STUDIES ON INDIGENOUS BACILLUS THURINGIENSIS ISOLATES ACTIVE AGAINST SELECTED INSECT PESTS
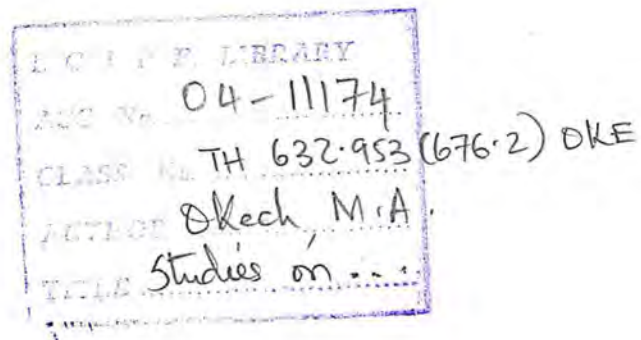
BY

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Declaration

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

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Dedication

This work has been dedicated to my father, the late
Romlus Omolo Orego

ACKNOWLEDGEMENTS

ABSTRACT

The overall goal was to contribute to crop-productivity by developing sustainable pest management and the objective was to select and identify an efficacious local *B. thuringiensis* isolate active against the selected stem borers, *C. partellus*, *E. saccharina*, *B. fusca* and *S. calamistis*.

Various isolates of *B. thuringiensis* were isolated from soils and dead insects collected from different places in Kenya. They were screened against various stem borers including *C. partellus*, *E. saccharina*, *S. calamistis* and *B. fusca*. Based on the relative potencies, seven isolates (ICIPE 001, 012, 023, 027, 040, 054 and 061 were screened further and their LT_{50} value determined. Three isolates, ICIPE 012, ICIPE 023 and ICIPE 054 were selected from the seven isolates shown above. These three, had LT_{50} values ranging from 1.40 to 1.59 days, tested against *C. partellus* and were chosen as being most active. The LC_{50} values of these three isolates were from 0.32 to 1.6 x 10^7 spores/ml on *C. partellus*, from 0.09 to 0.15 x 10^7 spores/ml on *E. saccharina* and from 0.4 to 1.8 x 10^7 spores/ml on *S. calamistis*. By the value LC_{50}, isolate ICIPE 023 was then selected for further studies (0.32 X 10^7 spores/ml).

These three isolates were serologically identified as being, *B. thuringiensis var. kurstaki*. To establish differences among these three isolates, biochemical test

using API analytical profile was carried out. The results showed differences in their reaction to citrate utilization, urea production and production of arginine dihydrolase. Only one of the isolates, ICIPE 023, was found to be different from the others in terms of composition of the cell wall proteins.

From the LC_{50} values of the three isolates, ICIPE 023 was found to be more active against C. partellus larvae than ICIPE 012 and ICIPE 054. This isolate was selected for all the subsequent experiments.

Using local raw materials, eight different media were formulated and tested for their support for growth, sporulation and δ-endotoxin production of the selected B. thuringiensis isolate, ICIPE 023. This isolate grew best in one medium composed of cow-dung (3%) and soya (3 against %) giving LC_{50} value of 0.042 x 10^8 spores/ml, when the first instar of C. partellus larvae were tested. Optimization of growth conditions for this isolate yielded the most potent toxin when cultured in 50 ml volume (in 250 ml Erlenmeyer flasks) and an agitation speed of 300 rpm.

Several UV-protectants namely, Congo red, clay soil and molasses were mixed · with B. thuringiensis isolate ICIPE 023 grown in the cow-dung/soya medium separately and exposed to sunlight. These were tested at intervals against C. partellus larvae. Results showed that isolate ICIPE 023 grown in the cow-

dung/soya medium exposed to the sunlight without UV-protectant retained activity of approximately 60% mortality after 120 hours exposure to natural sunlight.

Field trials were conducted to determine the effectiveness of isolate ICIPE 023 culture against *C. partellus*. Results showed no damage to the maize plants sprayed with isolate ICIPE 023 culture- broth but the infested non-*B. thuringiensis* -treated maize plants showed plant damage as evidenced by stem tunneling of approximately 29%. *B. thuringiensis* ICIPE 023 culture grown in the medium of cow dung/soya had a shelf-life of approximately one year and gave mortality of approximately 27% using a concentration of 0.025 ml/ ml of the insect diet after one year of storage at room temperature (25°C). Samples stored in the cold room (4°C) gave 63.3% mortality against *C. partellus* after one year. This showed that there was very little loss of activity when kept in the cold room.

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CHAPTER ONE

1. INTRODUCTION

The tropical countries have a great diversity of insect life (Odhiambo, 1973). Many of these species, especially the insect pests, are a major constraint to agricultural production in those countries (Odhiambo, 1973). Some of these pests belong to crop-borer complex and include *Chilo partellus* Swinhoe (the spotted stalk-borer), *Busseola fusca* Fuller (the maize stalk-borer), *Sesamia calamistis* Hampson (the pink stalk-borer) and *Eldana saccharina* Walker (the sugarcane stalk-borer). These borers are some of the major pests that infest cereal crops in Africa, especially maize (*Zea mays*, L.) and sorghum (*Sorghum bicolor*, L. (Moech)), which are the staple food for most people in this region.

Larvae of these insects cause damage by feeding on the leaf whorls of plants and by boring inside the stem resulting in "dead hearts" (Seshu Reddy, 1983). The severity and nature of stem borer damage depends upon the borer species, plant growth stage, the number of larvae feeding on the plant and the plants reaction to borer feeding. The stem borers attack almost all plant parts including leaves, stems, tassels and ears. According to one estimate, the crop borers alone are responsible for crop yield losses of between 18 to 88% on unprotected crops (Seshu Reddy *et al.*, 1990). These losses indicate the importance of stem borers as a limiting factor affecting crop productivity.

1. 1. Distribution and damage caused by stem-borers

1.1.1 *Chilo partellus* (Swinhoe) (spotted stem borer)

Chilo partellus belongs to the family Pyralidae and is one of the most widespread sorghum, maize, finger millet, sugar-cane and rice pests in the hot and humid lowland areas of Eastern Africa and Southern Asia (Ampofo, 1987). *C. partellus* is not an indigenous species but was accidentally introduced from Asia approximately 60 years ago and has become widely established in Eastern and Southern Africa (Appert, 1970; Girling, 1978). The larvae of these pests penetrate the major veins of older leaves, then bore the stem where they complete development.

Chilo partellus damage host plants by foliar damage occurring when the whorl-stage plants are attacked and when larvae attack the growing tip of the plant, the characteristic "dead-heart" condition occurs. Such damage usually results in total plant loss. Severe foliar damage may also result in the death of the plant. Older (third to sixth instar) larvae generally cause stem tunnelling, a second type of damage. *C. partellus* bore into the stem and eat their way destroying the central pith and conductive tissues, causing reduction in nutrient uptake. This also weakens the plant growth and results in wind susceptibility and poor yield (Ajala *et al.*, 1994)

1.1.2 *Eldana saccharina* (Walker) (African sugarcane borer)

Eldana saccharina belongs to the family Pyralidae, and was first observed in Tanzania in 1959 and in Uganda in 1967. Since then, it has not only become an important pest of maize (Girling, 1978), but it also attacks sorghum, and other

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cereal crops. It is well known as a stem-borer of sugarcane in West and S. Africa (Atkinson, 1980). The young larvae feed on epidermal tissues between the sheath and stem. During the second or third instar, they bore tunnels to penetrate the stem close to a node. The larvae attack older plants and the main effects are extensive stem tunnelling and consequent breakage. On maize, larva may feed directly on the developing grain in the cobs, where it becomes a serious pest.

1.1.3   *Sesamia calamistis* (Hampson) (the pink stalk borer)

Sesamia calamistis belongs to the family Noctuidae and is widespread in tropical and Southern Africa (Tams and Bowden, 1953). It attacks rice, maize, sorghum and sugar cane (Nye, 1960). In East Africa, *S. calamistis* is mostly found in maize and sorghum growing regions (Warui and Kuria, 1983)

1.1.4   *Busseola fusca* (the maize stalk borer)

Busseola fusca belongs to the family Noctuidae and is a major pest throughout the maize and sorghum growing areas of Africa (Seshu Reddy, 1985). In Kenya, this pest is dominant at higher and cooler altitudes (114 0-2500m) (Seshu Reddy, 1983)

The larval stages of *B. fusca* attack plants when they are three to five weeks old (between 26-45 cm tall) (Barrow, 1989) causing extensive foliar damage leading to production of many holes ("windows") on the leaves of infested plants. They penetrate into the stem by boring through the whorl base into the stem destroying it finally leading to the death of the shoot, a condition referred to as

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the "dead-heart" effect (Bowlen, 1973)

1.2   Management of cereal stem-borers

A number of methods have been developed for insect pest management programmes. For example use of chemical insecticides, biological control, and cultural measures - such as early planting, disposal of plant residue by burning, or burial by deep ploughing during off-season, removal and destruction of alternate host plants, and crop rotation. Among these methods, synthetic chemical insecticides have been used in large quantities (Knusli, 1978) and for a relatively long time.

The four major classes of chemical insecticides commonly used include organochlorines, organophosphates, carbamates, and pyrethroids. Use of these chemical insecticides has major drawbacks because of their wide spectrum of toxicity even to non-target insects. Their persistence in the field has lead to rapid development of resistance (Beroza, 1970), thereby reducing the number of pesticides that can effectively be used.

The backlash to chemical use was highlighted in Rachel Carson's book in 1962. She showed the dangers of organochlorines such as DDT and the impact that these type of chemicals were having on the environment in general. The outcry over the effect of organochlorines, highlighted in Carson's book, resulted in government, scientists and farmers looking for alternative methods of pest control, and stricter criteria on the use of chemicals for pest control.

With an increased growing concern (Brent, 1987) that chemicals were being misused or overused in the control of pests, scientists increased their efforts to look for alternative pest management strategies that are selective and environmentally safe and for which resistance is not developed too quickly. One such strategy involves the use of naturally occurring organisms as biological agents. Examples of such agents include microorganisms such as bacteria, fungi, viruses, and microsporidia. These entomopathogens have been studied with a view towards their incorporation into pest control programmes.

Among the bacteria, a number of *Bacillus thuringiensis* strains are currently being evaluated for use as biological control agents. The first commercial preparation was tested in France during the late 1930's and in the USA in 1958 (Motoyama, 1989). Nowadays *B. thuringiensis* is the principal biopesticide produced and used world-wide and commercial products based on this bacterium make up over 90% of all biological products used in plant protection (Vega, 1999).

1.3   Rationale for the Study

Since *B. thuringiensis* was first recognised by the Japanese biologist Ishiwata in 1902 as the causative agent of the sotto disease of silkworms (*Bombyx mori*), many such isolates have been described all over the world. The majority of these organisms have toxins, which are active against specific lepidopterous insects, although other strains are activity against Diptera (Goldberg and Margalit, 1977), Coleopteran, (Hofte and Whiteley, 1989) and parasitic nematodes (Edwards *et al.*,

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1988) have also been reported. Although the early *B. thuringiensis* isolates were pathogenic to insects, it is apparent that several *B. thuringiensis* isolates have no known target (Hansen, et al., 1996). This is probably a function of incomplete host range testing, rather than lack of biological activity *per se*. The list of *B. thuringiensis* targets is still increasing. Although knowledge of the activity of *B. thuringiensis* population in the environment is limited, a certain level of turn-over and vegetative growth must occur, as annual and seasonal variations in numbers and subspecies diversity of *B. thuringiensis* populations have been observed (Kim, et .al., 1998). There is therefore need for continued host range testing of the existing isolates as well as new strains which may have enhanced efficacy against the pest species in the country of isolation. The screening could also lead to the discovery of *B. thuringiensis* strain(s) with novel entomocidal specificities which may be the source of novel d-endotoxin genes potentially suitable for expression in transgenic plants

Strains may also be found which show better fermentation or storage characteristics enhancing their potential for widespread use. Furthermore, the existence of pest complexes necessitates the continued search for strains active against different insects.

Indigenous strains are important as there are restrictions on the introduction of exotic micro-organisms for pest control purposes (Brownbridge, 1989). The regulatory agency considers the benefits and the risks of using an introduced control agent. An exotic control agent introduced into an ecological system may

establish itself and become a serious and impossible organism to eradicate. This risk is much less with indigenous species that are merely cultured and released for the control of target pest at a propitious time.

Finally, when considering strain selection for control purposes, it is important to select the potent strains for the particular target pest. Commercially available *B. thuringiensis* products might not necessarily be active against a country's important pests. In most cases they have to be screened in quarantine against the pest before being applied. This is time consuming and the product might end up not being active against pest of interest.

It is desirable that local strains of *B. thuringiensis* become available for possible development of biopesticides for local use in the country. This requires the isolation of strains with high potency, active against indigenous insect pests.

The *B. thuringiensis*-based biopesticide product developed will contribute to a reduction of heavy losses caused by the stem-borers. Further, as a biological control agent, it will contribute to a reduction in the use of chemical insecticides, thus contributing to protecting the environment.

1.4   Objectives

1.4.1   General objective

The overall objective was to obtain an active *B. thuringiensis* isolate against *C. partellus, E. saccharina, S. calamistis* and *B. fusca.*

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1.4.2 Specific objectives

1. To screen and select efficacious *B. thuringiensis* isolate(s) or those with the desired host range against *C. partellus, E. saccharina S. calamistis* and *B. fusca*.

2. To characterize the active *B. thuringiensis* isolates using pathogenicity spectrum, serological identification and crystal protein pattern.

3. To optimize growth conditions of the selected *B. thuringiensis* isolate(s) using medium made from the local available raw materials.

4. To formulate the selected *B. thuringiensis* isolate and determine its shelf-life.

5. To evaluate the selected *B. thuringiensis* isolate(s) against *C. partellus* in the experimental screen house.

1.5. Hypothesis

The hypothesis of the project is that " A *Bacillus thuringiensis* isolate that is highly pathogenic to the major maize stem borers, (*C. partellus, E. saccharina, S. calamistis* and *B. fusca*) exists in soil samples obtained at various locations in Kenya".

CHAPTER TWO

2. LITERATURE REVIEW

2.1. General properties of *B. thuringiensis*

Bacillus thuringiensis is a gram-positive, rod-shaped, aerobic, motile, spore-forming, soil bacterium, very similar to *Bacillus cereus* Franklin and Franklin (Carlson and Kolsto, 1994; Carlson *et al.*, 1996). This bacterium was first isolated by Ishiwata in 1902 from a diseased silkworms (*Bombyx mori*) and given the name *Sotto bacillus* and subsequently named by Berliner as *B. thuringiensis* in 1915 (Angus, 1971).

The bacterium is about 1.0 to 1.2 μm wide and 3 to 5 μm long (Bai *et al.*, 1993). During sporulation, it synthesises a cytoplasmic inclusion containing one or more proteins that are toxic to insect larvae (Gibson and Gordon, 1974, Burges ed., 1981, Ellar, 1999). These are deposited as crystalline inclusions and are referred to as δ-endotoxin or insecticidal crystal protein (ICP). The presence of δ-endotoxin in the cells differentiates *B. thuringiensis* from *B.cereus* (Buchanan and Gibsons, 1974). It is also interesting to note the recent finding by Kostichka *et al.*, (1996) of soluble δ-endotoxin in some strains of *B. thuringiensis*. Such strains would be missed out or identified as *B. cereus* because of lack of δ-endotoxin in the cell. When the bacterium completes sporulation, the parent bacterium lyses, releasing the spore and the inclusion.

The toxins exist as inactive protoxins but when ingested by susceptible insect larvae, they dissolve in the midgut and the protoxin is converted to the active form by gut proteases (Ellar, 1999). Aronson *et al.*,(1995) showed that solubilization of the crystal by the gut protease is a key step in the overall toxicity and that it is highly dependent on protein contents and protoxin sequence; and the presence or accessibility of specific toxin receptors in the insect gut (Knowles *et al.*, 1986). High pH is also required for solubilization of the crystal (Monnerat *et al.*, 1999), except for *CryIII* toxin active against coleopteran larvae. *CryIII* toxin lacks the C-terminal segment found in other *Cry* proteins. The lack of C-terminal segments causes the *CryIII*-based crystals to be soluble in the acidic guts of the coleopteran larvae (Agaisse and Lereclus, 1995).

Since *B. thuringiensis* was first recognised many new strains producing δ-endotoxin active against representatives from the orders Coleopteran (Hofte and Whiteley, 1989), Diptera (Goldberg and Margalit, 1977) Hymenoptera, Arachnida, Acari and Nematoda (Edwards et al., 1988) have been isolated (Ellar, 1999). Majority of the early *B.thuringiensis* isolates were active against specific lepidopterous insects (Hofte and Whiteley, 1989).

In addition to the δ-endotoxin, *B. thuringiensis* produces several other cellular products. These include a secreted protein (α-exotoxin) with activity against both lepidoptera and mice (Krieg, 1971) and the heat stable β-exotoxin. β-exotoxin is an adenine nucleotide analogue secreted during vegetative growth by certain

strains, which can substitute for ATP in cellular reactions. For this reason the activity of the β-exotoxin is not restricted to insects and consequently the use of β-exotoxin producing strains in commercial preparation is prohibited.

The most important toxin produced by these organisms is δ-endotoxin, a proteinacous insecticidal crystal. The δ-endotoxin activity against insect pests, forms the basis of many *B. thuringiensis*-based biopesticides, employed worldwide to control lepidopteran pests (Hill and Pinnock, 1998, Cross *et al.*, 1999) and has also made the organism an important industrial microbe (Sudha *et al.*, 1999). Worldwide use of *B. thuringiensis* was established by the early 1980s (Van Frankenhuyzen, 1993). The majority of research and development have focused on use of the *B. thuringiensis var. kurstaki* (HD-1) for the control of lepidopterous larvae, against which the isolate is primarily active (Luthy *et al.*, 1982, Cross *et al.*, 1999). However, currently there are a number of commercial products based on different strains. These are presented in Table 1.

Table 1. Examples of some commercial *B. thuringiensis based* products.

Product name	Manufacturer	*B. thuringiensis* strain
Dipel	Abbott	*B. thuringiensis* HD1
Thuricide	Sandoz	*B. thuringiensis* HD1
Vectorbac	Abbott	*B. thuringiensis israelensis*
Bactimos	Novo	*B. thuringiensis israelensis*
Trident	Sandoz	*B. thuringiensis tenebranosis*
Novodor	Novo	*B. thuringiensis tenebranosis*

2.2 Classification and Identification of *B. thuringiensis*

According to *de* Barjac and Frachon (1990), *B. thuringiensis* is a *B. cereus* with the capacity to synthesis a protein "crystal" which enables the bacterium to kill larvae of various insects. As *B. thuringiensis* became commercially important, and numerous different isolates with different pathogenicity patterns were discovered and the need for a reliable mode of classification became apparent. The first effort was made by Heimpel and Angus, (1958), and it was based on morphological and biochemical characteristics while Krywienczyk and Angus (1960) used crystal serology. *de* Barjac (1981) developed identification and classification scheme based on serological analysis of vegetative cell, flagella antigen (H-antigen) plus biochemical characteristics. Norris (1964) based his identification on analysis of esterase patterns of vegetative cells by starch gel electrophoresis. Other workers (Ohba and Aizawa, 1978) used *B. thuringiensis* heat-stable somatic O-antigens; De

Lucca (1984) used lectins. Other identification and classification schemes such as high-performance liquid chromatography (HPLC); plasmid and DNA sequencing of crystal toxin genes (Kronstad and Whiteley, 1986) were tried.

Identification using crystal protein genes was tested using specific DNA probes (Visser, 1989). The DNA probes provided new powerful tools for comparing *B. thuringiensis* strains and their relative pathogenicity. But the coexistence in the same strain of several types of δ-endotoxin genes (Sanchis *et al.*, 1988) prevents the use of these characters as a basis for a general classification.

The most recent development has been the identification and sequencing of the genes that code for the crystal toxins (Hofte and Whiteley, 1989). They proposed a nomenclature and classification scheme for crystal genes (*cry*) based on their phenotype, types of crystal produced, and the host range as insecticidal toxins. In this system the genetic nomenclature relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes (Hofte and Whiteley, 1989). According to their classification, the *cryI* genes encoded proteins toxic to lepidopterans; *cryII* genes encode proteins toxic to lepidopterans and dipterans; *cryIII* genes encoded proteins toxic to coleopterans; and *cryIV* genes encoded proteins toxic to dipterans alone; and *cryVI* active against nematodes (Feitelson *et al.* 1992). This classification (Hofte and Whiteley, 1989) could not accommodate genes that were highly homologous to known genes but did not encode a toxin with a similar insecticidal spectrum.

B. thuringiensis pesticidal crystal protein (*Cry* and *cyt*) nomenclature was initially based on insecticidal activity (Hofte and Whitelely, 1989). Many exceptions to this systematic become apparent with the rapid discovery of proteins with new pesticidal properties, making the nomenclature system inconsistent. Crickmore *et al.* (1998) proposed a new nomenclature, based on amino acid sequence identity. Roman numerical was exchanged for Arabic numerical in the primary rank (e.g. *Cry*1Aa) to better accommodate the large number of expected new sequences. The symbol *cyt* designates crystal proteins showing a general cytolytic activity; *Cry* protein is a parasporal inclusion (crystal) proteins of *B. thuringiensis* that exhibits some toxic effect to a target organism.

The system by de Barjac (1981) is used most for identification of new isolates. It has been recognised and it is the most frequently used system, although not very easy for small local laboratories, to carry out serotyping as one requires a large number of reference *B. thuringiensis* sample. It is important for small *B. thuringiensis* laboratories to find other simpler methods by which new isolates can be differentiated from each other or into groups.

2.3.   *Bacillus thuringiensis* δ-endotoxin and its mode of action

Hannay and Fitz-James (1955) showed that δ-endotoxin was a protein in nature, and studies by Pfannenstiel *et al.* (1987) indicated that the δ-endotoxin is a glycoprotein, containing 1.7% amino sugars and 1% neutral sugars. δ-endotoxin is

a water-soluble protoxin which is solubilised in the alkaline conditions of the larval gut, with an exception of *Cry111* toxin (Agaisse and Lereclus, 1995), and activated by gut proteases (Knowles *et al.* 1984)

The δ-endotoxin is synthesised within the sporangium as a protoxin with a molecular weight between 70 KD and 135 KD (Bulla *et al.* 1980). It becomes a toxin when it is proteolytically degraded in the susceptible insect midgut. *Bacillus thuringiensis* produces large variety of proteolytic enzymes, especially during the stationary phase of growth (Agaisse and Lereclus, 1995).

The δ-endotoxin acts as an insect gut poison and is the major active component of commercial products (Burges and Pillai, 1987). According to de la Riva and Adang (1996), the mode of action of δ-endotoxin is still a matter of research, but in general when the toxin is ingested by a susceptible insect, the toxins are proteolytically converted into smaller toxic polypeptides in the midgut (Hofte and Whiteley, 1989). The toxins interact with cells of the insect midgut epithelium via high affinity binding sites, disrupting the cell membrane integrity. This then induces formation of small specific pores in the susceptible insect gut membrane cells resulting in the influx of ions and water thus leading to swelling and lysis of the cells, causing spilling of their cytoplasmic contents into the lumen (Heimpel and Angus, 1959; Osir and Vundla, 1997).

At a low dose of the toxin, spores germinate in the intestinal tract, causing

septicaemia and insect finally dies (Heimpel and Angus, 1959; Hofte and Whiteley, 1989; Knowles and Dow, 1993). The generalised mechanism of action of the δ-endotoxin is illustrated in Figure 1.

Figure 1 was modified from:
http://www.nysaes.cornell.edu/ent/biocontrol/pathogens/bacteria.html

Fig. 1. Mode of action of δ-endotoxin of *B. thuringiensis* in the midgut of a susceptible insect larvae

Bt crystaline toxin

Bt spore

Susceptible insect

High pH + enzymes

Bacteria invade body and multiply

Protoxin crystal — Protoxin activated — Toxin released — Receptor on epithelial cell membrane — Gut cell functions altered

Spore — Spores germinate — Bacteria — Gut wall perforated

Larva stops feeding

Larva dies
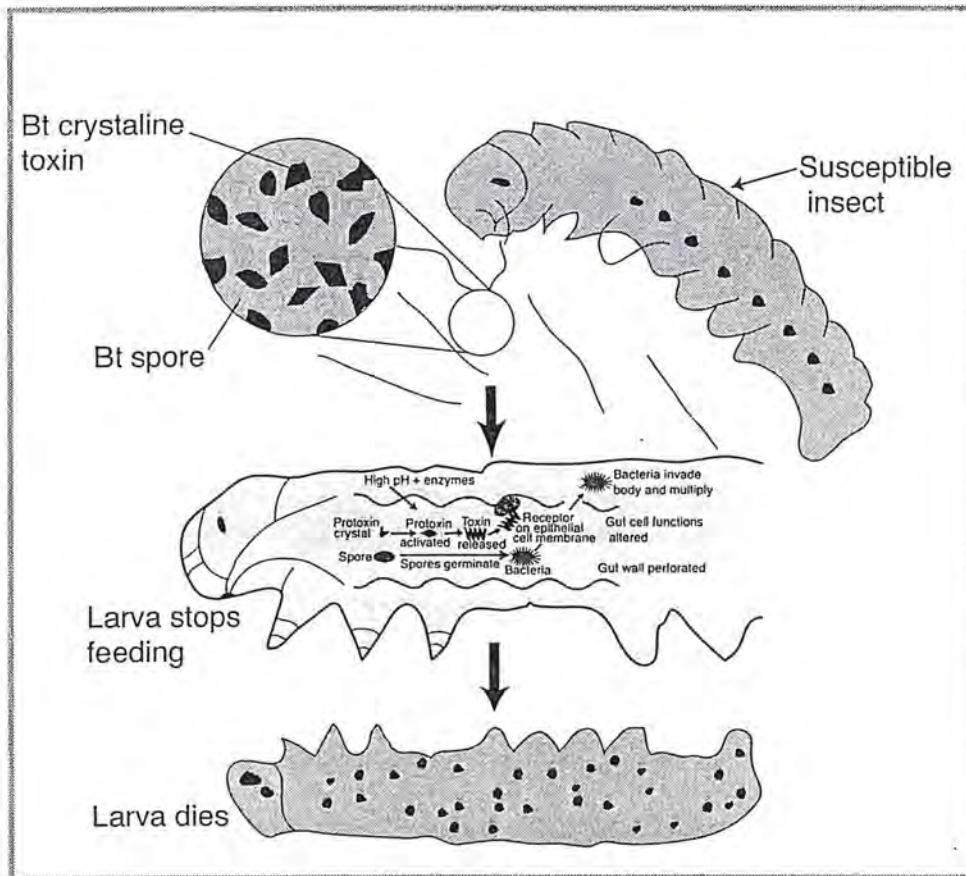
Most δ-endotoxin are active against larvae of lepidopteran, dipteran, and coleopteran species (Hofte and Whiteley, 1989). The discovery of new strains toxic to protozoan pathogens, animal-parasitic liver flukes, or mites (Feitelson *et al.*, 1992) has further broadened the potential uses of *B. thuringiensis*-based products

Although the bacterium usually produces one crystal, bipyramidal in shape in each cell, some strains such as *B. thuringiensis var. israelensis* produce irregular shaped crystals (Luthy *et al.*, 1982). Crystal toxins of all strains reduce or stop larval feeding, but the speed at which the toxin kills larvae varies greatly according to the dosage and bacterial strain (Lecadet and Martouret, 1987; van Frankenhyzen *et al.*, 1992; Tamez-guerra *et al.*, 1996). Another factor causing these differences is the fact that many strains produce several crystal proteins simultaneously and same or similar crystal proteins occur in some strains of different sub-species (Krywienzeyh, 1977). The toxicity spectrum of individual proteins is difficult to determine as many strains produce more than one crystal (Lecadet and Martouret, 1987).

Other factors influencing specificity are, first, differences in the quantity and quality of the δ-endotoxin produced by different strains ((McGaughey and Whalon, 1992). Second, differences in toxin level interacting with high affinity binding sites on insect midgut epithelium (Jacquet *et al.*, 1987; Hofmann *et al.*, 1988; Aronson *et al.*,, 1995). This is a key determinant of the host spectrum exhibited by any δ-endotoxin. Third, differences shown by strains in their activity and also in the

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relative potency.

It is also interesting to note that specificity can also differ in the same species of *B. thuringiensis*. For example, *B. thuringiensis var. tenebrionis* produces δ-endotoxin with different molecular weights during different phases of sporulation (Deml *et al.*, 1999). In an early stage, only a protein of about 73kD is synthesised whose concentration steadily declines during further sporulation, in favour of a polypeptide of about 67kD which is converted from the 73kD protein by a spore-associated proteinase.

2.4   *Bacullus thuringiensis* habitats

The normal habitat of *B. thuringiensis* is the soil (Dulmage and Aizawa, 1982, Smith and Couche, 1991, Ohba and Aizawa, 1986a,b; Martin and Travers, 1989; Chilcott and Wigley, 1993; Hossain *et al.*, 1997. The bacterium has also been isolated from leaf surfaces of plants (Smith and Couche, 1991) and stored products (Bernhard *et al.*, 1997). Ohba and Aizawa (1989) recovered *B. thuringiensis* from sericulture. According to Smith and Couche (1991) *B. thuringiensis* occurs naturally on the phylloplane.

2.5   Fermentation

The type of medium and growth condition used during fermentation directly affects bacterial growth, sporulation and δ-endotoxin production. Important conditions include temperature, aeration, pH and agitation. *B. thuringiensis* grows well at

temperatures of about 30^0C and pH of about 7.2

Most microorganisms require carbon and nitrogen for their growth and development. Dulmage (1981) showed that a balance of carbon and nitrogen levels is essential to prevent severe pH fluctuations during vegetative growth, which may inhibit the process of fermentation. Glucose and Yeast extract (carbon and nitrogen source respectively) used in commercial fermentation, are very expensive, making them unaffordable for third World large scale production. Several less expensive carbon and nitrogen sources, based on locally available substrates, have been tried.

In developing countries, for example in Nigeria, media containing cow blood or legumes such as groundnuts, cow pea, soya beans, or bambara beans in basal medium have been used with good results (Obeta and Okafor, 1984). Wheat bran, corn meal, soybean, defatted cottonseed cake and peanut bran have been used in China, which is one of the highest producers of *B. thuringiensis* bioinsecticides (Salama and Morris, 1993). In Egypt, fodder yeast and molasses are used for *B. thuringiensis* production (Salama and Morris, 1993). Other agro-industrial residues and by-products used in *B. thuringiensis* production includes starch, casein, cotton and soya bean seed meals, corn steep liquor, cheese whey, dried residues from chicken slaughter houses and coconut water (Salama, *et al.*, 1983a).

Foda *et al.*, (1993) recommended that developing countries should produce

bacteria for microbial control locally using available residues and by-products. This recommendation was due to the fact that such materials would lower the cost of production.

2.6 Formulation

The basic requirement for the successful use of *B. thuringiensis* as a biopesticide, is the development of effective formulations suited to the biology and habitat of the target pests. Factors influencing the use and performance of *B. thuringiensis* are the lack of residual activity on foliage due to degradation of the δ-endotoxin by sunlight (Ignoffo *et al.*, 1977; Morris, 1983, McKemy, 1990; Moore & Morley-Davies 1994), and washed off by rainfall (Sundaram *et al.*, 1993).

B. thuringiensis products are inadequately stable under field conditions and rapidly lose their biological activity (Pinnock *et al.*, 1974; Leong *et al.*, 1980). Natural sunlight, especially the UV portion of the spectrum (280 - 400 nm) has been shown to be responsible for inactivation of insect pathogens (Ignoffo *et al.*, 1977; Ignoffo *et al.*, 1981).

Whether spores and/or crystals are affected equally by sun's rays is unclear as, most researchers report that the main effect of UV is on spore while others associate it also with crystal toxin. Burges *et al.* (1976) reported that there is no effect of UV on crystal while Sneh *et al.* (1981) considered it to inactivate crystal toxin. The effectiveness of UV-absorbing materials to improve the efficacy of *B.thuringiensis*

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δ-endotoxin was investigated by Ignoffo and Garcia, (1978) who found that peroxides produced by UV-irradiation of amino acids are responsible for the sunlight inactivation of the field applied entomopathogens.

The stability of *B. thuringiensis* is also affected by the action of endogenous proteinases of the bacterium, which when adsorbed on the crystal surface perform limited proteolysis. Chestukhina *et al.* (1979) has shown that preparations of the bacterium crystals contain proteinases of several types capable of hydrolyzing crystal proteins. The intensity and extent of the proteolysis strongly depends on the content of the proteinases in the crystal, the conditions used for the protein solubilization, and the stability of the proteinases against the denaturing agents. The work by Alves *et al.* (1997) shows that high pH is optimal for the bacterium proteases and potentially damaging to the crystals. In this case fermentation liquid should be brought to neutral pH at harvest (Chestukhina *et al.*, 1979)

2.7 *Bacillus thuringiensis* research in Kenya

In Kenya, a few institutions are carrying out research on *B. thuringiensis* as a biological control agent. These includes the International Centre of Insect Physiology and Ecology (ICIPE) and the University of Nairobi. ICIPE has isolated over 100 isolates of *B. thuringiensis* from different parts of Kenya and screened them for activity against *C. partellus, E. saccharina, S. calamistis, B. fusca, Glossina* sp. and mosquitoes.

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ICIPE also initiated an investigation on *B. thuringiensis* based on *B. thuringiensis* var. *thuringiensis*, a strain that produces β-exotoxin. This research was funded by the Finnish International Development Agency (FINNIDA). The project successfully optimized the growth (agitation speed, aeration and pH) conditions for the production of β-exotoxin and carried out feasibility studies on the utilization of the product, which was then registered as DUDUstop, for control of flies (Musca species) in pit latrines in slum areas and refuge camps in Kenya.

The product, DUDUstop, was successful as it controlled the population of the filthflies in the test areas within two weeks of application. A number of refugee camps applied to get the product for continued use, but unfortunately, production within the country was limiting.

University of Nairobi, Department of Biochemistry started similar work on isolation of indigenous *B. thuringiensis* two years ago. They concentrated their isolation on soils from Kakamega and Machakos area. Their isolates are active against stem borers (*C. partellus, B. fusca, S. calamistis* and *Maruca testuralis*). They have developed some of the active isolates, fermenting using shake-flask and are testing these active isolates in farmers fields in Kakamega

Several commercial *B. thuringiensis*-based biopesticides are imported into Kenya. These includes Dipel (*B. thuringiensis var. kurstaki*, Abbot Labs),

Thuricide (*B. thuringiensis var. kurstaki*, Novartis AG), Xentari (*B. thuringiensis var. aizawai*, Abbot Labs), and Delfin (*B. thuringiensis var. kurstaki*, Novartis AG). The listed *B. thuringinsis*-based products are imported and distributed by Safina, Novartis East Africa and Bayer East Africa.

B. thuringiensis- based products, Dipel and Thuricide are used in the control of vegetable pests and coffee giant looper (*Ascotis seleraria*). It is interesting to note that these commercial *B. thuringiensis* based products have not been used in the control of cereal stem borers in Kenya, even though stem borers infesting cereals are quite susceptible to *B. thuringiensis*. Registration of *B. thuringiensis* products trials were once initiated at Kenya Seed Company. farms in Kitale but nothing further is on record.

It is very important to recognize that these imported *B. thuringiensis* -based biopesticides are expensive and cannot be afforded by the small- scale farmers therefore there is a great need for local isolates to be developed, using local raw materials for the local farmers.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1   Isolation of *B. thuringiensis*

3.1.1   Sample collection

Soil samples were collected from different districts within Kenya, mainly from maize and sorghum growing areas. These areas included Migori, Homa-Bay, Kisumu, Siaya, Kajiado, Mombasa, Mwingi , Nairobi and Suba district (fig. 2). The selected collection sites had no known history of *B. thuringiensis* treatment. A total of 100 samples from 30 different localities were randomly collected from the surface to about 10 cm below using a sterile spoon. These were stored in small sterile bags and transferred to the laboratory where they were kept at room temperature. Dead insects were also collected from the same sites.

Fig.2 . Map of Kenya showing districts where samples for isolation
of *B. thuringiensis* were collected

3.1.2. Media for isolation of *B. thuringiensis*

Commercial Nutrient Agar (Oxoid Ltd, Wade Road, Basingstoke, Hampshire, RG 24 OPW), composed of peptic digest of animal tissue (5g), Sodium chloride (5g), beef extract (1.5g), yeast extract (1.5g) and agar (15g) mixed in 1000 litre of distilled water was used in the isolation of *B. thuringiensis*. This was prepared according to manufacturer's instruction, autoclaved and then poured into sterile Petri dishes.

3.1.3. Isolation of *B. thuringiensis* from soil samples

Soil samples, (1g) were suspended in 10 ml sterile water contained in 50 ml shake-flasks. The flasks were placed in a shaker-incubator (30°C, 200 rpm) for one hour. The mixture was allowed to settle for 10 minutes. Aliquots of 0.5ml of the supernatant from each flask were diluted with 4.5 ml sterile saline, to attain a ten-fold dilution. The diluted and the remaining stock were heat-shocked at 80°C for 10 minutes to remove the vegetative, non-spore-forming cells. Loop-full of the heat-shocked solutions were streaked on two nutrient agar plates and incubated at 30°C for 3 days.

The resulting colonies, especially those rough and spread out, were sub-cultured on nutrient agar and incubated until sporulation. Isolates were examined under phase contrast microscope and the presence or absence of spore and crystal in the cells confirmed by staining with Smirnoff stain (Smirniff, 1962). The staining was

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carried out as follows: a few drops of solution A (1.5g Amido black dissolved in 50 parts 98% methanol, 40 parts dissolved water and 10 parts acetic acid) was added to the heat fixed slides of *B. thuringiensis* cells and washed off after 70 sec. Thirty percent of solution B (1g of basic Fuchsin dissolved in 10 ml of 95% ethanol and mixed with 5g of phenol dissolved in 90ml of distilled water) was then added to the slide and left for 20 sec. before washing in cold water and drying on filter paper. This procedure detects the presence of both crystal and spores in the culture; crystals appear blue/black while spores appear pale-pink with a clear hilac/red tint. The cells containing crystal inclusion in the sporangium were assumed to be *B. thuringiensis*. These colonies were sub-cultured on nutrient agar in order to get pure cultures and then stored in agar slants for further studies.

3.1.4. Isolation of *B. thuringiensis* from dead insects

Dead insects were first surface sterilised by washing in 0.5% Sodium hypochlorite for 2 minutes, then in sterile distilled water for 5 minutes and finally in 0.85% sterile saline. The insects were then crushed in 0.5 ml sterile saline using a homogenizer. The crushed insects were allowed to settle for 30 minutes and the supernatant solution removed. This solution was heat-shocked and inoculated onto nutrient agar plates in the same way as the soil samples. Colonies were subsequently identified as described in section 3.1.3

3.2.   Bioassays of *B. thuringiensis* isolates against insect pests

The tested isolates were given ICIPE code numbers, starting with the new isolates.

Fifteen new *B. thuringiensis* isolates together with 86 isolates already existing in

the ICIPE germplasm were screened for their activity against *C. partellus*, *E.*

saccharina, *B. fusca* and *S. calamistis* larvae.

3.2.1. Insect larvae

The insect larvae were obtained from the Animal Rearing and Quarantine Research

Unit at ICIPE, Duduville, Nairobi. The initial screening was carried out using third

instar of *C. partellus*, *E. saccharina*, *B. fusca* and *S. calamistis* larvae. These

insects were reared on artificial diet as described by Ochieng *et al.* (1985). The diet

composition is detailed in appendix 1. In the later bioassays, neonates were used

since they were easily available and were of uniform sizes

3.2.2. Preparation of *B. thuringiensis* for bioassay against the insect pests

All isolates were sub-cultured onto nutrient agar media and incubated for 72 hours.

A single colony from each culture plate was sub-cultured onto nutrient agar plates.

The culture plates were incubated for 18 hours then loopful from the growth was

inoculated into nutrient broth (100 ml of the media in 250 Erlenmeyer flask). The

flasks were incubated at 30°C (200 rpm, 72h) in rotary shaker incubator (Orbital

incubator, Lab-Line Instrument inc. Sporulation and lysis of cells were confirmed

by observing the cultures under the compound microscope. Accumulation of crystal protein in the cultures was confirmed microscopically before harvesting the culture. These cultures were then stored in the cold-room until required for toxicity test.

3.2.3. Bioassay procedure for selection of active *B. thuringiensis* isolates

Artificial insect diet was prepared as described by Ochieng *et al.* (1985). The diet was cooled in a water-bath to about $50^{\circ}C$. *B. thuringiensis* culture broth was (400 µl) added to 20 ml of the cooled diet. This was mixed well using a kitchen blender and then poured onto Petri-dishes and left to settle and harden. A total of 101 local isolates were screened against the four insect pests. Ten third instar larvae were placed into the Petri dishes containing the insect diet and *B. thuringiensis* culture. The plates were incubated at $25^{\circ}C$ and observation made after three days. Insect death was determined by unresponsiveness to touch. The isolates with the highest killing activity to the test insect species were selected for further study.

From the screening results, seven isolates with the highest killing activity to the test insects were selected. The following isolates showed the highest total killing, when their activity on each test insects were added together . The selected isolates, ICIPE 001, 012, 023, 027, 040, 054 and 061 are shown in table 4. Further tests were carried out to reduce this number and this was done by determining the time taken for the toxin of each of the selected seven isolate to kill

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50% of the tested insects (Lethal time LT_{50}). Daily mortality was recorded for three days. Using LT_{50} values determined by SAS probit method, three isolates were selected and a further reduction in number was done by determining the LC_{50} values.

3.2.4. Determination of LC_{50} values of the selected isolates

In order to select the most active isolate(s), the LC_{50} values of the isolates showing the lowest LT_{50} were determined. Each isolate to be tested was grown in nutrient broth to sporulation. The total number of spores in the culture was estimated using the pour plate method (Gerhardt eds. *et al.*, 1981). The samples were serially diluted in the artificial insect diet to a final volume of 40 ml, using approximately the following volumes of culture broth: 1, 0.5, 0.25, 0.13, 0.06 and 0.03 ml. One (ml) of protoxin/spore-diet mixtures of the different concentrations were placed in vials using a 100 ml syringe.

Single neonate larvae of *C. partellus* were picked with camel-brush and placed in each vial. Twenty neonate larvae were tested per dilution and each test repeated three times. The vials were arranged on trays and incubated in an incubator at 25°C. Control tests were set in the same way, but without the protoxin/spores mixtures. Mortality of *C. partellus* larvae was recorded after three days.

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3.3. Characterisation of the selected isolates

Three isolates designated as ICIPE 012, ICIPE 023 and ICIPE 054 were selected as the most active against *C. partellus*, *E. saccharina*, *S. calamistis* and *B. fusca*. The isolates were characterised using biochemical and serological techniques.

3.3.1. Biochemical characterisation of the selected isolates

Biochemical differentiation of the three selected isolates was carried out according to Berkeley *et al.* (1984) using API 20 E strips (API system - La Balme les Grottes - 38390 Montalieu Vercieu - France). The API 20 E strip is composed of a plastic strip with 20 cupules containing dehydrated substrates. The substrates include Ortho-Nitro-Phenyl-Galactsid (ONPG), Arginine, lysine, ornithine, Sodium citrate, Sodium thiosulphate, urea, trptophane (TDA), tryptohpane (IND), Sodium pyruvate, Kohn's gelatin, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose. The isolates to be tested were well isolated colonies sub-cultured in nutrient agar and incubated for 18 hours. Four colonies from the young growing cells (18 hour-old) were suspended in 0.85% sterile saline (10 ml). These were mixed thoroughly and then introduced in the ampoules using Pasteur pipette as per the instruction manual. After 24 hours incubation, biochemical reactions were revealed by colour changes (indicators) that were either spontaneous or revealed by addition of reagents

3.3.2. Determination of crystal protein pattern of the three active isolates using SDS-PAGE analysis

A single colony from 18 hour-old culture plate was inoculated in 200 ml nutrient broth in a flask and placed in a shaker-incubator (30° C and agitated, at 200 rpm). The cultures were incubated for 72 hours or until they sporulated). The sporulated cultures were pelleted by centrifugation (11,000 g, 15 min. 4°C) in a sorval GSA rotor (Dupont, Newton, CT, USA) and Sorvall RC-5C centrifuge. The crystals that settled at the bottom of the tube were washed three times with distilled water. Each washing cycle included centrifugation at 11,000 g. The crystals were lyophilized and stored at 4°C for further studies. The resulting crystals were freeze-dried overnight and protein estimations of each sample were carried out by bicinchoninic acid-binding protein analysis using Bovine Serum Albumen (BSA) (Sigma) method, described by Smith *et al.* (1985). The absorbencies were measured at 562 nm using a spectrophotometer (Beckman model).

Sodium Dodecyl Sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using gradient gel (4 - 15%). Freeze dried crystals (1mg/ml) were dissolved in equal volume of SDS-PAGE sample buffer which contained 0.5 M Tris-HCl, 10% glycerol, 0.5% β-mercaptoethanol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8. The samples were boiled for 5 minutes in a water bath and centrifuged for 3 minutes at 13.000 X g in an Eppendorf centrifuge (model) before being loaded on to the gel. The gels were calibrated using molecular weight standards (Pharmacia, low molecular weight standard):

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phosphorylase b, 94 Kd; albumin, 67 Kd; ovalbulmin, 43 Kd; carbonic anhydrate, 30 Kd; trypsin inhibitor, 20 Kd; A-lactalbumin, 14 Kd.

Electrophoresis was carried out at a constant current of 25 mA, at room temperature until the tracking dye reached the bottom of the gel. After electrophoresis, the gels were stained with 0.1% Coomassie blue in a solution containing 25% methanol and 10% acetic acid for 3 h at room temperature. The gels were subsequently destained with several changes of methanol:acetic acid: water (50:9.2:40.8) until the gel background was clear and the protein bands distinct. These gels were then preserved in 7% acetic acid in sealed polythene bags for photography. This procedure was repeated using whole cells (*B. thuringiensis* cells, toxin and spores) from culture plates.

The molecular weight of each fraction was determined from plots of relative migration of the standard band versus log molecular weight.

3.3.3. Serotyping of active isolates

Serotyping is one of the methods used in the identification of new isolates and it uses the immunological principal that antibodies agglutinate antigens that are the same as those used to raise it.

Serotyping of the selected active *B. thuringiensis* isolates (ICIPE 012, ICIPE 023 and ICIPE 054) was carried out by raising antibodies, in laboratory rabbits, to the known already characterized isolates (the known isolates are listed in the appendix

2) Antigens of the unknown isolates were prepared by heat treating the vegetative *B. thuringiensis* cells at 100° C for two hours. Serotyping was then carried out by reacting the antigen of the unknown to the antibody of the known *B. thuringiensis*. Agglutination is formed where an unknown is the same as the known. These tests were carried out by Jean Cabana of Ministre de L'Energie et des Resources service's des Laboratories in Canada (2700, rue Einstein, Sainte-Foy Quebec, G1P 3W8). They have a reference centre for classification of *B. thuringiensis* and have a large number of known *B. thuringiensis* antibodies that can be used for serotyping antigens of new unknown isolates.

3.4. Selection of medium and optimization of growth conditions for isolate ICIPE 023

Locally available, inexpensive materials were analysed with an aim to test their appropriateness as a carrier material for *B. thuringiensis*. The local materials were soya bean powder (purchased from a local market in Nairobi), molasses (from the Muhoroni sugar industry), cow-dung (from ICIPE cow-shades), chicken-droppings (from Ken-chick in Nairobi), "omena" (sadines) (purchased from a local market in Nairobi), blood meal and horn and hoof (from the Kenya Meat Commission Athi River). These were analysed at the Kenya Bureau of Standards (KBS) laboratories. Horn and hoof was not analysed because the amount available was not enough for the analysis.

The analysed raw materials were used to formulate media. Table 2 lists the compositions of 8 media A-H (the numbers indicate percentage, weight by volume) tested for growth, sporulation and production of δ-endotoxin.

Table 2: Composition of various medium combinations tested for growth, sporulation and δ-endotoxin production by *B.thuringiensis* (ICIPE 023)

	Amount percentage weight by volume							
Ingredient	A	B	C	D	E	F	G	H
Soya	3	3	-	3	-	-	-	-
Cowdung	-	3	-	-	3	-	-	-
Chicken droppings	-	-	8	2	3	-	-	-
Molasses	1	-	1	-	-	1	1	1
Phosphate	0.5	-	0.5	--	-	0.5	0.5	0.5
Horn and hoof	-	-	-	-	-	3	-	-
Omena	-	-	-	-	-	-	3	--
Blood meal	-	-	-	-	--	-	-	3

A -H = Medium depending on the composition; numbers stand for percent weight by volume

3.4.1. Procedure for selection of medium for optimisation studies

The test media were prepared by mixing the ingredients as shown in Table 2. A

hundred (100) ml of each medium was made in 250 ml Erlenmeyer flasks. The pH of each medium was adjusted to 7.2, using either NaOH or HCl, then sterilised by autoclaving (121°C, 15 min). The sterile cultures were cooled and then inoculated with 18 hour-old starter culture at 1% (v/v).

The starter cultures were prepared by inoculating 100 ml of sterilised nutrient broth in 250 ml Erlenmeyer flask with a loopful of isolates. The cultures were incubated at 30°C (200 rpm) in a shaker incubator. The culture growth in the different media was monitored daily, by observing under a phase contrast microscope. Monitoring continued until complete lysis of cells was observed. Growth was determined by carrying out insect bioassay to determine the activity of the δ-endotoxin produced. Spore counts were also determined, samples (3 ml) were heated in a water-bath at 80°C for 10 minutes. This heating kills the vegetative cells and liberates the spores. After serial dilution with distilled water, the number of spores was estimated using pour-plate method. Pour plate is carried out by putting one (ml) of the diluent into sterile Petri-dishes and then overlaid with molten (45^{0} C) sterile nutrient agar medium. The diluent and the agar is mixed well by swirling the plate gently for a short time. This is then left to settle, then incubated overnight at 32^{0} C. The resulting colonies indicates the number of spores present in the medium. Spore counts were used as an easily obtained estimate of the concentration of the spore-toxic crystal complex.

The potencies of each fermentation broth (media) were tested against first instar of *C. partellus* larvae. The final dilution was obtained by mixing the following

volumes: 1, 0.25, 0.125 and 0.063 ml of *B. thuringiensis* ICIPE 023 grown in different culture media (Table 2) with insect diet to give a final volume of 40 ml. Thorough mixtures were obtained using kitchen blender. Each concentrate was aliquoted (2ml) in 20 vials. The vials were left to settle before *C. partellus* neonates were introduced in them. The test vials were incubated at 25^0C for three days before mortality was recorded. Mortality data were analysed using a probit analysis and lethal concentration for 50% (LC_{50}) of the tested insect population were determined. Based on the results, one medium was selected for further testing. This medium was selected for its high spore yield, high efficacy and availability of ingredients.

3.4.2 Medium optimisation

Medium B was selected and used in the optimisation studies. The medium was prepared and pH adjusted to 7.2 and then sterilised. It was then inoculated with 1% broth of 18-hour old starter culture.

Different culture conditions were assessed. The effect of aeration was studied by varying the volume of broth (25, 50 and 100 ml) while keeping the volume of the (250 ml) flask constant. The effect of agitation was studied by varying the agitation speed (100, 200,and 300 rpm).

All cultures were incubated at 30^oC. At the end of each fermentation period, total number of spores was determined using the pour plate method. The level of δ-

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endotoxin production was determined using insect bioassay.

3.5   Ultraviolet-protection

Culture of *B. thuringiensis* ICIPE 023 isolate was grown in cow dung- soya

medium to sporulation and its activity determined using first instar *C. partellus*

larvae. The number of spores were determined using the pour-plate method.

The culture broth containing spores and crystals was mixed well with different

UV-protectants at 1% (w/v) and divided in ten samples of three (ml) final

volume. Each sample was set in triplicate. The samples were placed in separate

sterile glass Petri dishes (diameter nine cm) and exposed to the sun for different

periods, 6, 12, 24, 48, 60, 72, 84, 96, 108 and 120 hours. The hours shown

are cumulative values. A control was set in which no UV-protectant was

added. After the exposure, the plates were washed with seven (ml) sterile

saline. Each exposure test was repeated three times. The effect of UV-

protectant on the samples was determined by using insect bioassay.

The insect bioassay was carried out by feeding first instar larvae of *C. partellus*

on *B. thuringiensis* sprayed young maize leaves (two to four week-old plants).

The samples (three ml) were mixed with 1% sucrose and 1% glycerine. Maize

leaves from young maize plants (four week-old) were dipped in the samples

and then placed in vials. The larvae were then introduced into the vials. The

test vials were incubated at room temperature and evaluated after three days by

counting the number of live insects (those individuals showing movement when

touched). By subtraction from the number introduced, mortality was recorded. The dose-response function of the treatment was analysed using Probit analysis.

3.5.1 Evaluation of UV- absorption of a cow-dung/soya broth

To determine the UV absorption spectra of cow-dung/soya mixture, the solution (cow-dung 3%w/v plus soya 3% w/v) was filtered to remove most of the solid particles then scanned for its absorbency from 200 to 500nm using a (Beckman DU-65) spectrophotometer

3.6 Shelf-life of *B. thuringiensis* isolate ICIPE 023 grown in a cow dung /soya medium

Culture of *B. thuringiensis* ICIPE 023 was grown in soya and cow dung media to sporulation. The media was then divided into two sets. One set was stored at room temperature while the other was placed in the cold-room at 4° C. The activity of the culture broth was determined prior to storage using *C. partellus* neonates (first instar). The activities of the stored culture broth, both at room and cold-room temperatures were tested on their activity against *C. partellus* monthly over a period of 12 months.

3.7   Screen-house trials

The test on the ability of *B. thuringiensis* ICIPE 023 to protect maize infested with *C. partellus* was carried out in a screen house at the Duduville campus of ICIPE in Kasarani. The test was done using neonate larvae of *C. partellus*. Maize variety, Katumani, from the Kenya Seed Company, was planted with phosphate fertiliser in two blocks. The screen house was divided into two blocks and each block divided into three plots with 15 plants each. The plants were watered using sprinkler irrigation. At four weeks, plant heights were measured and recorded. The treatments (infested plants; infested and *B. thuringiensis* sprayed plants and control plants not infested) were randomly allocated to the blocks and each block had 45 plants. Thirty plants were randomly allocated to each treatment; each plant therefore served as a replicate. The plants were well tagged to show where treatments were to be applied. The applications were carried out very carefully to avoid contaminating neighbouring plants.

The plants were infested with active newly hatched larvae (first instar) of *C. partellus*, reared on artificial diet (Ochieng *et al.*, 1985). The larvae were placed on the leaf whorl of the plant, 20 larvae per plant and left to settle. After 24 hours, two of the plots per block were sprayed with a suspension of *B. thuringiensis* ICIPE 023 using a hand sprayer. The fermentation broth was mixed with 1% sucrose and 1% glycerol to act as a sticker and a spreader, respectively. The suspension was made up to 3.2×10^8 spores/ml and applied

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with a hand sprayer. The spray was directed into the leaf whorl where the larvae had been placed. The *B. thuringiensis* bioinsecticide treatment was carried out once. There were two controls, one infested and one not infested per block.

Plants were observed weekly for dead hearts formation. On completion of the experiment, plant height and extent of leaf damage were recorded. The plants were then dissected and records made on, number of larvae per plant, number of pupae per plant, and length of stem tunnels were recorded. The number of dead-hearts and dead plants in each plot were also recorded. The data was analysed using probit model (SAS Institute 1990).

CHAPTER FOUR

4. RESULTS

4. 1. Isolation of *B. thuringiensis* from the soils and dead insect cadaver

A total of fifteen *B. thuringiensis* isolates were recovered from the soil and one isolate from insect cadaver (*B. fusca*) from Nairobi area.

Table 3. *B. thuringiensis* (*B.t.*) isolated from different districts in Kenya.

District	Number of isolates	% isolated
Siaya	36	8.8
Homa bay	21	9.5
Kisumu	11	9.09
Suba	6	33.3
Nairobi	15	20
Mwingi	4	25
Migori	7	42.8

The highest percentage (42.8%) of isolates was recovered from Migori while the lowest recovery was from Siaya district. Fifteen insect cadavers were collected and out of these, only one *B. thuringiensis* was isolated from a dead *B. fusca* larva obtained in Nairobi district. The total average recovery of *B. thuringiensis* from the collected soil samples was 15%.

4.2 Bioassay (screening) of *B. thuringiensis* isolates

The distribution of the potency of the isolates against the test insect species is shown in Fig 3. The isolates tested were divided into 5 groups, according to their potency and the number of test insects they kill within three day-exposure. The following were the groupings 0 - 20, 20 - 40, 40 - 60, 60 - 80, 80 - 100%.

The distribution of insecticidal activity among the isolates tested showed that most isolates were more active against *C. partellus* and *E saccharina* (26) at a mortality of 80 - 100% while 81 isolates gave a low mortality of 0 –20% on *B. fusca*. Low mortality was also seen on *S. calamistis* (0 - 20%).

Busseola fusca was dropped as a test insect due to the following problems: firstly, the insectary had a protracted problem on the rearing of *B. fusca* colonies and was not able to supply the insects. Secondly, it was not possible to assess the activity of *B. thuringiensis* as *B. fusca* larvae avoided eating diet in which *B. thuringiensis* toxin had been incorporated. Observations showed that most of the test *B. fusca* larvae did not feed and were recovered above the diet. Recorded death therefore could have been due to starvation since the toxin is only effective when ingested by the target insect.

The seven isolates namely ICIPE 001, 012, 023, 027, 040, 054 and 061 showed the highest means in percentage kill on the tested insects: *E. saccharina, C. partellus and S. calamistis* and were selected as active isolates for further studies. The isolate ICIPE 027 had the highest killing ability against *B. fusca* while ICIPE 007 was high against *S. calmistis* (Table 4).

Table 4. Isolates with high activity on stem borers

Isolate	C. partellus	E. saccharina	B. fusca	S. calmistis	Total activity
ICIPE 047	70	70	0	0	140
ICIPE 003	90	80	10	0	180
ICIPE 012	100	100	0	20	220 ☺
ICIPE 054	90	100	10	50	250 ☺
ICIPE 027	90	100	30	10	230 ☺
ICIPE 029	80	80	10	10	180
ICIPE 018	70	70	0	20	160
ICIPE 042	60	100	10	20	190
ICIPE 017	70	100	20	20	210
ICIPE 007	90	50	0	60	200
ICIPE 011	100	90	0	10	200
ICIPE 015	70	90	0	40	200
ICIPE 052	90	80	0	20	190
ICIPE 023	100	100	10	40	250 ☺
ICIPE 016	90	80	0	40	210
ICIPE 062	100	100	0	30	230 ☺
ICIPE 061	100	100	0	10	210
ICIPE 040	100	90	10	20	220 ☺
ICIPE 045	100	100	0	10	210
ICIPE 001	100	100	10	10	220 ☺

Isolate shown as ☺ were selected for further studies as they showed high total value. The total activity value is obtained by adding all the activity in each raw.

4.2.1 Selection for active isolates

4.2.1.1 **LT_{50} determination**

Larval mortality of *C. partellus* was observed to occur from day one (Table 5)

after they were treated with *B. thuringiensis*. The mortality increased

differently under different treatments (Table 5). However, LT_{50} values (Table

5) differed between the treatment with the different *B. thuringiensis* isolates.

The LT_{50} value of ICIPE 001, ICIPE 012, ICIPE 023, ICIPE 027, ICIPE 040,

ICIPE 054 and ICIPE 061 were 1.92, 1.59, 1.40, 2.12, 2.13, 1.59 and 2.16

respectively. These results show that isolates with lower LT_{50} values had

rapid lethal activity. Using the SAS, probit method, the means of the different

treatments indicate that there are no significant differences between the

isolates, $P. > 0.8$ (Table 5). Since the activity of the seven isolates were very

similar the only criteria that could be used to reduce the number for further

studies was to compare their LT_{50} values. Using the LT_{50} values, the following

isolates were selected, ICIPE 012, ICIPE 023 and ICIPE 054. A further

selection was carried out by comparing the LC_{50} values of the above three

isolates

Table 5: Time taken (from day one to day three) for the selected isolates to kill 50% of *C. partellus* (LT$_{50}$ time is in days.)

Isolates	LT$_{50}$	(95% feducial limits)
ICIPE 001	1.92a	(1.53 - 2.09)
ICIPE 012	1.59a	(1.12 - 3.23)
ICIPE 023	1.40a	(1.32 - 2.98)
ICIPE 027	2.12a	(1.78 - 4.21)
ICIPE 040	2.13a	(1.80 - 4.34)
ICIPE 054	1.59a	(1.12 - 3.23)
ICIPE 061	2.16a	(1.93 - 4.56)

Means with the same letter in the same column are not significantly different P>0.8.

4.2.1.2   Lethal Concentration (LC$_{50}$)

The calculated LC$_{50}$ values for the isolates are presented in Table 6 and expressed in spores per milliliter (colony forming units/ml = cfu/ml).

Table 6. LC$_{50}$ values (95% fiducial limits) of three selected active isolates against *E. saccharina. C. partellus*, and *S. calamistis.* (X 10^7)

Isolates	*C. partellus*	*E. saccharina*	*S. calamistis*
ICIPE 012	0.40 (0.29 - 0.61)a	0.13 (0.10 -0.16)a	0.38 (0.26 - 0.64)a
ICIPE 023	0.32 (0.28 - 0.37)a	0.09 (0.07 - 0.01)b	0.49 (0.33 - 0.94)a
ICIPE 054	1.59 (1.34 - 1.98)b	0.14 (0.09 - 0.20)a	1.86 (1.52 - 2.41)b
HD1	1.08 (0.81 - 1.46)c	0.45 (0.33 - 0.47)c	1.01(0.61 - 1.65)c

Means with the same letter in the same column are not significantly different P>0.05.

Table 6 shows the most toxic isolate was ICIPE 023 with LC50 value of 0.32 X 10^7, 0.09 X 10^7 and 0.38 X 10^7 tested against *C. partellus, E. saccharina* and *S. calamistis* respectively. This isolate was therefore used in the optimization studies and evaluated in the screen-house trials.

4.3 Characterization of the selected active isolates

4.3.1   Biochemical characterization of the selected active isolates

.Standard *B. thuringiensis* var. *kurstaki*, HD1 is the standard organism used in most of the commercial preparation of *B. thuringiensis* -based insect pesticide

products. It had been thought to be the most active isolate and so all new isolates are compared to the HD1 isolate. It was therefore used to determine whether these isolates, ICIPE 012, 023 and 054 had similar biochemical reaction to it. The results could indicate the diversity of *B. thuringiensis var. kurstaki.*

Table 7 shows the results of biochemical tests of the selected three isolates and a standard *B. thuringiensis* var *kurstaki* using biochemical of API identification (20E) kit

Table 7. Results of biochemical reactions of selected active *B. thuringiensis* isolates

Reagents tested	*B.thuringiensis var. kurstaki* (HD1)	ICIPE 012	ICIPE 023	ICIPE 054
Citrate utilization	+	+	Trace	-
Urea production	Trace	+	+	+
Arginine dihydrolase	+	+	_	+

+ = positive reaction - = negative reaction Trace = a slight color change

The biochemical differences (Table 7) among the selected isolates, ICIPE 012, 023 and 054 were seen in the utilization of arginine and citrate. ICIPE 023 showed no ability to utilize arginine whereas ICIPE 054 utilized arginine but did not utilize citrate. Production of urea was observed in all the three local isolates. The standard *B. thuringiensis* var *kurstaki*, HD1 utilized both arginine and citrate but produced trace amounts of urease.

4.3.2 Crystal protein gel analysis

Analysis by means of SDS-PAGE gel electrophoresis carried out on the three active isolates *B. thuringiensis* ICIPE 012, ICIPE 054 and ICIPE 023 and HD1 showed no difference on the protein patterns, but differences were seen when whole cell (crystal, spore and cell debris) was used in the analysis. One of the isolates *B. thuringiensis* ICIPE 023 showed differences in the cell wall protein patterns (fig.4)

Bacillus thuringiensis var. kurstaki was included in this test because these three local isolates were serologically identified as *B. thuringiensis var. kurstaki*. The activity against test insects showed that there was variation among these isolates. It was therefore important to find out if the differences could be observed in their protein using SDS-PAGE.

Fig. 4. Electrophoresis of the *B. thuringiensis* crystal, freeze-dried pellet.
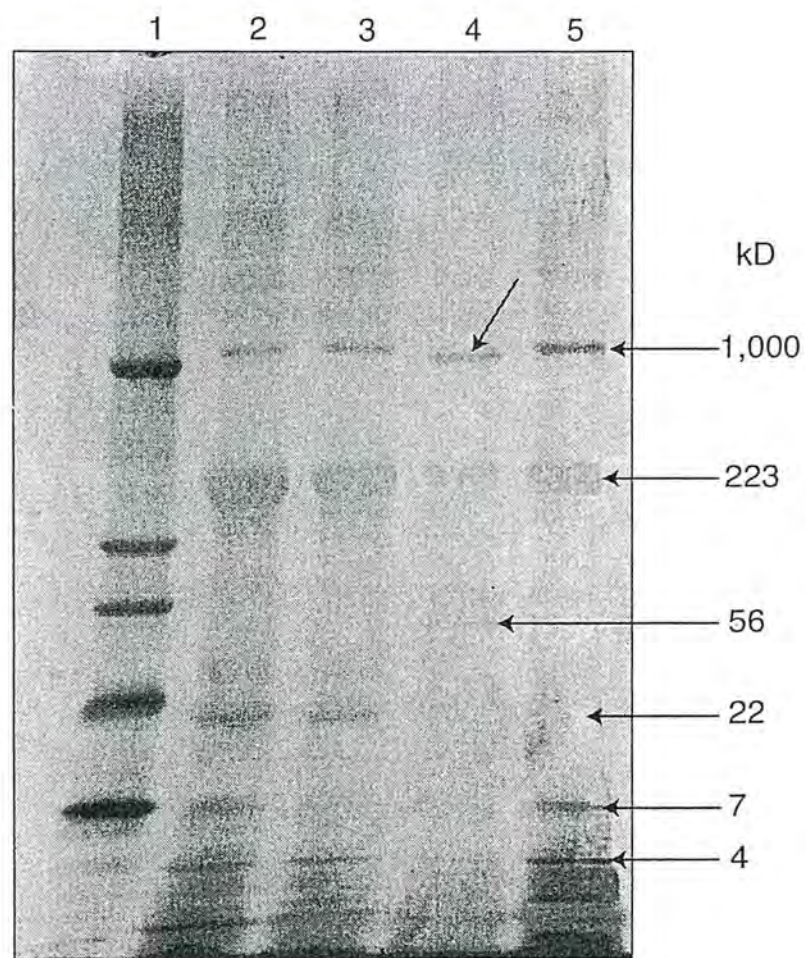
Samples were separated by SDS-PAGE (4 - 15%). (1)
Low molecular weight standards; (2) ICIPE 012; (3) B.
thuringiensis var. kurstaki (HD1); (4) ICIPE 023 and (5)
ICIPE 054.

4.3.3 Serotyping

The three active isolates (ICIPE 012, ICIPE 023 and ICIPE 054) were serotyped by Jean Cabana of Ministre de L'Energie et des Resources service's des Laboratories in Canada using H- antisera of the following strains: *B.thuringiensis thuringiensis, finitimus, alesti, kurstaki, sotto, kenyae, galleriae, canadensis, entomocidus, aizawai, morrisoni, ostriniae, tolworthi, darmstadiensis, toumanoffi, kyushuensis, thompsoni, pakistani, israelensis, dakota, indiana, tohokuensis, kumamotoensis, tochigiensis, yunnanensis, pondicheriensis, colmeri, shandongiensis, japonensis, neoleonensis, coreanensis, silo* and *mexicanensi.* The antigen of the three isolates, ICIPE 012, ICIPE 023 and ICIPE 054 gave a positive reaction with antibodies *B. thuringiensis* var. *kurstaki.* The results are shown in the appendix 2.

4.4 Optimization of growth conditions for ICIPE 023

4.4.1 Analysis of raw materials used in media development

The local raw materials used in the media development were analyzed at The

Kenya Bureau of Standard (KBS) in 1998; the results are shown in Table 8.

Table 8: Nutritional composition of the selected local raw materials used in media development

Source	protein	carbohydrate	phosphate	calcium	potassium
Soya	43.5	30	0.62	0.25	1.88
Molasses	3.0	50	0.08	0.74	3.67
Cow-dung	1.4	17.7	0.7	5.2	1.0
Chicken droppings	15.4	24.2	2.3	3.2	2.2
Omena(Sadine)	72	-	1.5	2.0	1.12
Blood meal	80	2.5	0.22	0.3	0.09

This is analysis of materials containing soluble products of protein,

carbohydrates, phosphates, calcium and potassium and is presented in %

weight by volume.

4.4.2. Potency of the different media made from the local raw material

The potency of eight different media types designated, A to H, tested against
C. partellus larvae are shown in Table 9.

**Table 9. Differences in potency value of ICIPE 023 isolate grown in
different medium types and tested against *C. partellus* larvae . The values
are ($X10^7$) spores /ml**

Composition of medium (medium type)	LC_{50} (95% fiducial limit)
Cow-dung/soya (B)	0.04 (0.03 - 0.05)a
Horn & hoof/molasses/phosphate (F)	0.06 (0.04 - 0.07)a
Blood meal/molasses/phosphate (H)	0.14 (0.12 -0.17)b
Omena/molasses/phosphate (G)	0.23 (0.15 -0.52)c
Soya/molasses/phosphate (A)	0.32 (0.19 -0.60)d
Chicken droppings/molasses/phosphate (C)	1.21 (1.00 -1.75)e
Soya/chicken droppings (D)	1.21 (1.00 - 1.75)e
Cow-dung/chicken droppings (E)	1.59 (1.12 - 3.19)f

*Means with the same letter in the same column are not significantly different
P>0.005*

The above results (Table 9) of the LC_{50} analysis show that medium F (Horn
and hoof 3% molasses 1% and phosphate 0.5%) tested against *C. partellus*,
had an LC_{50} of 0.06 X 10^7 spores/ml. Medium B (Cowdung 3% and soya 3%)
had LC_{50} value of 0.04 X 10^7 spores/ml. The medium with the lowest toxin
production was medium E (cow dung 3%, chicken droppings 3%) and gave an
LC_{50} value of 1.59 X 10^7 spores/ml. The LC_{50} values give the indication of the

quality of the δ-endotoxin production in each of the media tested against *C. partellus* .

The results (Fig. 6) showed that when *B. thuringiensis* isolate ICIPE 023 grown under agitation of 100 rpm and volume of medium at 100 ml, sporulation was 3.28 X 10^8 spores/ml and the time taken to sporulation was about 119 hours (Fig. 5). At the agitation speed of 100 rpm and medium volume of 25 ml, sporulation was 4.33 X 10^8 (Fig. 6) and sporulation time was about 44 hours (Fig 5). At the agitation speed of 300 rpm and medium volume of 100 ml, the amount of spores recorded was about 0.7 x 10^8 (Fig 6) while sporulation time was 43 hours (Fig. 5). At low volumes (25 ml, 50 ml) of broth and high agitation (200 rpm, 300 rpm), the development of ICIPE 023 took shorter time than when the volumes were high (100 ml) and agitation low (100 rpm).

The cultures were tested for their potency (Table 10) in order to find the best growth conditions for isolate ICIPE 023. The potency of the different cultures were determined using *C. partellus* in the insect bioassay. Cultures grown in 50 ml broth with agitation of 300 rpm LC_{50} activity required approximately 0.05 ml of culture broth mixed with 40 ml of insect diet. The lowest potency value (0.17 ml/40 ml insect diet) was obtained in the culture grown in 100 ml and the agitation of 100 rpm. Similar potency value was obtained when growth conditions were, agitation of 200 rpm and volume of 100 ml.

Fig 5. Sporulation time of ICIPE 023 grown under different growth conditions (agitation speed and aeration).
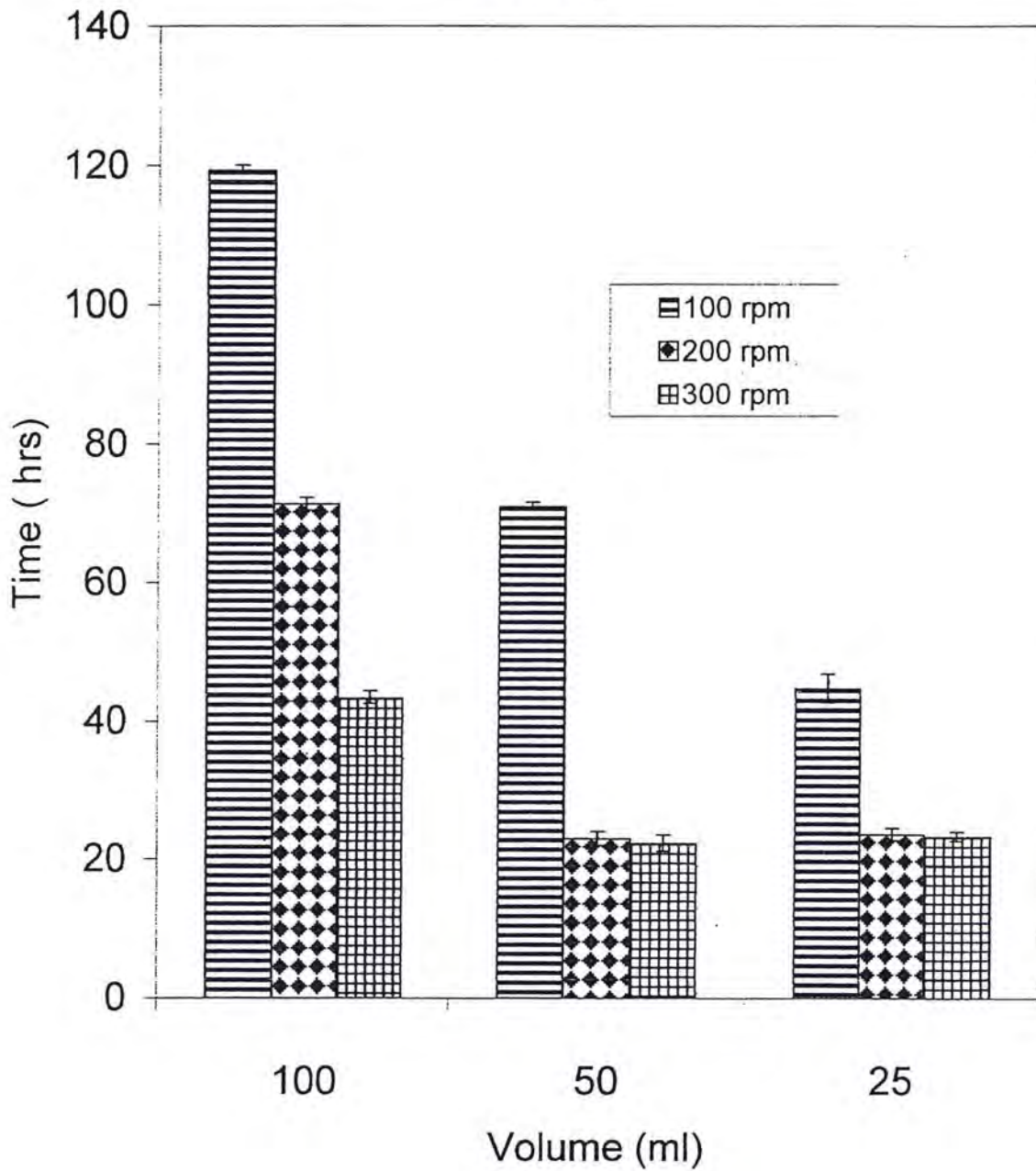
Fig 6. Spore quantity produced by ICIPE 023 grown under different growth conditions (agitation speed and aeration).
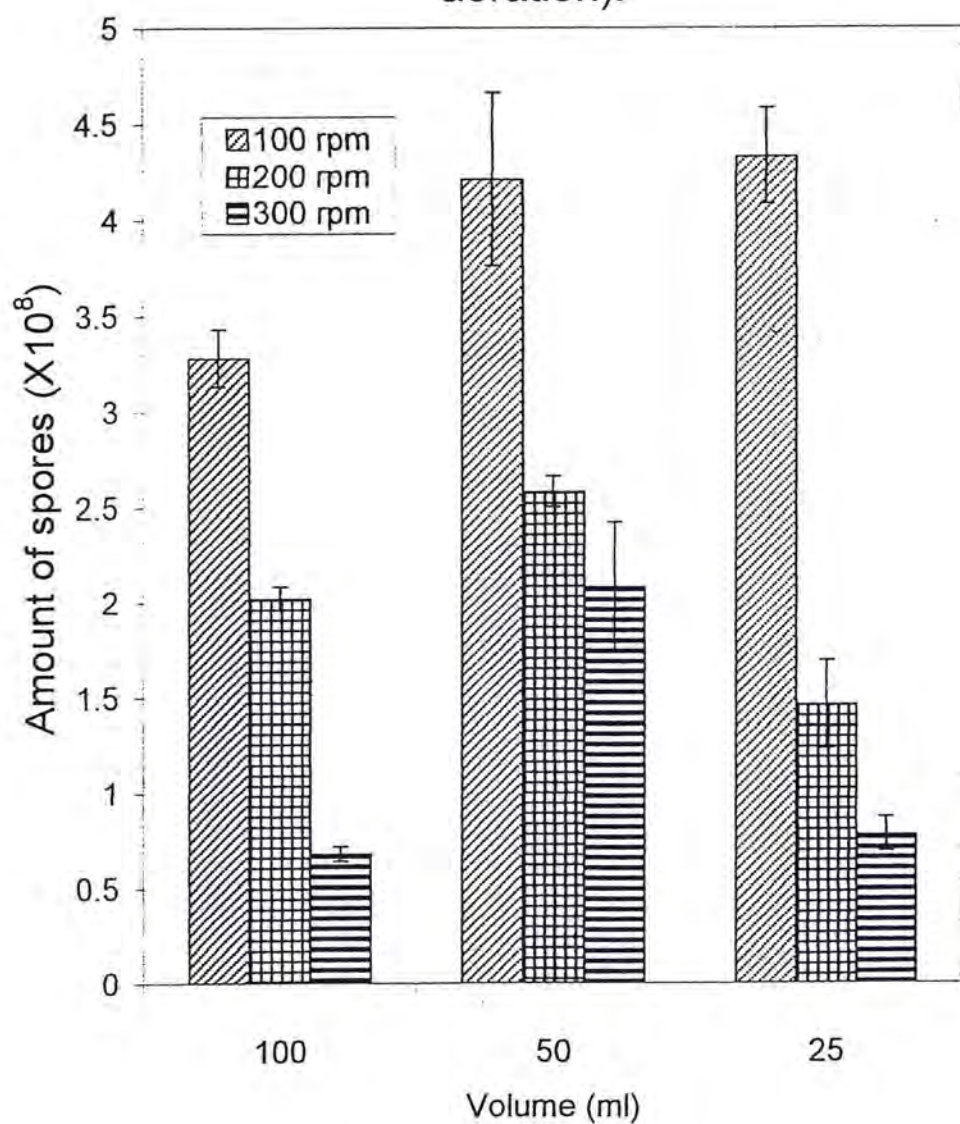
Table 10 Potency of ICIPE 023 grown under different growth culture conditions and tested against C. partellus (LC50 value ml/40ml of the insect diet)

Growth condition rpm/volume	LC50 (95% Fiducial limit)
100rpm/25ml	0.14 (0.11 - 0.17)a
200rpm/25ml	0.14 (0.07 - 0.22)a
300rpm/25ml	0.08 (0.06 - 0.09)b
100rpm/50ml	0.08 (0.06 - 0.1)b
200rpm/50ml	0.06 (0.05 - 0.06)b
300rpm/50ml	0.05 (0.05 - 0.06)b
100rpm/100ml	0.17 (0.09 - 0.26)a
200rpm/100ml	0.17 (0.09 - 0.26)a
300rpm/100ml	0.06 (0.04 - 0.08)b

Means with the same letter in the same column are not significantly different P>0.005.

4.5   ULTRAVIOLET- protection

The results in fig. 7 show the mean percentage mortality ($\pm$ S.E) of *C. partellus*

fed on *B. thuringiensis* ICIPE 023 culture samples protected by different UV-

protectants. As seen in the results, all the three UV- protectants used, clay soil,

congo red and molasses, show the ability to protect the toxin of *B.*

thuringiensis ICIPE 023 from UV destruction.

Fig.7 Mean percentage mortality of *C. partellus* larvae treated with *B. thuringiensis* ICIPE 023 after different hour exposure to sunlight.
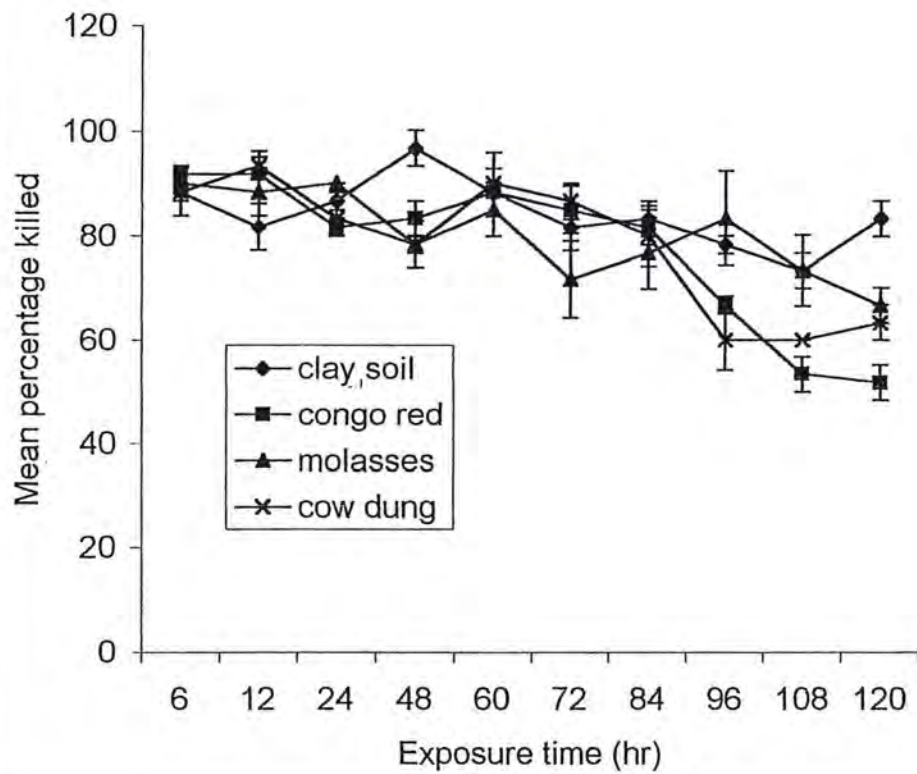
The sample protected with clay soil and exposed to the sunlight for 108 hours gave 73% mortality when fed to *C. partellus*. Samples with Congo red exposed to sunlight for 120 hours gave 51% mortality to *C. partellus* whereas the sample treated with molasses had a protection of 66% after 120 hour-exposure. The unprotected sample grown in cow dung/soya medium (unprotected sample) showed very similar protection with congo red, clay soil and molasses. This unprotected sample gave activity against *C. partellus* of about 60% after 120 hours exposure to sunlight. The results show that cow dung/soya medium was able to protect *B. thuringiensis* ICIPE 023 from destruction by ultraviolet rays present in the sun's rays.

4.6   Shelf-life

The result of the shelf-life determination of *B. thuringiensis* ICIPE 023 is shown in Table 11. Samples kept in the cold room, and tested against *C. partellus* neonates after a year later gave an average mortality value of 60%. The activity of room-temperature sample was down to 50% after 2 months and only 25%.activity after one year.

Table 11. Activity of ICIPE 023 culture stored at 4°C and 25°C (room temperature) against *C. partellus*

Storage	0 month	1 month	2 months	6 months	9 months	12 months
Cold-room (4°C)	19.7± 0.3	19 ±1	15.7± 0.9	14 ± 1.2	13.3± 0.3	12.3 ± 1.5
Room temperature (25°C)	20 ± 0	19.3± 0.3	11.3± 1.4	9.0 ± 0	8.3 ± 1.5	5.3 ± 1.4

4.7 SCREEN-HOUSE TRIALS

Table 12 Results of the screen house trials on the selected *B. thuringiensis* ICIPE 023 on *C. partellus* infested maize plants.

Treatment	Plant height	Stem tunneling	No. of larvae	No. of pupae
Infested maize	84.9 ± 9.62 (b)	21.8 ± 1.89	5.4 ± 0.55	1.66 ± 0.33
Infested and treated maize	170.06± 8.54 (a)	-	1	-
Control maize	178.6 ± 5.01 (a)	-	-	-

Means with the same letter in the same column are not significantly different P>0.001

Isolate *B.thuringiensis* ICIPE 023 controlled the stem borer larvae in the maize plants infested with *C. partellus* in screen-house. The effect of ICIPE

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023 against *C. partellus* was determined by comparing the results obtained from plants infested with *C. partellus* larvae treated with *B. thuringiensis* ICIPE 023 isolate , infested but not given *B. thuringiensis* ICIPE 023 treatment and control plants. Plant development showed no difference in plant height of the infested and treated plants from those non-infested control plants (Fig. 8). The plants in the infested non-treated controls only gained a height of 57 cm, whereas ICIPE 023 treated plants gained a height of 151 cm. Control plants gained a height of 145 cm on average. This showed how stunted the infested-non-treated control plants were.

Stem -tunneling was one of the important parameters noted (Table 12) in this study. The infested non-treated control plants had stem-tunneling height of 24 cm whereas *B. thuringiensis* ICIPE 023 treated plants had stem-tunneling height of about 0.2 cm. The non-infested control plants showed no stem-tunneling.

Dead heart formation was recorded in 70% of the infested non-treated control plants. There were no dead hearts seen in the infested *B. thuringiensis* ICIPE 023 treated plants.

There was only one larva found in the plants infested and treated with *B. thuringiensis* whereas there were at least an average of about five larvae per plant in the infested and not treated plants. Some pupae were also found in the infested plants.

Fig 8. Screen house trials of *B. thuringiensis* ICIPE 023 isolate on *C. partellus* infested maize
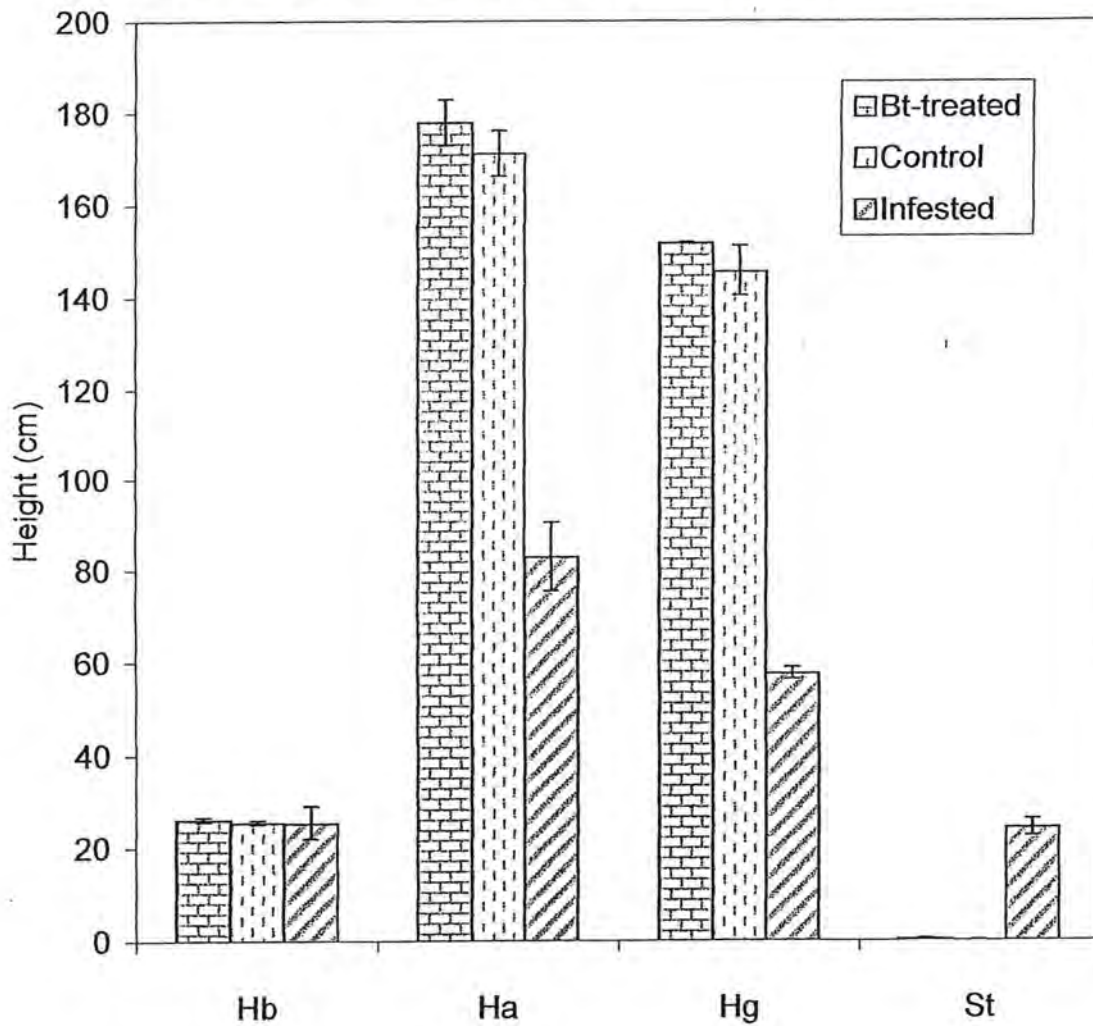
Hb= height of plants before infestation; Ha = height of plant at harvest time; Hg = height gained and St = stem tunnelling height.

CHAPTER FIVE

5. DISCUSSION

A total of fifteen *B. thuringiensis* were isolated from soil samples and dead insect cadavers In this isolation study, *B. thuringiensis* was found in 15 out of the 100 collected samples. Approximately 2% of all the samples collected had at least one *B. thuringiensis* isolate. Although the presence of *B. thuringiensis* was examined in the samples collected from various localities in Kenya, the frequency of occurrence was generally low (Fig. 2). We believe that *B. thuringiensis* isolates recovered from the soil samples were naturally occurring organisms and not residues from agricultural sprays. This is based on the low frequency of occurrence and the fact that there are no reports on the use of *B. thuringiensis* -based products for stemborer control in the localities examined. In addition, several of the isolates obtained were not active against the species of the Lepidoptera tested, and only one insect cadaver out of the fifteen collected was positive for *B. thuringiensis*.

Numerous studies have shown that grain dust and soils are environments in which *B. thuringiensis* is abundant (Meadows, 1993). For example, the presence of *B. thuringiensis* was assessed in grain dust and soil samples in Pakistan (Khan *et al.*, 1995). Martin and Travers (1989) isolated *B. thuringiensis* from 70% of soil samples, while Padua *et al.* (1982) found *B. thuringiensis* in 18% of soil samples in the Philippines. Comparing the finding of this study with those of researchers mentioned above, it may be concluded that the amount of *B. thuringiensis* recovered depends on the localities sampled

as different environmental conditions (soil fertility, pH, water holding capacity and temperature) can affect the micro flora.

Bioassays were carried out on the 101 *B. thuringiensis* isolates using selected stem borers (*C. partellus, E. saccharina, S. calamistis* and *B. fusca*). The results are presented as percentage killed (Fig. 3). About 26 isolates killed 80 to 100% of the third instar larvae of *C. partellus* and *E. saccharina*. Most of the isolates had little or no activity on *B. fusca* larvae, with between 0-20% being killed by about 80% of the isolates. Concerning the effect of *B. thuringiensis* on *B. fusca*, this study was not able to conclude whether *B. fusca* was susceptible to any of the local *B. thuringiensis* toxins tested. *B. thuringiensis* toxin must be ingested by the target insect to be effective but the observation showed that *B. fusca* avoided eating the *B. thuringiensis* - treated diet. The recorded death could very well be due to starvation(Table 4). There was a wide variation in the distribution of insecticidal activity among the isolates tested against the different stemborers studied. The number of isolates belonging to the groups of isolates active against *B. fusca* and *S. calamistis* were low (Fig. 3). It is interesting to note that isolates active against *C. partellus* and *E. saccharina* were more abundant, suggesting the presence of more selective genes against these two insect species (van Frankenhuyzen *et al.*, 1992, Attathom *et al.*, 1995) or the differences in the level of interaction between the gut of the target insect and the different toxin produced by the isolates (Chiang *et al.*; 1986; Haider *et al.*, 1986; Bai *et al.*, 1990 ; Ellar, 1999).

Biochemical testing of isolates represents a fast method used for *B. thuringiensis* identification. The ability to isolate a large number of *B. thuringiensis* requires an efficient identification method. In this study, biochemical tests were carried out on the isolates using API 20E analytical profile. Reactions for citrate utilization, urease production and arginine dihydrolase tests were used to differentiate the selected active isolates, namely, ICIPE 012, ICIPE 023, ICIPE 054 and the standard *B. thuringiensis*, HD1.

These biochemical tests were supported by serological investigations using the "H" - antigen. Serological classification of *B. thuringiensis*, based on flagella antigen, was introduced by *de* Barjac (1981). Serological tests classifies *B. thuringiensis* according to the nature of the antigen present on the flagella of the vegetative cells. The classification of *B. thuringiensis* using this method has been useful in the identification of isolates but it has some limitations, as seen in our results, not all isolates identified as being serologically similar gave the same biochemical reactions (Table 7). For example, the isolates ICIPE 012, 023, 054 and HD1 are all serologically identified as *B. thuringiensis var. kurstaki*, yet their biochemical results are different. De Barjac, (1981) has also recommended that the classification by "H" antigen should be confirmed by the biochemical characterization because each serotype has specific physiological characters and sometimes, one serotype can be divided into biotypes, on the basis of different enzymatic reactions.

In this study, further identification of the selected active isolates was carried

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out by analyzing the protein compositions of the crystals using SDS polyacrylamide gel electrophoresis. The results showed that the crystal protein bands are the same except ICIPE 023 which had a different pattern in terms of the composition of cell wall proteins (Figure 4). These results are in agreement with those of Lereclus *et al.,* (1993) who found that the protein compositions of the crystal by SDS-gel were characteristic of the strains.

Data presented in Table 9 shows the potency against *C. partellus* of the ICIPE 023 grown in various media. Several medium ingredients, such as soya, cow dung, chicken droppings, molasses, horn and hoof, sadine and blood meal were investigated for their ability to support δ-endotoxin production by *B. thuringiensis* ICIPE 023 isolated in Kenya. Culturing of ICIPE 23 in different media resulted in toxins with different potencies against the target insects. For example, medium B supported a good production of δ-endotoxin with high potency against *C. partellus* than medium E. Since the cultures were grown in shake flasks at air : medium ratio of 1.5:1, the potency differences could also have been due to other factors, for example, amino acid availability from a given protein source, or dissolution of protein source components affecting the rate of oxygen transfer and hence the oxygen availability to the cells. Salama *et al.* (1983) using different protein sources for growth of *B. thuringiensis* produced widely differing spore yields and activities towards several insect species. He showed that different protein source is among the factors that affect *B. thuringiensis* production of δ-endotoxin and therefore the potency of the cultures.

The yield of δ-endotoxin was relatively high in medium B (composed of cow dung and soya bean flour) and relatively low in medium E (cow dung and chicken droppings). The difference in these two media was the protein source (soya - medium B and chicken droppings - medium E). Cow dung had a higher protein content (43.5%) than chicken droppings (15.4%). Dulmage (1971) carried out similar studies and found that there were differences in potencies of δ-endotoxin crystals originating from different media. He showed that the choice of medium is important and that there is no single medium that can be predicted to be best.

This investigation shows that some of the tested medium combinations could be used for local production of a cheap *C. partellus* larvicide product. Other researchers have also shown the use of locally available raw materials for production of *B. thuringiensis*. For example, Obeta and Okafor (1984) reported a medium made up of dried bovine blood and extracts of leguminous seeds and used for the production of *B. sphaericus*. Ejiofer and Okafor (1989) reported a medium composed of cassava, whole maize and whole cowpeas used for the production of B. *thuringiensis* isolate. Roldan et al. (1988) reported a medium for *B. thuringiensis* using molasses, soya bean flour and corn steep liquor, while Moscardi (1988) used a medium made up of residues of paper, cellulose and soluble starch. These reported media composed of local raw materials are being used in the local production of *B. thuringiensis*-based biopesticides. The search for local medium for development of new potent

isolates is important since the yield of δ-endotoxin from any isolate can be improved by fermentation conditions (agitation, aeration and pH). The medium selected for a given isolate must be one that is suitable for its growth.

The amount of spore - δ-endotoxin complex produced by *B. thuringiensis* depends on the isolate, the medium used and the growth conditions. A maximum spore yield of 4.33×10^8 was obtained when ICIPE 023 cultures were grown under a medium volume of 25 ml and agitation speed of 100 rpm. On the other hand, the minimum spore yield of 0.683×10^8 was obtained under a medium volume of 100 ml and agitation speed of 300 rpm. The differences in volume may have caused the low yield due to limiting aeration. The time to sporulation was longer (119 h) in cultures under a medium volume of 100 ml and agitation of 100 rpm, whereas the sporulation time was shorter (22 h) under a medium volume of 50 ml and agitation of 300 rpm. The differences in sporulation time were remarkable (Fig. 5). The LC_{50} values of the ICIPE 023 grown under 100 rpm/100 ml (0.17 ml/ 40 ml diet) and 300 rpm/50 ml (0.05 ml/40 ml diet) culture conditions indicated that their potency were significantly different. Although the potency level when ICIPE 023 was grown under 100 rpm/100 ml was almost identical to that of the 200 rpm/100 ml, the relative time to the sporulation of 100 rpm /100 ml was longer.

The results presented in Table 10 show the effect of different growth conditions (agitation and aeration) on the potency of ICIPE 023 against *C. partellus*. From the results therefore, it can be concluded that selection of an

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effective *B. thuringiensis* isolate offers the potential for a highly potent end product and that the culture conditions drastically influence the ability of the isolate to produce the maximum potential potency (Bryant, 1994; Morris *et al.*, 1996). Additionally we conclude that if you have lower volume, it would be cost effective to shake at 200rpm rather than 300rpm because there was no difference in the results obtained. When working at higher volume, it would be recommended to shake at higher revolutions per minute. The investigation has also shown that toxin yield can be improved by manipulating fermentation conditions such as agitation and aeration.

Studies were carried out to establish the abilities of Congo red, clay soil and molasses to protect the loss of activity of ICIPE 023 toxin when exposed to natural sunlight. The results showed significant differences in the protection afforded to the samples treated with clay soil and molasses (Fig. 7). Samples treated with Congo red showed reduced activity after 96 h exposure to sunlight. However, since the untreated sample (*B. thuringiensis* ICIPE 023 grown in cow dung/soya medium) also showed reduced activity after 96 h exposure, it can be concluded that Congo red did not improve the product as the product was grown in cow dung/soya medium. Based on percent mortality of *C. partellus*, 1 % clay soil or 1% molasses ranked first as efficient UV-protectants. On the other hand, cow dung/soya medium had effect as UV-protectant against inactivation by the natural sun's ultraviolet rays. A number of studies were carried out by Ignoffo *et al.* (1981); Sneh *et al.* (1981) and Liu. *et al.* (1993) searching for methods to protect *B. thuringiensis*-based products

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from the effect of sunlight. In their studies they found out that the UV-absorbing materials improve the efficacy of *B. thuringiensis*. Radiation of sunlight at wavelength ranging from 250 to 380 nm has a detrimental effect on the viability and toxicity of *B. thuringiensis* (Ignoffo, *et al.*, 1977)

Screen-house trials were conducted to determine the effectiveness of ICIPE 023 against *C. partellus* larvae. The isolate ICIPE 023 was found to significantly protect the maize plants from pest damage. Stem tunnelling was zero, no larvae was found, and no pupae in plants protected with ICIPE 023. Plants with no ICIPE 023 protection had 21.8 ± 1.89 (cm) stem tunnelling length, 5.4 ± 1.89 larvae and, 1.66 ± 0.33 pupae. *B. thuringiensis* has previously been used to control the European corn borer (*Ostrinia nubilalis*) and the Asian corn borer (*O. furnacalis*) (Schreiner and Nafus, 1987). The results of the current work support these observations and underscores the fact that the stemborer control by *B. thuringiensis* is feasible as long as the right strain is used. Although the number of larvae used in this study (20, neonate larvae/ plant) were sufficient to cause high level of damage to plants, the application of *B. thuringiensis* almost eliminated the risk (Fig.8). The results of the screen house trials demonstrated the potential of *B. thuringiensis* strain (ICIPE 023) as a biological control agent against *C. partellus*.

6. CONCLUSION

Through this study several local *B. thuringiensis* have been isolated and tested for their activity against *C. partellus*, *E. saccharina*, *S. calamistis* and *B. fusca* and the active isolated were identified. The study demonstrated that a local *B. thuringiensis* isolate ICIPE 023 had potential for controlling stem borers, especially *C. partellus* and *E. saccharina*.

Bacillus thuringiensis isolate can be grown in cow dung/ soya medium, which are cheap and locally available. Cow dung could sell for fifty Kenya shillings per kilogram. The study has not only identified cow dung/soya medium as a good medium for the development of *B. thuringiensis* ICIPE 023 but also that the medium has qualities of being a protectant against sunlight. Cow dung/soya being natural product has no polluting effect to the environment. If *B. thuringiensis* ICIPE 023 can be produced in the cow dung/soya medium, the resulting product would be cheaper than if grown in commercial media. It would also be available to the local farming communities in the developing countries.

The fact that *B. thuringiensis* ICIPE 023 is an indigenous isolate with activity against the major stem borers suggests that it could be exploited commercially. The use of an indigenous isolate is advantageous since registration procedures of a product derived from it would be easy. If *B. thuringiensis* is cheaply produced, and is available to farmers, the developing countries would make a

big saving on hard currency which would otherwise be used in importing chemical insecticides.

By finding specific *B. thuringiensis* ICIPE 023, active against *C. partellus* and *E. saccharina* , it is now possible for molecular biologist to genetically engineer the *cry* toxic gene into maize plants. Isolate ICIPE 023 would be the 'tailored' *B. thuringiensis* to the local target insect pests.

7 FURTHER WORK

Bacillus thuringiensis ICIPE 023 produced in cow-dung/soya medium should be further evaluated under field conditions in different agroecological zones. Although cow-dung/soya medium was identified as a good medium for the - production of ICIPE 023, additional work may be needed to develop other media that may be economical for large-scale production. In addition, further work is needed on formulations, with the specific aim to improve persistence of the toxin in the field. Historically, *B. thuringiensis* is dispensed manually by spraying, typically mixed with UV- absorbers,stickers and spreaders. Manual application fosters unevenly distribution of the toxin on the plants. New delivery systems of *B. thuringiensis* are being developed through genetic engineering. Genetic engineered plants have advantage in that it eliminates the effect of UV destruction and expresses the toxin throughout the plant

Our observation has shown that *B. fusca* avoids eating diet in which *B. thuringiensis* is incorporated. This is an important observation, therefore it would be important to carry out a study to validate the reason(s) why *B. fusca* avoids eating a diet mixed with *B. thuringiensis*. Would it do the same to *B. thuringiensis* engineered cereals?

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APPENDIX 1

Composition of *C. partellus* diet

Ingredients	Quantity (%) w/v
Fraction A	
1. Distilled water	46.52
2. Benlate (g)	0.02
3. Grabacin (g)	0.003
Fraction B	
4. Bean powder (g)	10.2
5. Sorghum leaf powder (g)	3.72
6. brewer's yeast (g)	0.74
7. Sorbic acid (g)	0.09
8. Ascobic acid (g)	0.17
9. Methyl-p-hydroxybenzoate (g) (dissolved in 62.5 ml ethanol)	0.15
10. Vitamin E (g)	0.1
11 Formaldehyde 40% (ml)	0.19
Fraction C	
12. Agar technical no. 3 (g)	0.88
13. Distilled water (ml)	37.22

Fraction A: Distilled water is boiled, cooled to 60°C and mixed with weighed benlate and grabacin in the blender for 1 min.

Fraction B: Powdered ingredients are pre-mixed in a clean container and mixed together with ingredients of fraction A in the blender for 1 min. Formaldehyde 40% is added into the blender at this stage and mixed. The dissolved Methyl -p-hydroxybenzoate is also added.

Fraction C: Agar powder is weighed in a separate container, added to cold distilled water in a separate sauce pan, boiled while stirring periodically, cooled to 60 °C and mixed together with ingredients of fractions A and B in the blender for 1 min.

Composition of *S. calamistis, B. fusca and E. saccharina* diet

Ingredients	Quantity

Fraction A

1. Distilled water	800 ml
4. Bean powder	175 g
5. Sorghum leaf powder	50 g
6. Brewer's yeast	45 g
7. Sorbic acid	1.3 g
8. Ascobic acid	5 g
9. Methyl-p-hydroxybenzoate	
(dissolved in 62.5 ml ethanol)	2 g
10. Vitamin E)	4.2 g
11. sucrose	70 g

Fraction B:

12. Agar technical no. 3	25 g
13. Distilled water (ml)	800 ml

Fraction C

11 Formaldehyde 40%	2 ml

The preparation is the same as that of *C. partellus* diet.

Appendix 2.

Results of the serotyping tests made on three isolates (ICIPE 012, 023 and 054)

H. antigen	serovar	ICIPE 012	ICIPE 023	ICIPE 054	B..tk	Bti
1	*thuringiensis*	-	-	-	-	-
2	*finitimus*	-	-	-	-	-
3a	*elesti*	-	-	-	-	-
3a,3b	*kurstaki*	+	+	+	+	-
4a,4b	*sotto*	-	-	-	-	-
4a,4c	*kenyae*	-	-	-	-	-
5a,5b	*galleriae*	-	-	-	-	-
5a,5c	*canadensis*	-	-	-	-	-
6	*entomocidus*	-	-	-	-	-
7	*aizawai*	-	-	-	-	-
8a,8b	*morrisoni*	-	-	-	-	-
8a,8c	*ostriniae*	-	-	-	-	-
9	*tolworthi*	-	-	-	-	-
10	*darmstadiensis*	-	-	-	-	-
11a,11b	*toumanoffi*	-	-	-	-	-
11a,11c	*kyushuensis*	-	-	-	-	-
12	*thompsoni*	-	-	-	-	-
13	*pakistani*	-	-	-	-	-
14	*israelensis*	-	-	-	-	+

- indicates negative reaction

+ indicates positive reaction

Btk = Bacillus thuringiensis kurstaki; Bti = B. thuringiensis israelensis

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Appendix (2 cont.)

H. antigen	serovar	ICIPE 012	ICIPE 023	ICIPE 054	*Btk*	*Bti*
15	*dekota*	-	-	-	-	-
16	*indiana*	-	-	-	-	-
17	*tohokuensis*	-	-	-	-	-
18	*kumamotoensis*	-	-	-	-	-
19	*tochigiensis*	-	-	-	-	-
20a,20b	*yunnanensis*	-	-	-	-	-
20a,20c	*pondicheriensis*	-	-	-	-	-
21	*colmeri*	-	-	-	-	-
22	*shandongiensis*	-	-	-	-	-
23	*japonensis*	-	-	-	-	-
24	*neoleonensis*	-	-	-	-	-
25	*coreanensis*	-	-	-	-	-
26	*silo*	-	-	-	-	-
27	*mexicanensis*	-	-	-	-	-

-   indicates negative reaction

+   indicates positive reaction

Btk = Bacillus thuringiensis kurstaki; Bti = B. thuringiensis israelensis