indicated by a positive AMC test. This was consistent with findings of other investigators such as, Kwan-Hee *et al.*, (1996), Sergio *et al.*, (1992) and Hossain *et al.*, (1997), indicating that indeed *B.t* was isolated.

5.3: pH tolerance of the *B.t* Isolates

The various *B t* isolates of both locations were tested for their pH tolerance and this indicated some differences. Isolate K10-2 from Kakamega could only grow from pH 6.0 and above, while isolate 12F-K grow at pH 6.0 and above, whereas isolate K13-1 grew at pH 7.6. Growth at pH 7.6 was high which is in accordance with the optimal pH of *B.t*, which is 7.4 ± 0.2. On the other hand, three of the *B.t* isolates from Machakos (V24-M, 1M, 45M) grew from pH 6.5 and above while only three (35M, V14-M, and 44M) grew from pH6.0 and above. Isolates VM-10, V15-M, 34M, grew at only pH 7.6. Maximum growth was also observed at pH 7.6. There was growth at pH 8.0, though it was minimal

than that observed at pH 7.6. In a study by Hossain *et al.*, (1997), they reported that although the *B.t* isolates grow well between pH 7.5 and 8.2. They found *B.t* in soils with pH values ranging from 5.3-7.95.

In the current study, all the *B.t* isolates grew well at pH 7.6 and showed lower growth rates below and above pH 7.6. Kiselek, (1974) and Sekijima *et al.* (1977) have reported that viable spores survive in the soil for only 3 to 16 months, under natural conditions. Hossain *et al.*, (1997), also reported that *B.t* does not survive if the spores do not survive and the distribution *of B.t* spores in the soil mainly depends on ecological factors that affect viability of *B.t* spores.

In this study the tolerance of *B.t* to the different pH ranges could be related to the abundance of *B.t* in the two localities. Fewer isolates were obtained from Kakamega whose soil was more acidic than Machakos. The pH of former was (4.28 - 5.65), which could mean that fewer vegetative cells of *B.t* survive upto sporulation, hence fewer spores. which also means fewer isolates of *B.t*. The pH of Machakos soil samples was slightly acidic to alkaline (5.42 -7.32). More vegetative cells of *B.t* can survive to sporulation. hence more spores and more *B.t* isolates. Therefore this study concluded that there were several factors that could have played a role in the presence of *B.t* in the different soil samples owing to the different geographic conditions of the two localities, which experience different ecological factors.

5.4 Bioassays on artificial diet

5.4.1 Bioassays on *C. partellus*

Ranges of dilutions were prepared for each of the *B.t* isolates in the bioassays. This was to determine which concentration was more potent against *C. partellus.* Isolates

1M and VM-10 were the most potent and caused 100% larval mortality within 72 hrs at 0.5 and 0.86 mg/ml toxin concentration respectively. Their LD_{50} values were 0.004 mg/ml and 0.04 mg/ml respectively. When the isolates were tested at the same concentration of 8.6 mg/ml against first instar larvae of *C. partellus,* isolates VM-10 and 1M were still the most potent and caused 100% larval mortality within 72 hrs. *B. thuringiensis* isolates were most toxic to *C. partellus* larvae than to the commercial product "Thuricide ®" and International Centre of Insect Physiology and Ecology *B.t* strain MJ 99 2, which caused 69% and 58% larval mortality within 72 hrs respectively.

The *B.t* isolates were found to be highly toxic against 2nd instar larvae at a concentration of 8.6 mg/m. This indicated that the 2nd instar larvae were susceptible to the *B. t* toxin of the isolates. However, mortality of the 2nd instar larvae was high than that of the 1st instar, for all other isolates, except for isolates VM-10 and 1M. This might be a consequence of the different characteristics of the stages of the larvae. Larval mortalities of the 2nd instar were slightly high than those of 1st instar larvae. Second instar larvae feed actively than the neonates. However the two larval stages were susceptible to the *B.t* toxin of the different isolates.

These results are related to the findings of Karamanlidou *et al.*, (1991). He reported that mortality levels of *Drosophila oleae* caused by the toxin of different isolates of *B.t* are always higher if the experiments are carried with larvae rather than the adults. This was also reported by Kahindi (1987) and Kariuki (1987) working on Mosquito and *C. partellus* larvae respectively. They found that young larvae (1st and 2nd instars) were more susceptible than mature larvae and succumbed to infection earlier than the mature larvae. Older larvae (3rd and 4th instars) of these target pests feed minimally as they are

ready to pupate, hence do not feed, compared to 1st and 2nd instars that feed more. They should thus be targeted during a pest control program. The data obtained clearly indicates there is great potential of using *B.t* for the control of cereal pests. It was evident that increase in toxin (spore-crystal complex) concentration resulted in an increase in larval mortality.

5.4.2 Bioassays on *S. calamistis.*

Bioassays with the 1st instar larvae of *Sesamia calamistis* (pink stem borer) showed that the *B.t* isolates were toxic to *S. calamistis.* The pests, on introduction to the artificial diet, positioned themselves above the diet for about 4 hrs without feeding. Unlike with *C. partellus* where death was observed after 24 hrs, death of *S. calamistis* was observed after 48 hrs. At a concentration 0.86 mg/ml there was significant increase in larval mortality with time for all the isolates (Fig.34). However at 8.6 mg/ml, increase in incubation time from 72 hrs to 96 hrs did not seem to increase the larval mortality (Figs. 36 and 37). Isolate 44M showed the highest mortality at the two concentrations tested: 0.86 mg/ml and 8.6 mg/ml with larval mortalities of 54% and 73% after 96 hrs respectively.

The results indicated that, increase in toxin concentration resulted in an increase in larval mortality for most of the isolates except for isolate K10-2. Isolates VM-10 and 1M also showed high level activity of 48% and 47% at 0.86 mg/ and 64% and 62% at 8.6 mg/ml, at 72 hrs respectively. However, it was noted that, at 96 hrs of incubation at 8.6 mg/ml concentration, there was no further increase in larval mortality for the isolates. Isolates 44M, VM-10 and V14-M showed a slight increase in percentage larval mortality. Increase in toxin concentration did not seem to affect the activity of isolate K10-2. At 72

hrs. its larval mortality was 33% and 31% at a concentration of 0.86 mg/ml and 8.6 mg/ml respectively. At 96 hrs, it had killed 60% of the larvae at 0.86 mg/ml compared to 31% at 8.6 mg/ml at the same time.

4.3 Bioassays on *B. fusca*

Bioassays with 1st and 2nd instar larvae of *B. fusca* showed a lower toxicity of the isolates against the stem borer. Isolate 44M had the highest larval mortality of 51% after 6 days. This was at a concentration of 8.6 mg/ml (Fig. 38). The percentage mortality of *B. fusca* larvae for all the isolates was low compared to that of *C. partellus* and *S. calamistis* larvae. This shows that, the two pests could be more susceptible to the toxin than *B. fusca*. Cherry *et al.*, (1999) reported that the concentration required to cause 50% larval mortality was higher for *B. fusca* than that for other species.

This could also be explained by the fact that, *B.t* exhibits anti- feedant effects, which may inhibit full ingestion leading to variability in the amount of toxin obtained by larvae. This was also observed during the current study, where the larvae, on introduction to the tubes with treated artificial diet, positioned themselves on top of the tube (near the gauze) for almost 4 hrs during which there was minimal feeding or none. For most of the isolates mortality was observed after 48 hrs compared to 24 hrs with *C. partellus*.

5.5 Leaf disc bioassays

Leaf disc bioassays were carried out using young maize leaves of about 5 weeks old. The bioassays indicated some degree of differences in comparison with the artificial diet bioassays. The leaf disc bioassays were carried out with 1st instar larvae of *C. partellus*. Unlike in the artificial diet bioassays where the larvae took some time before

they started feeding, larvae in the leaf disk bioassays started feeding immediately after introduction.

The 100% mortality by 72 hrs indicates that natural diet is more preffered than artificial diet. However larvae in the treated leaves were feeding minimally as compared to the controls. All the larvae in both the treatments and the controls preferred the under side of the leaf disc. The live larvae were creamish, but turned darkish after death. Larval mortality was 100% within 48 hrs for all the isolates (Fig. 33). However isolate 1M had the highest percentage larval mortality of 71% after 24 hrs compared to the other isolates.

The diet preference may have contributed to the high mortality rate within 48 hrs in comparison with the artificial diet where larval mortality records went upto 6 days. There were no larval mortalities among the controls. More toxic isolates caused less leaf area damage than the less toxic ones. The most leaf area damage was in the controls where no toxin was applied. Thus this clearly indicates that the mortality of the insects was as result of the *B.t* toxin.

5.6 Toxicity of different *B.t* isolates to different insect pest species.

From this study it was observed that different *B.t* isolates have different levels of toxicity, even for the same pest species under test. This was consistent with findings of (Brownbridge, 1992). All the *B.t* isolates showed marked differences in their levels of activity against the three pest species (*C. partellus, B. fusca* and *S. calamistis*) (Table 9). Each isolate showed different levels of toxicity against the three pest species. Isolate VM-10 and 1M, were the most potent against *C. partellus*, whereas 44M was the most potent against *S. calamistis* and. *B. fusca*. Martin and Travers, (1989), found a correlation

between origin or sample type and insecticidal activity. This was also evident in this study, as isolates from Machakos appeared to be more potent than those from Kakamega.

Differences in the levels of toxicity to the three pest species demonstrated the need for screening a large number of *B.t* strains when considering their use in a microbial control programme. It is also important to screen the *B.t* isolates, against a wide range of insect pests as one isolate that is not toxic to a certain pest may be highly toxic to another. This calls for the need to screen the isolates against many lepidopteran, dipteran and coleopteran insect pests. This is because *B. t* toxin is highly specific and is a narrow spectrum biopesticide. Some preliminary tests done with the isolates showed that they are toxic to *Helicoverpa armigera*. Such inter-varietal differences have been observed by a number of other workers (Amonkar *et al.*, 1985; Jarret and Burges, 1986).

5.7 SDS-PAGE Analysis of crystal proteins of the different *B.t* isolates.

Different *B.t* varieties produce δ- endotoxins that differ in their biochemical properties and host specificity. This has been reported by Dulmage, (1975), Tyrell *et al.*, (1981), and Knowles *et al.*, (1986). Chilcott and Ellar, (1988), reported that analysis of *B.t* crystals using SDS-PAGE, revealed that most Lepidopteran- active crystals contain 130- and/ or 65 kDA proteins. Mosquito active crystals were 130- 65-, and 28 kDA proteins. Chilcott and Wigley, (1993) also reported that Lepidopteran active crystals contain 130 kDA and or 65 kDA proteins. The major protein bands in this study were of molecular weights, ~28 kDA, ~65 kDA and ~130 kDa for all the isolates except isolate 1M, which showed two protein bands of molecular weight ~28 kDA- and ~ 65 kDA (Fig. 40). The protein pattern of the crystals described in this report was thus similar to those of other varieties active against Lepidopteran larvae.

5.8 Conclusions

Increase in the number of *B. t* collections has led to an increase in the discovery of new *B.t* isolates with insecticidal activity against a diverse range of insects or with increased insecticidal activity, (Martin and Travers, 1989). This study indicates the need for isolation of more local *B.t* isolates, as this would provide a large genetic resource base for the utilization of *B.t* as a microbial insecticide or the incorporation of the gene coding for the toxic proteins into the plants and or other microorganisms. It would also be important to find out if the isolates are toxic to mosquito larvae, as they expressed the proteins active against them as reported by Chilcott and Ellar, (1988). Screening the isolates against other insect pests of other orders such as Coleoptera and Diptera would be important as *B.t* is host specific, and non-toxicity to one insect pest does not completely rule out its toxicity to other insect pests of other orders. Even in the same order, different *B.t* strains show different host specificity.

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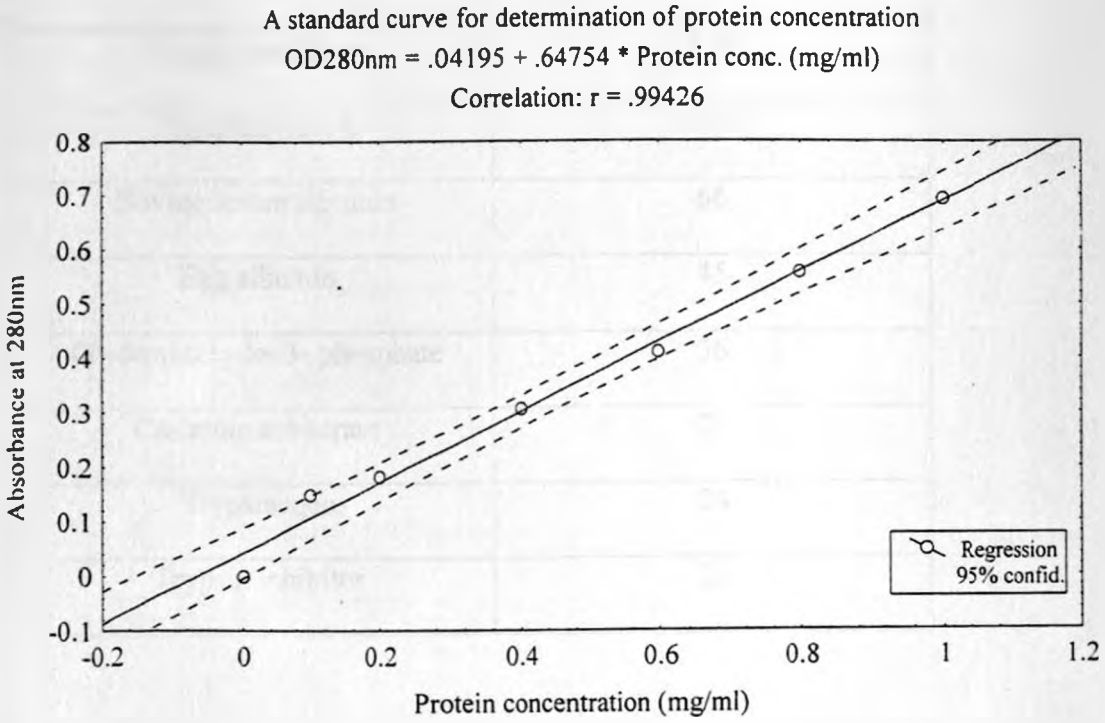
APPENDIX

Appendix I

Standard curve for determination of protein concentration



Appendix II

Molecular weight markers employed for estimation of molecular weight by SDS-PAGE

Marker	Molecular weight (kDA)
Myosin	200
β- galactosidase	116
Phosphorylase b	97.4
Bovine serum albumin	66
Egg albumin	45
Glyceraldehyde- 3- phosphate	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20

Appendix III

Percentage mortality of 1st instar larvae of *C. partellus* exposed to 8.6 mg/ml delta-endotoxin of different *B.t* isolates at 48 and 72 hrs. The mean is calculated from three replicates by artificial diet bioassay method

Isolates	48 hrs		72 hrs	
	%Mean	%SD	%Mean	%SD
K10-2	60	7	91	15
V24-M	60	0	77	4
V14-M	33	0	78	4
VM-10	64	4	100	0
1M	76	10	100	0
12F-K	76	8	93	12
Thuricide®	56	14	69	10
MJ992	53	7	58	10

Appendix IV

Percentage mortality of 2nd instar larvae of *C. partellus* exposed to 8.6 mg/ml delta-endotoxin of different *B.t* isolates at 48 and 72 hrs by artificial diet bioassay method. The mean is calculated from three replicates.

Isolates	48 hrs		72 hrs	
	%Mean	%SD	%Mean	%SD
K10-2	49	4	93	7
V24-M	60	7	93	7
V14-M	76	4	100	0
VM-10	38	4	73	7
1M	58	4	87	18
44M	60	7	93	7
12F-K	87	12	93	12

Appendix V

Percentage mortalities of the different larval stages of *C. partellus* exposed to 8.6 mg /ml of delta- endotoxin of different *B. t* isolates at 48-to120 hrs

***B.t* isolates**	**Larval stage**	**48 hrs**		**72 hrs**		**96 hrs**		**120 hrs**	
		% Mean	% SD	% Mean	% SD	% Mean	% SD	% Mean	% SD
12F-K	L1	29	14	76	8	93	12	100	0
	L2	47	7	87	12	93	12	93	12
VM-10	L1	64	4	100	0	100	0	100	0
	L2	38	4	73	7	93	5	91	8
V14-M	L1	33	0	78	3	93	5	93	5
	L2	76	3	100	0	100	0	100	0
V24-M	L1	60	0	84	4	93	7	96	8
	L2	60	7	93	7	93	12	100	0
K10-2	L1	60	7	91	15	93	12	100	0
	L2	49	4	93	7	98	4	100	0
44M	L1	64	4	80	12	91	10		
	L2	60	7	93	7	100	0		
1M	L1	76	10	100	0	100	0	100	0
	L2	58	4	87	18	93	7	100	0

Appendix VI

Percentage mortality of 1st instar larvae of *C. partellus* exposed to 8.6 mg/ml of delta-endotoxin of different *B. t* isolates by the leaf disc bioassay method

B.t isolate	24 hrs		48 hrs	
	%Mean	%SD	%Mean	%SD
K10-2	67	7	100	0
V24-M	29	15	100	0
1M	71	21	100	0
44M	53	12	100	0
VM-10	49	14	100	0
12F-K	49	17	100	0
V14-M	60	7	100	0

Appendix VII

Percentage mortality of 1st instar larvae of *S. calamistis* exposed to different concentrations of delta- endotoxin of different *B. t* isolates at 72 hrs by artificial diet bioassay method

B.t isolate	0.86mg/ml		8.6mg/ml	
	%Mean	%SD	%Mean	%SD
K10-2	29	4	31	19
V24-M	38	3	58	4
1M	47	5	62	14
44M	54	13	73	12
VM-10	19	17	64	14
12F-K	18	9	47	18
V14-M	43	3	49	23

Appendix VIII

Percentage mortality of 1st instar larvae of *S. calamistis* exposed to different concentrations of delta- endotoxin of different *B. t* isolates at 96 hrs by artificial diet bioassay method

***B.t* isolate**	**0.86mg/ml**		**8.6mg/ml**	
	%Mean	%SD	%Mean	%SD
K10-2	54	4	31	19
V24-M	48	3	58	4
1M	61	13	62	14
44M	77	16	76	14
VM-10	48	4	69	21
12F-K	27	0	47	18
V14-M	55	3	60	18

Appendix IX

Percentage mortality of 1st instar larvae of *B. fusca* exposed to 8.6 mg/ml of delta-endotoxin of different *B. t* isolates by the artificial diet bioassay method

B.t Isolates	Cumulative % larval mortality with time					
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**	**144 hrs**
44M	0	13	20	29	31	36
V14-M	0	11	11	16	18	27
VM-10	4	9	13	13	22	22
12F-K	0	3	3	3	8	8
K10-2	4	7	9	9	11	13
V24-M	0	0	2	9	13	27
1M	0	11	18	27	21	32

APPENDIX X

List of reagents and suppliers

Reagent	Suppliers
Gel filtration molecular weight standards	SIGMA chemicals co., USA
Glutaraldehyde	SIGMA chemicals co., USA
Coomassie brilliant blue	SIGMA chemicals co., USA
Nutrient Broth (CM1)	OXOID Ltd., ENGLAND
Nutrient Agar (CM3)	OXOID Ltd., ENGLAND
Glucose	AnalaR
Tris	Genpark Ltd.,UK