ISOLATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* AND ITS LARVICIDAL ACTIVITY AGAINST MOSQUITO

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ISOLATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* AND ITS LARVICIDAL ACTIVITY AGAINST MOSQUITO

M.Sc. DISSERTATION

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

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DECLARATION

I declare that the work in this dissertation entitle "ISOLATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* AND ITS LARVICIDAL ACTIVITY AGAINST MOSQUITO" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

ABDULHAKEEM BELLO _____ _____

M.Sc./SCI/177/2011-2012 Signature Date

CERTIFICATION

This dissertation entitled "ISOLATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* AND ITS LARVICIDAL ACTIVITY AGAINST MOSQUITO" by ABDULHAKEEM BELLO meets the regulations governing the award of the Master of Science degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Application of chemical insecticides poses a wide range of problems in the environment. They are non specific and are implicated as carcinogens hence the need to search for an eco-friendly biological control agent such as the use of *Bacillus thuringiensis* habouring insecticidal crystals. In this study, *Bacillus thuringiensis* were isolated from different soil types in Zaria. The 12 isolates from these obtained from different soil types were screened for the presence of *cry* gene by PCR using primers specific for *cry*2 and *cry*4 (diptera-active *cry* genes). Of the 12 isolates, 1 isolate had only *cry*2 gene, 4 isolates had *cry*4 gene, 1 isolate had both of the genes while 6 isolates had none of the two genes. Bioassay to assess the insecticidal activity of the isolates was carried out using *Culex quinquefasciatus* and *Aedes aegypti* larvae using 3 different concentrations of spore crystal mixture (100, 75 and 50 ppm) alongside one control. In each case, 10 larvae of *Cx. quinquefasciatus* and *A. aegypti* were exposed to spore crystal mixture in triplicates for both. The isolates differ greatly in their larvicidal activity against the larvae of *Cx. quinquefasciatus* and *A. aegyti*. The mortality of *Cx. quinquefasciatus* larvae when exposed to 100ppm concentration of the isolates' spore crystal mixture ranged between 33.33% and 96.00% while the range of mortality for *A. aegyti* was between 40.00% and 100.00%. The concentrations of the spore crystal mixture which kill 50% of the exposed populations in standard bioassays (LC_{50}) were determined by probit analyses. The LC_{50} using *Culex quinquefasciatus* was between 135.95ppm and 37.48ppm while it was between 118.03ppm and 55.79ppm for *Aedes aegypti*. The results of this research shows that the isolates habouring the diptera-active *cry* gene from the soils in Zaria can serve as biocontrol agent for the control of mosquito by targeting their larvae stage hence controlling the diseases they spread.

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CHAPTER ONE

1.0 INTRODUCTION

Bacillus thuringiensis belongs to the family Bacillaceae*;* which taxonomists consider as sub-species of *Bacillus cereus* because they are closely related (Leonard *et al.,* 1997; Helgason *et al.,* 2000; Chen and Tsen, 2002). According to Priest (2000), the genotypic and phenotypic characteristics of *Bacillus thuringiensis* are very similar to *Bacillus cereus*; the only difference between these two species being the formation of large proteinaceous parasporal inclusions observed in *Bacillus thuringiensis.*

All over the world, the medical and economic burden caused by vector-borne diseases continues to increase as current control measures are not adequate to cope with the trend of the diseases. In view of this development, there is an urgent need to identify new control strategies that will remain effective, even in the face of growing insecticide and drug resistance (Achs and Malaney, 2002). One such strategy is vector control. Vector control strategies include chemical based control measures, non - chemical based control measures and biological control agents (Poopathi and Tyagi, 2006). Repetitive use of man-made insecticides for mosquito control disrupts natural biological control systems and leads to reappearance of mosquito populations. It also results in the development of resistance, detrimental effects on non-target organisms and human health problems and subsequently these necessitate a search for alternative control measures (Das *et al*., 2007; Zhang *et al*., 2011).

The use of biological control agents such as predatory fish (Legner, 1995), bacteria (Becker and Ascher, 1998), protozoa (Chapman, 1974), fungus (Murugesan *et al*., 2009) and nematodes (Kaya and Gaugler, 1993) have shown promising results for the control mosquito populations.

The development of new strategies, including naturally occurring larvicides to control mosquitoes, is important in order to counter the evolution of resistance in target populations and the possible effects on non target organisms (Cetin and Yanikoglu, 2006).

Mosquitoes, which are vectors for many diseases belong to the family Culicidae in the insect order of true-flies or two-winged flies called Diptera. The family is large, occurring throughout the temperate and tropical regions of the world, and well beyond the Arctic Circle, and from lowlands to the peaks of high mountains (Harbach, 2008).

The mouthparts of female mosquitoes are long and adapted for piercing and sucking blood from vertebrate hosts (Schowalter, 2000; Verma and Jordan, 2003). The blood is required by female mosquitoes to supply essential proteins for egg development prior to the initial and for subsequent ovipositions (Chapman, 1982). During these blood meals, a female mosquito could transmit agents of disease to man. These include, arboviruses (disease agents for yellow fever, dengue, encephalitis, chikungunya, rift valley fevers) transmitted by female culicine mosquitoes; protozoa (etiological agents for malaria) by female anopheline mosquitoes; and nematodes (causal agents for filariasis) by females of both culicine and anopheline groups (Metcalf and Luckmann, 1994; CDC, 2007). Some of the diseases transmitted by mosquitoes are known to affect between 500 million to a billion people each year (Manga *et al*., 2012).

Insect pests of crops and forest plants and vectors of disease of humans and other animals are serious threat to agriculture and public health. Worldwide, about US $8000 billion is spent on insecticides and estimates reveal that US $2700 billion can be substituted by the use of the biopesticide *B. thuringiensis* (Krattiger, 1997). Besides their exorbitant cost, resistance and resurgence of the different pests, chemical pesticides are the single main cause of health and

environmental hazards (Krattiger, 1997). The situation demands that safer pesticides and biopesticides are the most desired alternatives. Bacteria, especially *B. thuringiensis* and *B. sphaericus,* are the most potent and successful group of organisms for effective control of insect pests and vectors of diseases (Krattiger, 1997; Chatterjee *et al*., 2007).

Research of almost 85 years reveals that *Bacillus* spp. especially *B. thuringiensis* and *B. sphaericus* are the most potent biopesticides (Boucias and Pendland, 1988). Available information depicts that *Bacillus thuringiensis* is a versatile pathogen capable of infecting protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards (Feitelson *et al.*, 1992). *Bacillus thuringiensis* has been obtained from soil, phyllosphere, diseased insects, stored products, dumping pits, excreta of vegetarian animals etc. and about 30-100% spore formers of phyllosphere were found to be *B. thuringiensis* (Martin and Travers, 1989; Boucias and Pendland, 1988).

An analysis of 27,000 isolates collected from 100 soil samples all over the world showed that *B. thuringiensis* might be found anywhere, including desert, beach and tundra habitats (Martin and Travers, 1989; Attathom *et al*., 1995). *Bacillus thuringiensis* accounts for about 5-8% of *Bacillus* spp. population in the environment (Hastowo *et al*., 1992; Chatterjee *et al*., 2007).

Till date more than 130 species of lepidopteran, dipteran and coleopteran insects are known to be controlled by *B. thuringiensis*. So far, 68 serotypes (81 serovars/varieties) of *B. thuringiensis* having wide array of host range have been isolated and characterized and some of them have already been commercially exploited directly as native form or indirectly as transgenic microbes or plants (Krattiger, 1997).

Potentiality of *B. thuringiensis* as larvicide of *Culex* in India has been demonstrated by Ghosh *et al*. (2012). *Bacillus thuringiensis* has certain advantages for exploitation as biopesticide viz. It can be used directly and as transgenic microbes and plants; being a prokaryote there is no dominant or recessive allele; highly vulnerable to genetic manipulation and the toxin gene is coded by single gene (monocistronic); *B. thuringiensis* is fermentation friendly and therefore commercially exploitable and it is host specific or has narrow host range (Chatterjee *et al.,* 2007).

These advantages favoured the development of about 100 formulations (Federici, 1993) and commercialization of 40 *B. thuringiensis* products internationally and eight products in India exclusively by multinational organizations (Saxena, 2000). However, none of the formulations, if any, marketed in Nigeria is an indigenous strain. Since 1996, insect-resistant transgenic crops, known as *Bacillus thuringiensis* crops, have expanded around the globe and are proving to be quite efficient and helpful in reducing the use of chemical insecticides (Qaim and Zilberman, 2003). Additionally, latest estimates indicate that more than 50% of the cotton and 40% of the corn planted in the US are genetically engineered to produce *Bacillus thuringiensis* insecticidal toxins (Mohamed *et al.*,2010).

Present study was envisaged to isolate and identify the *B. thuringiensis* of indigenous soils of Zaria, Kaduna State, Nigeria and characterize the polymorphic crystal producing strains, which was exploited for biological control of mosquito insect-pests which are disease vectors.

1.1 Statement of Research Problem

The application of chemical insecticides in the control of insect pests on farmers' fields and insect vectors of diseases has remained the major practice in Nigeria. Despite recorded successes following the application of these chemicals, there are abundant reports to the effect that, these practices pose a wide range of problems in the environment (Pimentel, 1991). Many of the insecticide formulations that are locally available and in current application in the environment are recalcitrant and their residues are left in the environment long after their application. There are also reports that such residues could accumulate in the tissues of living organisms in such a way as to be biomagnified. Reported consequences of bioaccumulation of pesticide residues in human could range from direct poisoning to carcinogenic and teratogenic effects (Cremlyn, 1991).

Owing to their non-specificity, both target and non target organisms are often impacted upon with undesirable consequences. As a result, organisms that play an important role in the environment could be detrimentally affected. Key insect pollinators and other resourceful life forms could be wiped out (Biswas, 1994). Both surface and underground water bodies could become heavily contaminated with insecticide residues. This could pose a serious threat to public health and aquatic life forms. (Cremlyn, 1991). These problems are further compounded by exorbitant cost of procurement and almost total lack of proper knowledge of their application among the general populace (Biswas, 1994).

In view of the aforementioned problems associated with the use of chemical insecticides, it is pertinent to conduct researches with the sole aim of seeking a biological alternative for the control of insect pests and vectors in the environment. The current trend employs *Bacillus thuringiensis,* a soil bacterium that has been shown to possess high level of insecticidal potency.

Mosquitoes are the most serious indirect cause of morbidity and mortality among humans when compared to other groups of organisms (Verma and Jordan, 2003; Harbach, 2008). Malaria and other vector-borne diseases contribute substantially to the global burden of diseases and disproportionately affect poor and under-served populations living in tropical and sub-tropical regions of the world (Beier *et al.,* 2008).

1.2 Justification

Due to their high specificity, their safety to most non-target organisms and to the environment in general, *Bacillus thuringiensis* crystal proteins are preferred and widely used as an alternative to chemical pesticides in pest management strategies against insect pests of agricultural crops (Roh *et al.,* 2007; van Frankenhuyzen, 2009) and vectors of important human diseases (Ohba *et al.,* 2009).

Bacillus thuringiensis strains are ubiquitous in the environment and is naturally found in soils (Martin and Travers, 1989), aquatic environments (Ichimastu *et al.,* 2000), plants (Maduell *et al.,* 2002), insects (Cavados *et al.,* 2001) and animal faeces (Lee *et al.,* 2003). These discoveries stimulated the development of worldwide screening programs for new *B. thuringiensis* isolates which have led to more than 300 characterized crystal proteins (Crickmore *et al.,* 2000). Besides insecticidal activity, certain *Bacillus thuringiensis* strains with activity against protozoa, mites and nematodes have also been reported (Feitelson *et al.,* 1992; Schnepf *et al.,* 1998).

Given the undesirable effects of chemical insecticides and public health problems associated with their application in tropical countries, these biopesticides present the advantage of having only a minor impact on the environment and have thus come to occupy a stable, although modest position in the insecticide market. The biopesticides market currently accounts for 2% of the

worldwide crop protection market of about 600 million US dollars, with about 90% of all biopesticides sales involving products based on *Bacillus thuringiensis* (*Bt*) (Glazer and Nikaido, 1995).

There are many reasons for this success: the larvicidal activity of *Bacillus thuringiensis* is rapid but sustained; *Bt* can be applied with standard equipment and its effects on beneficial insects and non-target organisms are negligible, as such the advantages of *Bt* have not escaped biotech companies, which began introducing *Bt* genes into many crop plants, including cotton and maize, at the end of the 1980s (Sanchis and Bourguet, 2008). The insertion of these genes leads to the production of *Bacillus thuringiensis* toxins in various tissues, protecting the plant against attacks by several highly damaging pests (Sanchis and Bourguet, 2008).

Bacillus thuringiensis is fermentation friendly and therefore commercially exploitable and it is host specific or has narrow host range. These advantages favoured the development of about 100 formulations (Federici, 1993) and commercialization of 40 *Bacillus thuringiensis* products internationally and eight products in India exclusively by multinational organizations (Saxena, 2000). However, if available, none of the formulations marketed in Nigeria is an indigenous strain.

Present study was aimed at isolating, identifying and characterizing the *Bacillus thuringiensis* that are indigenous to soils of Zaria, Kaduna State, Nigeria, which could be exploited for biological control of a wide range of insect-pests and disease vectors. On the long run it will provide information on indigenous strains that might be used for the control of these pests in our local environment.

1.3 Aim:

The aim of this study was to isolate and characterize *Bacillus thuringiensis* and to assess its larvicidal activity against mosquito.

1.4 Objectives:

The objectives of this research are:

1. To isolate *Bacillus thuringiensis* from soil samples at various sites in Zaria.
2. To characterize the isolated *Bacillus thuringiensis* from the soil using microscopic and biochemical methods.
3. To detect diptera-active *Cry* genes in the isolated *Bacillus thuringiensis* using PCR.
4. To evaluate the larvicidal effect of diptera-active *Cry* genes harboring *Bacillus thuringiensis* against mosquito larvae.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Genus *Bacillus*

The genus *Bacillus* is made up of saprophytic bacteria capable of producing endospore (Slepecky and Leadbetter 1994). Members of this genus are rod-shaped, usually Gram-positive, catalase-positive, and aerobic or facultatively anaerobic (Thiery and Frachon, 1997).

Based on the shape of their spores and swelling of the sporangium, *Bacillus* has been divided into three morphological groups (Çinar, 2005). Group I is characterized by the presence of ellipsoidal spores that do not swell the mother cell (Priest, 1993). This group comprises a large number of species living in soil such as *Bacillus thuringiensis*, *B. sphaericus*, *B. subtilis*, *B. anthracis* and *B. cereus*. Some of these species are very closely related and form 3 different groups within the group I. One of these subgroups includes the *B. cereus* group (Çinar, 2005).

The genetic and phenotypic characteristics of *Bacillus thuringiensis* are very similar to *B. cereus* (Priest, 2000). The only difference between these two species is the formation of large proteinaceous parasporal inclusions observed in *B. thuringiensis.* These inclusion bodies, crystals have unique toxic activities against certain insects and some other invertebrates (Charles *et al.,* 2000), against human cancer cells (Mizuki *et al.,* 2000), and human pathogenic protozoa (Kondo *et al.,* 2002).

2.2 *Bacillus thuringiensis*

Scientific Classification of *Bacillus thuringiensis*

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

Species: *Bacillus thuringiensis* (Carson *et al.*, 1996)

Bacillus thuringiensis is an aerobic, Gram-positive, rod-shaped, spore-forming bacterium. This bacterium has filamentous appendages (or pili) on the spores. Colonies have a dull or frosted glass appearance and often andulate margin from which extensive outgrowths do not develop (Çinar, 2005).

Under aerobic conditions, *B. thuringiensis* grows in a simple culture medium such as nutrient broth. After nutrients are depleted, it produces spores along with one or several parasporal crystals. There are seven stages during the sporulation. Parasporal protein synthesis starts at about stage II or III of sporulation, and the crystal reaches its maximum size (approximately spore size) by stage V. The crystals are made of proteins varying in size. These crystal proteins

are called δ-endotoxins or insecticidal crystal proteins. When the spore matures, cells lyse. Then, free spores and crystals are released into the environment (Asano *et al*., 2003).

2.2.1. General Characteristics of *Bacillus thuringiensis*

Bacillus thuringiensis, like other members of the genus *Bacillus,* has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents. The spore formation of the organism varies from terminal to subterminal in sporangia that are not swollen, therefore, *B. thuringiensis* resembles other *Bacillus* species in morphology and shape (Stahly *et al.,* 1991). The width of the rod varies 3-5 μm in size when grown in standard liquid media. The most distinguishing feature of *B. thuringiensis* from closely related species of *Bacillus* (e.g. *B. cereus, B. anthracis*) is the presence of a parasporal crystal body that is near to the spore, outside the exosporangium during the endospore formation (Bulla *et al*., 1995).

It is thought that *B. thuringiensis* is an insecticide-producing variant of *B. cereus*. Plasmids coding for the insecticidal toxin of *B. thuringiensis* have been transferred into *B. cereus* to make it a crystal producing variant of *B. thuringiensis* (Çetinkaya, 2002). Molecular methods, including genomic restriction digestion analysis and 16S rRNA sequence comparison, support that *B. thuringiensis, B. anthracis* and *B. cereus* are closely related species and they should be considered as a single species (Helgason *et al*., 2000).

2.2.2 Morphological Properties of *Bacillus thuringiensis*

Colony morphology can help to distinguish *B. thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough colonies, which spread out and can expand over the plate very quickly. *Bacillus thuringiensis* strains have unswollen and ellipsoidal spores that lie in the

subterminal position. The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *B. thuringiensis* from other closely related *Bacillus* species. The morphology, size, and number of parasporal inclusions may vary among *B. thuringiensis* strains. However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to *Cry* 1 proteins; cuboidal inclusions related to *Cry* 2 proteins and usually associated with bipyramidal crystals; amorphous and composite crystals releated to *Cry* 4 and Cyt proteins; and flat, square crystals, related to *Cry* 3 proteins. Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains (Çetinkaya, 2002).

There is a relationship between toxic activity and crystal shape, so that the observation of crystal morphology by phase contrast microscopy can provide important clues. For instance, Maeda *et al*. (2000), collected 22 isolates of *B. thuringiensis* from marine sediments in Japan. Two isolates of *B. thuringiensis* subsp. *kurstaki,* which are toxic to lepidopteran larvae formed typically bipyramidal inclusions, whereas isolate *higo*, which is toxic to mosquitoes, formed spherical crystals (Bernhard *et al*., 1997).

The observation of crystal morphology is the first step for establishing *B. thuringiensis* strain collections. Bernhard *et al*. (1997) isolated 5303 *B. thuringiensis* from 80 different countries and 2793 of them were classified according to their crystal shape. They reported that the proportion with bipyramidal shaped crystals was 45.9%, while 14 % were spherical and 4 % rectangular (Çetinkaya, 2002).

2.2.3 Natural Habitats and Prevalence of *Bacillus thuringiensis*

Bacillus thuringiensis occurs naturally and it can also be added to an ecosystem artificially to achieve insect control. For this reason, the prevalence of *B. thuringiensis* in nature can be defined

as “natural” and “artificial”. The habitat is considered as natural when *B. thuringiensis* can be isolated when there is no previous record of application of the organism for insect control. The artificial habitats of *B. thuringiensis* are areas sprayed with *B. thuringiensis* based insecticides (usually a mixture of spores and crystals) (Stahly *et al*., 1991).

Bacillus thuringiensis is indigenous to the soil, insect cadavers, phylloplanes of many plants and in freshwater. This shows that this organism is found among the predominant sporeformers in natural environments. The Coleopteran and Lepidopteran-active *B. thuringiensis* subspecies are mainly associated with soil and phylloplane, while the Dipteran active ones are mostly found in aquatic environments (Çinar, 2005).

B. thuringiensis has been isolated from marine sediments (Maeda *et al*., 2000), and also from the soils of Antarctica (Forsty and Logan, 2000). Thus, it is obvious that *B. thuringiensis* is widespread in nature even though the normal habitat of the organism is soil. The organism grows naturally as a saprophyte, feeding on dead-organic matter, therefore, the spores of *B. thuringiensis* persist in soil and vegetative growth occurs when nutrients are available. Because of this, *B. thuringiensis* can also be found in dead insects.

Although, it produces parasporal crystal inclusions that are toxic to many orders of insects, many *B. thuringiensis* strains obtained from diverse environments show no insecticidal activity. For example, Maeda *et al*. (2000) has found that *B. thuringiensis* strains obtained from marine environments of Japan exhibit no insecticidal activities.

2.3 Genetics of *Bacillus thuringiensis*

2.3.1. Genetic Diversity of *Bacillus thuringiensis*

The genetic diversity of *B. thuringiensis* arises from the presence of many different plasmids in each strain, conjugation transfer mechanism, and the transposon-like inverted repeats flanking the endotoxin genes, facilitating a high frequency of DNA rearrangements. Horizontal transfer of protoxin encoding-plasmids may lead to strains producing two different parasporal inclusions. In most species, the major protoxin gene is carried on a low copy number large plasmid (one plasmid per cell). The number and sizes of plasmids vary. There is a very broad range in size, from 4 to 100 MDa. Protoxin genes are often found in plasmids which are >30 MDa (Çinar, 2005).

The *B. thuringiensis* species have transposable elements, including insertion sequences and transposons. Insertion sequences (IS) are especially found in large plasmids and many of these sequences carry protoxin genes. Plasmids that do not include protoxin genes also play a role in the regulation of protoxin synthesis. Plasmids also enhance and provide supplementary growth factors when nutrients are limited. If protoxin gene is found on a transposable element, it can move into and out of the chromosome. Because of this movement, protoxin sequences may sometimes be present in the chromosome of some subspecies (Çinar, 2005).

The numbers of both large and small plasmids are between 2 and 11 in one cell (Gonzales *et al*. 1981, Lereclus *et al*., 1993). If plasmids are lost, it will be impossible to distinguish *B. thuringiensis* from *B. cereus* (Thorne, 1993; Crickmore *et al*., 1995).

2.3.2 *Bacillus thuringiensis* Genome

The genome of *B. thuringiensis* strains is about 2.4 to 5.7 Mb. Most *B.thuringiensis* isolates have several extra-chromosomal elements (plasmids) ranging in size from 2 to >200 kb. Some of these plasmids are circular and some are linear. The parasporal crystal proteins are generally encoded by large plasmids. Sequence hybridizing studies with *Cry* gene probes have been shown that *Cry* genes are also found in the bacterial chromosome. *Bacillus thuringiensis* also contains large variety of transposable elements. These transposable elements are thought to be involved in the amplification of the *Cry* genes in the bacterial cell (Çetinkaya, 2002).

Another possible role of these elements could be mediating the transfer of plasmid by a conduction process involving the formations of co-integrate structures between self conjugative plasmids and chromosomal DNA or non conjugative plasmids. The last function of these elements may be the horizontal dissemination of genetic material, including *Cry* genes, within *B. cereus* and *B. thuringiensis* species (Schnepf *et al*., 1998).

2.3.3 The *Cry* Genes

The genes coding for the insecticidal crystal proteins are normally associated with plasmid of large molecular mass. Many *Cry* protein genes have been cloned, sequenced, and named *Cry* and *cyt* genes. To date, over 100 *Cry* gene sequences have been organized into 32 groups and different subgroups on the basis of their nucleotide similarities and range of specificity. For example, the proteins toxic for lepidopteran insects belong to the *Cry* 1, *Cry* 9, and *Cry* 2 groups. The toxins against coleopteran insects are the *Cry* 3, *Cry* 7, and *Cry* 8 proteins and *Cry*1Ia1, which is a subgroup of *Cry* 11 proteins. The *Cry* 5, *Cry* 12, *Cry* 13 and *Cry* 14 proteins are nematocidal, and the *Cry* 2Aa1, which is a subgroup of *Cry* 2 proteins, *Cry* 4, *Cry* 10, *Cry* 11,

Cry 16, *Cry* 17, *Cry* 19, and Cyt proteins are toxic to dipteran insects (Zeigler, 1999; Çetinkaya, 2002).

Each of the *B. thuringiensis* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal proteins. Transfer of plasmids among *B. thuringiensis* strains is the main mechanism for generating diversity in toxin genes (Thomas *et al*., 2001).

2.3.4 *Cry* Gene Expression

The insecticidal crystal proteins are synthesized during the stationary phase of the bacterial life cycle growth. These proteins generally accumulate in the mother cell, accounting for up to 25% of the dry weight in sporulated cells of *B. thuringiensis*. The high level of crystal protein synthesis in *B. thuringiensis* is controlled by a variety of mechanisms. These mechanisms may occur at the transcriptional, post transcriptional and post-translational levels (Agaisse and Lereclus, 1995).

The expression of *Cry* gene is controlled by the sporulation-specific genes. However, some of *Cry* gene expression occurs during the vegetative growth. Thus, the expression of *Cry* gene mechanisms has been grouped in two groups; sporulation-dependent and sporulation-independent. The *Cry* 1Aa gene, encoding toxins active against lepidoptera, is a typical example of a sporulation-dependent *Cry* gene. This gene is only expressed during the sporulation phase. On the other hand, *Cry* 3Aa gene, isolated from the coleopteran-active *B. thuringiensis* var. *tenebrionis*, is expressed during the vegetative growth and also during the stationary phase. In the stationary phase, the expression of this gene has been found to be less than the vegetative phase (Çetinkaya, 2002).

The stability of *Cry* mRNA is an important contributor to high levels toxin production at the post-transcriptional level. The half-life of *Cry* mRNA is about 10 minutes, which is at least fivefold greater than the half-life of an average bacterial mRNA. The putative transcriptional terminator of the *Cry*1Aa (a stem loop structure) acts as a positive retroregulator. The fusion of a DNA fragment carrying this terminator to the 3′ end of the heterogenous genes increases the half-life of their transcripts by two to threefold. The stability of *Cry* mRNA is also increased by the 3′-stem loop structures. Three-fold structure reduces the movement of 3′-5′ exoribonucleases. For example, the *Cry* 1Aa transcriptional terminator sequence increases *Cry* mRNA stability by protecting it from exonucleolytic degradation at the 3' end (Schnepf *et al*., 1998; Çetinkaya, 2002).

The crystal proteins are generally found in the form of crystalline inclusion in the mother cell compartment. The crystal shape depends on the protoxin composition. This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. The factors, including the secondary structure of the protoxin, the energy of the disulphide bonds and the presence of additional *B. thuringiensis* specific components affect the structure and the solubility characteristics of *Cry* proteins (Schnepf *et al*., 1998).

2.4 *Bacillus thuringiensis* δ-endotoxin

Bacillus thuringiensis is an insecticidal bacterium producing toxins which are used commercially in pest control in the agricultural field. *Bacillus thuringiensis* strains produce two types of δ–endotoxin, namely *Cry* and Cyt proteins. Each insecticidal crystal protein is the product of a single gene. The genes responsible for the synthesis of these endotoxins are often located on large, transmissible plasmids. *Cry* and Cyt proteins differ structurally. The most important

feature of these proteins is their pathogenicity to insects and each crystal protein has its distinct host range (Çinar, 2005).

The number and type of δ-endotoxins produced determine the bioactivity of a *B. thuringiensis* strain (Kumar *et al*., 1996, Schnepf *et al*., 1998). Based on the amino acid homology, over 300 *Cry* genes have been classified into 47 groups and 22 *cyt* genes have been divided into two classes (Web_2, 2005).

2.4.1 The Crystal Proteins

The predominant type of δ-endotoxins is the *Cry* proteins, a crystal protein encoded by *Cry* genes. The accumulation of *Cry* protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells (Agaisse and Lereclus, 1995; Baum and Malvar, 1995).

Each Crystal protein has its own insecticidal spectrum. Therefore, *Cry* proteins have been classified on the basis of their host specificity and their amino acid compositions (Jensen *et al*., 2003). The crystal proteins have different forms such as bipyramidal (*Cry*1), cuboidal (*Cry*2), flat rectangular (*Cry*3A), irregular (*Cry*3B), spherical (*Cry*4A and *Cry*4B), and rhomboidal (*Cry*11A) (Schnepf *et al*., 1998). *Cry*1, *Cry*2, and *Cry*9 proteins show strongest toxicity to Lepidopterans (Crickmore, 2000). Proteins belonging to the class *Cry*4 and *Cry*11 are specifically toxic to Dipterans.

*Cry*3, *Cry*7, *Cry*8, *Cry*14, *Cry*18, *Cry*34, and *Cry*35 (Ellis *et al*., 2002, de Maagd *et al*., 2001) proteins show insecticidal activity against Coleopterans. Some *Cry* proteins on the other hand display toxicity to more than one insect order. For example, *Cry*1I is both active against Lepidopterans and Coleopterans (Tailor *et al*., 1992), whereas *Cry*1B shows toxicity against Lepidoptera, Coleoptera, and Diptera (Zhang *et al*., 2000).

2.4.2 The Cyt Proteins

Some *B. thuringiensis* strains also synthesize cytolytic proteins encoded by *cyt* genes in addition to *Cry* proteins. *Cyt* means a parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein (Crickmore *et al*., 1998). This class of δ-endotoxins differs in amino acid composition and action mechanism from *Cry* toxins (Butko *et al*., 1997). These toxins act synergistically with mosquitocidal *Cry* toxins (Poncet *et al*., 1994).

Cyt toxins differ from the *Cry* toxins; the protoxin mass of Cyt toxins (30 kDa) is smaller than the *Cry* toxins (Du *et al*. 1999). The Cyt toxins are only found in Diptheran specific strains, while the *Cry* toxins are present in many *B. thuringiensis* strains with wide host range. One Cyt toxin is found in a given *B. thuringiensis* strain, but two or more subclasses of *Cry* toxins can exist in a strain. Although both the activated forms of these toxins can lead to pores in lipid bilayers, only the Cyt toxins cause the cytolysis of various eukaryotic cells including erythrocytes (Gill *et al*., 1992). Cyt toxins may be used to overcome insecticide resistance and to increase the activity of microbial insecticides (Guerchicoff *et al*., 2001).

Cyt1 and Cyt2 are two cytolytic classes of Cyt toxins that have been identified on the basis of the amino acid identity and are divided into 22 subclasses (Web_2, 2005). Among these subclasses, Cyt1Aa and Cyt2Aa display the highest mosquitocidal activity (Koni and Ellar 1994). Cyt1A may be used as a practical tool to manage resistance against *B. sphaericus*, which is also a mosquitocidal bacterium. Also other Cyt proteins may increase the insecticidal activity of non-Cyt proteins to other insects (Wirth *et al*., 2000).

2.4.3 Insecticidal Spectrum of *Bacillus thuringiensis* δ-endotoxins

More than 3000 insect species included in 16 orders have been found to be susceptible to different crystal proteins (Lin and Xiong, 2004). Insecticidal crystal proteins are toxic to insects within the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga as well as non-insect organisms such as nematodes, mites, protozoa and platyhelminthes (Feitelson *et al.*, 1992; Çinar, 2005).

The toxicity is high against the insects belonging to the first three orders. Lepidopteran and Coleopteran insects are leaf-feeders with chewing mouthparts, whereas Dipterans feed by filtering water. These two different feeding behaviours provide the possible intake of *B. thuringiensis* spores /crystals (Çinar, 2005).

2.4.4 Mechanism of Action of δ – endotoxins

The crystal proteins of *B. thuringiensis* show host specificity. For this reason, each type of *Cry* protein can be toxic to one or more specific insect species. Because of this specific toxicity, they do not affect many beneficial insects, plants and animals including humans. The specificity of these insecticidal crystal proteins (ICPs) is derived from their mode of action (Adang, 1991; Gill *et al*., 1992).

The crystal protein of *B. thuringiensis* acts as a protoxins, for this protoxins to become active; a suspectible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, enzymes in midgut (proteases) convert the protoxins into active toxins. This active toxin then binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). This pore formation leads to

gut paralysis. Finally, insect larvae stop feeding and die from lethal septicemia (Adang, 1991; Gill *et al*., 1992).

The mode of action of Cytolytic toxins has not been fully determined. It has been suggested that these toxins could also be involved in colloid-osmotic lysis like *Cry* toxins but the formation of lesions in the cell membrane may be different (Crickmore *et al*., 1995; Butko *et al*., 1996; Butko *et al*., 1997). All Cyt toxins react directly with phospholipids without the need for a membrane protein receptor (Çinar, 2005).

Serine proteases such as chymotrypsin, thermolysin and elestase are important in both solubilization and activation of protoxins (Dai and Gill, 1993). Besides these digestive proteases, a novel DNase from an insect has been found to act synergistically with the crystal protein and to convert it to the active DNA-free toxin in the larval gut (Clairmont *et al*., 1998; Milne and Kaplan, 1993).

Spores are known to synergize the insecticidal activity of crystals when tested against insects. This may be related to the invasion of haemocele through the ulcerated midgut, and the subsequent development of septicemia (Çinar, 2005). The efficiency and potency of *Cry* toxins in insects control could be increased by the addition of enzyme chitinase in *B. thuringiensis* preparations. The chitinase acts on the peritrophic membrane which is composed of a network of chitin and proteins (Çinar, 2005). This enzyme hydrolyses the β-1,4 linkages in chitin so it may distrupt the peritrophic membrane by creating holes and facilitates the contact between δ-endotoxins and membrane receptors in the midgut epithelium (Regev *et al*., 1996). Some factors such as pH, enzymes, peritrophic membrane, enzyme detoxification, and antimicrobial characteristics of gastric juice of insect gut make insects resistant to the toxin (Davidson, 1992).

2.5 Other Pathogenic Factors of *Bacillus thuringiensis*

Aside the δ-endotoxin, certain strains of *B. thuringiensis* produce extracellular compounds during the active growth cycle, which might contribute to it virulence. These extracellular compounds include phospholipases, β-exotoxins, proteases, chitinases and vegetative insecticidal proteins (VIPs). *B. thuringiensis* also produces antibiotic compounds having antifungal activity. However, the *Cry* toxins are more effective than these extracellular compounds and allow the development of the bacteria in dead or weakened insect larvae (Schnepf *et al*., 1998; Çetinkaya, 2002).

Some strains of *B. thuringiensis* produce a low molecular weight, heat stable toxin called β-exotoxin, which has a nucleotide-like structure. Because of its nucleotide like structure it inhibits the activity of DNA-dependent RNA polymerase of both bacterial and mammalian cells. *B. thuringiensis* strains also produce a protease, which is called inhibitor A. This protein attacks and selectively destroys cecropins and attacins which are antibacterial proteins in insect resulting in calapse of the defense response of the insect. The protease activity is specific, because it attacks an open hydrophobic region near the C-terminus of the cecropin and it does not attacks the globular proteins (Çetinkaya, 2002).

Other important insecticidal proteins, unrelated to *Cry* proteins, are vegetative insecticidal proteins (VIPs). These proteins are produced by some strains of *B. thuringiensis* during vegetative growth. These VIPs do not form parasporal crystals and are secreted from the cell. For this reason, they are not included in the *Cry* protein nomenclature. For example, the VIP 1A gene encodes a 100 kDa protein which is processed from its N-terminus. This processing produces an 80 kDa product, which has been shown to be toxic to western corn root warp larvae (Schnepf, 1998; Çetinkaya, 2002).

2.6 Safety of *Bacillus thuringiensis*-Biopesticides

The use of *Bacillus thuringiensis*-based insecticides commercially in the control selected insect pests is on the increase. The use of *Bacillus thuringiensis*-based insecticides over the years has caused no adverse effects on human health or environment.

In the 1990s, the development of natural and recombinant *B. thuringiensis* products have broadened the insect host range in pest management programs. New formulations based on conventional or genetic engineering methods (encapsulation of the toxins and/or feeding stimulants to increase ingestion), screening of the interactions of *B. thuringiensis* with insect herbivores and plant allelochemicals or natural enemies of the pests to improve the formulation of biological control strategies, and information and management of insect resistance increased the uses of *B. thuringiensis* (Navon, 2000). Short persistence and complete biodegradability are other benefits of *B. thuringiensis* toxins (Bohorova *et al*., 1997, Copping, 1998).

Over synthetic pesticides, the advantages of this organism include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammalians, birds, amphibians and reptiles as well as its relatively low costs of development and registration (Flexner and Belnavis, 1999).

Recently, the gene(s) encoding the insecticidal proteins have been cloned and expressed in genetically modified plants to make them naturally resistant against harmful insects. Cotton, potato, sugarcane, tomato, peanut, and rice expressing *Cry* genes have been produced (Çinar, 2005).

2.7 Mosquitoes

Mosquitoes are the oldest human enemy and represent a significant threat to human health because of their ability to vector pathogens that cause diseases that afflict millions of people worldwide (WHO, 1998). Mosquitoes are rank first amongst the arthropod vectors of diseases because of their efficiency in the transmission of malaria, Brugian and Bancroftian filariasis, dengue, dengue haemorrhagic syndrome, yellow fever and several other arboviruses that take appalling toll of human lives (Adebote *et al.,* 2011).

Mosquito have four distinct stages in their life cycle: egg, larva, pupa and adult. Depending on the specie a female lays between 30 and 300 eggs at a time on the surface of the water, singly (*Anopheles*), in floating rafts (*Culex*) or just above the water line or on wet mud (*Aedes*). Once hatched the larvae grow in four different stages (instars). The first instar measures 1.5 mm in length, the fourth instar about 8-10 mm. The fully grown larvae then changes into a comma shaped pupa. When mature, the pupal skin splits at one end and a fully developed adult emerges. The entire period from egg to adult takes about 7-13 days under good conditions (Adebote *et al.,* 2011).

Eliminating the source of infection is an essential component for the control of mosquito borne diseases. During the past several decades, many synthetic organic insecticides have been developed and effectively used to eliminate mosquitoes. Unfortunately, the management of this disease vector by using synthetic insecticides has failed in part because the continuous and indiscriminate use of conventional chemical insecticides has resulted in the development of physiological resistance (Mabaso *et al.,* 2004). In addition, there are long-term harmful effects on non-target organisms and the environment.

Most of the mosquito control programmes target the larval stage of the mosquitoes in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (Adebote *et al*., 2011). Personal protective measures, including repellents and larvicides are widely used to prevent the transmission of arthropod-borne-diseases by minimizing the contact between humans and vectors. In contrast to vaccines and chemoprophylaxis as means of personal protection, repellents and larvicides are convenient, inexpensive, and offer advantages in protection against a wide range of vectors (WHO, 1998). They are also the primary means of mosquito-borne disease prevention available in areas where vector control is not practical.

2.7.1 Economic Importance of mosquitoes

Mosquitoes are important vectors of several tropical diseases as they suck blood from human and animals. They are vectors of multiple of diseases of man through transmision of pathogenic viruses, bacteria, protozoa and nematodes (Priest, 1992). From the medical point of view, mosquitoes are among the most important insects due to their capacity to transmit human diseases such as malaria and dengue. Mosquitoes are important vectors of various diseases of economic importance in humans causing nuisance, local skin and systematic reaction (Govindarajan, 2010).

*Cx. quinquefasciatus*is an important vector of periodic filariasis caused by *Wuchereria bancrofti* a disease which is a major health challenge (WHO, 1996) and other diseases like Western equine encephalomyelitis and St. Louis encephalitis have been isolated from this species and it has been implicated as a vector of dog heart worm, Lymphatic filariasis, Japanese encephalitis, other viral diseases.

Aedes vittatus (Rock pool mosquito) and *Aedes aegypti* (Yellow fever mosquito) constitute another biting nuisance and diseases like chikungunya virus (CHIKV) and yellow fever virus (YFV) have been isolated from these species. *Aedes* species are the vector diseases such as Yellow fever, dengue, dengue hemorrhagic fever, other viral diseases and lymphatic filariasis. Mosquitoes transmit more diseases, compared to other arthropod group (Ghosh *et al*., 2012).

2.7.2 *Aedes aegypti*

Aedes aegypti (Linnaeus) belongs to the family Culicidae and the order Diptera. It is the dengue fever vector. The female *A. aegypti* preferably lay eggs in artificial collections of water. The hatched larvae undergo growth and metamorphosis. In their life cycle, four larval stages and the pupal stage are aquatic and their adults are aerial. Growth changes in form and size occur during their larval development. The first instar *A. aegypti* larva is only about 1 mm length, whereas in the fourth instar stage it reaches a length of approximately 8 mm (Schaper and Hernandez-Chavarria, 2006).

On shedding the IV instar larval cuticle, pupa emerges with most of the adult organs and after the pupal moult a complete mosquito appears. Identification of *A. aegypti* larvae, pupae and adults by their morphologic features immediately after collection is of considerable value in recognizing vector prevalence. Most of the identification keys of the *A. aegypti* are based exclusively on the adult characteristics and on the 4th instar larvae whereas I, II and III instar larvae are also available in samples which need identification. In larval collections of *Aedes aegypti*, various other species of larvae (eg. *A. albopictus, A. vittatus*) also co-exist (Choudhury, 1997). It becomes very difficult to identify all species of mosquito larvae in the samples because taxonomic keys are on all the larval instars are not included (Bar and Andrew, 2013).

The morphology of *A. aegypti* larval body parts of head, neck, thorax and abdomen like mouth brush, palatum, preclypeal spines, mentum, compound eye, antenna, comb spines, siphon tube, pecten teeth and anal papilla were described by various researchers. In Ist instar stage *A. aegypti* larval head is narrow and triangular. In later stage, in the head capsule, convexity appears. The head becomes large and attain globular shape (Bar and Andrew, 2013).

Klingenberg and Zimmermann (1992) applied Dyar's rule on the head width of water striders, *Gerris* and *Aqzlarius* (Heteroptera: Gerridae) and found that the data followed the rule in some areas. The growth ratio differed between moults. Mohammadi *et al.* (2010) used Dyar's rule on the size of the sclerotized body parts of different larval instars of cotton bollworm, *Helicoverpa armigera* and found that the ratio of the size of the sclerotized body parts were in a constant range. Ghafoor (2011) applied Dyar's rule on the width of head capsule of larval instars of *Agrotis ipsilon* and found that the head capsule width was 0.28 mm in I instar and 3.42 mm instar VI. In this study, Dyar's rule was used on the width of head capsule, neck, thorax and abdomen.

The antennae are smooth and cylindrical in shape. It has a single hair (Bar and Andrew, 2013). In mosquito larval head a pair of large compound eyes are present below the antennae on the lateral side. *A. aegypt*i larvae are found in different aquatic habitats mainly in small water collections. Various environmental factors like temperature, salinity, pH, dissolved nutrients and gases in the aquatic habitat influence the growth of mosquito larvae. Extremes of temperature, lack of food (Bar and Andrew, 2013) and increased salinity (Clark *et al.*, 2004) result in reduced *A. aegypti* larval growth and delayed development. Variations in the larval, pupal and adult morphology were also described by different authors. Various larval forms like sensu strictu, the type form, *formosus* (walker) and *queenslandensis* (Theobald) were reported within *A. aegypti* (Bar and Andrew, 2013).

2.7.3 *Culex quinquefasciatus*

Culex quinquefasciatus is one of the medically important species of mosquito that has been implicated in the transmission of several diseases amongst human and animal populations. In particular, the species has gained notoriety as the dominant culicid constituting biting nuisance to humans in several urban centers in Nigeria, where it breeds predominantly in foul aquatic environments (Adebote *et al*., 2011).

Culex quinquefasciatus has an important role in the spread of diseases worldwide and this species is the major vector of lymphatic filariasis which remains an endemic disease in some urban areas. Generally, the status of *Culex sp.* as a disease vector has greatly increased in recent years in the spread of the West Nile virus in the Americas. However, field trials have proved its effectiveness for reducing population density in areas where *Culex* is a source of nuisance or vector of diseases (Hougard *et al.,* 1993; Kumar *et al.,* 1996; Regis *et al.,* 2000).

Recently, the bacterial mosquito larvicides, *Bacillus thuringiensis var. israelensis* and *Bacillus sphaericus*, are identified as alternate tools and are being used for effective control of vector mosquitoes of filariasis (Gunasekaran *et al.,* 2000; Lee and Zairi, 2005; Medeiros *et al.,* 2005). It has been proved to be very effective against *Cx. quinquefasciatus,* the vector of bancroftian filariasis, breeding in habitats prevalent in urban and peri-urban areas (Gunasekaran *et al.,* 2000; Medeiros *et al.,* 2005; Mwangangi *et al.,* 2011).

After its application, the spores and crystals of the bacterium are ingested by mosquito larvae present in the breeding habitats and are eventually killed by the action of the crystal toxins. Apart from the larvicidal effect, reduced infection and infectivity of *Wucheriria bancrofti* filarial parasite is reported in *Cx. quinquefasciatus* that emerged from natural breeding habitats treated

with B. sphaericus (Gunasekaran *et al.,* 2000). There are some previous reports that crystal proteins of *B. sphaericus* and *B. thuringiensis* are toxic to parasitic nematodes (Kotze *et al.,* 2005). Besides, anti-parasitic molecules have been reported to be upregulated in mosquitoes, especially in *Cx. quinquefasciatus*, after infection with filarial parasite (Paily *et al.,* 2007).

2.8 Need for eco-friendly approach in the control of mosquitoes

Diseases caused by mosquito are prevalent in more than 100 countries across the world, with a global morbidity burden of over 700 million people annually .The approach most commonly used to control mosquito vectors is the application of insecticides (Ghosh *et al.,* 2012). Different strategies have been devised to reduce the prevalence of insect-borne disease, but these strategies have their limitations. One of such strategies is the use of synthetic insecticides which has suffered from major disadvantages of environmental pollution and physiological resistance by vectors. These limitations have created the need for environmentally safe, degradable and target-specific insecticides against these insect vectors. The search for such compounds has been directed extensively to the plant kingdom.

The misuse of insecticides in agriculture and public health program results in many health and environment related problems like insecticides resistance, resurgence of pest species toxic hazards to human and non-target organisms and environmental pollution (Junwei *et al*., 2006). In other to overcome these hazards, researchers should innovate biological or plant based products that can provide an effective alternative approach to synthetic insecticides without side effects.

There is a need to provide alternative environmentally safe, degradable and target-specific insecticides against insect vectors. Biological control becomes the most eco-friendly alternative,

therefore it has become the central focus of the control programme in lieu of the chemical insecticides.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

Samples of organic matter-rich soil were collected from various site in Zaria namely Agricultural field, Refuse dump site and Cow rangeland. From each location, samples of about l0g were collected from five spots. Samples were taken from one inch below the surface by scrapping off surface material with a spatula. The samples were brought to the Environmental laboratory of Microbiology department and stored at 4^{o}C until analyzed.

3.2 Isolation Procedure

Bacillus thurigiensis were isolated according to the method described by Travers *et al.* (1987). For each sample, 0.5 g soil was added in 10 ml of Luria Bertani (LB) medium (Tryptone 10g /L, yeast extract 5g/L, NaCl 5g/L) to which 0.25M sodium acetate was added and incubated in shaking incubator at 30°C and 250 rpm for 4 hours. From each sample, 2ml was taken and heat shocked in a water bath at 80°C for 20 minutes. Serial dilutions of treated samples were prepared and spread on T3 agar ((Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, $MnCl_2$ 0.005g/L, Sodium phosphate 0.05M and Agar15g/L) and incubated for 2days at 26°C. Colonies with *B. thuringiensis* like morphology (entire margin, off white color, dry and rich growth of colony) were picked up at random and purified by streaking them LB agar plates (Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, $MnCl_2$ 0.005g/L, NaH_2PO4 6.9g/L, Na_2HPO4 8.9g/L and Agar15g/L).

3.3 Microscopic Characterization of *Bacillus thuringiensis*

The suspected isolates of *Bacillus thuringiensis* were identified by following the diagnostic plan specified in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The isolate were characterized using Gram reaction and endospore staining, motility, growth above 45 °C, catalase activity, Voges Proskauer test and Microgen^{TM} Bacillus-ID.

3.3.1 Gram staining

Using a sterile cooled loop, a loopful of distilled water was dropped on a slide and a minute amount of the colony was aseptically transferred from the Petri dish and smeared to form a thin layer on the slide then allowed to air dry. The smear was then heat-fixed by passing the slide through an open flame. The slide was then stained with crystal-violet for 1 minute and rinsed with distilled water. The slides was then stained with Gram's iodine (1% iodine, 2% potassium iodide in water) for 1 minute to fix the dye and then rinsed with distilled water. The stain was then decolourized with 95% ethanol and then rinsed with distilled water. The smear was then counterstained with Safranin for 1 minute, rinsed with water and then air dried. The stained slides were then viewed using light microscopy under oil immersion. *Bacillus thuringiensis* appears as dark purple bacilli (Cowan and Steel, 2003).

3.3.2 Endospore stain (Schaeffer-Fulton staining method)

Using a sterile cooled loop, a loopful of distilled water was dropped on a slide and a minute amount of the colony was aseptically transferred from the Petri dish and smeared to form a thin layer on the slide then allowed to air dry. Once dried the slides were flooded with Malachite green (made by dissolving 5.0g in distilled water, made up to 100ml) and immediately steamed over a water bath for 5 minutes. The slides were rinsed with sterile water. The slides was then

counterstained with Safranin O (made by dissolving 0.5 grams Safranin O powder in distilled water, made up to 100ml) for 2 minutes and then rinsed with sterile water. Once dried, the slides were viewed under the microscope with oil immersion. The isolates having visible parasporal crystals next to the spore in the sporangium cells were identified as *Bacillus thuringiensis.* The endospores stains green with malachite green while the vegetative tissue stains pink with safranin. (Holt *et al.,* 1994).

3.4 Biochemical Characterization of *Bacillus thuringiensis*

The isolates were characterized biochemically using the following biochemical tests:

3.4.1 Motility Testing

The test organisms were stab-inoculated in tubes of motility medium to a depth of about 5 mm. The tubes were then incubated at the optimum growth temperature (37^0C). Motile organisms migrate throughout the medium, which becomes turbid; growth of non-motile organisms is confined to the stab inoculums (Cowan and Steel, 2003). Isolates that were motile were assumed to be *Bacillus thuringiensis.*

3.4.2 Growth above 45 °C

Isolates were incubated in nutrient broth at a temperature exceeding 45°C for a 5 day period. Cultures that showed signs of growth and thus were capable of reproducing at such high temperatures were assumed not to be *Bacillus thuringiensis* and were eliminated as putative *Bacillus thuringiensis* isolates (Cowan and Steel, 2003).

3.4.3 Catalase test

The test involves adding 3% hydrogen peroxide to each sample of *Bacillus thuringiensis.* A small sample of 24 hours cultures of the isolates were smeared onto a clean slide. A drop of 3% hydrogen peroxide was added onto the bacterial wet mount preparation and observed for effervescence. The presence of bubbles indicates the ability of the isolate to break down hydrogen peroxide into water and oxygen (Cowan and Steel, 2003).

3.4.4 Oxidase Test

This test was performed by adding 1 -2 drops of Oxidase Reagent directly to colonies from a culture plate on filter paper. A positive reaction is a purple color change occurring within 30 seconds. Oxidase- positive colonies typically take 10 seconds to produce a positive color reaction; reactions occurring between 30 and 60 seconds were classified as a delayed positive and retested. A negative result is no color change after 1 minute or a color change that occurs after 1 minute. (Cowan and Steel, 2003).

3.4.5 Voges-Proskauer and Methyl Red Tests

Voges-Proskauer (VP) is used to identify organisms that are able to produce neutral end-products such as acetyl-methyl-carbinol and 2,3-butanediol from the degradation of glucose during 2,3-butanediol fermentation. Acetyl-methyl-carbinol is converted into diacetyl through the action of potassium hydroxide and atmospheric oxygen.

This test was carried out by inoculating 5ml of MR-VP broth with the suspected *Bacillus thuringiensis* isolates and incubated at 35^0C for 48-72 hrs. After 24 or 48 hrs of incubation, about 1ml of the cultured broth was transferred to a small test tube to which 2-3 drops of methyl red

indicator was added. Formation of red colour on addition of the indicator signifies a positive methyl red test and a yellow colour signifies a negative test.

To the rest of the broth, 2 drops of 40% potassium hydroxide was added followed by 2 drops of 5% α Naphthol in ethanol. The tube was shaken and placed in a slope. Development of a red colour starting from the liquid – air interface within 1 hour indicates a VP positive test. No colour change indicates VP negative test (Cowan and Steel, 2003).

3.4.6 Esculin Hydrolysis

The suspected isolates were inoculated on bile esculin agar and incubated at 37^{o}C for 24hrs. A positive test was indicated by a dark brown to black colouration of the whole medium after 24hrs incubation. A negative reaction is indicated by lack of colour change throughout the medium (Cowan and Steel, 2003).

3.5 Confirmation of Isolates Using Microgen^{TM} Bacillus-ID

Bacillus thuringiensis isolates were confirmed using Microgen^{TM} Bacillus-ID (MID-66) according to the manufacturer's instructions. Each Microgen Bacillus-ID test consists of the following 24 biochemical reactions: fermentation of Arabinose, Cellobiose, Inositol, Mannitol, Mannose, Raffinose, Rhamnose, Salicin, Sorbitol, Sucrose, Trehalose, Xylose, Adonitol, Galactose, Methyl-D-Mannoside, Methyl-D-Glucoside, Inulin and Melezitose, Indole test, ONPG Hydrolysis, Nitrate, Arginine Dihydrolase, Citrate Utilisation, Voges Proskauer.

3.6 Molecular Characterization of *Bacillus thuringiensis* strains Isolated

3.6.1 DNA extraction

PCR was done using crude DNA. Overnight culture of *B. thuringiensis* LB broth was transferred into Eppendorf tubes and centrifuged at 1000rpm for one minute. The supernatant was discarded and the pellet was resuspended in 50µl of lysis buffer, vortex mixtured and incubated at 37^0C overnight. The resulting cell lysate was briefly centrifuged for 10 seconds at 10,000 rpm and then supernatant was used for the Polymarse Chain Reaction.

3.6.2 Detection of cry genes by PCR

PCR reactions were carried out as described by Khojand *et al.* (2013). The reaction was done in 20 µl reaction mixture containing 5 µl template DNA, 150 mM dNTPs, 20 pM of each of the 4 primers (Table 3.1) and 0.5U of *Taq* DNA polymerase. Amplification was carried out in a DNA thermocycler with the program: one denaturing cycle at 94ºC for 4 minutes, 35 cycles (94ºC for 45 seconds, annealing at 48-55ºC for 45 seconds and 1 minute at 72ºC). The *cry* gene bands were visualized through agarose gel electrophoresis. The gels were stained with ethidium bromide and documented with a 100bp molecular weight marker.

Table 3.1: Primer sets used in the PCR assay

Cry genes	Primer Sequence (5'-3')	product size (bp)	Reference
*Cry*2	F GTTATTCTTAATGCAGATGAATGGG R CGGATAAAATAATCTGGGAAATAGT	689	Khojand *et al.* (2013)
*Cry*4	F GCATATGATGTAGCGAAACAAGCC R GCGTGACATACCCATTTCCAGGTCC	440	Khojand *et al.* (2013)

F = Forward primer

R = Reverse primer

3.7 Preparation of Spore Crystal Mixture

To obtain the spore-crystal mixtures, single colonies from overnight LB plates were inoculated into 10ml T3 sporulation medium (per litre 3g of tryptone, 2g of tryptose, 1.5g of yeast extract,0.05m sodium phosphate (pH 6.8) and 0.005g $MnCl_2$) and cultured for 60 hours in a shaker incubator at 30^0C. Spores and crystals were harvested by centrifugation at 7000g for 10 minutes and washed twice with sterilized distilled water, the pellet spore crystal mixture were stored at low temperature until required (Carrozi *et al*., 1991; Gorashi *et al*., 2012).

3.8 Raising and Collection of Mosquito Larva

The third instar larvae (L3) of *Aedes aegypti* were collected from discarded containers and stagnant water within the main campus of Ahmadu Bello University. As for *Culex quinquefasciatus,* blood fed adult female were trapped with test tubes and introduced into entomological cages containing bowls of distilled water in the laboratory to facilitate oviposition.

Eclosed larvae from the eggs were nurtured on a diet of biscuit and bakers yeast (Ratio of 3:1) until they moult into the third instar larvae. The third instar larvae were used for bioassay (Adebote *et al*., 2011).

3.8.1 Larval selection and counting

Early third instar (L3) Larvae of *Aedes aegypti* and *Culex quinquefasciatus,* used in the bioassay were selected using a modified pasteur pipette and placed into test tubes. Ten early L3 larvae were counted into each test tube and the volume was made up to 10 ml using distilled water. Twenty four tube tubes per *Bacillus thuringiensis* isolate i.e. 12 for *Aedes aegypti* (3 different concentrations and 1 control all in triplicate) and another 12 *Culex quinquefasciatus* (3 different concentrations and 1 control all in triplicate).

3.9 Determination of the Larvicidal Activity of *Bacillus thuringiensis* Against *Aedes aegypti* and *Culex quinquefasciatus* Larvae.

The mosquitocidal assay was performed using three concentrations of spores and crystal mixture (100ppm, 75ppm and 50ppm). Each concentration was replicated three times and one test tube with 10 (L3) larvae was used as the control. Mortality of the treated larvae were recorded after 24 hours (Naiema *et al.,* 2012).

3.10 Data Analysis

Results were presented in tables, graphs and charts where applicable. Using probit analysis, the mean lethal concentration, LC_{50} was determined, a graph of empirical Probit of mortality was plotted against the logarithm of concentration used and a regression equation was obtained for each of the isolates used. ANOVA was also used to compare the mean mortaility of the isolates at different concentrations of spore crystal mixture of each of the isolates.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *Bacillus thuringiensis* from Different Soil Types

Table 4.1 shows the result of the colonial morphology, microscopic and biochemical characterization of the isolates showing characteristics of *Bacillus thuringiensis* colonies on T3 agar. Colonies that appear off white, dry and with entire margin on T3 agar were regarded as suspected *Bacillus thuringiensis*. Isolates with this colonial morphology were then characterized biochemically.

Table 4.2 shows the frequency and percentage of isolates with the desired colonial morphology, microscopic and biochemical characterization. Table 4.3 shows the result of confirmation of *Bacillus thuringiensis* isolates using Microgen^{TM} Bacillus-ID system kit. Table 4.4 shows the distribution of *Bacillus thuringiensis* isolates in soil from different soil type namely agricultural soil, refuse dump site and cow rangeland. A total of 30 soil samples were collected, 10 from each of the three different soil types. Five isolates of *Bacillus thuringiensis* were gotten from agricultural soil, 2 from refuse dump site and 5 from cow range land giving a total of 12 isolates.

4.2 Detection of *Cry* Genes by PCR

The 12 isolates identified using cultural, microscopic and biochemical characterization were screen for the presence of diptera active *cry* genes namely *cry* 2 and *cry* 4 using specific primers by PCR. Plate I showed result of the polymerase chain reaction using the primer for *Cry 2* with an amplicon size of 700bp. Two of the isolates show bands corresponding to 700bp. Plate II shows result of the polymerase chain reaction using the primer for *Cry 4* with an amplicon size of 450bp. Five of the isolates show bands corresponding to 450bp.

Table 4. 1: Colonial morphology, microscopic and biochemical characterization of the isolates that showed characteristics of *Bacillus thuringiensis* colonies on T3 agar.

	Isolates																		
	A1	B1	C2	D1	D2	E2	F2	G1	N1	N2	N3	N4	L1	L2	L3	L4	L5	L6	L7
*Colonial morphology on T3 agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gram reaction	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape of cells	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Chains of cells	+	NA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endospore stain	+	NA	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+
Motility	+	NA	+	+	+	+	+	NA	+	+	+	NA	+	+	+	NA	NA	+	+
Growth at 45^0C	-	NA	-	-	-	-	+	NA	+	-	-	NA	-	-	-	NA	NA	-	-
Catalase	+	NA	+	+	+	+	NA	NA	NA	+	+	NA	+	+	+	NA	NA	+	+
Oxidase	+	NA	+	+	+	+	NA	NA	NA	+	+	NA	+	+	+	NA	NA	+	+
Methyl Red	-	NA	-	-	-	-	NA	NA	NA	-	-	NA	-	-	-	NA	NA	-	-
Voges Proskauer	+	NA	+	+	+	+	NA	NA	NA	+	+	NA	+	+	+	NA	NA	+	+
Esculin hydrolysis	+	NA	+	+	+	+	NA	NA	NA	+	+	NA	+	+	+	NA	NA	+	+
Remark	Bt	NBt	Bt	Bt	Bt	Bt	NBt	NBt	NBt	Bt	Bt	NBt	Bt	Bt	Bt	NBt	NBt	Bt	Bt

* = colonies that appear off white, dry and with entire margin on T3 agar were regarded as suspected *Bacillus thuringiensis*.

+ = positive reaction. **-** = negative reaction. NA = not applicable. R = rod.

Bt = *Bacillus thuringiensis.* NBt = Not *Bacillus thuringiensis.*

Table 4. 2: Frequency and percentage of isolates with the desired colonial morphology, microscopic and biochemical characterization.

Test	Result expected for *Bacillus thuringiensis*	Number of isolates tested	Number with the desired result (percentage)
*Colonial morphology on T3 agar	Off white dry colonies with entire margin	30	19(63.33%)
Gram reaction	Gram positive	19	18(94.74)
Shape of cells	Rod shaped	19	19(100.00%)
Chains of cells	Rods in chain	18	18(100.00%)
Endospore stain	Green spores with pink vegetative tissue	18	14(77.78%)
Motility	Motile organism	14	14(100.00%)
Growth at 45^0C	No growth at 45^0C	14	12(85.71%)
Catalase	Positive reaction (presence of bubbles)	12	12(100.00%)
Oxidase	Positive reaction (purple color change)	12	12(100.00%)
Methyl Red	Negative reaction (No Colour change)	12	12(100.00%)
Voges Proskauer	Positive reaction (red colour)	12	12(100.00%)
Esculin hydrolysis	Positive reaction (Black colouration)	12	12(100.00%)

Table 4. 3: Identification of *Bacillus thuringiensis* isolates using MicrogenTM Bacillus-ID system kit.

Isolates	Octal code	*Final identification	Percentage probability
A1	00360013	*Bacillus cereus* group	59.68%
C2	00263013	*Bacillus cereus* group	90.00%
D1	00261013	*Bacillus cereus* group	91.69%
E2	70261013	*Bacillus cereus* group	97.67%
D2	40260013	*Bacillus cereus* group	63.57%
N2	00560013	*Bacillus cereus* group	93.08%
N3	00160013	*Bacillus cereus* group	95.74%
L1	00263013	*Bacillus cereus* group	90.00%
L2	00760013	*Bacillus cereus* group	58.78%
L3	00360013	*Bacillus cereus* group	59.68%
L6	70261013	*Bacillus cereus* group	97.67%
L7	00261013	*Bacillus cereus* group	91.69%

* *B. cereus* group consists of *B. cereus, B. thuringiensis, B. mycoides* and *B. weihenstephanensis.* On the basis of routinely employed biochemical tests, these species are indistinguishable. But on the basis of motility test and use of specific media, this species are distinguishable.

Table 4. 4: Occurrence of *Bacillus thuringiensis* in soil from various sources.

Sources of Soil samples	Number of samples Analysed	Frequency of occurrence of *Bacillus thuringiensis* N (%)
Agricultural field	10	5 (50)
Refuse dump site	10	2 (20)
Cow rangeland	10	5 (50)
Total	30	12 (40)

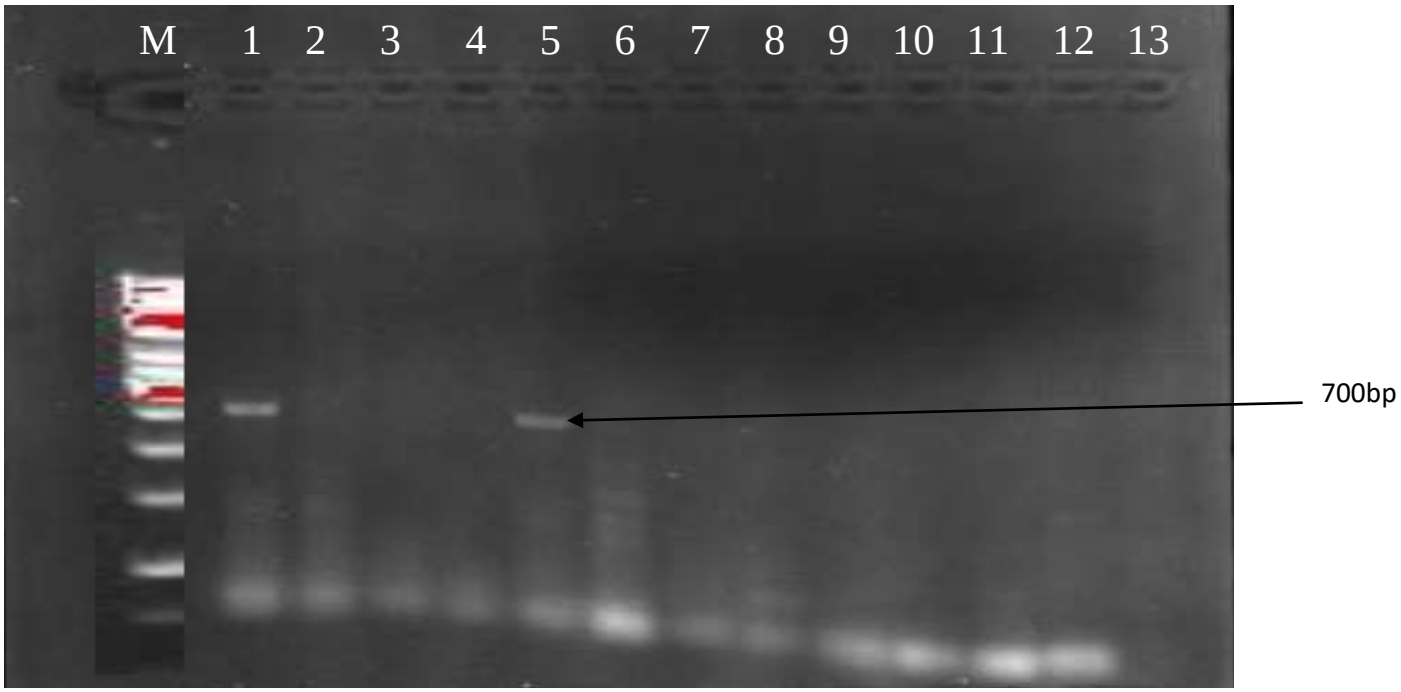

Plate I: Agarose gel electrophoresis of PCR products obtained with the primers for *cry2* genes in the *B. thuringiensis* isolates (M is Marker (100 bp DNA ladder) and lanes 1-13 PCR amplicons).

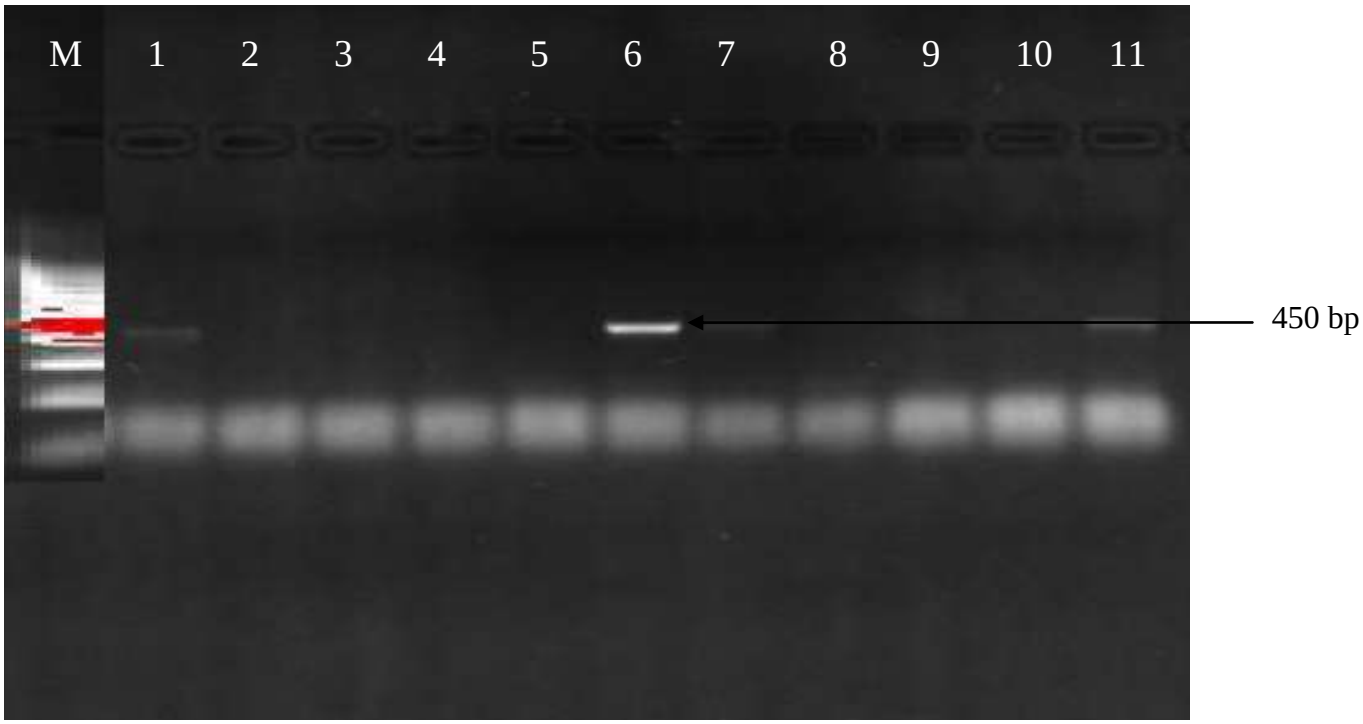

Plate II: Agarose gel electrophoresis of PCR products obtained with the primers for *cry4* genes in the *B. thuringiensis* isolates (M is Marker (100 bp DNA ladder) and lanes 1-11 PCR amplicons).

4.3 Distribution of the *Cry* genes among the isolates.

The PCR result shows that some of the isolates harbour none of the *cry* gene screened for. One of the isolates harbour the two genes while 5 others harbour at least one of the two genes. Table 4.5 shows the distribution of the *Cry* genes among the 12 isolates of *Bacillus thuringiensis*.

4.4 Frequency of occurrence (%) of the *Cry* genes among the 12 *Bacillus thuringiensis* isolates.

The frequency of occurrence (%) of the two genes is shown in figure 4.1. *Cry* 4 was present in 34% of the isolates, 8% of the isolates harbour *Cry* 2, 50% of the isolates harbour none of the genes while both genes were found in 8% of the isolates.

4.5 Bioassay with *Culex quinquefasciatus* larvae.

Isolate L3 shows the highest larvicidal activity (96.67% mortality) at 100ppm concentration of spore crystal mixture while isolate D2 shows the lowest larvicidal activity (33.33) against *Culex quinquefasciatus* larvae. The differences observed between the mean mortality of the isolates at different concentration of the spore crystal mixture for *Culex quinquefasciatus* were statistically significant.

Figure 4.2 shows the larvicidal activity of the isolates against *Culex quinquefasciatus* larvae to different concentrations (100ppm, 70ppm and 50ppm) of *Bacillus thuringiensis* spore crystal mixture of the 16 isolates after 24 hours of exposure.

Table 4. 5: Distribution of the *Cry* genes among the 12 *Bacillus thuringiensis* isolates from different soil sites.

Isolates	*Cry 2*	*Cry4*
L3	+	+
N3	-	-
L2	-	-
D2	-	-
N2	+	-
L7	-	+
L1	-	+
L6	-	-
A1	-	+
C2	-	-
D1	-	+
E2	-	-

+ mean the *cry* gene is present

- mean the *cry* gene is absent

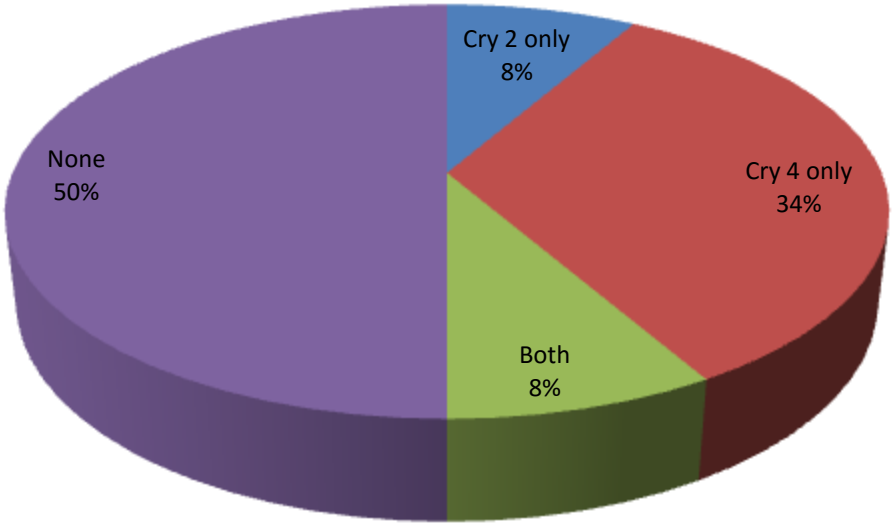

Figure 4. 1: Frequency of occurrence the *Cry* genes among the 12 *Bacillus thuringiensis* isolates from different soil sites.

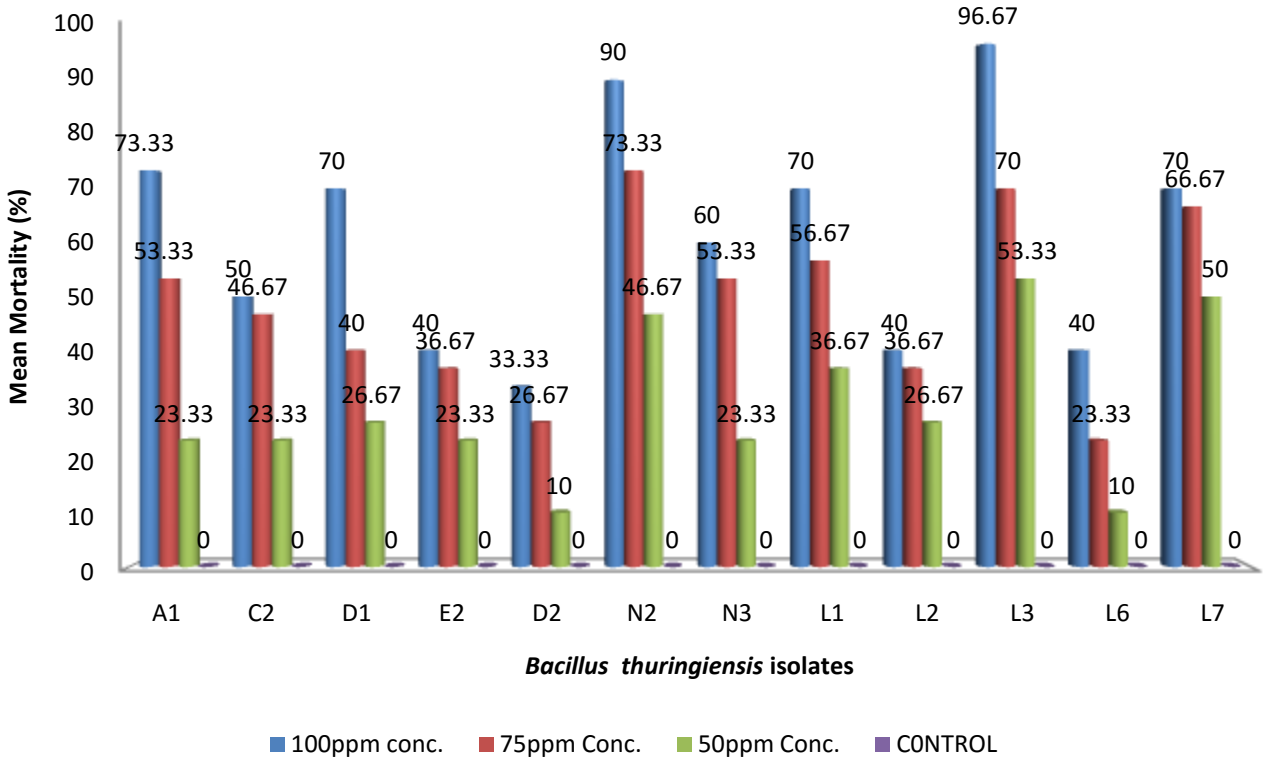

Figure 4. 2: Mean mortality of *Culex quinquefasciatus* larvae to different concentrations of *Bacillus thuringiensis* spore crystal mixture after 24 hours of exposure.

Table 4. 6: Mortality of *Culex quinquefasciatus* larvae on exposure to various concentrations of twelve isolates of *Bacillus thuringiensis.*

Isolates	Conc. (ppm)	No. of larvae		Mean mortality(95% CI ±SE)	ANOVA	
		Exposed*	Dead		F-Value	P-Value
A1	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	124.422	$4.73x10^{-7}$
	50	30	7	2.33^{c} (0.89-3.77±0.34)		
	75	30	16	5.33^{b} (3.88-6.77±0.34)		
	100	30	22	7.33^{a} (5.88-8.77±0.34)		
C2	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	24.4278	$2.216x10^{-4}$
	50	30	7	2.33^{b} (-0.53-5.18±0.66)		
	75	30	14	4.67^{a} (3.22-6.11±0.34)		
	100	30	15	5.00^{a} (2.51-7.48±0.00)		
D1	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	32.523	$7.865x10^{-5}$
	50	30	8	2.67^{b} (1.22-4.11±0.34)		
	75	30	12	4.00^{ab} (1.51-6.48±0.58)		
	100	30	21	7.00^{a} (7.00-7.00±0.00)		
E2	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	111.789	$7.189x10^{-7}$
	50	30	7	2.33^{c} (0.88-3.77±0.34)		
	75	30	11	3.67^{b} (2.22-5.11±0.34)		
	100	30	12	4.00^{a} (1.51-6.48±0.34)		

D2	0 (Control)	30	0	0.00^{c} (0.00-0.00±0.00)	16.801	$8.174x10^{-4}$
	50	30	3	1.00^{b} (1.00-1.00±0.00)		
	75	30	8	2.67^{a} (1.22-4.11±0.34)		
	100	30	10	3.33^{a} (0.47-6.18±0.66)		
N2	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	110.683	$7.467x10^{-7}$
	50	30	14	4.67^{b} (3.22-6.11±0.34)		
	75	30	22	7.33^{a} (5.88-8.77±0.34)		
	100	30	27	9.00^{a} (6.51-11.48±0.58)		
N3	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	207.152	$6.388x10^{-8}$
	50	30	7	2.33^{c} (0.88-3.77±0.34)		
	75	30	16	5.33^{b} (3.88-6.77±0.34)		
	100	30	18	6.00^{a} (1.70-10.29±1.00)		
L1	0(Control)	30	0	0.00^{b} (0.00-0.00±0.00)	25.7684	$1.830x10^{-4}$
	50	30	11	3.67^{a} (2.22-5.11±0.34)		
	75	30	17	5.67^{a} (2.81-8.52±0.66)		
	100	30	21	7.00^{a} (2.70-11.29±1.00)		
L2	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	27.3988	$1.467x10^{-4}$
	50	30	8	2.67^{b} (1.22-4.11±0.34)		
	75	30	11	3.67^{b} (2.22-5.11±0.34)		
	100	30	12	4.00^{a} (1.51-6.48±0.58)		
L3	0(Control)	30	1	0.00^{b} (0.00-0.00±0.00)	76.3103	$3.155x10^{-6}$
	50	30	16	5.33^{a} (2.47-8.18±0.66)		

	75	30	21	7.00^{a} (7.00-7.00±0.00)		
	100	30	29	9.67^{a} (8.22-11.11±0.34)		
L6	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	15.4026	$1.096x10^{-3}$
	50	30	3	1.00^{bc} (-1.48-3.48±0.58)		
	75	30	7	2.33^{ab} (0.88-3.77±0.34)		
	100	30	12	4.00^{a} (1.51-6.48±0.58)		
L7	0(Control)	30	0	0.00^{b} (0.00-0.00±0.00)	82.1062	$2.379x10^{-6}$
	50	30	15	5.00^{a} (5.00-5.00±0.00)		
	75	30	20	6.67^{a} (5.22-8.11±0.34)		
	100	30	21	7.00^{a} (7.00-7.00±0.00)		

Means followed by the same superscript within the same isolate are not significantly different ($P>0.05$).

* 10 larvae were exposed in triplicate giving a total of 30.

4.6 Determination of median lethal concentration (LC_{50}) of the isolates against *Culex quinquefasciatus* larvae

The probit analysis is used to assess the potency of toxins on living organisms (as such it was used to assess the potency of the spore crystal mixture of the isolates on mosquito larvae); the probit table was used to get the Empirical Probit of kill for each of the concentrations using their corresponding mortality. The empirical probit of kill was plotted against the log of concentration to derive the regression equation. The coefficient of regression (R^2) was also generated from the graph. The regression equation was used to calculate the median lethal concentration (LC_{50}) which is the concentration that will kill 50% of the exposed population. The table below shows the median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Culex quinquefasciatus* larvae obtained by probit analysis.

4.7 Bioassay with *Aedes aegypti* larvae

In the case of bioassay using *Aedes aegyti,* isolate L3 had the highest activity (100% mortality) at 100ppm concentration of spore crystal mixture, followed by L1 (93.33% mortality) at the same concentration. Isolate D2 shows the lowest larvicidal activity (40.00%) at 100% concentration. The differences observed between the mean mortality of the isolates at different concentration of the spore crystal mixture for both *Aedes aegyti* and *Culex quinquefasciatus* were statistically significant.

Figure 4.3 shows the larvicidal activity of the isolates on *Aedes aegypti* larvae using different concentrations (100ppm, 75ppm and 50ppm) of *Bacillus thuringiensis* spore crystal mixture of the 16 isolates after 24 hours of exposure.

Table 4. 7: Median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Culex quinquefasciatus* larvae obtained by probit analysis.

Isolates	Conc. (ppm)	Log of conc	Mort. (%)	EPK	RE	R^2	LC_{50} ppm
A1	100	2.00	73	5.61	Y= 4.3673x-3.1107	0.9991	71.96
	75	1.87	53	5.08			
	50	1.67	23	4.26			
	0	-	0	-			
C2	100	2.00	50	5.00	Y= 2.4663x-0.1558	0.8937	92.06
	75	1.87	47	4.92			
	50	1.67	23	4.26			
	0	-	0	-			
D1	100	2.00	70	5.25	Y= 2.7263x-0.256	0.9656	84.68
	75	1.87	40	4.75			
	50	1.67	27	4.39			
	0	-	0	-			
E2	100	2.00	40	4.75	Y= 4.2263x-2.986	0.9036	77.53
	75	1.87	37	4.67			
	50	1.67	23	4.26			
	0	-	0	-			
D2	100	2.00	33	4.56	Y= 2.7724x-0.9148	0.9443	135.95
	75	1.87	27	4.39			
	50	1.67	10	3.72			
	0	-	0	-			

N2	100	2.00	90	6.28	Y= 4.3528x-2.4639	0.9929	51.84
	75	1.87	73	5.61			
	50	1.67	47	4.92			
	0	-	0	-			
N3	100	2.00	60	5.25	Y= 8.2105x-9.81	0.9095	63.63
	75	1.87	53	5.08			
	50	1.67	23	4.26			
	0	-	0	-			
L1	100	2.00	70	5.52	Y= 0.4175x+4.093	0.0561	148.73
	75	1.87	57	5.18			
	50	1.67	37	4.67			
	0	-	0	-			
L2	100	2.00	40	4.75	Y= 3.5158x-1.656	0.8650	78.18
	75	1.87	37	4.67			
	50	1.67	27	4.39			
	0	-	0	-			
L3	100	2.00	97	6.88	Y= 1.2407x+3.0472	0.8589	55.79
	75	1.87	70	5.52			
	50	1.67	53	5.08			
	0	-	0	-			

L6	100	2.00	40		4.75	Y= 3.3026x-1.8775	0.9958	120.89
	75	1.87	23	4.26				
	50	1.67	10	3.72				
	0	-	0	-				
L7	100	2.00	70		5.52	Y= 0.9078x+3.5475	0.4101	39.81
	75	1.87	67	5.44				
	50	1.67	50	5.00				
	0	-	0	-				

Conc. = concentration, Log of conc = logarithm of concentration, Mort. = mortality, ACM = Abbott's corrected mortality, EPK = Empirical probit of kill, RE = Regression equation R^2 = Coefficient of determination LC_{50} = Median Lethal concentration.

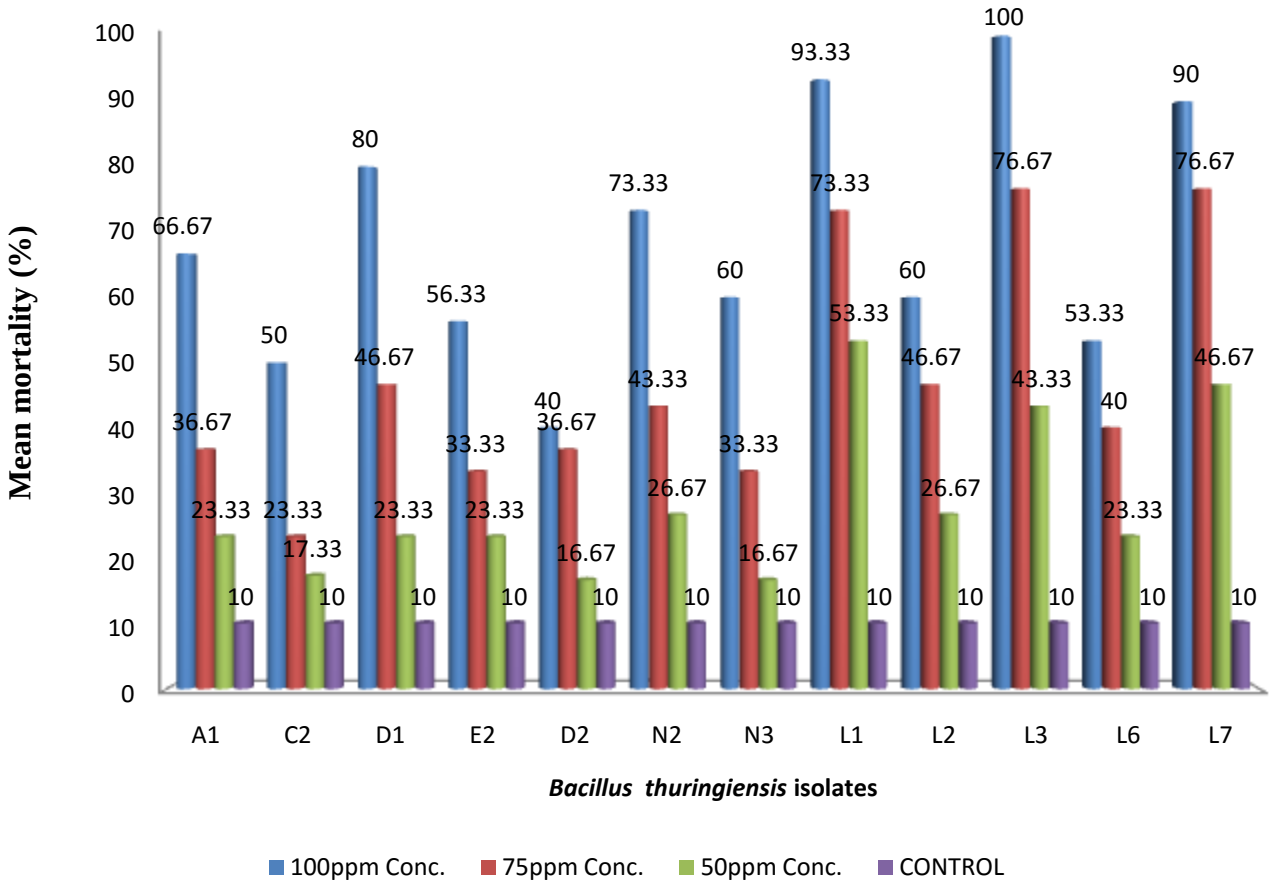

Figure 4. 3: Mean mortality of *Aedes aegypti* to different concentrations of *Bacillus thuringiensis* spore crystal mixture after 24 hours of exposure.

Table 4. 8: Mortality of *Aedes aegypti* larvae on exposure to various concentrations of twelve isolates of *Bacillus thuringiensis.*

Isolates	Conc. (ppm)	No. of larvae		Mean mortality(95% CI ±SE)	ANOVA	
		Exposed*	Dead		F-Value	P-Value
A1	0(Control)	30	3	1.00^{c} (1.00-1.00±0.00)	35.4222	$5.744x10^{-5}$
	50	30	7	2.33^{bc} (-0.52-5.18±0.66)		
	75	30	11	3.67^{b} (2.22-5.11±0.34)		
	100	30	20	6.67^{a} (5.22-8.11±0.34)		
C2	0(Control)	30	3	1.00^{b} (1.00-1.00±0.00)	26.1948	$1.725x10^{-4}$
	50	30	6	2.00^{b} (-0.48-4.48±0.58)		
	75	30	7	2.33^{b} (0.88-3.77±0.34)		
	100	30	15	5.00^{a} (5.00-5.00±0.00)		
D1	0(Control)	30	3	1.00^{d} (1.00-1.00±0.00)	42.4804	$2.923x10^{-5}$
	50	30	7	2.33^{c} (0.88-3.77±0.34)		
	75	30	14	4.67^{b} (1.81-7.52±0.66)		
	100	30	24	8.00^{a} (5.51-10.48±0.58)		
E2	0(Control)	30	3	1.00^{c} (1.00-1.00±0.00)	19.0021	$5.359x10^{-4}$
	50	30	7	2.33^{b} (0.88-3.77±0.88)		
	75	30	10	3.33^{a} (1.88-4.77±0.34)		
	100	30	16	5.33^{a} (3.88-6.77±0.34)		
D2	0(Control)	30	3	1.00^{c} (1.00-1.00±0.00)	24.9854	$2.044x10^{-4}$

	50	30	5	1.67^{b} (0.22-3.11±0.34)		
	75	30	11	3.67^{a} (2.22-5.11±0.34)		
	100	30	12	4.00^{a} (1.51-6.48±0.58)		
N2	0(Control)	30	3	1.00^{d} (1.00-1.00±0.00)	43.8119	$2.604x10^{-5}$
	50	30	8	2.67^{c} (1.22-4.11±0.34)		
	75	30	13	4.33^{b} (2.88-5.77±0.34)		
	100	30	22	7.33^{a} (4.47-10.18±0.66)		
N3	0(Control)	30	3	1.00^{b} (1.00-1.00±0.00)	16.2346	$9.182x10^{-4}$
	50	30	5	1.67^{b} (0.22-3.11±0.34)		
	75	30	10	3.33^{a} (1.88-4.77±0.34)		
	100	30	18	6.00^{a} (1.70-10.29±1.00)		
L1	0(Control)	30	3	1.00^{d} (1.00-1.00±0.00)	76.2826	$3.159x10^{-6}$
	50	30	16	5.33^{c} (3.88-6.77±0.34)		
	75	30	22	7.33^{b} (5.88-8.77±0.34)		
	100	30	28	9.33^{a} (6.47-12.18±0.66)		
L2	0(Control)	30	3	1.00^{c} (1.00-1.00±0.00)	21.857	$3.287x10^{-4}$
	50	30	8	2.67^{b} (1.22-4.11±0.34)		
	75	30	14	4.67^{ab} (1.81-7.52±0.66)		
	100	30	18	6.00^{a} (3.51-8.48±0.58)		
L3	0(Control)	30	3	1.00^{d} (1.00-1.00±0.00)	111.707	$7.204x10^{-7}$
	50	30	13	4.33^{c} (2.88-5.77±0.34)		
	75	30	23	7.67^{b} (4.81-10.52±0.66)		

	100	30	30	10.00^{a} (10.00-10.00±0.00)		
L6	0(Control)	30	3	1.00^{b} (1.00-1.00±0.00)	5.6209	0.022
	50	30	7	2.33^{ab} (-1.47-6.13±0.88)		
	75	30	12	4.00^{ab} (-0.96-8.96±1.16)		
	100	30	16	5.33^{a} (2.47-8.18±0.66)		
L7	0(Control)	30	3	1.00^{c} (1.00-1.00±0.00)	56.9693	$9.661x10^{-6}$
	50	30	14	4.67^{b} (1.81-7.52±0.66)		
	75	30	23	7.67^{a} (6.22-9.11±0.34)		
	100	30	27	9.00^{a} (6.51-11.48±0.58)		

Means followed by the same superscript within the same isolate are not significantly different (P>0.05).

***** 10 larvae were exposed in triplicate giving a total of 30.

4.8 Determination of the median lethal concentration (LC_{50}) of the isolates against *Aedes aegypti* larvae

The probit analysis is used to assess the potency toxin on living organisms (as such it was used to assess the potency of the spore crystal mixture of the isolates on mosquito larvae) the probit table was used to get the Empirical Probit of kill for each of the concentrations using their corresponding mortality. The empirical probit of kill was plotted against the log of concentration to derive the regression equation. The coefficient of regression (R^2) was also generated from the graph. The regression equation was used to calculate the median lethal concentration (LC_{50}) which is the concentration that will kill 50% of the exposed population. Table 4.1 shows the median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Aedes aegypti* larvae obtained by probit analysis.

4.9 Iarvicidal Activity of the *B. thuringiensis* Isolates obtained From the Three Soil Types on *Culex quinquefasciatus* larvae

In terms of the degree of the larvicidal activity of the isolates, no apparent larvicidal activity was observed at the range of 0.00%-25.00% against *Culex quinquefasciatus* was seen in all the isolates. Three isolates obtained from agricultural soil and two from cow rangeland had larvicidal activity between 25.01%-50.00% against *Culex quinquefasciatus* so also two isolates from these two sites and one from waste dump soil had activity between 50.01%-75.00% against *Culex quinquefasciatus.* One isolate from refuse dump soil and cow rangeland had activity between 75.01%-100.00% against *Culex quinquefasciatus* all at 100ppm concentration (Figure 4.4).

Table 4. 9: Median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Aedes aegypti* larvae obtained by probit analysis.

Isolates	Conc. (ppm)	Log of conc	Mort. (%)	ACM	EPK	RE	R^2	LC50 ppm
A1	100	2.00	67	63	5.33	Y= 4.4594x-3.6881	0.9561	88.78
	75	1.87	37	30	4.48			
	50	1.67	23	14	3.92			
	0	-	10	0	-			
C2	100	2.00	50	44	4.85	Y= 3.3198x-1.9727	0.7802	125
	75	1.87	23	14	3.92			
	50	1.67	20	11	3.77			
	0	-	10	0	-			
D1	100	2.00	80	78	5.77	Y= 5.8906x-6.0973	0.9806	76.54
	75	1.87	47	41	4.77			
	50	1.67	23	14	3.92			
	0	-	10	0	-			
E2	100	2.00	53	48	4.95	Y= 3.4711x-1.8265	0.8257	92.59
	75	1.87	53	48	4.95			
	50	1.67	23	14	3.92			
	0	-	10	0	-			
D2	100	2.00	40		4.75	Y= 2.3315x-0.169	0.8973	118.03
	75	1.87	37		4.67			
	50	1.67	17		4.05			
	0	-	0		-			

N2	100	2.00	73	70	5.52	Y= 4.4257x-3.4323	0.9541	80.40
	75	1.87	43	37	4.67			
	50	1.67	27	19	4.12			
	0	-	10	0	-			
N3	100	2.00	60	56	5.15	Y= 4.9856x-4.8732	0.9900	95.56
	75	1.87	33	26	4.36			
	50	1.67	17	8	3.59			
	0	-	10	0	-			
L1	100	2.00	93	92	6.41	Y= 4.6142x-2.9249	0.9529	52.17
	75	1.87	78	70	5.52			
	50	1.67	53	48	4.95			
	0	-	10	0	-			
L2	100	2.00	60	56	5.15	Y= 3.3404x-1.5109	0.9967	88.94
	75	1.87	47	41	4.77			
	50	1.67	27	19	4.12			
	0	-	10	0	-			
L3	100	2.00	100	100	8.72	Y= 12.589x-16.989	0.8589	55.79
	75	1.87	77	74	5.64			
	50	1.67	43	37	4.67			
	0	-	10	0	-			
L6	100	2.00	53	48	4.95	Y= 3.337x-1.7079	0.9979	102.35
	75	1.87	40	33	4.56			

	50	1.67	23	14	3.92				
	0	-	10	0	-				
L7	100	2.00	90	89	6.23	Y= 4.7173x-3.1961	0.9997	54.62	
	75	1.87	77	74	5.64				
	50	1.67	47	41	4.77				
	0	-	10	0	-				

Conc. = concentration, Log of conc = logarithm of concentration, Mort. = mortality, ACM = Abbott's corrected mortality, EPK = Empirical probit of kill, RE = Regression equation R^2 = Coefficient of determination LC_{50} % = Median Lethal concentration.

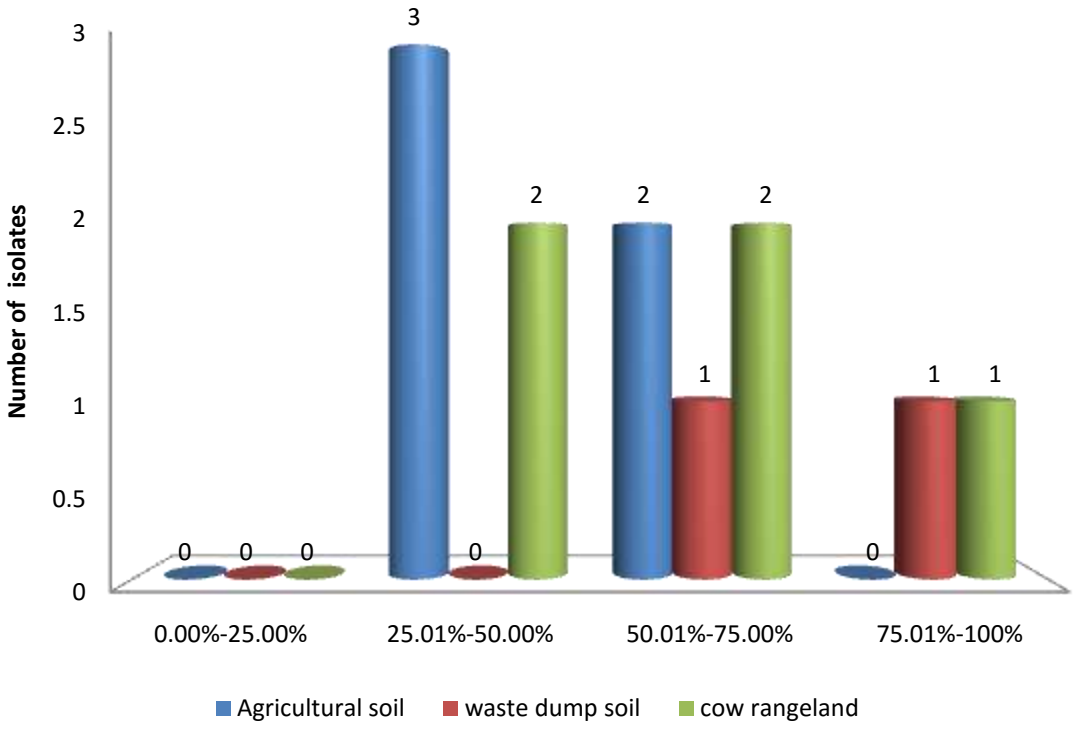

Figure 4. 4: Insecticidal activity of the *B. thuringiensis* isolates obtained from the three soil types against *Culex quinquefasciatus* using 100ppm spore crystal mixture of the isolates.

4.10 Larvicidal Activity of the *B. thuringiensis* Isolates obtained From the Three Soil Types on *Aedes aegypti* larvae

At 100% spore crystal mixture of the isolates, none of the isolates from the three soil type had larvicidal activity between 0.00%-25.00% against *Aedes aegypti*. Two isolates from agricultural soil had activity between 25.01- 50.00% while none from the other soil type had activity within that range. Two isolates from all the soil types had activity between 50.01-75.00%. Three isolates from cow range land had insecticidal activity between 75.01%-100.00% against *Aedes aegypti*. One isolate from agricultural soil had activity between 75.01%-100.00% while none from waste dump soil had activity within this range against *Aedes aegypti* (Figure 4.5).

4.11 Comparison of the Insecticidal Activity of the Isolates against *Cx. quinquefasciatus* and *A. aegypti* larvae

A comparative analysis of the susceptibility of the two mosquitoes species shows that on the average *Aedes aegyti* is more susceptible to most of the isolates compared to *Culex quinquefasciatus* which tends to resist the activity of some of the isolates. Eight of the twelve isolates showed higher larvicidal activity against *Aedes aegyti* compared to *Culex quinquefasciatus* (Figure 4.6).

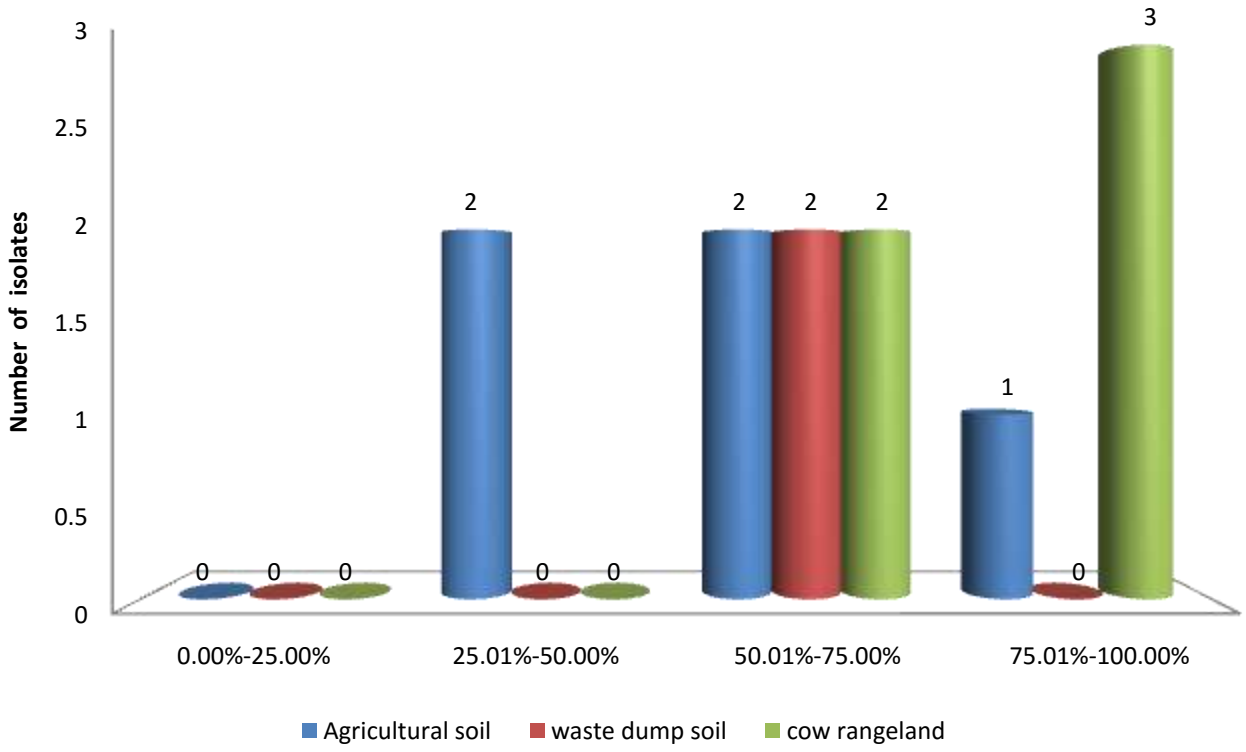

Figure 4. 5: Insecticidal activity of the *B. thuringiensis* isolates obtained from the three soil types against *Aedes aegypti* using 100ppm spore crystal mixture of the isolates.

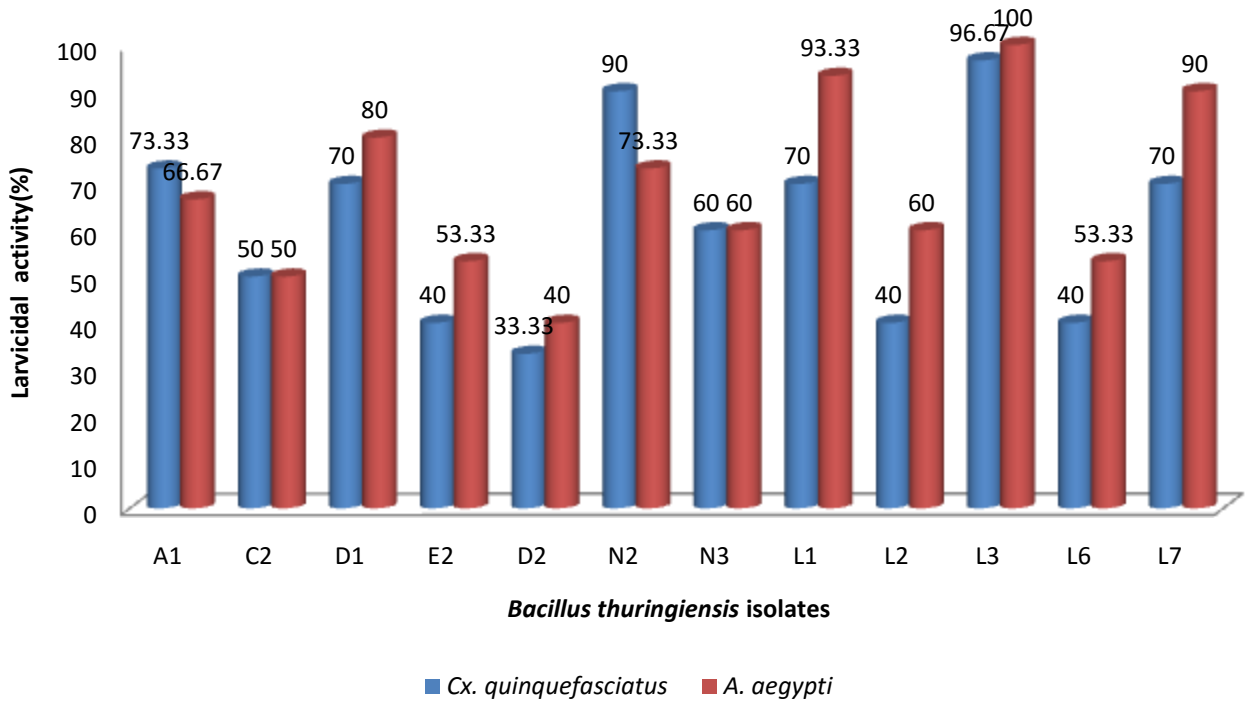

Figure 4. 6: A comparative analysis of the insecticidal activity of 100ppm spore crystal mixture of the isolates to *Cx. quinquefasciatus* and *A. aegypti* larvae after exposure for 24hours.

4.12 Iarvicidal Activity of *B. thurimgiensis* against *Cx. quinquefasciatus* larvae

Based on the larvicidal activity of the isolates against *Cx. quinquefasciatus*; none (0%) of the isolates had activity between 0.00-25.00%, 41% had activity between 25.01%-50.00%, activity between 75.00%-100.0% was seen in 17% of the isolates, while activity between 50.01%-75.00% was seen in 42%. It then means that most of the isolates had activity between 25.00 and 75.00% mortality (Figure 4.7).

4.11 Distribution of the larvicidal activity of *Bacillus thuringiensis* to *Aedes aegypti* larvae

Based on the larvicidal activity of the *Bacillus thuringiensis* isolates against *Aedes aegypti*; none of the isolates had activity between 0.00-25.00%, 17% had activity between 25.01%-50.00%, activity between 75.00%-100.0% was seen in 33% of the isolates, while activity between 50.01%-75.00% was seen in 50%. It then means that most of the isolates had activity between 25.00 and 75.00% mortality as seen also in *Cx. quinquefasciatus* (Figure 4.8).

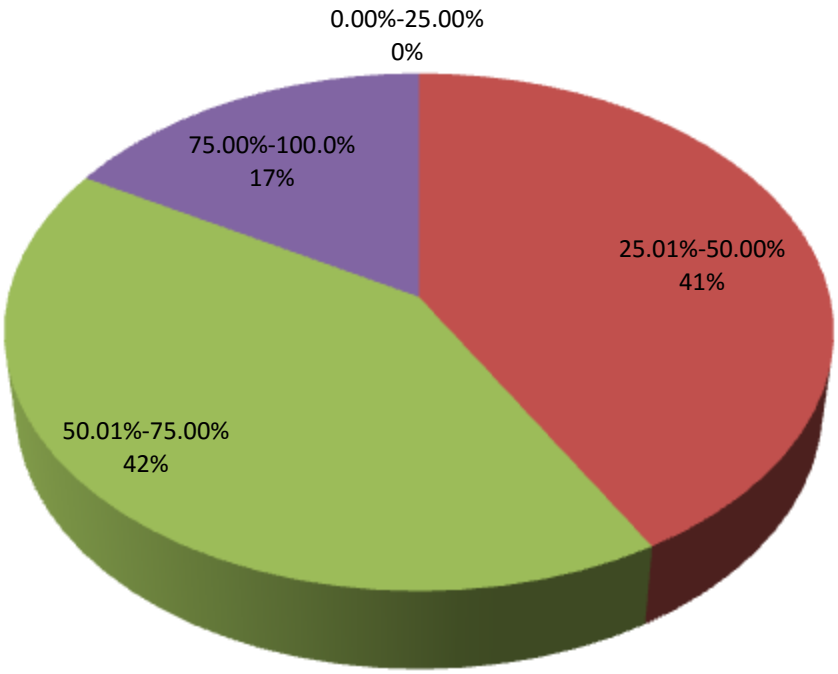

Figure 4. 7: Larvicidal activity (%) of 100ppm spore crystal mixture of the isolates to *Cx. quinquefasciatus* larvae.

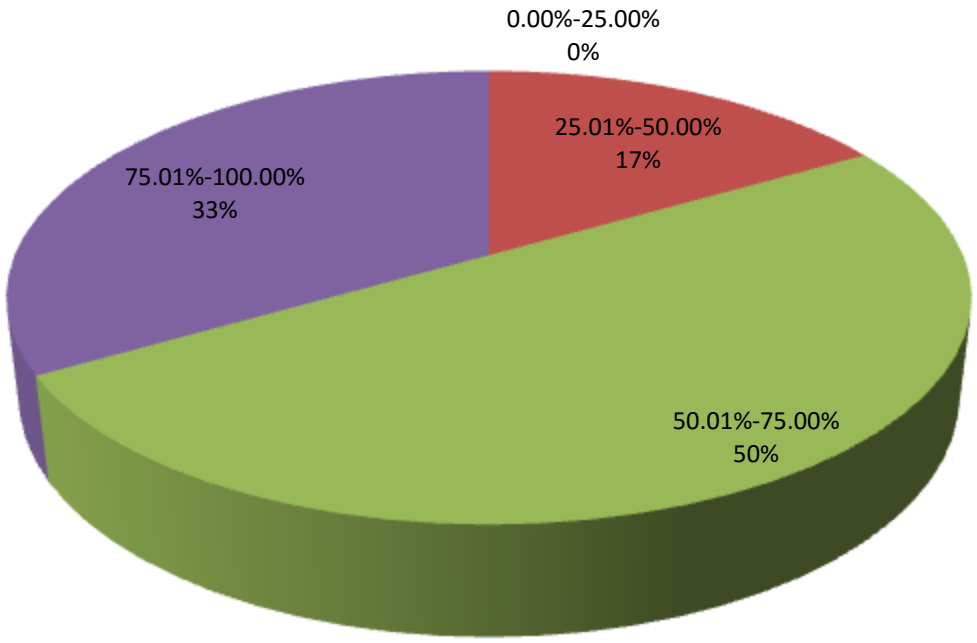

Figure 4. 8: Larvicidal activity (%) of 100ppm spore crystal mixture of the isolates to *Aedes aegypti* larvae.

CHAPTER FIVE

5.0 DISCUSSION

Bacillus thuringiensis is one of the bacterial biolarvicides, which presents an alternative for controlling several mosquito species. *Bacillus thuringiensis* produces a component of a spherical parasporal body and composed of many toxin proteins encoded by the *cry* gene.

An alternative approach for mosquito control is the use of natural products such as plant and microorganisms. The microbial pesticides are essentially nontoxic to humans as such there are no concerns for human health effects with *Bacillus thuringiensis*. Researchers have also shown that microbial larvicides do not pose risk to wildlife, non-target species or the environment and retain a good activity in polluted water.

The search for alternative biocontrol agents such as *Bacillus thuringiensis*-based biopesticides is increasingly attracting interest. This bacterium produces parasporal crystalline inclusions (Cry proteins) which is toxic to many important agricultural pests. The Cry proteins are encoded by *cry* genes and so far, many *cry* genes have been identified in different *B. thuringiensis* strain collections. *Cry2* and *cry4* are toxic to dipteran insects (Zeigler, 1999; Çetinkaya, 2002).

The higher frequency of *B. thuringiensis* isolated from agricultural soil and cow rangeland soil could be attributed to the fact that these soil type have higher organic matter content which favour *B. thuringiensis* that grows naturally as a saprophyte, feeding on dead-organic matter.

In this study the isolates were screened for the presence of diptera-active *cry2* and *cry4* genes. Whereby, 6(50%) of the 12 isolates were found to be good candidates in the search for biocontrol agents mosquito since they habour the diptera-active genes. One of the isoloates was

found to contain the two genes, one other isolate also contain only *cry2* while 4 other isolates habour only *cry4* gene.

Cry 2 gene was found in 2 (L3 and N2) of the 12 isolates while *Cry 4* gene was present in 4 (L3, L7, L1, A1 and D1) of the 12 isolates. One (L3) of the isolates habours both *Cry2* and *Cry 4* genes. None of the genes was present in six isolates namely: N3, L2, D2, L6, C2 and E2.

The most common *cry* gene among the isolates in this study is *cry4* gene (54%) while *cry2* had a lower occurrence (22%). This agrees with the work of Cinar (2005) where 57% of his isolates had *cry4* gene while 20% had *cry2* genes.

The presence of multiple *cry* genes in one strain could be due to genetic information exchange between different strains. Such isolates could show simultaneous toxicities towards different insect families and are good candidates in the search for biocontrol agents covering a wider spectrum of action (Khojand *et al.,* 2013).

Effectiveness of the *Bacillus thuringiensis* against mosquitoes depends on efficient application and long retention in the larval feeding zone until lethal doses are ingested. Mosquito larvae collect particles from the air-water interface and rapidly ingest them, this suspended particles are filtered at low rates.

The bioassay for the larvicidal activity of the isolates on *Culex quinquefasciatus* and *Aedes aegypti* larvae using different concentrations of the spore crystal mixture shows that the higher the concentration of spore crystal mixture, the more the larvicidal activity.

The isolates differ greatly in their larvicidal activity against the larvae of *Culex quinquefasciatus* and *Aedes aegypti,* wherein the mortality ranged between 33.33% (D2 with LC_{50} = 135.95ppm)

and 96.00% (L3 with LC_{50} = 37.48ppm) for *Culex quinquefasciatus* while the range of mortality for *Aedes aegyti* is between 40.00% (D2 with LC_{50} = 118.03ppm) and 100.00% (L3 with LC_{50} = 55.79ppm) at 100ppm concentration of spore crystal mixture.

Isolate L3 shows the highest larvicidal activity (96.67% mortality) at 100ppm concentration of spore crystal mixture while isolate D2 shows the lowest larvicidal activity (33.33%) against *Culex quinquefasciatus* larvae. In the case of bioassay using *Aedes aegypti,* Isolate L3 had the highest activity (100% mortality) at 100ppm concentration of spore crystal mixture, followed by L1 (93.33% mortality) at the same concentration. Isolate D2 shows the lowest activity (40.00%) at 100ppm concentration. The differences observed between the mean mortality of the isolates at different concentration of the spore crystal mixture for both *Aedes aegypti* and *Culex quinquefasciatus* were statistically significant.

The concentrations of the spore crystal mixture which kill 50% of the exposed populations in standard bioassays (LC_{50}) were determined by probit analyses with triplicate bioassay. This concentrations range between 37.48ppm and 135.95ppm for *Culex quinquefasciatus* while the range observed for *Aedes aegypti* was between 52.17ppm and 125.89ppm.

The high larvicidal activity shown by isolate L3 against *Culex quinquefasciatus* and *Aedes aegypti* in this study could be attributed to the presence of the two diptera-active cry genes in this particular isolate. So also, isolates harbouring either of the two genes also had larvicidal activities higher than those isolates with neither of the genes.

The differences in potency of the isolates observed in this study could be attributed to the differences in susceptibility of the mosquito larvae as reported by Misztel *et al*. (1996) who explained the connection between insect mortality and exposure time. This author reported that

highly susceptible insects stopped feeding within 60 minutes and died within 1-7 hours after ingestion of the toxin, less susceptible ones ceased feeding after 3-4 hours, and died after 2-7 days, while slightly susceptible insects stopped feeding after 10-15 hours and died after 2-3 weeks.

None of the isolates from all the soil type had insecticidal activity between 0.00%-25.00% against *Culex quinquefasciatus*. Three isolates from agricultural soil and two from cow rangeland had insecticidal activity between 25.01%-50.00% against *Culex quinquefasciatus* so also two isolates from these two sites and one from waste dump soil had activity between 50.01%-75.00% against *Culex quinquefasciatus.* One isolate from refuse dump soil and cow rangeland had activity between 75.01%-100.00% against *Culex quinquefasciatus*.

None of the isolates from the three soil type had insecticidal activity between 0.00%-25.00% against *Aedes aegypti*. Two isolates from agricultural soil had activity between 25.01- 50.00% while none from the other soil type had activity within that range. Two isolates from all the soil types had activity between 50.01-75.00%. Three isolates from cow range land had insecticidal activity between 75.01%-100.00% against *Aedes aegypti*. One isolate from agricultural soil had activity between 75.01%-100.00% while none from waste dump soil had activity within this range against *Aedes aegypti*.

On the average *Aedes aegyti* is more susceptible to most of the isolates compared to *Culex quinquefasciatus* which tends to resist the activity of some of the isolates. Eight of the twelve isolates showed higher larvicidal activity against *Aedes aegyti* compared to *Culex quinquefasciatus.* The larvicidal activity of most of the isolates (42%) against *Cx. quinquefasciatus* is between 50.01% - 75.00% while none of the isolates (0%) had activity

between 0.00%-25.00%. The larvicidal activity of most of the isolates (50%) against *Aedes aegypti* is between 50.01 and 75.00%. None of the isolates had activity between 0.00 and 25.00%. The larvicidal activity of 33% of the isolates lies between 75.01 and 100.00%.

Aside the *cry2* and *cry4* genes that are diptera-active, *Bacillus thuringiensis* also habour cytolytic proteins that are also diptera-active. This account for the larvicidal activity observed in those isolates that harbor none of the *cry* gene.

Chemical insecticides provide many benefits to food production and human health and have proven very effective at increasing agriculture and forestry productivities. However, they also pose some hazards such as contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistant to the chemical insecticides (Wojciech and Korsten, 2002).

Long term exposure to the modern synthetic insecticides has been associated with cancer, liver damage, immune-toxicity, birth defects and reproductive problems in humans and other animals (Kegley and Wise, 1998). The value of *Bacillus thuringiensis* in controlling insects that transmit human diseases and destroy crops is well established. The aim of this study was therefore to isolate strains of *Bacillus thuringiensis* from different soil types and to test for their larvicidal activity against the larvae of *Aedes aegypti* and *Culex quinquefasciatus*.

Mosquitoes are top most insect vectors related to human health (Chakkaravarthy *et al.,* 2011). Different mosquito species like *Aedes aegypti* and *Culex quinquefasciatus* are widely distributed in tropical and subtropical zones and serve as vectors of disease pathogens like dengue, filariasis, Japanese encephalitis, yellow fever and chikungunya (Redwane *et al.,* 2002).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In conclusion, the result of this research shows that *Bacillus thuringiensis* isolated from different soil types in Zaria habour diteraactive *cry* genes so also these isolates are promising biocontrol agent for mosquito.

A total of 12 *Bacillus thuringiensis* isolates were isolated from different soil types. The isolates were screened for diptera-active *cry* genes namely, c*ry*2 and *cry*4 by PCR. Most of the isolates had at least one of the *cry* genes; one had both of the genes while others had none of the genes.

Bioassay to screen for the larvicidal activity of the isolates against *Culex. quinquefasciatus* and *Aedes aegypti* showed that the isolates are promising biocontrol agent for the control of these mosquitoes. The bioassay also showed that most of the *Bacillus thuringiensis* isolates from the different soil harbor diptera-active c*ry* hence they are good biocontrol agent for mosquitoes. The bioassay result using *Culex quinquefasciatus* and *Aedes aegypti* suggest the use of *cry* gene habouring *Bacillus thuringiensis* in the control of these mosquitoes by targeting the larva stages.

However, this is not the first report on microbial larvicide of *Bacillus thuringiensis* effective against the larval stage of *Culex. quinquefasciatus* and *Aedes aegypti*. The abundance of literature with promising findings suggests that *Bacillus thuringiensis* parasoral proteins (*cry* protein) may be useful in the control of mosquitoes and the diseases they transmit.

6.2 RECOMMENDATIONS

1. *Bacillus thuringiensis* habouring diptera active *cry* genes may be used as biocontrol agent owning to the advantages.

2. *Bacillus thuringiensis* isolates should also be screened for other *cry* genes that are active against other insects for biocontrol purposes.

3. Research in area of mutation should be carried out to produce mutants with improve spore crystal production ability.

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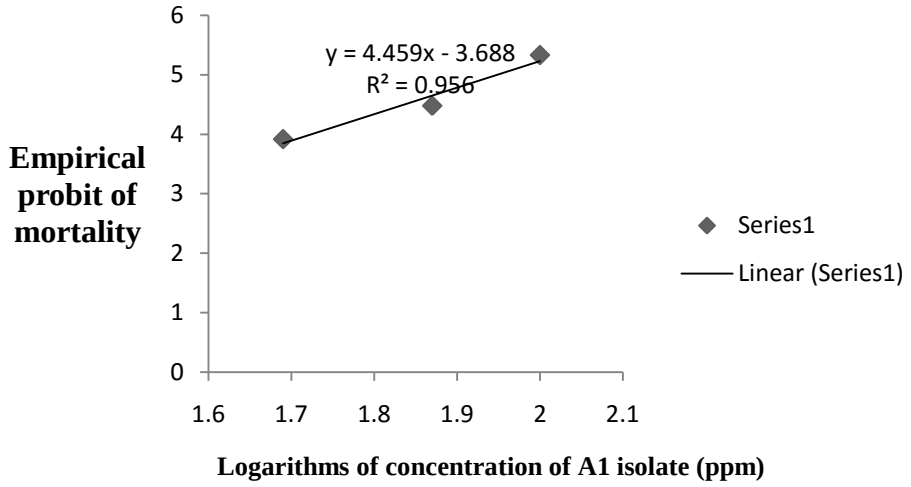
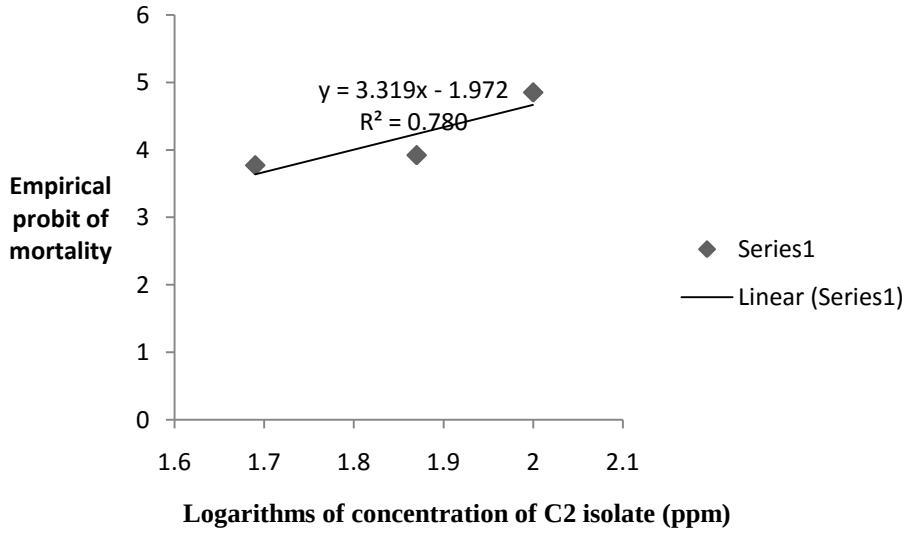
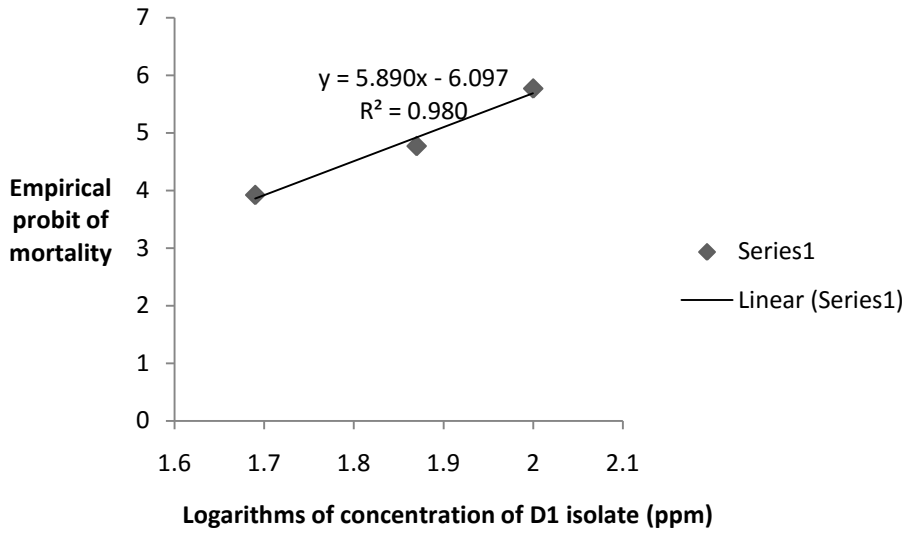
APPENDICES



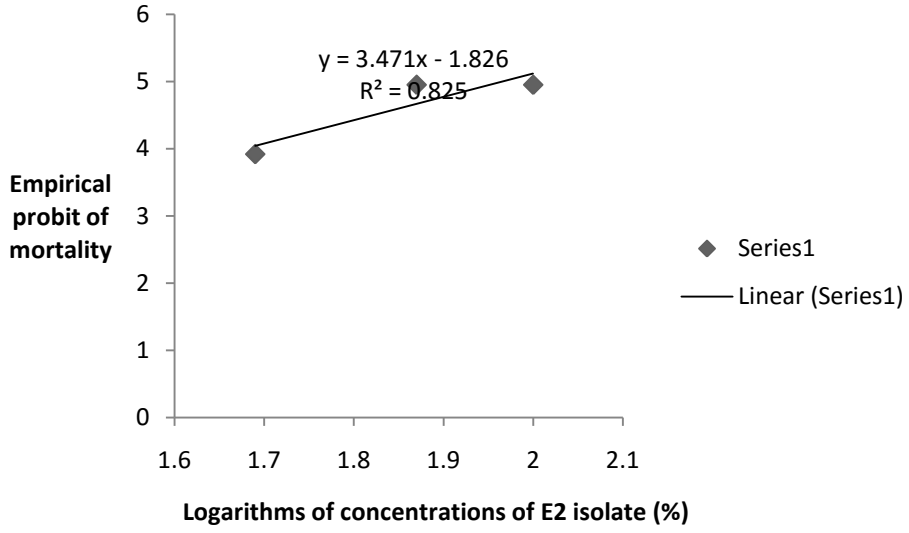
Appendix I: Relationship between concentration of A1 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



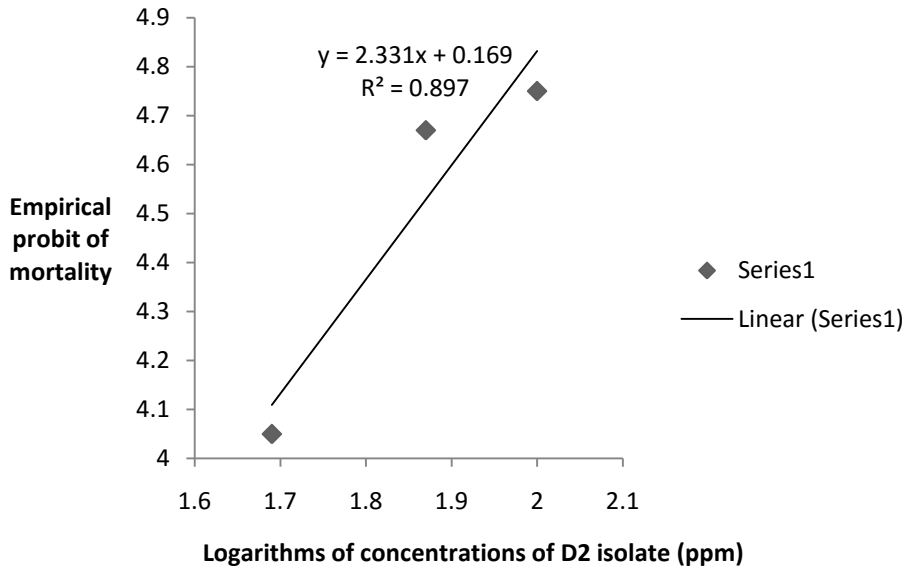
Appendix II: Relationship between concentration of C2 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



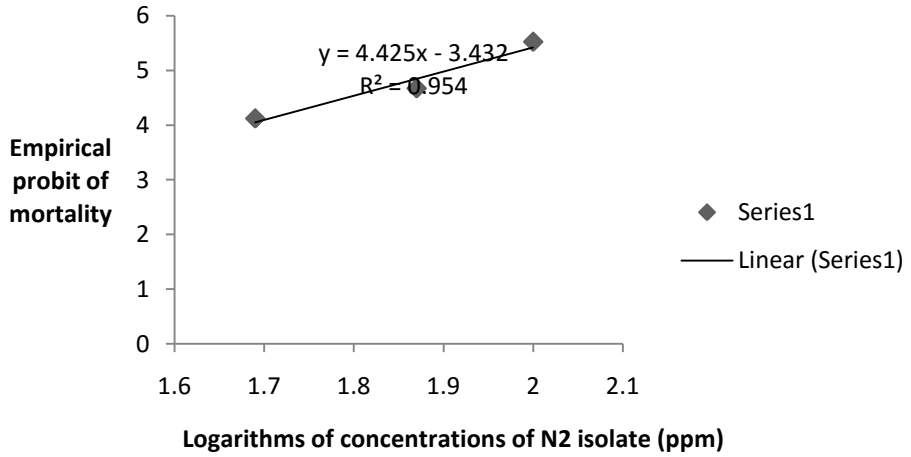
Appendix III: Relationship between concentration of D1 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



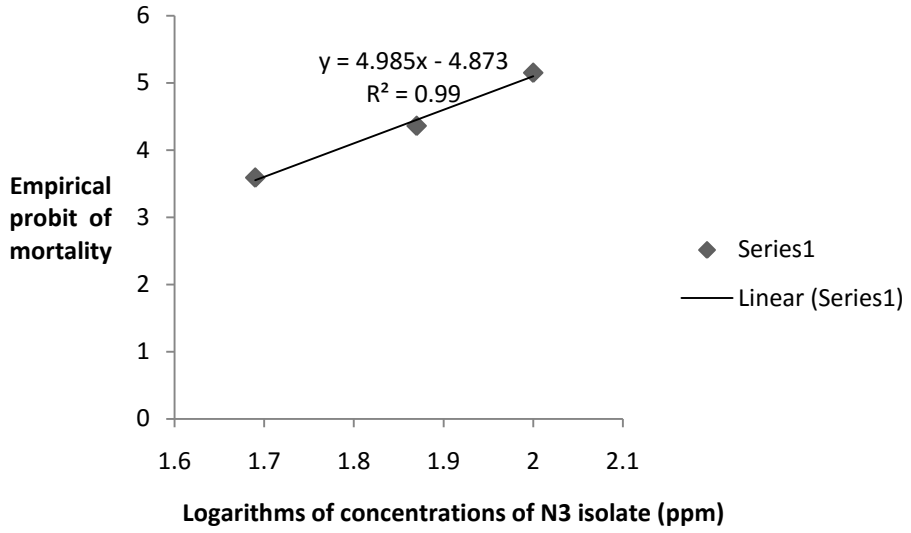
Appendix IV: Relationship between concentration of E2 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



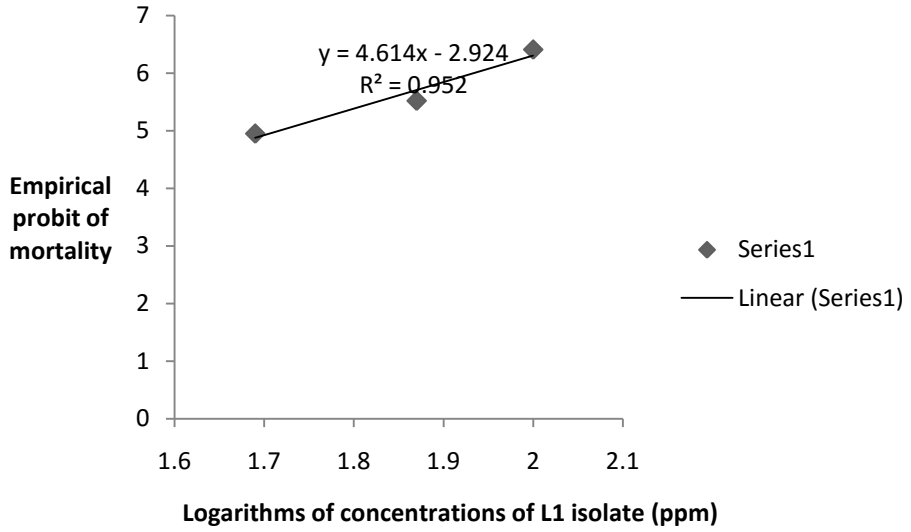
Appendix V: Relationship between concentration of D2 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



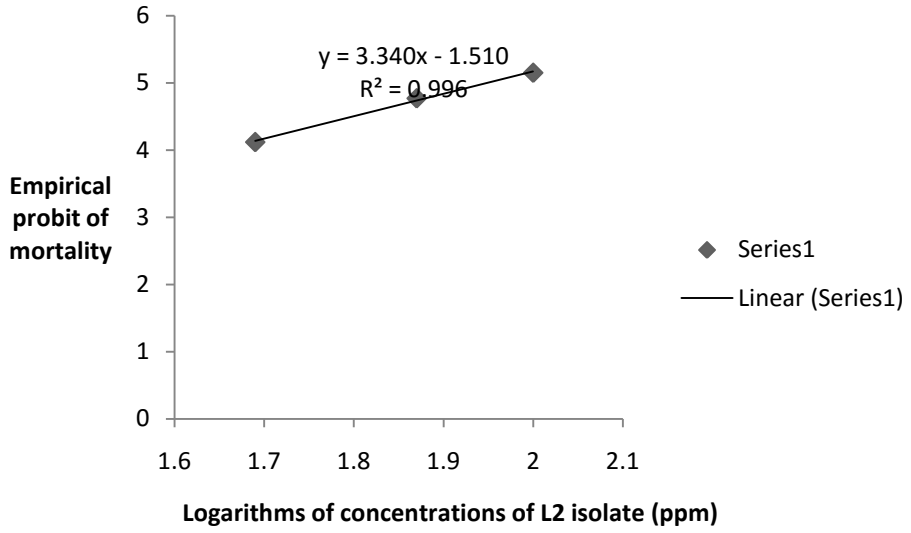
Appendix VI: Relationship between concentration of N2 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



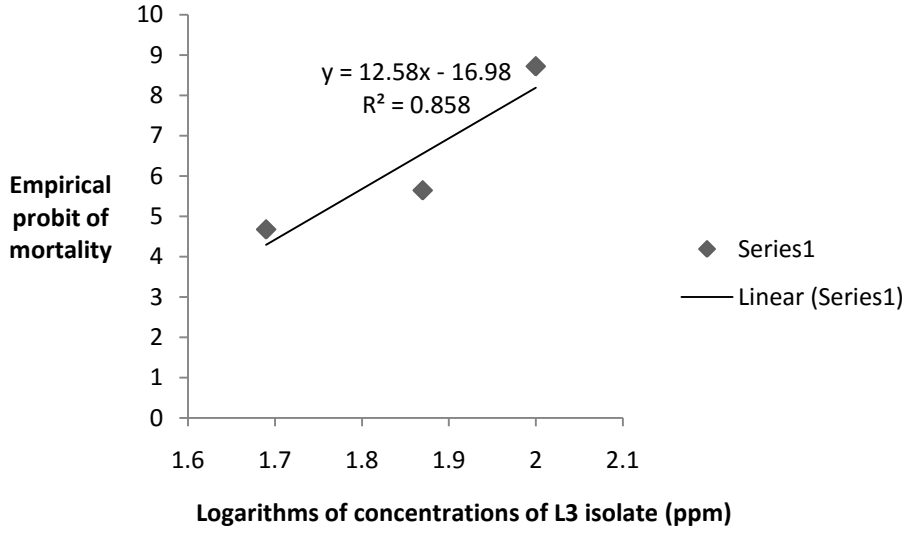
Appendix VII: Relationship between concentration of N3 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



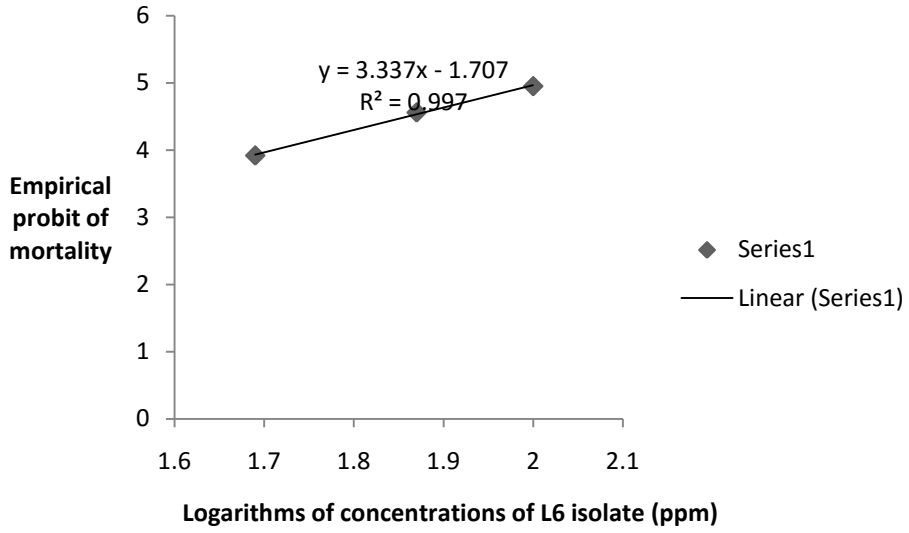
Appendix VIII: Relationship between concentration of L1 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



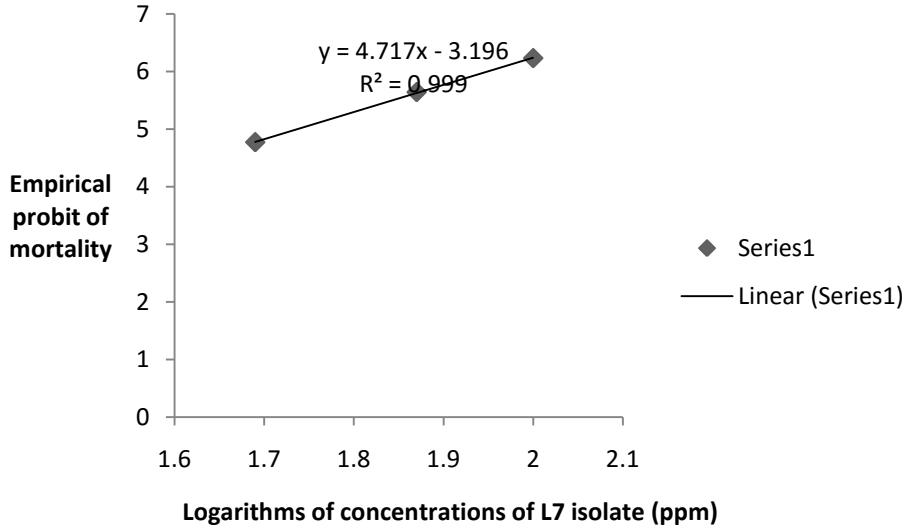
Appendix IX: Relationship between concentration of L2 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



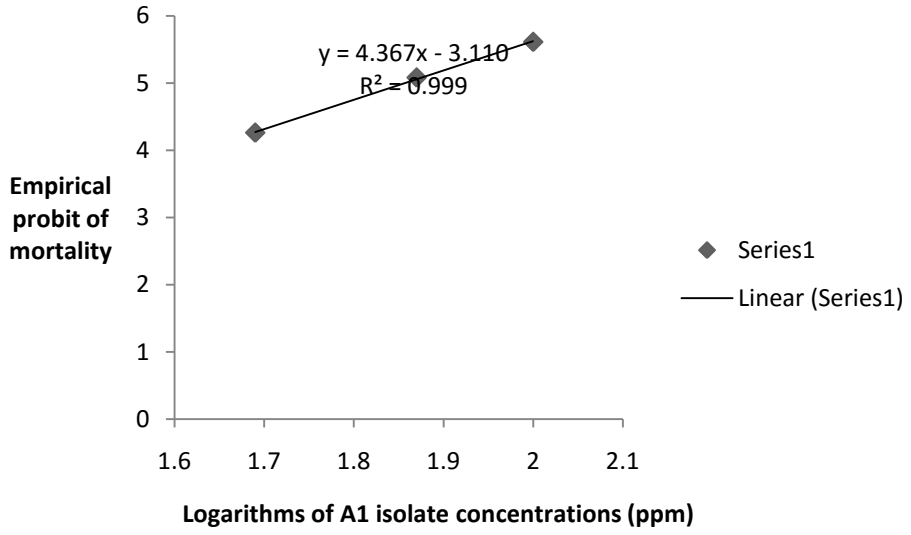
Appendix X: Relationship between concentration of L3 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



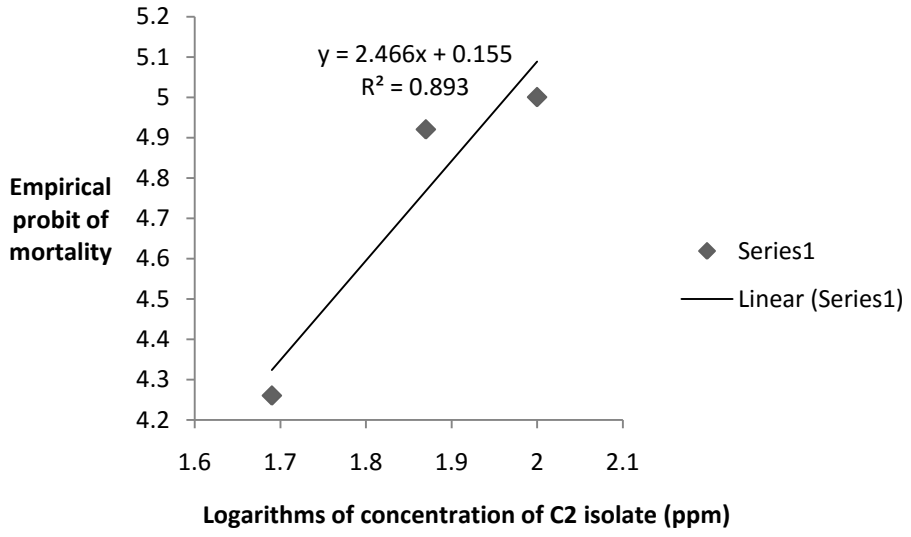
Appendix XI: Relationship between concentration of L6 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



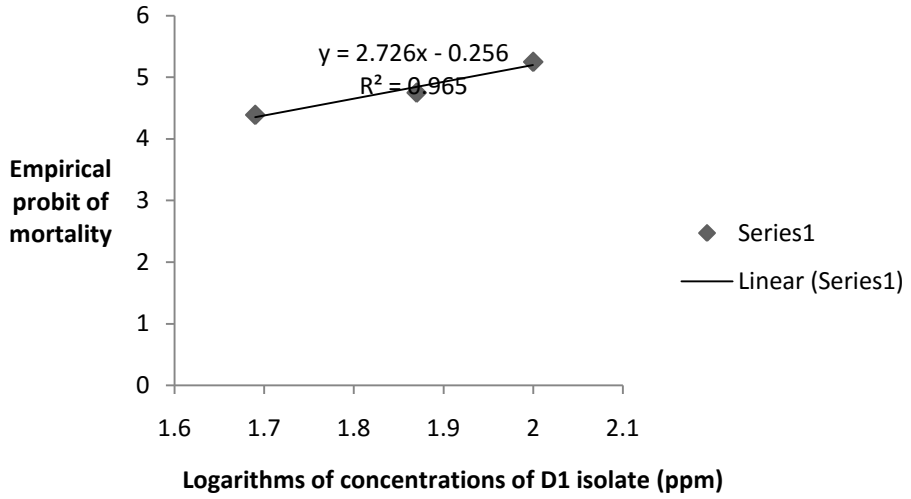
Appendix XII: Relationship between concentration of L7 isolate of *Bacillus thuringiensis* and probit of mortality of *Aedes aegypti* larvae, showing probit regression line.



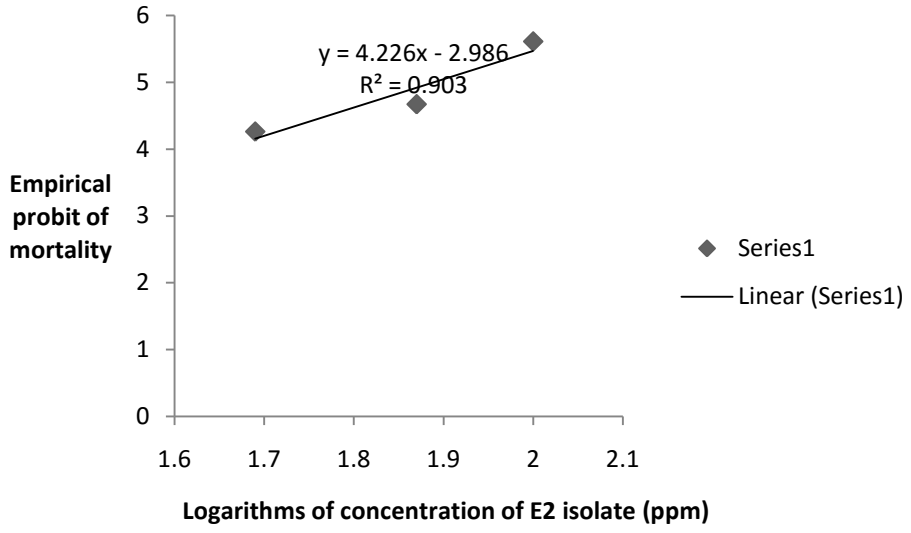
Appendix XIII: Relationship between concentration of A1 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



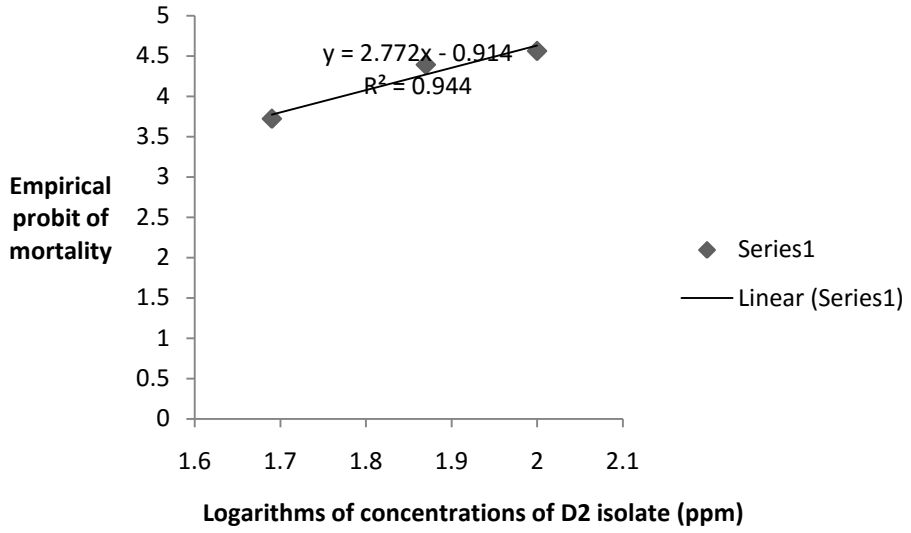
Appendix XIV: Relationship between concentration of C2 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



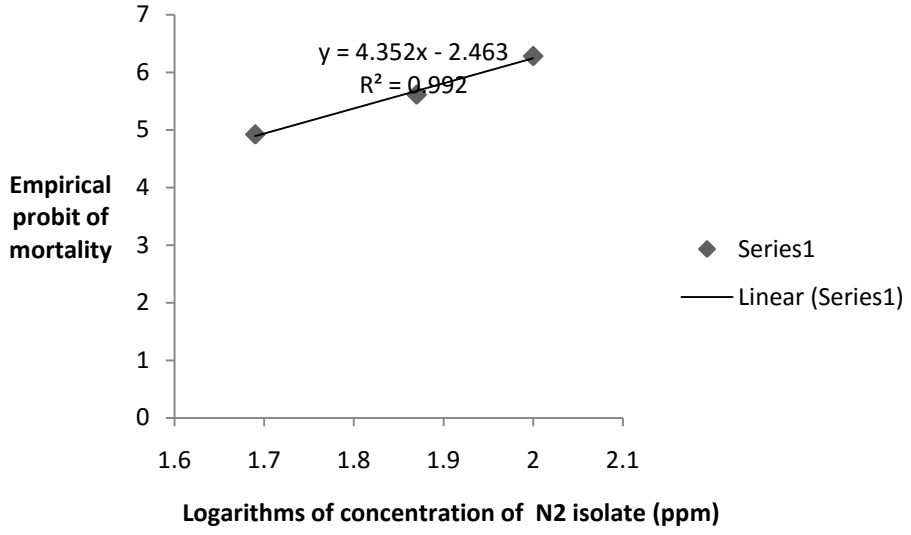
Appendix XV: Relationship between concentration of D1 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



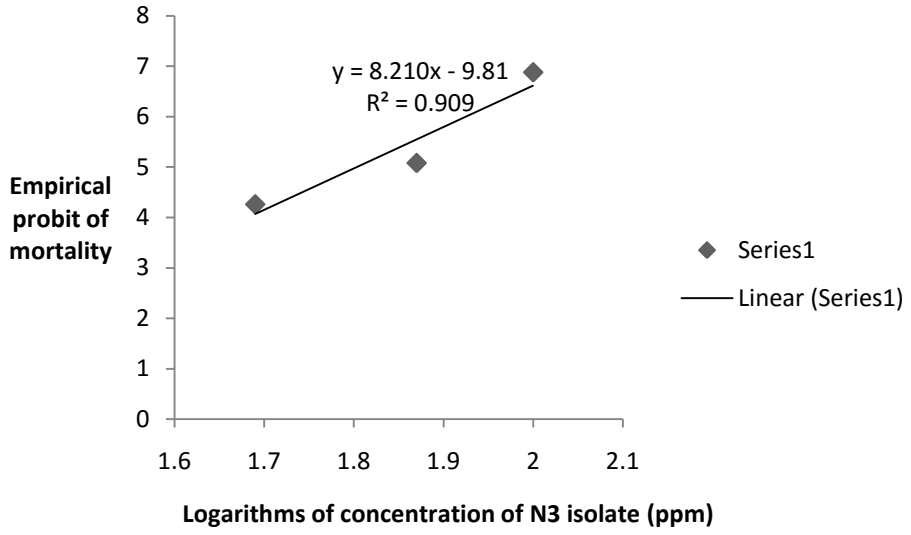
Appendix XVI: Relationship between concentration of E2 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line



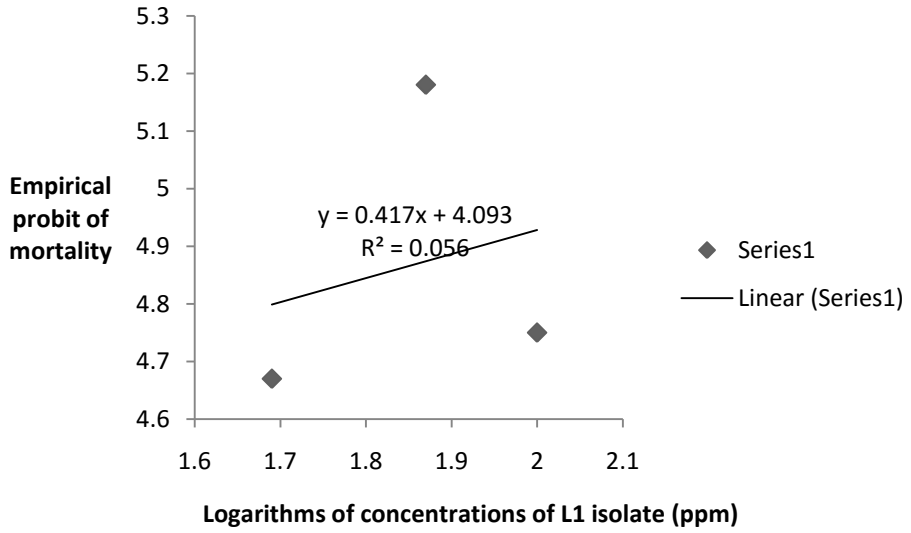
Appendix XVII: Relationship between concentration of D2 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



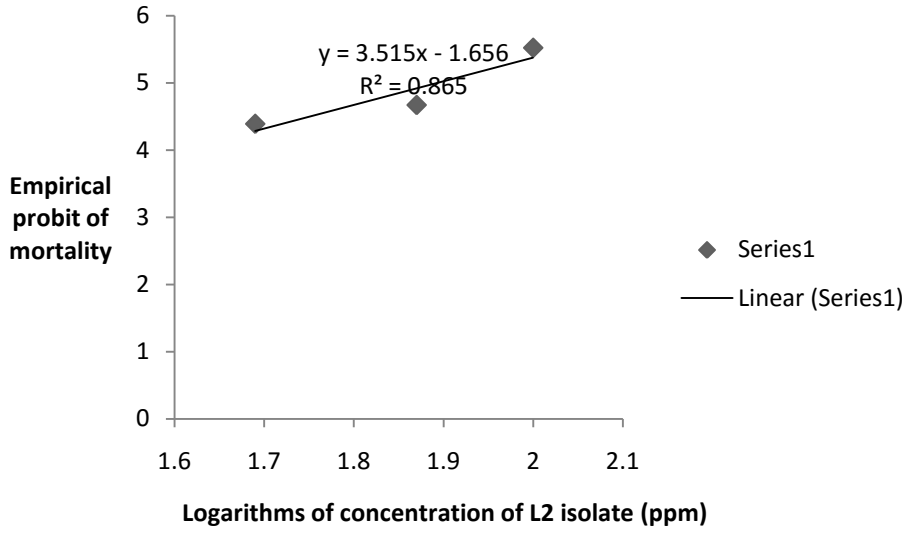
Appendix XVIII: Relationship between concentration of N2 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



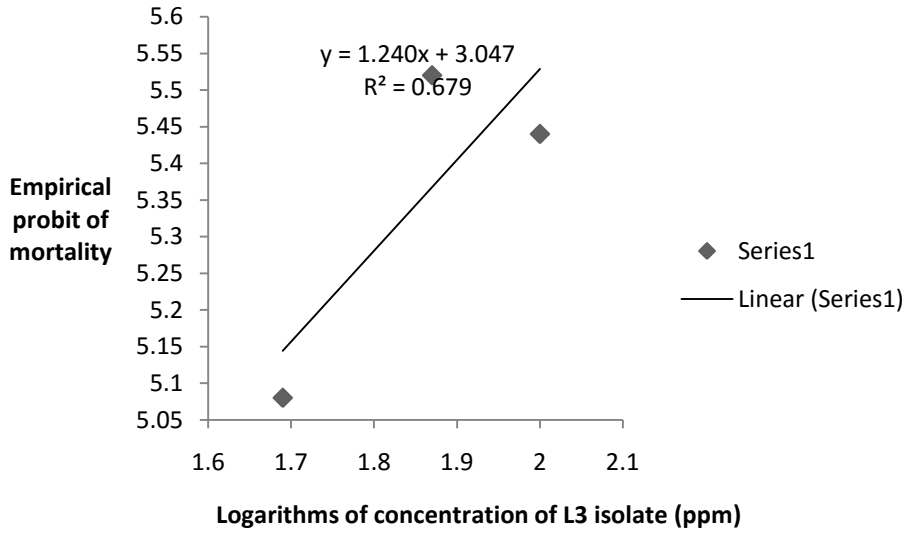
Appendix XIX: Relationship between concentration of N3 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



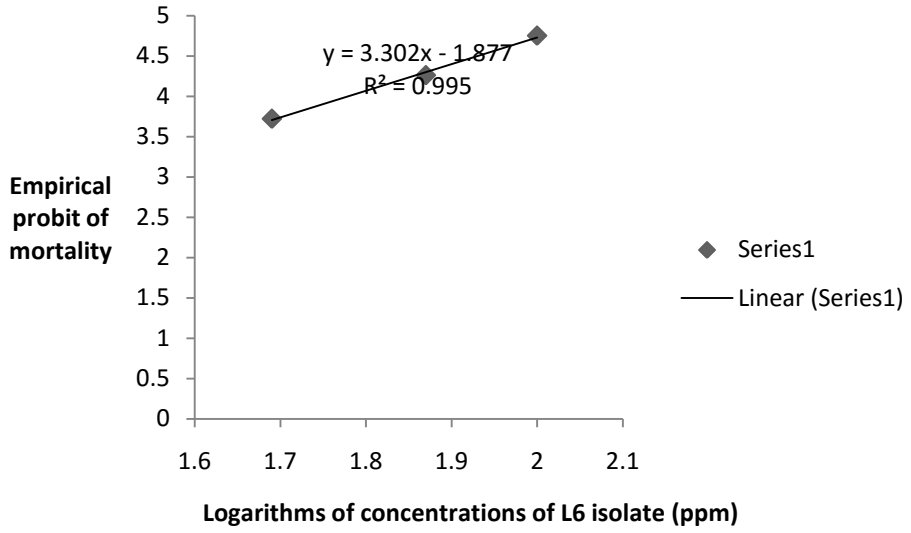
Appendix XX: Relationship between concentration of L1 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



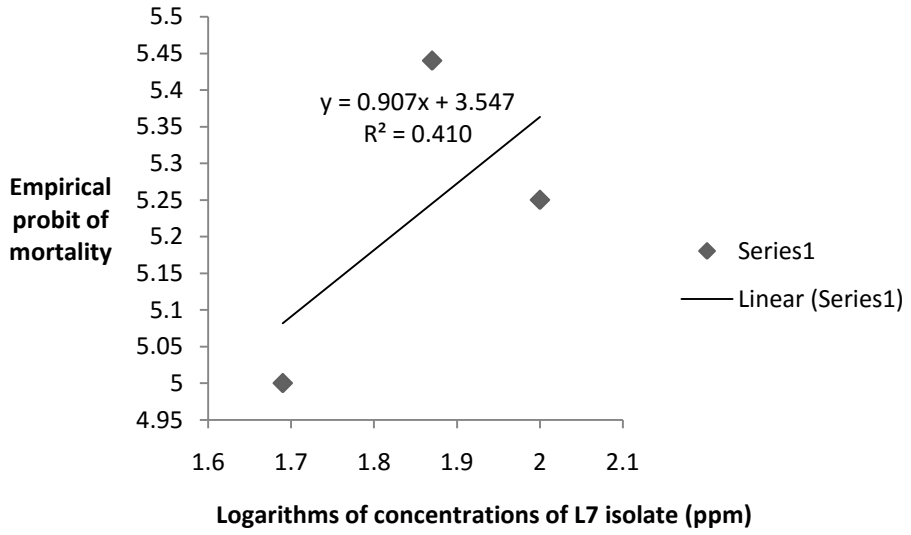
Appendix XXI: Relationship between concentration of L2 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



Appendix XXII: Relationship between concentration of L3 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



Appendix XXIII: Relationship between concentration of L6 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.



Appendix XXIV: Relationship between concentration of L7 isolate of *Bacillus thuringiensis* and probit of mortality of *Culex quinquefasciatus* larvae, showing probit regression line.